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Functional genomics of the fibroblast growth factor receptor 4 and the FES tyrosine kinase

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dedicated to my son

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I. Introduction

Live takes place in an ordered and regulated fashion. Especially eukaryotic organisms are very complex life forms that are not able to survive without regulating and controlling mechanisms. This is true during the embryonic development as well as during the adult life. This regulation is achieved by utilisation of different mechanisms. Signals can be transmitted when cells come into contact with other cells or when signalling molecules are released from cells. These molecules (e.g. hormones, growth factors) are able to bind to their receptors at the target cells and thereby induce specific biological processes like cell proliferation, migration, differentiation or apoptosis. This whole network of regulation and signalling processes is of tremendous complexity and thus it is not astonishing, that changes in this network, resulting in deregulation, leads to diseases like diabetes (Malecki and Klupa, 2005), cardiovascular disorders , immune deficiencies (Notarangelo et al., 2001) or cancer (Bertram, 2000).

One of the most frequent mechanisms of signalling is obtained by phosphorylation of proteins, a reversible process which is catalyzed by protein kinases (van der Geer et al., 1994). The direct counter players of these enzymes are protein phosphatases, which are able to remove phosphate groups from proteins (Streuli, 1996). Phosphorylation and dephosphorylation modifies the function of proteins by modulating their biological activity, by stabilizing or degrading them, by alteration of their cellular localisation or by interfering with protein-protein interactions.

Today it is known, that there are 518 putative protein kinase genes and 130 protein phosphatases (Blume-Jensen and Hunter, 2001; Manning et al., 2002). According to their substrate specificity the kinase and phosphatase entirety can be subdivided into either tyrosine- or serine/threonine –kinases and –phosphatases.

1. The human tyrosine kinome

The entirety of the tyrosine kinases in man are combined as the “human tyrosine kinome”, which can be again subdivided into receptor tyrosine kinases and non receptor tyrosine kinases (Robinson et al., 2000). The human genome contains 90 tyrosine kinases of which 58 encode for receptor tyrosine kinases (RTK’s). With the help of advantages in sequencing

technologies the human genome project could be completed and the human kinase complement could be identified (Arena et al., 2005; Manning et al., 2002).

The tyrosine kinases can not only be classified according to their cellular localisation, they can also be grouped into different families of kinases due to their structural properties.

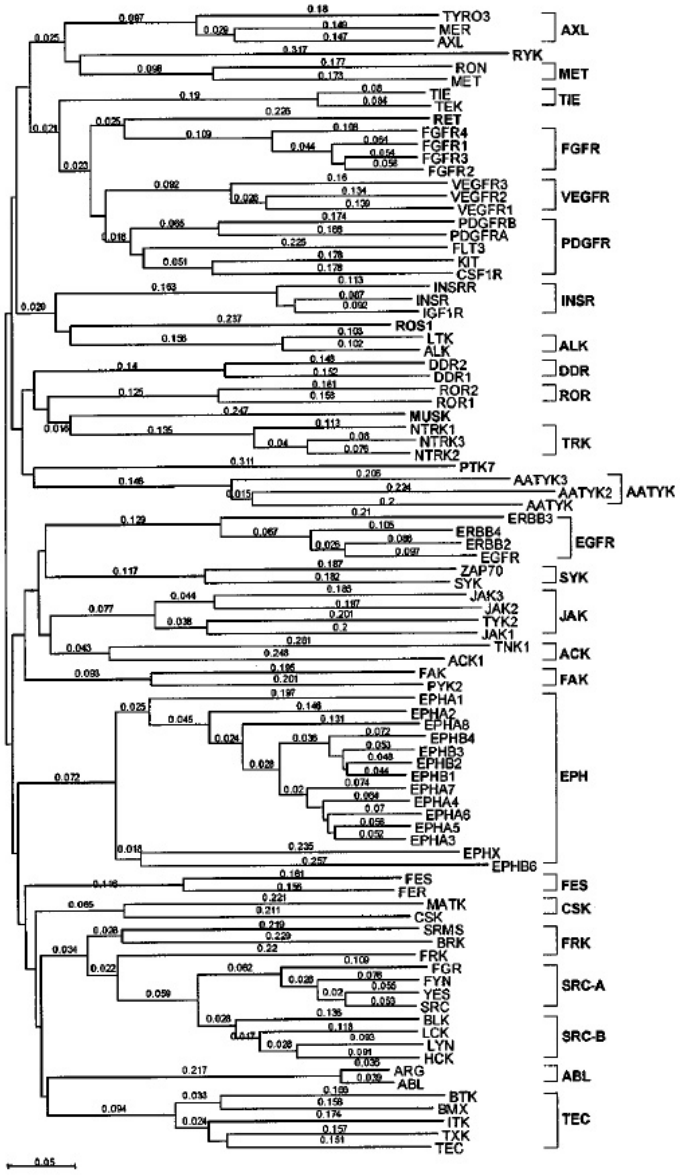


Figure 1: Phylogram of the human tyrosine kinase protein family inferred from amino acid sequencesof the kinase domains. The numbers at each node are indicating the evolutionary disctance, calculated by the Tamura-Nei algorithm (Robinson et al., 2000).

1.1. Structure of the receptor tyrosine kinases

Receptor Tyrosine Kinases (RTKs) are membrane associated proteins with a cytoplasmic tyrosine kinase domain. The general assembly of RTKs includes a glycosylated extracellular part, which is connected to a single transmembrane helix, followed by the intracellular carboxyterminal part, which carries the catalytic centre of the enzyme. The extracellular domains are structurally very diverse, which is the cause for the substrate specificity and the basis for the classification of the RTKs. Typical domains which build the extracellular portion of the RTKs are immunoglobulin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains and EGF (epidermal growth factor)-like Domains. In contrast the intracellular part of a RTK is more simple, consisting of a juxtamembrane Domain, a catalytic tyrosine kinase domain and a carboxyterminal part (Blume-Jensen and Hunter, 2001; Plowman et al., 1993; van der Geer et al., 1994).

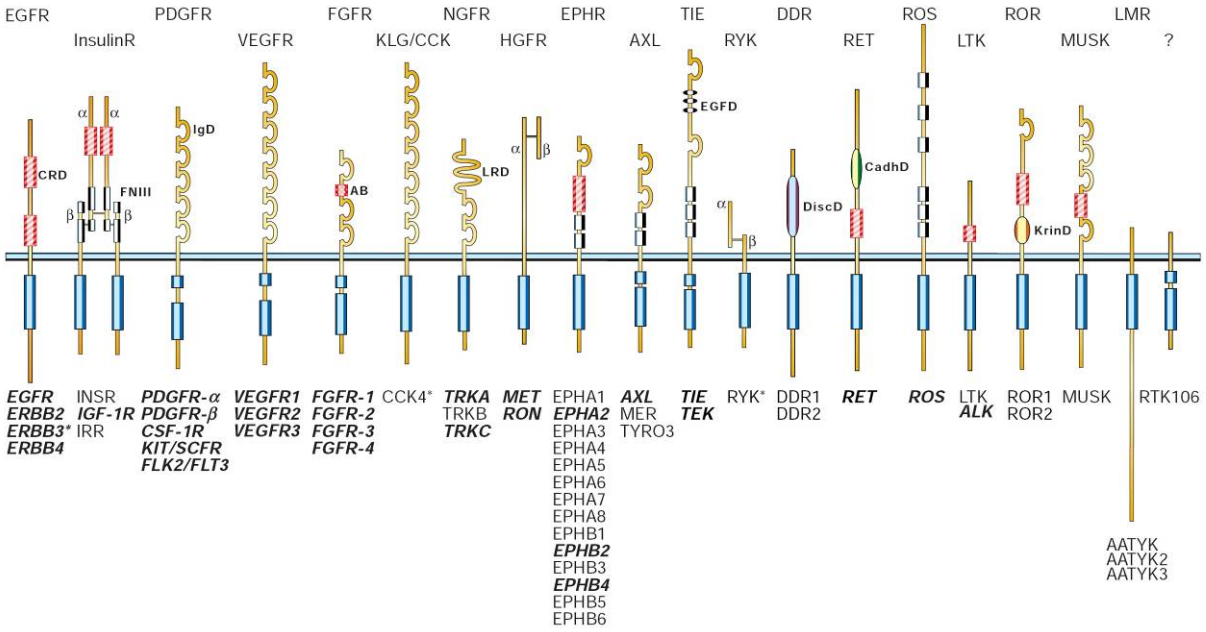


Figure 2: Structure and domains of the receptor tyrosine kinase family Abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD; discoidin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RTK subunits (Blume-Jensen and Hunter, 2001).

1.2. Structure of the non receptor tyrosine kinases

The structure of non receptor tyrosine kinases (NRTKs) is lacking some parts known from the RTKs, especially the extracellular domains as well as the membrane spanning part. Common to the RTKs they possess a tyrosine kinase domain and in addition NRTKs contain domains, which mediate protein-protein, protein-lipid or protein-DNA interactions. The most frequently occurring protein-protein interaction domains are the Src homology 2 (SH2) and 3 (SH3) domains. Other NRTKs that lack SH2 or SH3 domains harbour different sub-family specific protein-protein interaction domains. On the basis of these domains the NRTKs are classified into different subgroups (Robinson et al., 2000).

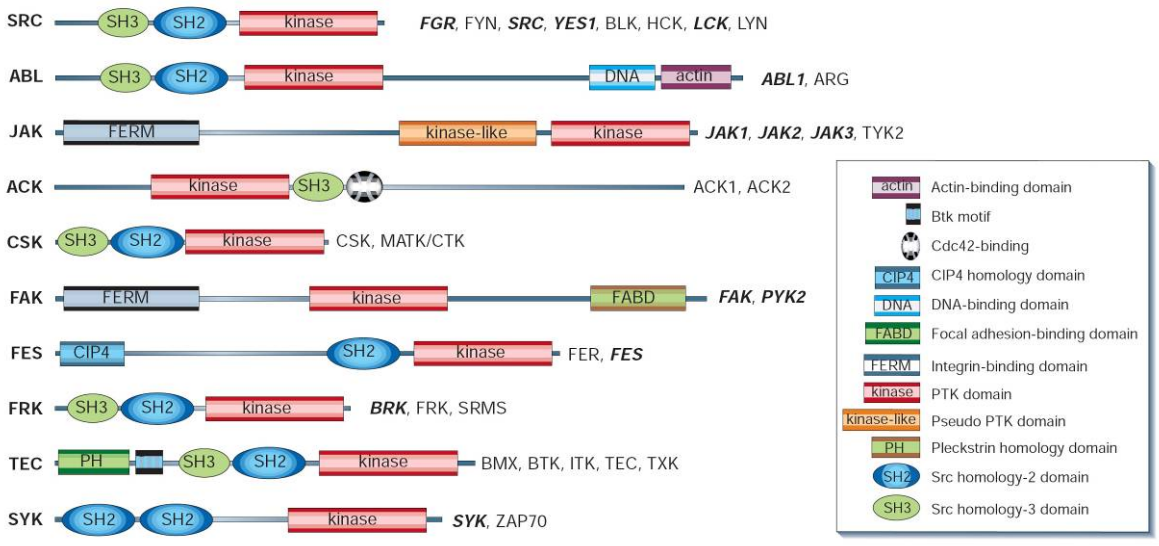


Figure 3: Structure and Domains of the non receptor tyrosine kinase family The left panel indicates the family name, on the right side the family members are indicated, the NRTKs shown in bold are implicated in human malignancies (Blume-Jensen and Hunter, 2001).

1.3. Mechanisms of receptor tyrosine kinase activation

The activation process of receptor tyrosine kinases requires ligand-binding to the protein and thereby allowing dimerisation of the receptor. The consequence of this binding and the dimerisation are autophosphorylation events inside the cell at the receptor. Tyrosines within the activation loop of the kinase domain become phosphorylated, which results in an activation of the enzymatic phosphotransferase activity and in mutual tyrosine

phosphorylation in the juxtamembrane domain, the kinase domain and the carboxyterminal region (Lemmon and Schlessinger, 1994). This process generates docking sites for phosphotyrosine specific adaptor domains, e.g. SH2 or phosphotyrosine-binding (PTB) domain (Songyang et al., 1993). The dimerisation of the receptor can be a result of the association of two identical monomers of a RTK, but it has also been shown, that two different members of a RTK family are able to form dimers (e.g. Her2 and the EGFR) (Lemmon and Schlessinger, 1994; Ullrich and Schlessinger, 1990; Wallasch et al., 1995). Besides ligand induced dimerisation, RTKs can also be transactivated independent of a ligand. This has been shown for the epidermal-growth-factor receptor (EGFR) and the platelet-derived-growth-factor receptor (PDGFR) after activation of G-protein coupled receptors (Daub et al., 1996; Linseman et al., 1995). In addition the inactivation of tyrosine phosphatases may be sufficient to phosphorylate tyrosine kinases (Herrlich and Bohmer, 2000).

1.4. Mechanisms of non receptor tyrosine kinase activation

The activation of NRTKs and the activation of RTKs have in common, that the phosphorylation in the activation loop of the kinase domain leads to an increase in enzymatic activity. The phosphorylation of the activation loop occurs in trans. The phosphorylation of tyrosine residues outside of this region can result in a negative regulation of the kinase activity. Besides binding to cell surface receptors, NRTKs are localized at several subcellular sites including the nucleolus, cytoplasm, mitochondria, the endoplasmatic reticulum and the cell membrane through amino-terminal modifications, such as myristylation or palmitoylation (Hantschel and Superti-Furga, 2004).

2. The genesis of Cancer

The integrity of the balance between cell-cycle progression, cell proliferation and programmed cell death is essential for the existence of live. The signalling processes that maintain this balance are often driven by tyrosine kinases (Bertram, 2000; Blume-Jensen and Hunter, 2001). However, if this tightly regulated system is disturbed at critical points, it can lead to hyper proliferative diseases like cancer. Cancer is comprised of about 100 different

subtypes and is one of the most common diseases of the industrialised countries (Bertram, 2000). The critical early event in cancerogenesis is the loss of genetic integrity in one cell resulting in genomic destabilisation and progression through accumulation of oncogenic alterations. These alterations are usually somatic events, but germline predisposition may also be a cause for familial cancer. These alterations are occurring in oncogenes, tumour suppressor genes or microRNA genes (Croce, 2008). The pathological phenotype of cancer is characterised by several "hallmarks" including aneuploidy, genetic instability, evasion from the immune defence, loss of contact inhibition, uncontrolled growth, anti-apoptosis, angiogenesis and metastatic spread of fully malignant cells (Hanahan and Weinberg, 2000). Ocogenes encode proteins that control cell growth or apoptosis. Cancer can arise in many sites and behaves differently depending on its organ of origin. Tumorigenesis is a multistep process involving genetic alterations that drive the progressive transformation of normal cells into highly malignant derivatives (Vogelstein and Kinzler, 1993).

3. The role of genetic alterations in cancer

The genesis of cancer as already described is the accumulation of genetic alterations in a normal cell, leading to a malignant transformation of this cell. There are different types of genetic alterations that can occur. On the one hand amplification of genes can occur and proteins are in an unusual way abundant in the cell (Dixon and Kopras, 2004). This has been shown for the gene encoding the RTK Her2, which is amplified in 20 to 30 % of breast cancers and therefore acts as a marker for prognosis and can be targeted in anti cancer treatments (Gschwind et al., 2004; Kamath and Buolamwini, 2006; Press et al., 2002; Slamon et al., 1987). On the other hand chromosomal aberrations are disruptions of the normal chromosomal content of the cell. These translocations, chromosomal inversions or abnormal numbers of chromosomes or chromosome sets may lead to genetic disorders like cancer. All these alterations cause either a change in the oncogene structure or an increase and deregulation of its expression (Bishop, 1991). Besides sporadic genetic changes due to cancer genome instability there are also DNA sequence polymorphisms that have been linked to cancer properties (Bange et al., 2002).

Centrally important proteins regulating signal transduction pathways are protein tyrosine kinases (PTKs), which are tightly regulated in normal cells (Alroy and Yarden, 1997; Laird and Shalloway, 1997). These proteins are common targets for oncogenic modifications as

already indicated by the retroviral oncogenes v-src, v-fms, v-erbB and v-kit, which were discovered in the late 1970ies and early 1980ies. Many of the receptor type and cytosolic PTKs have been found either in mutated or overexpressed forms in diverse types of human cancer (Arena et al., 2005; Dixon and Kopras, 2004). These proto-oncogenes do accumulate gain-of-function mutations and genetic alterations, which transform them to an oncogene.

The course of cancer is not only characterised by PTKs - tumour supressors gather loss-of-function alterations that makes them unable to keep the regulated balance upright. Especially, this has been shown for many mutations in the gene encoding for the tumour suppressor protein p53 (Ishii and de Tribolet, 1998; Striteska, 2005).

Since the DNA sequencing techniques improved and large scale screening methodologies arose, more and more became known about mutations and genetic alterations in cancer and other diseases. Screening of primary tumour samples or evaluated cell culture material for yet unknown genetic changes in proto-oncogenes yielded a lot of datasets of uncharacterised mutations (Arena et al., 2005; Maser and DePinho, 2002). Often the biological significance of the genetic alteration remained for the moment unsolved. To predict the impact of a newly discovered PTK gene mutation is not trivial. There are some basic rules to follow, but the in vitro or in vivo biological significance can only be proved by performing adequate experiments. Approaches to predict functional consequences involves the localisation of the alteration within the gene and comparisons to known oncogenic mutations within the PTK family or other PTK genes (MacAuley and Ladiges, 2005).

Anticancer Drug	Target	Disease
Monoclonal antibodies		
Trastuzumab (Herceptin, Genentech)	ERBB2	Breast cancer
Cetuximab (Erbitux, ImClone)	EGFR	Colorectal cancer
Bevacizumab (Avastin, Genentech)	VEGF	Colorectal cancer, non–small-cell lung cancer
Small molecules		
Imatinib (Gleevec, Novartis)	ABL, PDGFR, KIT	Chronic myelogenous leukemia, gastrointestinal stromal tumors, chordoma
Gefitinib (Iressa, AstraZeneca)	EGFR	Non–small-cell lung cancer
Erlotinib (Tarceva, Genentech)	EGFR	Non–small-cell lung cancer
Sorafenib (Nexavar, Bayer/Onyx)	VEGFR, PDGFR, FLT3	Renal-cell carcinoma
Sunitinib (Sutent, Pfizer)	VEGFR, PDGFR, FLT3	Gastrointestinal stromal tumors, renal-cell carcinoma

Figure 4: Cancer therapies targeting oncogenes. The common therapies against oncogenic proteins are utilising monoclonal antibodies or small molecule inhibitors (Croce, 2008).

4. Biological large scale screens

Today in the area of biological research there are more and more large scale or high throughput screens coming up. With advancing methodologies in in vitro diagnostics and increasing computing power in the field of systems biology the screening of huge sample datasets is made possible (Lea et al., 2007; Merrell and Camilli, 2002). Much effort was put into the completion of the human genome project, which serves now as the basis for large scale screens in the area of genomics. The screens are performed using on the one hand tissue culture material, which serves as a well established tool in molecular biology. On the other hand primary tissue samples derived from patients are analyzed, which brings insights into the actual circumstances in cancer patients (Bardelli et al., 2003; Bardelli and Velculescu, 2005). In a study performed in 254 established tumour cell lines the astonishing number of 155 polymorphisms and 234 somatic mutations could be detected. This data is now available in an internet based data platform called Tykiva (tyrosine kinome variant) (Ruhe et al., 2007). There are many databases which have been created to collect the mutation and single nucleotide polymorphism (SNP) abundances in specific genes. These databases serve today as information reservoirs. One of these databases is the International Agency for Research on Cancer TP53 Mutation Database, which is a set of data dealing with the tumour suppressor gene p53. Another general database is the Genetic Alterations in Cancer (GAC) database (Garnis et al., 2004).

5. The FES tyrosine kinase

The feline sarcoma oncogene (FES) tyrosine kinase is a non receptor tyrosine kinase, which was originally isolated as a retroviral oncogene in avian and feline retroviruses (Groffen et al., 1983; Hampe et al., 1982). FES, also known as fujinama poultry sarcoma (FPS), was amongst the first members of the PTKs to be characterised as a dominant acting oncoprotein. FPS/FES and the FES related protein (FER) are the only two members of a unique family of cytoplasmic PTKs (Roebroek et al., 1987). It was shown that FES is expressed in the myeloid lineage, in vascular endothelial, epithelial and neuronal cells and that FER is ubiquitously expressed (Feldman et al., 1985; Haigh et al., 1996; MacDonald et al., 1985; Pawson et al., 1989).

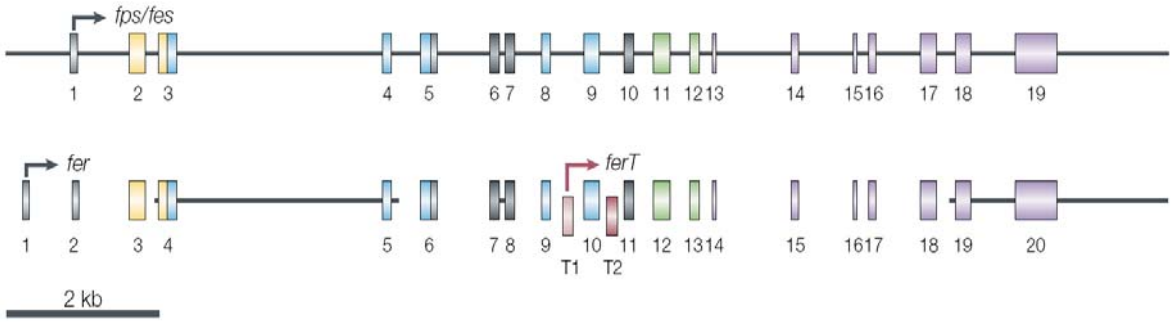


Figure 5: Chromosomal organisation of the FES tyrosine kinase gene. The FPS/FES loci is localized to the human chromosome 15.q26q1 The complete locus of the FPS/FES gene is located within 13 kb and harbours 19 exons. The FER locus is located on chromosome 5q.21. The exon structure of FER is essential identical to the structure of FPS/FES with an additional 5'-non coding exon and an internal testes-specific promoter.

Structurally the FES kinase consists of a FPS/FES/FER/CIP4 homology domain, a SH2 protein binding domain, some coiled-coil domains and a kinase domain at the carboxyterminal region (Greer, 2002; Roebroek et al., 1987).

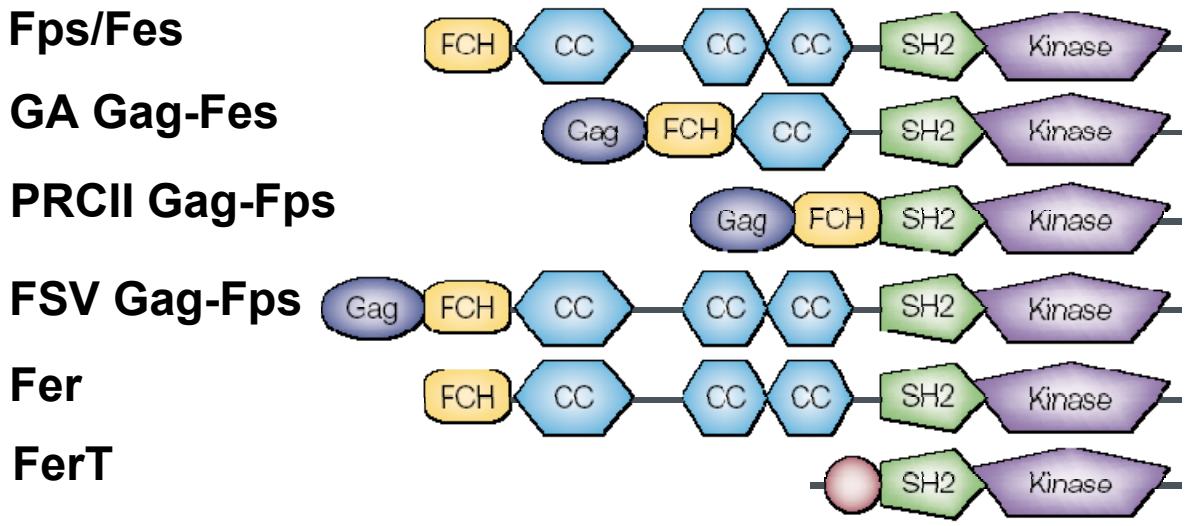


Figure 6: The domain structure of the FPS/FES tyrosine kinase family of non receptor tyrosine kinases. Abbreviations: FCH, FPS/FES/FER/CIP4 homology domain; CC, coiled-coil domain; SH2, src-homology-2 domain; FSV, Fujinami sarcoma virus; FERT, testes specific FER; FPS, fujinami poultry sarcoma; PRC, Poultry Research Center strain (Greer, 2002).

Known ligands for the Protein are the granulocyte-macrophage colony-stimulating-factor and Interleukin-6 and 4 (Izuhara et al., 1994; Linnekin et al., 1995). After stimulation with these agonists FES becomes phosphorylated and activated. Some reports could demonstrate a physiological association between the β-chain of the granulocyte-mocrophage colony-stimulating factor-1-receptor (GM-CSF-Receptor) and the FES Kinase (Greer, 2002). The

Fusion of the viral GAG-Protein at the NH2-terminus of FES results in an unregulated kinase activity, what leads to several biological consequences: one major issue, which could be demonstrated is the cytokine independent differentiation of hematopoietic progenitor cells and reduced growth factor requirements (Greer et al., 1988; Hampe et al., 1982; Veronese et al., 1983). The process of differentiation changes the expression pattern of alternative splicing forms of the FES protein. The p93/p74/p70 pattern is predominant in immature progenitor cells, whereas the p67/p62 pattern is typically for differentiated myeloid cells. In murine model systems it was demonstrated that v-FPS, which is the viral oncogene of the FES kinase, induces tumours in lymphoid and mesenchymal tissues, when transgenically expressed in mice. If the FES kinase is targeted to the cell membrane transgenic mice develop vascular tumours (Greer et al., 1994). This can be explained by the fact that FES is linked to FGF-2 induced chemotaxis and tube-formation of capillary endothelial cells (Kanda et al., 2007).

5.1. Genetic variations of the FES tyrosine kinase

Until 2003 no activating mutations of the FES gene were known in human cancer. In a sequence based study, where 14 PTK genes were analysed in 143 colorectal cancers 4 mutations in the FES gene could be detected in more than one sample (Bardelli et al., 2003).

Gene	Group	Mutation (amino acid)
NTRK3	TK	G608S, I695V, R731Q, K732T, L760I
FES	TK	M704V, R706Q, V743M, S759F
KDR	TK	G800D, R819STP, A1073T
EPHA3	TK	S792P, D806N
NTRK2	TK	T695I, D751N
MLK4	TKL	H261Y, H261Q, G291E, A293E, W296STP, R470C, R553STP, N596I, K629E
GUCY2F	RGC	D225Y, A360T, Q361H, F390L, R492H, R545S, E624D, E778G, V1026M, exon 17 donor splice site

Figure 7: genetic alterations discovered by Bardelli and colleagues (Bardelli et al., 2003). The alterations were identified in a screen were colorectal cancer samples were sequenced for changes in genes of tyrosine kinases.

The authors classified the different mutations into 3 groups: some mutations could not be grouped into a family, whereas others were taken together because of the fact, that the affected amino acid is evolutionary conserved. A third group summarizes mutations where equivalent residues in other kinases are known to be pathogenic. Besides the observed genetic variations in the FES gene other PTKs like the NTRK3, the KDR or the MLK4 gene were changed on the genetic level in the cancer samples, too.

By the means of these large scale screens for tumour disease associated variations in the human genome interesting candidates arise, which can be used in the search for cancer treatments and intervention strategies. The goal of improving the conditions of patients suffering under a specific type of cancer requires not only the identification of possible transforming mutations, but necessarily the functional consequences of the mutations have to be demonstrated. On that basis the targeting of oncogenes may lead to new strategies of interfering with transforming signalling pathways as it was exemplified for the development of the breast cancer therapeutic Herceptin (Fischer et al., 2003).

6. The fibroblast growth factor receptor family

The family of the fibroblast growth factor receptors (FGFRs) consists of 4 RTKs: fibroblast-growth-factor receptor 1 (FGFR1), fibroblast-grwoth-factor receptor 2 (FGFR2), fibroblast-growth-factor receptor 3 (FGFR3) and fibroblast-growth-factor receptor 4 (FGFR4) (Dionne et al., 1990; Keegan et al., 1991; Partanen et al., 1991). The four proteins share a common domain structure. The extracellular part consists of 3 immunglobulin (IG) like domains, which is followed by a conserved helical transmembrane domain and a cytoplasmic region with a tyrosine kinase domain that is separated by a short kinase insert-sequence. The IG-domains 2 and 3 have been shown to be necessary for the binding of the fibroblast-growth-factors (FGFs) and the attachment of heparin. This ligand binding is negatively regulated by a short acid box, which is located between the IG-domain 1 and the IG-domain 2.

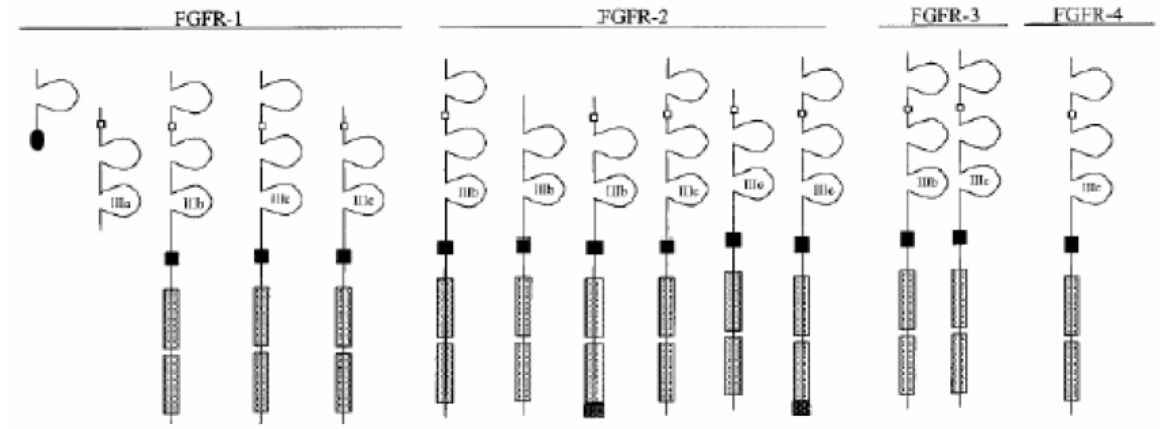


Figure 8: structural organisation of the domains within the family members of the fibroblast growth factor receptor family. The loops are indicating the IG-domains. The transmembrane domain is located at the black square and the lower panel shows the tyrosine kinase domain, which is divided by the short insert-sequence.

There are different isoforms of the FGFR 1, 2 and 3, which leads to a wide spread possibility of the FGFR related signalling pathways (Champion-Arnaud et al., 1991; Dionne et al., 1990). They differ in their extracellular parts, which can consist of two or three immunoglobulin like domains. The isoforms are generated via alternative mRNA splicing in the IG-domain 3, which has an impact on ligand specificity.

6.1. The fibroblast growth factor receptor 4

The FGFR4 gene was cloned out of the cDNA of the cell line K562 at the beginning of the 1990s (Partanen et al., 1991). The protein is composed of an amino acid sequence, which is 55% homologous to the already known FGFR1 and FGFR2 (Dionne et al., 1990). The structural components of the receptor are FGFR family specific: it consists of three immunoglobulin-like domains in the extracellular part and a split tyrosine kinase domain with a short kinase insert. The 11.3 kb FGFR4 gene is located at the chromosome 5q35.1-qter (Kostrzewa and Muller, 1998). The FGFR4 is expressed in the kidney, the intestinal tract, the pancreas, the spleen the liver and in high intensity in the lung. In the bloodstream FGFR4 is expressed too, but mainly in the media, which consists of smooth muscle cells (Hughes, 1997; Partanen et al., 1991). Although the genes of FGFR3 and FGFR4 were cloned out of the same cDNA of the cell line K562 the proteins differ in their expression profile (Keegan et al.,

1991). In embryonic mice FGFR4 is additionally expressed during the development of muscle cells (Stark et al., 1991).

Figure9: Domain structure of the fibroblast-growth factor receptor 4. Abbreviations: IG, immunoglobulin-like domain; TM, transmembrane domain; JM, juxtamembrane domain; TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2; KI, kinase insert.

7. Signalling pathways of the FGFR family

The fibroblast growth factor receptors are regulating a variety of different processes during the embryonic development and the homeostasis of tissues. If the expression of FGF-receptors is altered from its normal level the biological consequences may lead to distinct pathological changes and cancer.

During the embryogenesis the FGF-receptors are capable of differentiating and organising cells into various tissue types and they allow stimulation of proliferation processes (Basilico and Moscatelli, 1992). After fibroblast growth factor stimulation of osteoblasts and endothelial cells, they start to enter cell cycle, what results in enhanced proliferation as well as enhanced motility (Boilly et al., 2000). Stimulation of mesodermal and ectodermal cells with FGF-2 results in an increasing angiogenic and mitogenic strength (Burgess and Maciag, 1989). In other cell types the signalling via the FGF-receptors yields an arrest of the cell cycle and even a pro-apoptotic effect (Hondermarck et al., 1994; Sahni et al., 1999).

On the molecular level fibroblast growth factor receptors are able to influence many different signalling molecules. The combination of cell type, ligand, receptor and the available downstream signalling partners makes it possible to activate various biological processes.

In chondrocytes it has been demonstrated that FGF-receptors are able to activate the mitogen activated protein kinase pathway (ERK1/2) intensively, leading in a growth arrest instead of activating the proliferation. Further FGF-receptors are involved in the development of the skeleton (Naski and Ornitz, 1998), in tissue regeneration and wound healing.

The activation pattern of FGF-receptors involves ligand binding at the extracellular part and autophosphorylation of the receptor at specific sites in the intracellular region. Thereby

signalling proteins are recruited to the tyrosine autophosphorylation sites and docking proteins become phosphorylated. Together with other signalling partners a complex is formed, which is responsible for the further transmitting of the signals. In the juxtamemrane domain of FGF-receptors additional regulatory and highly conserved sequences, which serve as phosphotyrosine binding domains for FRS2α and FRS2β, are located. FRS2α and FRS2β belong to the family of FRS2 family of docking proteins (Eswarakumar et al., 2005).

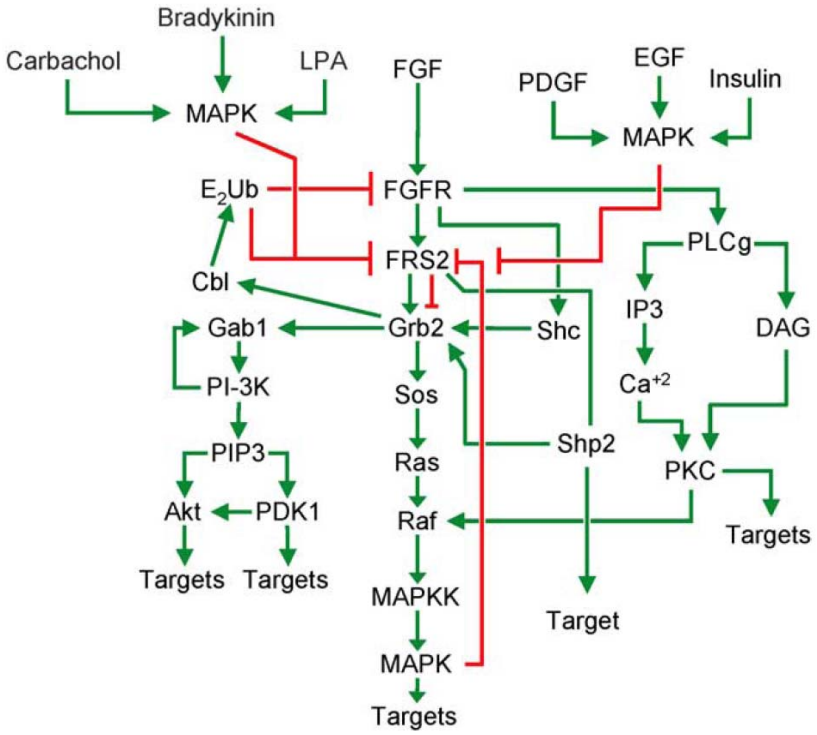


Figure 10: Organigram of the signalling pathways downstream of the FGFR family. Signalling downstream of FGFRs, that is dependent on tyrosine phosphorylation of FRS2α after FGF-stimulation, is highlighted with the green arrows. Negativ signals mediated by FRS2α are shown by red arrows (Eswarakumar et al., 2005).

The phosphorylation of FRS2α and FRS2β results in the recruitment of Grb2/Sos complexes, which leads to the activation of the Ras/Map kinase signalling pathway and finally to the activation of transcription factors. The autophosphorylation of FGFR1 creates a binding site for the SH2 domain of the phospholipase Cγ (PLCγ), which then activates the signalling pathway leading to the activation of the protein kinase C (PKC) (Kouhara et al., 1997). The FGF induced phosphorylation of FRS2 can also lead to tyrosine phosphorylation of the docking protein Gab1, followed by the recruitment of the PI3-kinase resulting in activation of the anti-apoptotic Akt pathway (Hadari et al., 2001).

8. Biological responsibilities of the fibroblast growth factor receptor 4

In comparison to the other FGFR family members the FGFR4 has the unique property that there is a specific ligand FGF19. Otherwise heparin is able to activate the FGFR4, which was the first evidence that a RTK can be stimulated by a polysaccharide (Gao and Goldfarb, 1995). In our days much effort is put into the research to determine the defined biological functions of the FGFR4 in completion. Whereas FGFR1 and FGFR3 knock out mice inherit a very abnormal phenotype the FGFR4 knock out mice are showing no significant abnormalities (Yu et al., 2000). The FGFR4 is very important during the developmental stages but also for the homeostasis of the adult organism. In the liver the FGFR4 is responsible for the conversion of cholesterol to bile acid. FGFR4 knock out mice are showing a tremendous increase of white fat cells and an altered glucose metabolism, resulting in increased blood glucose values and impaired insulin sensitivity. The FGFR4 takes also part in an interaction with NCAM and N-Cadherin, resulting in an adhesive effect for the cells.

9. Deregulation of FGFR family signalling in tumour and non tumour related diseases

Like other RTKs, the fibroblast growth factor receptors have been linked to diseases in humans like skeletal disorders and cancer. Since now many mutations in FGFR genes have been reported, that are responsible for the deregulation of genes and pathologies of different diseases. It has been demonstrated that point mutations in the FGFR1, 2 and 3 are the cause for severe impairment in cranial, digital and skeletal development, which results in premature fusion of cranial structures (craniosynostosis syndrome) and skeletal dysplasia (dwarfism) (Eswarakumar et al., 2005).

Genetic alterations of FGFRs in human skeletal disorders

Disease	Description	Gene	Activating mutation
Crouzon syndrome	Synostosis of coronal sutures, midface hypoplacia, ocular proptosis	*FGFR2*	Multiple; about 39 different mutations were reported, e.g. Cys278Phe, Cys342Tyr, Ser347Cys
Jackson–Weiss syndrome	Craniosynostosis with foot abnormalities	*FGFR2*	Ala344Gly; Cys342Ser or Arg
Beare-Stevenson cutis gyrata	Cloverleaf skull, over growth of skin with furrowed palms and soles, prominent umbilical stump	*FGFR2*; *FGFR3*	Ser372Cys; Tyr375Cys; Pro250Arg
Apert syndrome	Severe and symmetric fusion of hands and feet, craniosynostosis	*FGFR2*	Ser252Trp; Pro253Arg
Pfeiffer syndrome	Craniosynostosis, broad thumbs and toes	*FGFR1*; *FGFR2*	Pro252Arg Multiple; 36 mutations reported
Muenke syndrome	Unilateral or bilateral coronal synostosis; thimble like middle phalanges (hands)	*FGFR3*	Pro250Arg
Saethre-Chotzen-like syndrome	Craniofacial and limb defects	*FGFR2*; *FGFR3*	ValVal269-70del Pro250Arg
Achondroplasia	Short statue, midface deficiency	*FGFR3*	Gly346Glu; Gly375Cys; Gly380Arg
SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans)	Short limbs, developmental delay, acanthosis nigricans	*FGFR3*	Lys650Met
Thanatophoric dysplasia type I	Curved short femurs; lethal	*FGFR3*	Multiple; 9 different mutations reported
Thanatophoric dysplasia type II	Stright femurs with cloverleaf skull; lethal	*FGFR3*	Lys650Glu
Hypochondroplasia	Short limbs but less severe than achondroplasia, large head circumference	*FGFR3*	Multiple; 9 different mutations reported

Figure 11: Genetic alterations in the FGFR family members that lead to human skeletal disorders (Eswarakumar et al., 2005)

Constitutive activation of tyrosine kinases as fusion proteins with other genes due to chromosomal translocations plays an important role in the development of many haematological malignancies, especially in myeloproliferative syndromes (MPS). Translocation and fusion of FGFR1 with other genes causes a specific type of MPS syndrome called '8p11 myeloproliferative syndrome' (EMS) which is characterized by myeloid hyperplasia, eosinophilia and lymphoblastic lymphoma. So far, five different fusion partners for FGFR1 have been identified. The generation of these fusion proteins induce constitutive tyrosine kinase activation of FGFR1 by oligomerization. Although the majority of FGF signals are transduced via the docking protein FRS2, these fusion kinases seem to utilize pathways that are independent of FRS2 (Eswarakumar et al., 2005). For instance, the ZNF-198-FGFR1 and Bcr-FGFR1 fusion kinases lack the juxtamembrane domain, which is required for FRS2 binding. Translocation and fusion of FGFR3 has been demonstrated to be associated with multiple myeloma (MM) and peripheral T cell lymphoma.

Genetic alterations of FGFRs in human cancers

Cancer	Gene alteration
8P11 myeloproliferative syndrome (EMS)	Translocation and fusion of *FGFR1* with *ZNF-198* (also called *FIM* or *RAMP*) t(8;13); fusion of *FOP* with *FGFR1* t(6;8); fusion of *FGFR1* with *CEP 110* t(8;9); fusion of *FGFR1* with endogenous human retroviral sequence t(8;19); and fusion of *FGFR1* with *BCR* t(8;22)
Breast cancer	Over expression of FGFR1
Pancreatic adenocarcinoma	Abnormal exprssion of FGFR1 and FGFR4
Prostate cancer	Class switch of FGFR2 from IIIb isoform to IIIc isoform Abnormal expression of FGFR1c in prostate epithelial cells
Astrocytoma	Elevated expression of FGFR1 in white matter and down regulation of FGFR2 in malignant astrocytomas
Gastric cancer	*FGFR2* splice site mutation (940-2A → G) and Ser267Pro mutation
Transitional cell carcinoma of bladder	Frequent *FGFR3* mutations: Arg248Cys; Ser249Cys; Gly372Cys; Lys652Glu
Thyroid carcinoma	Over expression of FGFR3
Cervical carcinoma	Low frequency of *FGFR3* mutation: Ser249Cys
Colorectal cancer	Aberrant splicing and activation of cryptic splice sequences in *FGFR3*
Peripheral T cell lymphoma	Tanslocation and fusion of *FGFR3* with *ETV6* t(4;12)
Multiple myeloma	Activating mutations of *FGFR3* (Lys650Glu; Lys650Met) associated with chromosomal translocation t(4:14) (p16.3;q32.3)
Head and neck squamous cell carcinoma	FGFR4 polymorphism: Gly388Arg

Figure 12: Genetic alterations in the FGFR family members that lead to human cancer (Eswarakumar et al., 2005)

The FGFR4 polymorphism resulting in a conversion from glycin to arginin at amino acid position 388 (Bange et al., 2002) was linked to a poor prognosis of patients suffering under breast cancer and head and neck squamous cell carcinoma (Streit et al., 2004).

10. The FGFR4 G388R polymorphism

The discovery of a polymorphism in the FGFR4 gene, which results in a conversion from a glycin to an arginin at position 388, took place in 2002. It was demonstrated that the existence of this SNP correlates with a very aggressive progress and poor prognosis for patients suffering under breast cancer (Bange et al., 2002). Surprisingly the distribution of this SNP within the population is almost 50% and therefore the FGFR4 388R has no influence on the initiation of a tumour. In melanomas the FGFR4 388R allele correlates with thickness and invasivity (Streit et al., 2006). In patients with head and neck squamous cell carcinoma which are carrying the FGFR4 388R allele have a poor prognosis for the development of the disease, especially if the FGFR4 is massively expressed. The poor prognosis for arginin allele carriers is also true in the case of prostate cancer. In this case it could also be demonstrated that the arginine allele is involved in the tumour initiation. In vitro wound closure assays with immortalised prostate epithelial cells (PNT1a) verified that the FGFR4 388R enhances the migration and the wound closure (Wang et al., 2004a).

II. Specific Aims

The research to understand the cellular functions of the PTKs is a big and growing field in science. The involvement of PTKs in the initiation and progression of tumours in human cancer lead already to the development of several anti-cancer drugs and to an improvement of cancer treatment. With the help of the knowledgebase of the human genome sequences one is able to identify changes within these sequences. The understanding of the consequences of these changes is very important because the alterations may possibly be oncogenic. .

In this thesis the goal of our studies was to identify and characterise genetic alterations that may have an impact in cancer initiation or progression and to establish a workflow that is suitable to be applied to different newly discovered genetic alterations. The already known genetic alterations in the FES tyrosine kinase were investigated to find out whether they may represent oncogenic conversions

In an additional part a newly discovered genetic alteration in the FGFR4 gene was investigated. The goal was to identify the physiological consequence and connection to cancer in the affected cancer cell line.

III. Material and Methods

1. Material sources

1.1 *Laboratory chemicals and biochemicals*

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3'-triphosphate)	Pharmacia, Freiburg
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
CHAPS	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
Ki16425	Kirin Laboratories, Tokyo
L-Glutamine	Gibco, Eggenstein
Lysozym	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen

Na-DOC (Sodium-desoxycholat)	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen

All other chemicals were purchased from Merck (Darmstadt).

1.2 Enzymes

Alkaline Phosphatase (CIAP)	Roche, Mannheim
LA-Taq-DNA Polymerase	Takara, Japan
Pfu-DNA Polymerase	Roche, Mannheim
Restriction Endonucleases	Pharmacia, Freiburg
	Roche, Mannheim
	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
T7-DNA Polymerase	Pharmacia, Freiburg
Taq-DNA Polymerase	Roche, Mannheim
	Takara, Japan
Trypsin	Gibco, Eggenstein

1.3 "Kits" and other materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg
PuReTaq Ready-To-Go PCR Beads	Amersham Biosciences, Piscataway, NJ
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
TOP10/P3 One Shot™	Invitrogen, USA
Transwells	Schubert & Weiss, Munich
Whatman 3MM	Whatman, USA

1.4 Growth factors and ligands

Interleukin 6	Sigma, Taufkirchen
FGF19	U3Pharma, Martinsried

2. Media

2.1. Bacterial Media

LB or 2xYT media were used for cultivation of all *Escherichia coli* strains. If and as required 100 µg/ml Ampicillin or 70 µg/ml Kanamycin were added to media after autoclavation. For the preparation of LB-plates 1.5% Agar was also added.

LB-Medium	1.0 % Tryptone
	0.5 % Yeast extract
	1.0 % NaCl
	pH 7.2
2xYT-Medium	1.6 % Tryptone
	1.0 % Yeast extract
	1.0 % NaCl
	pH 7.2

2.2. Cell culture media

All cell culture media and additives are from Gibco (Eggenstein), fetal calf serum (FCS) was purchased from Sigma and Gibco.
Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 mg/ml glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.
RPMI 1640 medium supplemented with 2 mM L-glutamine and 1 mM sodium pyruvayte.
Nutrient mixture F12 (HAM) supplemented with 2 mM L-glutamine and DMEM supplemented with 4.5 mg/ml glucose, 2 mM L-glutamine, 1 mM sodium pyruvate mixed 1:1.
Freeze medium containing 90 % heat-inactivated FCS and 10 % DMSO.

3. Stock solutions and buffers

BBS (2x)	50 mM BES 280 mM NaCl 1.5 mM Na2HPO4 pH 6.96
Collecting gel buffer (4x)	0,5 M Tris/HCl pH6.8 0,4 % SDS
HBS (2x)	46 mM HEPES, pH 7.5 274 mM NaCl 1.5 mM Na2HPO4 pH 7.0
HNTG	20.0 mM HEPES, pH 7.5 150 mM NaCl 0.1 % TritonX-100 10 .0 % Glycerol 10.0 mM Na4P2O7
DNA loading buffer (6x)	0.05 % Bromphenol blue 0.05 % Xylencyanol 30.0 % Glycerol 100.0 mM EDTA pH 8.0
Laemmli buffer (3x)	100 mM Tris/HCl pH 6.8 3.0 % SDS 45.0 % Glycerol 0.01 % Bromphenol blue 7.5 % ß-Mercaptoethanol
NET	50.0 mM Tris/HCl pH 7.4 5.0 mM EDTA 0.05 % Triton X-100 150.0 mM NaCl
	PBS 137.0 mM NaCl 27.0 mM KCl 80.9 mM Na2HPO4 1.5 mM KH2PO4 pH 7.4
SD-Transblot	50.0 mM Tris/HCl pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS
Separating gel buffer (4x)	0,5 M Tris/HCl pH 8.8 0,4 % SDS
"Strip" buffer	62.5 mM Tris/HCl pH 6.8 2.0 % SDS 100.0 mM β-Mercaptoethanol
TAE	40.0 mM Tris/Acetate pH 8.0 1.0 mM EDTA
TE10/0.1	10.0 mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0

Tris-Glycine-SDS	25.0 mM Tris/HCl pH 7.5 200.0 mM Glycine 0.1 % SDS
RIPA lysis buffer	1 % NP40 1 % Na-DOC 0.1 % SDS 150 mM NaCl 10 mM NaPO4, pH 7.2 2 mM EDTA 5 mM β-Glycerophosphat 4 mM VaO5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin 1mM DTT
SD-Transblot	50.0 mM Tris/ HCl, pH 7.5 40.0 mM Glycine 20.0 % Methanol 0.004 % SDS

4. Cells

4.1. Bacteria strains (E. coli)

E. coli strain	Description	Origin/ Reference
DH5aF'	F'/endA1 hsd17 (rk-mk-) supE44 recA1 gyrA (Nal) thi-1 (lacZYA-argF)	Genentech, USA
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB $lacI_qZ\Delta M15$ $Tn10\ (Tet_r)$]	Stratagene, NL
TOP10/P3	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG P3: KanR AmpR (am) TetR (am)	Invitrogen, USA

4.2. Eukaryotic cell lines

Cell line	Description	Origin/ Reference
BT474	Human mammary carcinoma	ATCC, USA
HEK293	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC, USA
MDA-MB 231	Human mammary carcinoma	ATCC, USA
MDA-MB-361	Human mammary carcinoma	ATCC, USA
MDA-MB 453	Human mammary carcinoma	ATCC, USA
NIH 3T3	Mouse fibroblasts, Clone 7 C. Sherr	ATCC, USA
Phoenix E, A	Retrovirus producer cell lines for the generation of helper free ecotropic and amphotropic retroviruses based on HEK-293	Nolan, USA
ZR751	Human mammary carcinoma	ATCC, USA

All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and grown as recommended by the supplier.

5. Antibodies

5.1. Primary antibodies

The following antibodies were used in immunoprecipitation experiments or as primary antibodies in immunoblot analysis.

Antibody	Description	Origin/ Reference
Akt1/2	Rabbit, polyclonal/ AA 345-480 of human Akt1	Santa Cruz, USA
FGFR4	Rabbit, polyclonal, recognises the FGFR4	U3Pharma, Germany

P-Akt/PKB	Rabbit, polyclonal/ phospho-Akt (Ser-473); Recognizes p-Akt of human, rabbit and rat origin	NEB, Germany
P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/ Tyr-204) MAPK	NEB, Germany.
GFP	mouse, monoclonal; recognizes the green Fluorescent protein (GFP)	G. Gerisch
HA	Mouse, monoclonal; recognizes the influenza hemagglutinin epitope (HA)	Babco, CA, USA
P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, USA
FES	Mouse, monoclonal; recognizes the FES Tyrosine kinase	Santa Cruz, USA

5.2. Secondary Antibodies

For western blot analyses secondary antibodies, conjugated with horseradish peroxidase (HRP) were utilized.

Antibody	Description	Origin/ Reference
Goat anti-mouse	1 : 10,000	Sigma, Germany
Goat anti-sheep	1 : 25,000	Dianova, Germany
Goat anti-rabbit	1 : 25,000	BioRad, Germany

6. Plasmids and oligonucleotides

6.1. Primary Vectors

Vector	Description	Origin/ Reference
pcDNA3	Mammalian expression vector, Ampr, CMV promotor, BGH pA, high copy number plasmid	Invitrogen, USA
pLXSN	Expression vector for retroviral gene transfer, Ampr, Neor, origin from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, USA
pGEX5x-3	Bacterial expression vector for GST-fusion proteins, pBR322 origin, tac promoter, Ampr,, lac Iq gene, protease recognition sites	Amersham, USA

6.2. Constructs

Vector	Description	Origin/ Reference
pcDNA3-HA	C-terminal HA-tag	MPI of Biochemistry Dept. Molecular Biology
pLXSN-HA	C-terminal HA-tag	this study

6.3. Important oligonucleotides

Sequence (description)	Name
5’ CGA CCA CCT ACT GAG CAC C 3’	fes-pcdna3-for
5’ GAG CTG TCA GCA TAT GAG CTG G 3’	fes-pcdna3-rev
5’ GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA GCC CAA GGC CAG TTC TCC 3’	fes-plxsn-fwd
5’ G GAA TTC GCC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CC 3’	fes-plxsn -rev
5’ ATA AGA ATG CGG CCG CGC CAC CAT GAA GCT GCG GCT CC 3’	her2-pLXHA-for
5’ ACG CGT CGA CCA CTG GCA CGT CCA GAC 3’	her2-plxha-rev
5’ CGG AAT TCG CCA CCA TGG GTA GCA ACA AGA GCA AG 3’	src-plxha-for
5’ ACG CGT CGA CGA GGT TCT CCC CGG G 3’	src-plxha-rev
5’ ATA AGA ATG CGG CCG CGC CAC CAT GCG ACC CTC CGG 3’	egfr-plxha-for
5’ ACG CGT CGA CTG CTC CAA TAA ATT CAC TGC 3’	egfr-plxha-rev
5’ ATA AGA ATG CGG CCG CGC CAC CAT GCA GAC GAA GGT GCT G 3’	kd-plxha-for
5’ ACG CGT CGA CAA CAG GAG GAG AGC TCA GTG TG 3’	kd-plxha-rev
5’ CGG AAT TCG CCA CCA TGG GCC CAG GAG TTC 3’	fms-plxha-for
5’ CGG GAT TCG CGC AGA ACT GAT AGT TGT TGG G 3’	fms-plxha-rev
5’ TC CTC GAG TCC AGA TCC AGA GTG TGG GTG CAG TTC TTC 3’	fes-pcdna3HA-fwd
5’ CCG CTCGAG GT ATT GTA AAA AAG AGC AAA CTT GCT 3’	fes-pcdna3HA -rev
5’ CCC AAGCTT CTA AGA TGA TTC CAG GTA CTC C 3’	fgfr4-pcdna3-fwd
5’ AGAGTGGTGAAAATAGCAGG 3’	fgfr4-pcdna3-rev
5’ TGCTATTTTGACCACTCTGGATCCCAGGAGAGGCCAAG GAGAGAGGAGCAAG 3’	fgfr4-y367c-fwd

5’ GAA TTC GCC ACC ATG ATT GTA AAA AAG AGC AAA CTT GCT AA 3’	fgfr4-y367c -rev
5’ GAA TCA TCT TAC CCG TAC GAT GTC CCG GAC TAC GCG TAG CTC GAG 3’	Fes-seq-1
5’ G GAA TTC GCC ACC ATG GAT CAA AAT AGA GC 3’	Fes-seq-2
5’ CT GAA TTC AGA GTC TGA AAT TCA TCC TTG 3’	Fes-seq-3

7. cDNA samples used for large scale sequencing

The cDNA used for the large scale sequencing screens was prepared by Tatjana Knyazeva using the following cell lines:

Squamous Cancer:

FaDu;
UM-SCC-10A;
UM-SCC-10B;
UM-SCC-17A;
UM-SCC-17B;
UM-SCC-22A;
UM-Scc-22B; KB;
Hlac-78; Hlac-79;
Hep-2; Scc-25;
HaCat; A-431;
Colo-16

Bladder cancer:

RT-4; SCaBER;
T-24; TCCSUP;
HAT-1376

Breast cancer:

SK-Br-3;
BT-549;
DAL;
MCF-7;
DU-44-75;
BT-483;
T-47D;
ZR-75-1;

ZR-75-30;
MDA-MB-468;
MDA-MB-453;
MDA-MB-175VII;
MDA-MB-415;
MDA-MB-231;
MDA-MB-435S;
MDA-MB-361;
MDA-MB-157;
Hs-578T

Ovarian Cancer:

2774;
2780;
SK-OV-3;
SK-OV-6;
SK-OV-8;
OVCAR-3;
OAW-42;
PA-1;
CaOv-3;
CaOv-4;
IGROV-1

Leukemia:

HL-60;
NB-4;
MV4-11;
MOLM

Cervical Cancer:

C-33A;
ME-180;
MS-751;
SiHa;
CaSki;
HeLa S3

Kidney Cancer:

A-498;
A-704;
769-P;
768-O;
SW-13;
ACHN

Stomach Cancer:

Hs-746T;
KATO III;
RF-1;
RF-48;
AGS;
MKN-1;
MKN-28;
STK-2

Melanoma:

WM-1341D;
WM-115;
HS-695T;
C-8161

Lung Carcinoma:

Calu-1;
Calu-3;
Calu-6;
SK-LU-1;
NCI-H596;
NCI-H460;
NCI-H146

Glioblastoma:

U-118

Pancreatic Cancer:

PANC-1;
Capan-1;
Capan-2;
DANG-G;
CFPAC-1;
AsPC-1

Endometrium Cancer:

KLE; RL95-2

8. Enzymatic manipulation of DNA

8.1. Plasmid preparation

Small amounts of plasmid DNA were prepared using the Qiagen Plasmid Mini Kit, larger amounts of DNA were obtained with the Qiagen Plasmid Maxi Kit following the manufacturer's instructions.

8.2. Restriction digestion of DNA

The ratio of Enzyme/DNA, the temperature, the buffer and the time of incubation were adjusted according to manufactures instruction. Usually, incubations for 2 hour at 37°C with a calculated 5-fold over digestion and the buffers as supplied by the manufacturers were chosen.

8.3. Dephosphorylation of DNA 5'-termini

In order to prevent self-ligation of vector termini generated by restriction digest, 5´-termini of vectors were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP). This phosphatase removes 5´-phosphate residues from DNA as well as RNA. For dephosphorylation, 1 µg of cut vector DNA was incubated with 5 units CIAP in adequate reaction buffer (e.g. 50 mM Tris/HCl pH 8.0, 0.1 mM EDTA pH 8.5) at 37°C for 10 minutes. Either reactions were stopped by heat inactivation at 85°C for 10 minutes or DNA was directly purified using the QIAquick PCR Purification Kit.

8.4. Ligation of vector and insert

Purified, digested and dephosphorylated vector DNA (40 ng), the designated insert DNA, 1 µl 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 unit T4 DNA Ligase were combined. A molar ratio between insert and vector of 3 to 1 was usually chosen. Reactions were either left on 14°C over night or at 37°C for 2 hours and subsequently transformed into competent bacteria.

8.5. Agarose gel electrophoresis

Depending on the size of the fragments of interest 0.7-2% agarose gels were prepared in horizontal chambers. TAE buffer was used for the electrophoresis. Voltage was usually set to 4-10 V per cm width of the gel. After separation, DNA fragments were stained by gently agitating gels in TAE containing 0.5 µg/ml ethidium bromide and were subsequently viewed under UV light.

8.6. Isolation of DNA fragments from agarose gels

Following gel electrophoresis gel slices bearing DNA fragments of interest were cut out of the gel. Agarose was dissolved and DNA was purified using the QIAquick Gel Extraction Kit following Qiagen's protocol.

8.7. Preparation of competent cells

The preparation of competent cells followed the procedure described by Chung and Miller (Chung and Miller, 1993). Competent cells were shock frozen in liquid nitrogen and stored for up to one year at –70°C. Transformation frequency ranged between 10^5 and 10^7 colonies/µg DNA.

8.8. Transformation of competent cells

A 50 µl aliquot of competent bacteria was added to a 50 µl mixture of DNA ligation cocktail, 10 µl 5x KCM solution (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and water. After thoroughly mixing samples were incubated on ice for 20 minutes and 10 minutes at room temperature. Then, 300 µl LB broth were added and samples were incubated at 37°C for 1 hour while constantly shaking. Bacteria were streaked out on appropriate agar plates containing ampicillin for the selection of transformants.

8.9. Enzymatic amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction mixture was prepared using the following protocol.

1 µl	template DNA, 1-10 ng
1 µl	"forward" oligonucleotide, 10 pmol/µl
1 µl	"reverse" oligonucleotide, 10 pmol/µl
2.5 µl	10x PCR buffer II containing 20 mM $MgCl_2$
2 µl	dNTP-Mix, 2.5 mM each
0.5 µl	Taq DNA Polymerase (5 U/µl)
ad 25 µl	H_2O

PCR reactions were carried out using an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to each specific application:

first denaturation:	3 min 94°C
amplification 25-30 cycles:	1 min 94°C (denaturation)
	1 min 54°C (annealing)
	1 min / kb product 72°C (extension)
last extension:	7 min 72°C

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified or directly purified with QIAquick Gel Extraction or PCR Purification Kit.

8.10. Polymerase chain reaction (PCR) in large scale screening

The polymerase chain reaction mixture, used to amplify DNA fragments for the large scale sequencing screen was set up in a 96 well plate, using the following protocol.

1 µl	template cDNA, 1-10 ng
2 µl	"forward" oligonucleotide, 10 pmol/µl
2 µl	"reverse" oligonucleotide, 10 pmol/µl
2 µl	20 mM $MgCl_2$
5 µl	10x PCR buffer
5 µl	dNTP-Mix, 2.5 mM each
1 µl	KOD Hot Start DNA Polymerase (5 U/µl)
ad 50 µl	H_2O

PCR reactions were carried out using an automated 96 well thermal cycler („EPmaster“, Eppendorff). The following standard protocol was adjusted to each specific application:

first denaturation:	2 min 96°C
amplification 30-40 cycles:	15 sec 96°C (denaturation)
	30 sec 58-64 °C (annealing)
	20 sec / kb product 68°C (extension)
last extension:	2 min 68°C

PCR products were directly purified with PCR Purification Kit in 96 well standard.

8.11. DNA sequencing

DNA sequencing was performed according to the "Big Dye Terminator Cycle Sequencing Protocol" (ABI). The following mix was subjected to a sequencing-PCR run:

0.5 µg DNA of interest
10 pmol oligonucleotide
4 µL Terminator Ready Reaction Mix
ad 20 µL H_2O
25 cycles: 30 sec 94°C
15 sec 45-60°C
4 min 60°C

The sequencing products were purified by sodium acetate/EtOH precipitation, dissolved in 20 µL HPLC grade ddH_2O and analyzed on a 310-Genetic Analyzer (ABI Prism).

8.12. Large scale DNA sequencing

DNA sequencing in 96 well standard was performed according to the "Big Dye Terminator Cycle Sequencing Protocol 3.1" (ABI). The following mix was subjected to a sequencing-PCR run:

0.5 µg DNA of interest
5 pmol oligonucleotide
2 µL Terminator Ready Reaction Mix
ad 10 µL H_2O
25 cycles: 30 sec 94°C
15 sec 50-60°C
4 min 60°C

The sequencing products were purified using sepharose chromatography and the volume was set to 20 µL. The samples were analysed on a ABI – 48 well sequencing instrument (ABI Prism).

9. Methods in mammalian cell culture

9.1. General cell culture techniques

Cell lines were grown in a humidified 93 % air, 7 % CO2 incubator (Heraeus, B5060 Ek/CO2) at 37° C and routinely assayed for mycoplasma contamination using a bisbenzimide staining kit (Sigma, Karlsruhe). Before seeding, cells were counted with a Coulter Counter (Coulter Electronics). Cells were cultured in the medium recommended by the manufacturer. The following cell lines required special media additives:
SCC9 Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 medium 1:1 containing 0.5 mM sodium pyruvate, 2 mM L-glutamine, 400 mg/L Hydrocortisone and 10 % FCS.
MCF10A Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 1:1 containing 0.5 mM sodium pyruvate, 5 % horse serum, 80 U/L Insulin, 1 mg/ml Hydrocortisone, 500 µg/ml Choleratoxine, 100 mg/ml EGF.
BT20, A498 Eagle's minimum essential medium (MEM) containing 10 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1 mM non-essential amino acids.
BT549 RPMI 1640 containing 10 % FCS, 2 mM L-glutamine and 2.67 U/L Insulin.
HS578T Dulbecco's modified Eagle medium (DMEM) containing 10 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 10µg/ml Insulin.

9.2. Transfection of cell lines with calcium phosphate

HEK-293T cells in six-well dishes were transfected transiently at about 70% confluency with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described previously (Chen and Okayama, 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells.
The transfection mix of DNA and CaCl2 in water was prepared as follows:

Dish	6-well	6 cm	10 cm
Area	10 cm2	21 cm2	57 cm2
Volume of medium	1 ml	2 ml	4 ml
DNA in H2Obidest	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M CaCl2	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction the indicated volume of 2x BBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified container at 3% CO2 overnight. One day after transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS. For transfection of Phoenix cells HBS was used instead of BBS.

9.3. Transfection with Lipofectamine®

Cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described. For transfections in 6-well dishes, 90 µl of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA in 100 µl serum-free medium were mixed. After 20 min the transfection mixture was added to 800 µl serum-free medium per well.
After 4 h the transfection mixture was replaced by normal growth medium and 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

8.4 Transfection with Lipofectamine 2000®

Cells were transiently transfected using Lipofectamine 2000® (Gibco-BRL) essentially according to the manufacturer's recommendations. For transfections in 6-well dishes, 2 µg of total plasmid DNA were diluted into 250 µl of serum-free medium. 5 µl Lipofectamine 2000® (Gibco-BRL) were also diluted into 250µl of serum-free medium and allowed to incubate at room temperature for 5-10 min. After mixing of DNA and transfection reagent, the mixture

was added to 2 ml of antibiotic-free, but serum containing medium per well. After 4 h the transfection mixture was removed and fresh media containing serum was added. After 20 h, cells were washed and cultured for a further 48 h in serum-free medium until lysis.

9.4. Retroviral gene transfer in cell lines

The ecotropic packaging cell line Phoenix (Nolan, Stanford, USA) was transfected with pLXSN retroviral expression plasmids (Clontech, Palo Alto, CA), encoding the genes for the analysed proteins, by the calcium phosphate/ chloroquine method as described previously (Swift et al., 2001). 24 h after transfection the viral supernatant was collected and used to infect NIH3T3 cells (5x 104 cells/6-well plate). 4 to 12 h later, retroviral supernatant was replaced with fresh medium. Selection for stable expression was started 48 h post infection with the respective antibiotic.

10. Protein analytical methods

10.1. Lysis of eukaryotic cells

Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the Figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in Triton X-100 lysis buffer. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4° C. For the dimerisation experiments of FGFR4 RIPA lysis buffer was used.

10.2. Determination of protein concentration in cell lysates

The „Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer's recommendations. For samples containing glycerol the BioRad Protein Assay (BioRad Laboratories GMBH, Munich) was used according to the manufacturer's recommendations.

10.3. Immunoprecipitation of proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 µL of protein A- or G-Sepharose for 4 h at 4°C. Precipitates were washed four times with 1 mL of HNTG buffer, suspended in 3× Laemmli buffer, boiled for 5 min, and subjected to SDS-PAGE.

10.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990). The following proteins were used as molecular weight standards:

Protein	MW (kD)	Protein	MW (kD)
Myosin	205.0	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

10.5. Colloidal Coomassie staining

Protein samples intended for further analysis were in-gel stained with a Roti®-Blue (Roth, Germany) colloidal Coomassie staining as recommended by the manufacturer.

10.6. Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 2 h at 0.8 mA/cm2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2 g/l in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

10.7. Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25 % gelatine for at least 4 h. The membrane was then probed with primary antibody (typically overnight at 4° C). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25 % gelatin. The membrane was washed 3x 20 min in 1x NET, 0.25 % gelatin, incubated for 1 h with secondary antibody and washed again as before. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films or a digital camera unit. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50° C. Stripped membranes were blocked and reprobed with different primary antibody to confirm equal protein loading.

11. Biochemical and cell biological assays

11.1. Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 24 or 48 h cells were treated with inhibitors and agonists as indicated in the Figure legends, washed with cold PBS and then lysed for 10 min on ice.

11.2. ERK 1/2 and AKT/PKB phosphorylation

For determination of Erk 1/2 and Akt phosphorylation, approximately 20 µg of whole cell lysate protein/lane was resolved by SDS-PAGE and immunoblotted using rabbit polyclonal phospho-specific Erk/MAPK antibody. Akt phosphorylation was detected by protein

immunoblotting using rabbit polyclonal anti-phospho-Akt antibody. Quantification of Erk 1/2 was performed using the Luminescent Image Analyis System (Fuji). After quantification of Erk 1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-Erk 1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

11.3. Focus formation assay

NIH3T3 cells were infected with different proteins and different oncogenic controls. V-src-infected cells were utilized as a positive control. Cells were cultured for 2-3 weeks until foci were visible and subsequently fixed and stained with crystal violet. (0.5 % crystal violet, 20 % methanol).

11.4. Soft-agar colony-formation assay

To determine the capability of cells to grow without adhesion to a matrix soft-agar colony-formation assays were performed. 4% agarose was heated and dissolved with water to 1,4%. 1 ml medium and 1 ml agarose were mixed and plated in a 6-well. For the top-agar layer 4% agarose was heated and dissolved to a concentration of 0.4% with water. 0,75 ml of medium containing $0{,}8x10^5$ cells was mixed with the agarose (top agar) and the mixture was added to the bottom agar layer. After hardening of the agarose mixture the cells were put into the incubator and after 14 days stained with MTT solution. The analysis was done with the help of microscopic pictures.

11.5. Proliferation assay

In a 96-well flat bottom plate (Nunc, Naperville, Ill.) approximately 2,000 cells/100 µl of cell suspension were seeded. Upon serum-starvation for 24 h cells were incubated with inhibitors and growth factors for the indicated times. MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide; thiazolyl blue, Sigma, St. Louis, MO) was added to each well to a final concentration of 1 mg/ml MTT. Plates were incubated in the presence of

MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is solubilized (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on an micro-plate reader.

A non-toxic alternative to MTT is Alamar Blue™ (Biosource, Camarilla, CA, USA). According to the manufacturer's recommendations, 10 µl of Alamar Blue™ are added to each well and absorbance at 590 nm can be read out after different times, because the cells are not affected.

11.6. In vitro wound closure assay

The assay was performed as previously described (Fishman, 2001) with some modifications. Confluent monolayers of breast cancer cells were wounded with a uniform scratch, the medium was removed and cells were washed twice with PBS. Medium was added to the cells containing FCS. Cells were permitted to migrate into the area of clearing for 8-24 h. Wound closure was monitored by visual examination using a Zeiss microscope.

11.7. Migration of Cancer cells

Cell migration assays were performed using Transwells (Sieuwerts et al., 1997). Serum free medium containing interleukin or serum as a chemo attractant was added to the lower well of a chamber. $1x10^5$ cells in exponential growth were harvested and then preincubated with the respective inhibitor for 20 min and added to the upper well of the chamber in serum free medium. The chambers were incubated for 6-24 h in a humidified 7 % CO2, 37° C incubator. Finally, the cells that have migrated to the lower surface of the membrane were stained with crystal violet and counted under the microscope. Alternatively, cells migrated to the lower surface were fixed with methanol and stained with crystal violet. The stained cells were solubilized in 10 % acetic acid, and the absorbance at 570 nm was measured in a micro-plate reader.

IV. Results

For the understanding of the importance of genetic alterations in respect for the development of human diseases like cancer or cardiovascular diseases, it is necessary to elucidate the biological output of a genetic change in a protein, as well as to get a broad overview of frequent genetic changes in such disease cells. In this work the search for the abundance of genetic alterations, which were already described to be existent in colorectal cancer patients, in cancer cell lines is described in a high throughput manner. In a second part the elucidation of the biological and physiological consequences of representative, already published mutations is shown. In a further study a yet unknown mutation was investigated as a follow up study to the large scale sequencing.

1. High throughput sequencing of cancer cell lines

1.1. Screen for known mutations

In previous studies in our and other labs different genetic alterations, that may lead to an oncogenic transformation of proto-oncogenes or cancer related behaviour of the protein carrying this alteration were described. In 2003 Bardelli and colleagues published a group of mutations in different proteins which they detected in samples of patients suffering under colorectal cancer as well as in cell lines (Bardelli et al., 2003). To test whether these mutations may have an effect on oncogenicity we used high throughput sequencing technologies to screen in a setup of cell lines different that are available in our laboratories for these genetic alterations.

Gene	Group	Mutation (amino acid)
NTRK3	TK	G608S, I695V, R731Q, K732T, L760I
FES	TK	M704V, R706Q, V743M, S759F
KDR	TK	G800D, R819STP, A1073T
EPHA3	TK	S792P, D806N
NTRK2	TK	T695I, D751N
MLK4	TKL	H261Y, H261Q, G291E, A293E, W296STP, R470C, R553STP, N596I, K629E
GUCY2F	RGC	D225Y, A360T, Q361H, F390L, R492H, R545S, E624D, E778G, V1026M, exon 17 donor splice site

Figure 13: Mutational screen in colorectal cancer. Bardelli and colleagues investigated mutational changes in colorectal cancer samples in a large screen. Affected residues that are evolutionary conserved are highlighted in blue and affected residues that have been shown to be pathogenic in other proteins are highlighted in orange (Bardelli et al., 2003)

1.1.1. The FES tyrosine kinase

Bardelli and colleagues described the abundance of the mutations methionine to valine at position 704 and arginine to glutamine at position 706 which they classified as mutations where residues are affected, that are in other kinases known to be pathogenic (Bardelli et al., 2003). The amplification of a DNA Fragment of the FES tyrosine kinase using primers that are flanking the mutated region was used to gain enough material to perform a direct sequencing using a 48 capillary sequencing instrument. After optimising the conditions for the polymerase chain reaction, using cDNA as template material, we were able to sequence and analyse the electropherograms using DNASTAR computational analysis Software. In this screening we could demonstrate that both the mutations, we were looking for, are not present in our setup of cell lines.

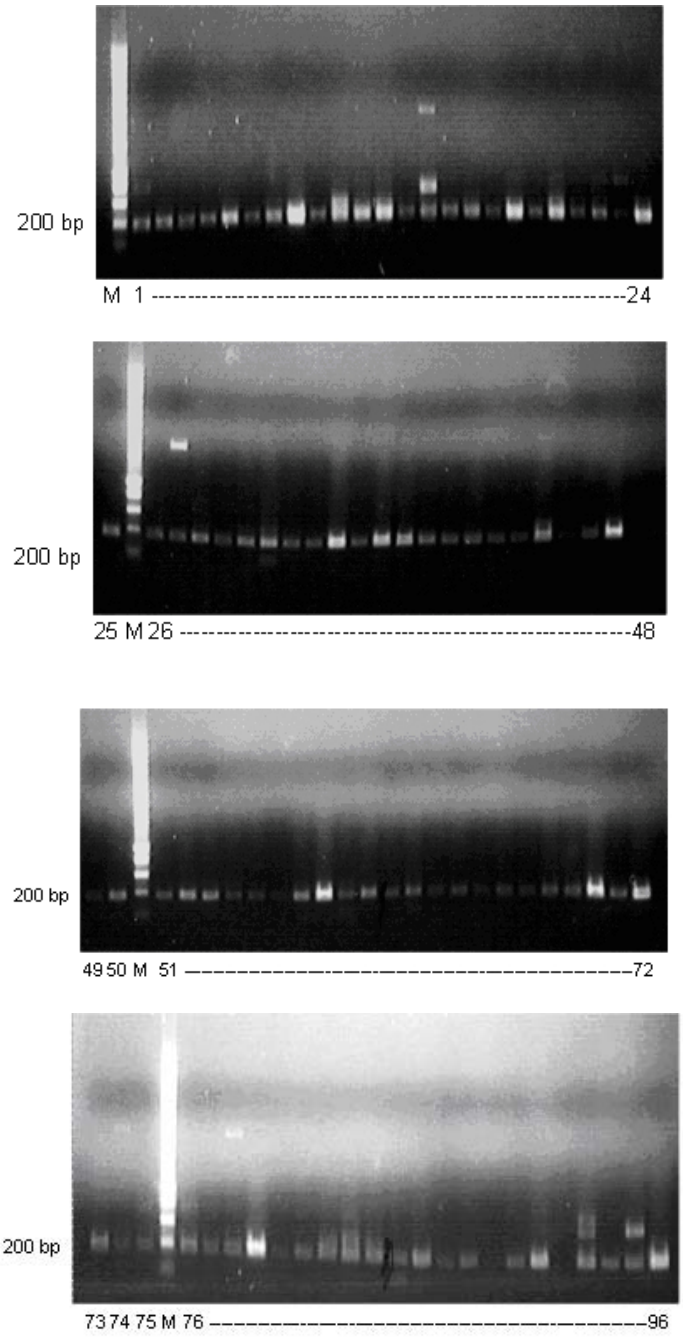


Figure 14: PCR based amplification of the regions of interest in the FES tyrosine kinase. The amplification of the desired 200 bp fragment for the subsequent sequencing is demonstrated in the agarose gel analysis. M – dna size standard; 1-96 – amplified DNA fragments out of cancer cell line cDNA

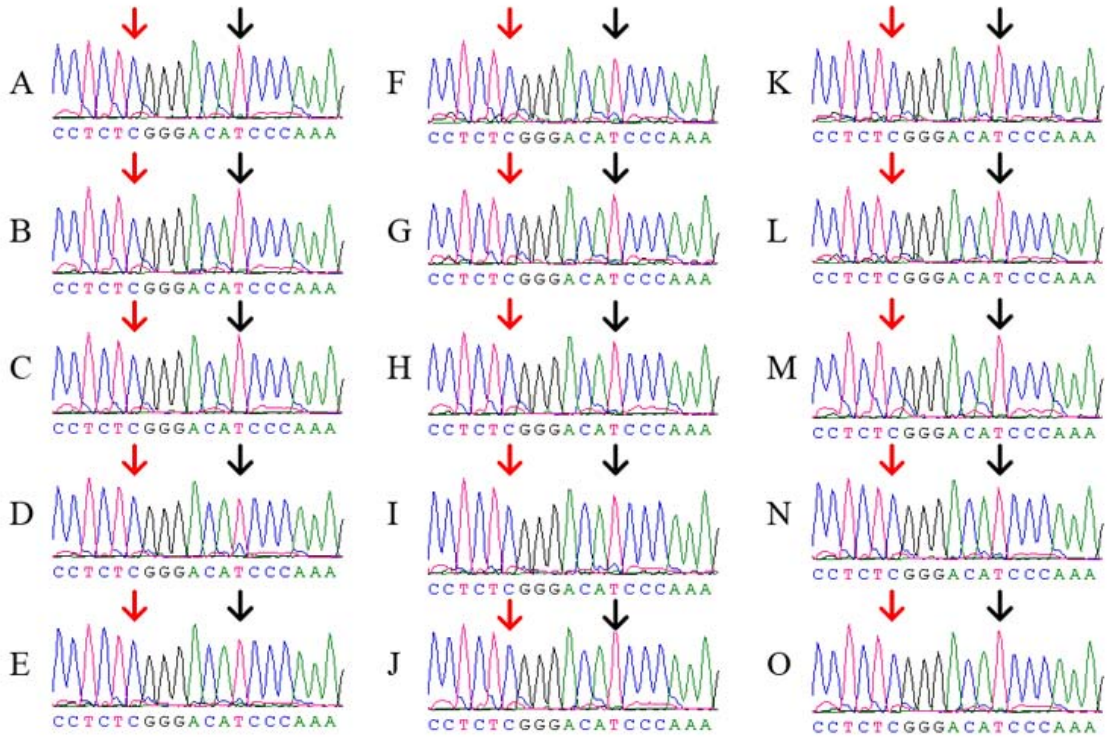


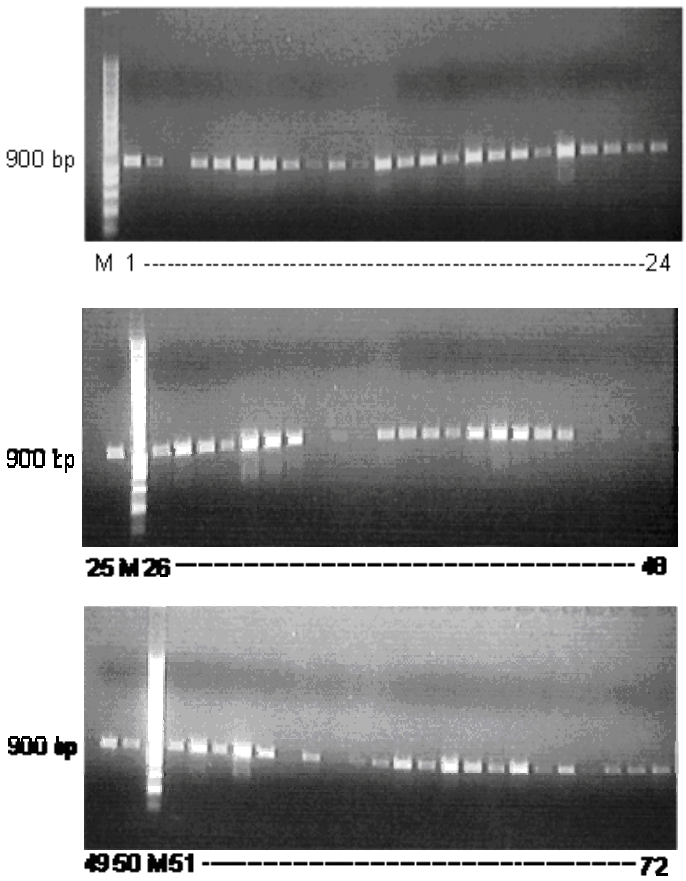
Figure 15: Example of the sequencing results (not all electropherograms are shown). In an example it is shown that the investigated mutations M704V and R706Q were not detectable. The black arrow indicates the position that should be changed if the M704V mutation occurs (T to C) and the red arrow indicates the position that should be changed if the R706Q mutation occurs (C to T). A: Hek293; B: MCF-10A; C:MDA-MB453; D: SKOV3; E: SKOV6; F: FaDu; G: UM-SCC-10A; H: UM-Scc10B; I: RT-4; J: SCaBER; K: 2774; L: 2780; M: HL-60; N: NB-4; O: MV4-11.

The whole entirety of the sequencing data was analysed individually and neither a complete exchange of the methionine at position 704 or the arginine at position 706 could be detected nor was an additional peak visible.

Additionally to the sequencing in cancer cell lines we performed a screen for the mutations in the FES tyrosine kinase in primary tumour samples, too. Therefore we took cDNA of 96 breast cancer samples, which were provided by Iacobelli and Höfler. In all the analysed samples the FES tyrosine kinase gene could be amplified and further analysed by direct sequencing. Unfortunately, also in these samples the observed mutations were not detected (data not shown).

1.1.2. The mixed-lineage kinase 4 gene

Another example of genetic changes that were described by Bardelli and their colleagues are alterations in the mixed lineage kinase 4 gene. The gene product belongs to the group of tyrosine kinase like proteins and is a serine/threonine kinase. In other studies the involvement of the MLK4 protein in neurodegenerative diseases could be demonstrated (Wang et al., 2004b). The mutational screen in colorectal cancers could show the existence of 2 mutations, changing residues which are again known to be pathogenic in other kinases. The mutations are localised in the kinase domain and were found in 10 out of 147 colorectal cancers. On the basis of this fact we decided to investigate the conversion resulting in the exchange of glycine to glutamic acid at position 291 and the substitution of alanine to glutamic acid at position 293. We designed primer that are flanking the mutated regions and performed polymerase chain reactions followed by subsequent sequencing of the purified samples.



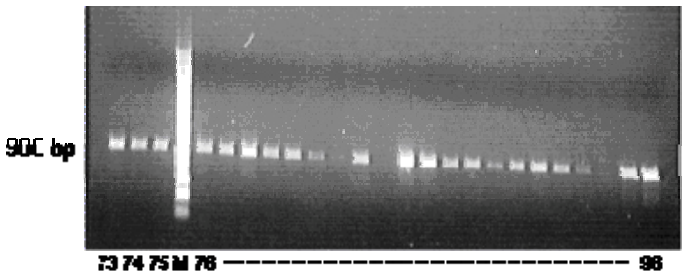


Figure 16: The sequencing results are shown exemplarily to demonstrate that in the used setup of cDNA of cancer cell lines the investigated mutations G291E and A203E are not detectable. The amplification of the desired 900 bp fragment for the subsequent sequencing is demonstrated in the agarose gel analysis. M – dna size standard; 1-96 – amplified DNA fragments out of cancer cell line cDNA

After optimisation of the PCR conditions the amplified DNA Fragment of 900 base pairs could be purified and used as template for the following sequencing reaction. The analysis of the electropherograms revealed that the two mutations we were looking for are absent in the cDNA of the cell lines we used in this study, and as we could show for the FES tyrosine kinase the mutations in the MLK4 kinase are not even present in a heterozygous manner.

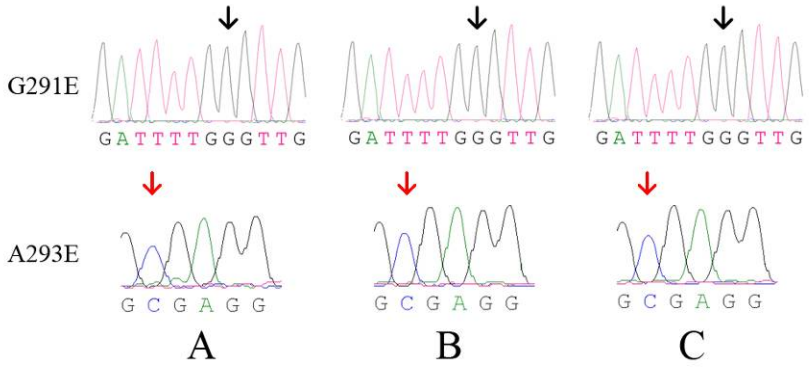
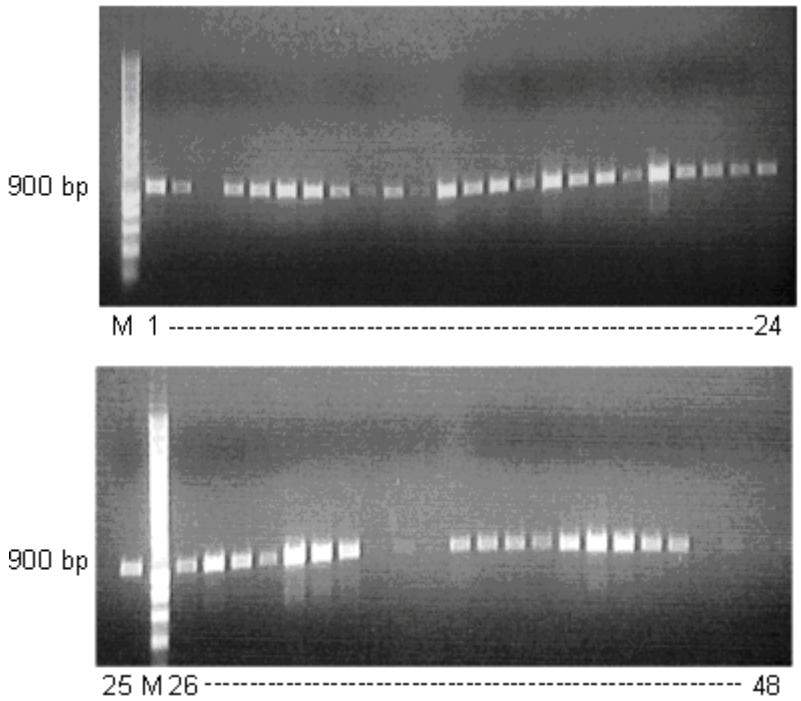


Figure 17: Example of the sequencing results (not all electropherograms are shown). In an example it is shown that the investigated mutations G291E and A293E were not detectable. The black arrow indicates the position that should be changed if the G291E mutation occurs (G to A) and the red arrow indicates the position that should be changed if the A293E mutation occurs (C to A). A: Hek293; B: SKOV3; C: SCaBER.

1.1.3. The kinase insert domain receptor (KDR)

The third mutated protein, which was described by Bardelli et. al, we were interested in further investigation is the KDR tyrosine kinase. Here our focus was lying in all the detected mutations, which they classified as mutations occurring at amino acid positions that are evolutionary conserved for this protein. They could see an exchange of glycine to aspartatic acid at position 800 and an exchange of alanine to threonine at position 1073. Additionally they found a mutation leading from an arginine at position 819 to an early stop codon. The KDR tyrosine kinase, also known as the vascular-endothelial growth factor receptor 2 (VEGFR2) plays a very important role in angiogenesis in humans and is closely connected to cancer as a direct transducer of pathological angiogenesis (Shibuya, 2006). This makes the Receptor a very potent target for cancer treatments. Again we were interested if these mutations are reproducible for our screening system and if we are able to see these mutations occurring in our cell lines. After designing the necessary primers, optimising and performing the polymerase chain reactions, the purified samples were used for the sequencing. Unfortunately again we were not able to detect any of these genetic alterations in our cell lines.



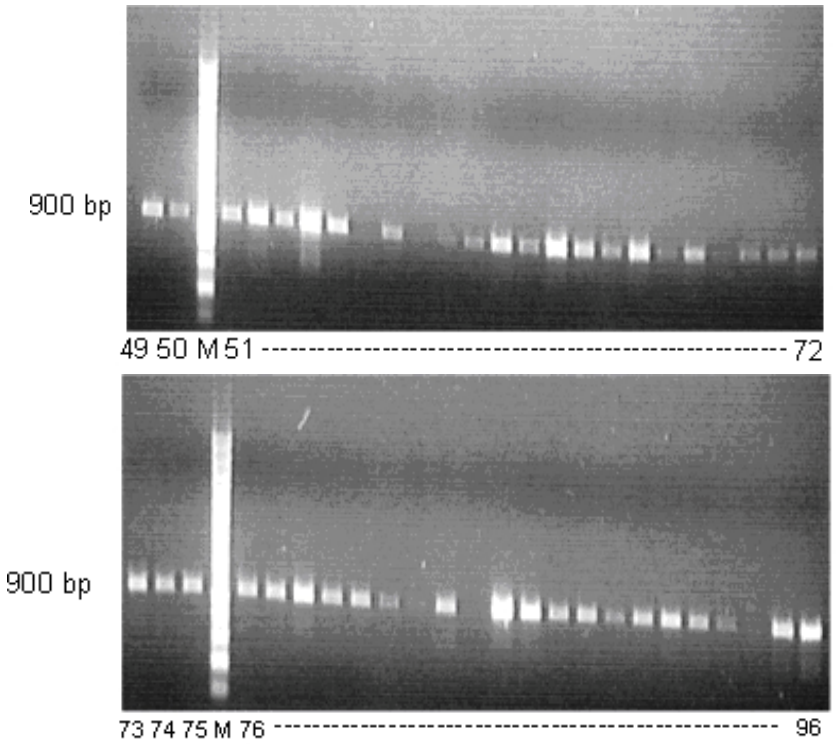


Figure 18: The sequencing results are shown exemplarily to demonstrate that in the used setup of cDNA of cancer cell lines the investigated mutations G291E and A203E are not detectable. The amplification of the desired 900 bp fragment for the subsequent sequencing is demonstrated in the agarose gel analysis. M – dna size standard; 1-96 – amplified DNA fragments out of cancer cell line cDNA

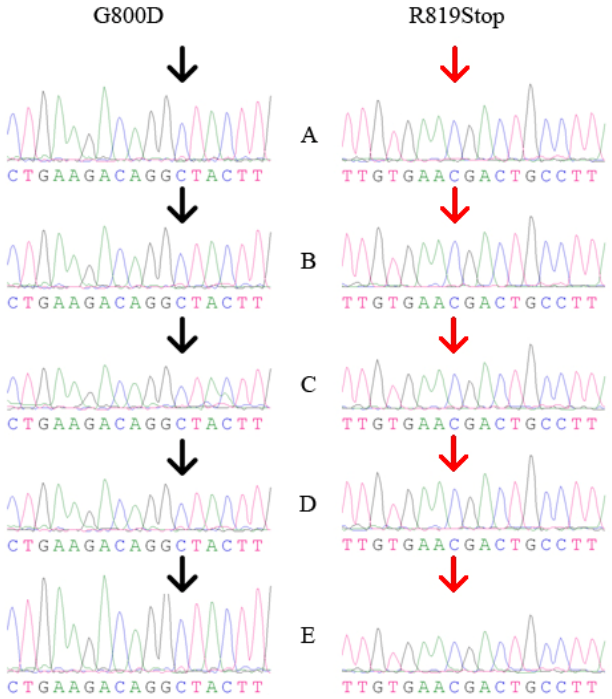


Figure 19: Example of the sequencing results (not all elektropherograms are shown). In an example it is shown that the investigated mutations G800D and R819Stop were not detectable. The black arrow indicates the position that should be changed if the G800D mutation occurs (G to A) and the red arrow indicates the position that should be changed if the R819Stop mutation occurs (C to T). A: Hek293; B: SKOV6; C: FaDu; D: UM-SCC-10A; E: SCaBER.

1.2. Tyrosine kinome wide screen for unknown genetic alterations

Cell lines in today's laboratory daily work are a very strong and potent tool which is now established since decades. With the help of tissue culture experiments a lot of progress in the field of cancer biology has been made. Many efforts have been put into genomic analysis of primary tumour material but less and insufficient work has been made to analyse cell lines for their genomic background (Dixon and Kopras, 2004; Garnis et al., 2004). Due to the promising data and the potent candidates for cancer research arising in such a screen the Singapore Oncogenome Project (SOG) has been set up. In collaboration with the Max-Planck-Institute of Biochemistry (Department Molecular Biology) a large scale screen for genetic alterations in the tyrosine kinase transcriptome was performed, were 254 established tumour cell lines were investigated in respect to their genetic fundamentals. The data of this screen was made available in a database called "Tykiva" (tyrosine kinome variant). The number of 155 polymorphisms and 234 somatic mutations could be identified in this screen (Ruhe et al., 2007).

Table 1: listed are cell lines that harbour more than 20 genetic alterations of somatic origin. The high throughput sequencing was performed in Singapore (Singapore Oncogenome Project) in collaboration with the Max-Planck-Institute of Biochemistry (Dept. Molecular Biology); the indicated alterations are either heterozygous or homozygous, when marked with # (Ruhe et al., 2007).

cell line	properties		genetic alterations - somatic origin			
DLD1	ATCC® Number:	CCL-221™	CSK Q26* #	FER I240T #	FES L690M #	FYN E521K #
	Organ:	colon	SYK A353T #	LMTK2 L879M #	HER3 N126K #	HER3 R667H #
	Disease:	colorectal adenocarcinoma	EPHB3 A517V #	FGFR1 A268S	ROR1 S870I #	VEGFR1 G203W
	Tumor Stage:	Duke's type C	LCK F151S #	HER3 P1142H #		
MKN1	HSRRB Number:	JCRB0252	FAK Q440R #	SYK A353T #	HER3 N126K #	HER3 R667H #
	Organ:	stomach	EPHA2 G391R #	EPHA4 S803A #	EPHA6 N291H #	EPHB2 A83V #
	Disease:	adenosquamous carcinoma,	FGFR1 A268S	IGF1R N209S #	ROR1 S870I #	HER3 R1089W #
			EPHB2 V136M #			
HCT15	ATCC® Number:	CCL-225™	ARG Q994H #	CSK Q26* #	FER I240T #	JAK2 L383P #
	Organ:	colon	SYK A353T #	TYRO3 P822L #	HER3 N126K #	HER3 R667H #
	Disease:	colorectal adenocarcinoma	FGFR1 A268S	ROR1 S870I #	FYN E521K #	HER3 P1142H #
	Tumor Stage:	Duke's type C				
LNCAP	ATCC® Number:	CRL-1740™	ABL1 N789S #	ACK1 R748W #	FER Q599R #	JAK3 DEL288-328 #
	Organ:	prostate	BMX A150D	LMTK2 G518V #	LMTK2 D523Y #	HER2 E930D #
	Disease:	carcinoma	FGFR1 R78H #	FLT3 D358V #	FYN D506E #	EPHB4 D576G #
	derived from:	left supraclavicular lymph node				
Jurkat	DSMZ Number:	ACC-282	ACK1 S699N #	FES V724M #	SYK INSnt242 G->GG (GGM..VVN 32-635 GGHE)	ZAP70 T155M #
	obtainded from	Ambion	TEC W531R	EPHA5 N81T #	EPHB6 A369T	CCK4 Q913H #
	Organ:	blood	VEGFR1 A673V #	VEGFR1 M938V #	ITK R448H #	RYK N96S
	Disease:	acute T cell leukemia				
IGROV1	obtainded from	Inst. G. Roussy, France Dr. J. Benard	JAK1 DELnt2614-2614 (KKN..LLK 847-1142 KKT..SAS)	TYK2 R901Q #	EPHB4 A955V #	EPHB6 DEL353-471 #
	Organ:	ovary	ROR1 R429Q #	TIE M871T #	RET A750T #	
	Disease:	adenocarcinoma				
LS180	ATCC® Number:	CL-187™	ABL1 DELnt3083-3083 (GGT..VQR 882-1149 GAP..SPE) #	LYN F130V #	SYK INSnt242 G->GG (GGM..VVN 32-635 GGHE) #	CARK INSnt2401 A->AGGA..CTAT (SAG..DSS 784-835 RMVH)
	Organ:	colon	EPHB3 DEL6-6	ROR1 R185H #	TEK A615T #	CARK N590S
	Disease:	colorectal adenocarcinoma				
	Tumor Stage:	Dukes' type B				
A498	ATCC® Number:	HTB-44™	ACK1 S985N	TYK2 P1104A #	SYK R520S #	ZAP70 DEL296-301 #
	obtained from:	Sugen	NTRK2 DEL387-398 #	FGFR2 I526T #		
	Organ:	kidney				
	Disease:	carcinoma				
NCI-H661	ATCC® Number:	HTB-183™	JAK3 E698K	TEC P587L	EPHA10 L629P #	ROR2 DELnt1369-1402 (DVP..LEA 390-943 EWG..GDS) #
	Organ:	lung	ROS A1443S			
	Disease:	large cell lung cancer				
	derived from:	lymph node				
MonoMac6	DSMZ Number:	ACC 124	TYK2 P1104A #	FLT3 V592A		
	Organ:	blood				
	Disease:	human acute monocytic leukemia				
SW1088	ATCC® Number:	HTB-12™	EPHB6 DEL353-471 #	ROS I537M #		
	obtained from:	Roche Bioscience				
	Organ:	brain				
	Disease:	astrocytoma				
U1242	obtained from:	SUGEN	/			
	Organ:	brain				
	Disease:	glioblastoma cells				
A549	ATCC® Number:	CCL-185™	/			
	obtained from:	DKFZ Heidelberg				
	Organ:	lung				
	Disease:	carcinoma				

Table 2: listed are cell lines that harbour more than 20 genetic alterations of germline origin. The high throughput sequencing was performed in Singapore (Singapore Oncogenome Project) in collaboration with the Max-Planck-Institute of Biochemistry (Dept. Molecular Biology); the indicated alterations are either heterozygous or homozygous, when marked with # (Ruhe et al., 2007).

cell line	properties		genetic alterations - somatic origin			
DLD1	ATCC® Number:	CCL-221™	FRK G122R	AATK G600C	LMTK2 L780M	ALK K1491R
	Organ:	colon	EGFR R521K #	HER2 P1170A #	HER3 S1119C #	EPHA10 G749E #
	Disease:	colorectal adenocarcinoma	FGFR1 E633K #	RON R1335G #	ROR2 V819I #	NTRK3 SUB402-410V
	Tumor Stage:	Duke's type C	ALK D1529E	EPHA2 R876H #		
MKN1	HSRRB Number:	JCRB0252	FRK G122R	LMTK2 L780M	ALK K1491R #	EGFR R521K #
	Organ:	stomach	EPHA2 R876H #	RON INSnt2465 A->ACCA (R 813 TR)	RON R1335G #	STYK G204S #
	Disease:	adenosquamous carcinoma,	HER2 P1170A #			
HCT15	ATCC® Number:	CCL-225™	FRK G122R	AATK G600C	LMTK2 L780M	ALK K1491R
	Organ:	colon	EGFR R521K #	HER2 P1170A #	EPHA2 R876H #	RON INSnt2465 A->ACCA (R 813 TR)
	Disease:	colorectal adenocarcinoma	ROR2 V819I	STYK G204S #	ALK D1529E	RON R1335G #
	Tumor Stage:	Duke's type C				
LNCAP	ATCC® Number:	CRL-1740™	ABL1 S991L #	HER2 P1170A	HER3 S1119C #	EPHA1 V900M #
	Organ:	prostate	EPHA7 I138V	FLT3 M227T #	NTRK1 H604Y	NTRK1 G613V #
	Disease:	carcinoma	EPHA10 G749E #			
	derived from:	left supraclavicular lymph node				
Jurkat	DSMZ Number:	ACC-282	ACK1 P725L #	PYK2 DEL739-780 #	PYK2 K838T #	TXK DELnt1326-1444 (YVL..ETW 414-527 SFN..NAF) #
	obtainded from	Ambion	ALK K1491R	ALK D1529E	MER V870I #	HER2 P1170A
	Organ:	blood	NTRK3 SUB402-410V #	^NTRK3 INSnt2288 G >GCTC..TGAG (R 711 RLF..WCE) #	LMTK2 L780M #	ROR2 V819I
	Disease:	acute T cell leukemia				
IGROV1	obtainded from	Inst. G. Roussy, France Dr. J. Benard	ABL1 S991L #	ACK1 P725L #	PYK2 K838T	TXK R336Q #
	Organ:	ovary	ALK D1529E #	TYRO3 I346N #	HER2 P1170A	HER3 S1119C #
	Disease:	adenocarcinoma	FGFR4 G388R #	RON INSnt2465 A->ACCA (R 813 TR)	RON R523Q #	RON R1335G #
			LMTK2 L780M	EPHA10 G749E	ROR1 M518T	
LS180	ATCC® Number:	CL-187™	PYK2 K838T	TYK2 V362F #	AATK G600C #	AATK F1163S #
	Organ:	colon	EGFR R521K #	HER2 P1170A	EPHB6 S309A #	FGFR4 V10I #
	Disease:	colorectal adenocarcinoma	ROR1 M518T	ROR2 V819I #	TEK V600L	LMTK2 L780M #
	Tumor Stage:	Dukes' type B	RON R523Q			
A498	ATCC® Number:	HTB-44™	TNK1 DEL472-473 #	FAK INSnt2929 C->CCATGGAGGC (L 926 PWRL) #	FES INSnt1311 G->GCCT..TCAG (SEQ..RHR 412-822 SPG..TTY)	FRK G122R
	obtained from:	Sugen	AATK G600C	LMTK2 L780M #	ALK K1491R #	EGFR R521K #
	Organ:	kidney	EPHA3 W924R	RON R523Q	ROR1 M518T	ROR2 V819I
	Disease:	carcinoma	NTRK3 INSnt2288 G->GCTC..TGAG (R 711 RLF..WCE) #	TYK2 V362F #	HER2 P1170A	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347)
NCI-H661	ATCC® Number:	HTB-183™	PYK2 K838T	TYK2 V362F	AATK G600C	LMTK2 L780M
	Organ:	lung	HER2 P1170A #	FGFR1 E633K #	FGFR4 L136P	RON R1335G
	Disease:	large cell lung cancer	ROR1 M518T #	ROR2 T245A	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347)	ROS S1109L
	derived from:	lymph node	ALK K1491R #	RET G691S #	NTRK3 SUB402-410V	
MonoMac6	DSMZ Number:	ACC 124	ACK1 P725L #	PYK2 DEL739-780 #	PYK2 K838T #	TYK2 V362F #
	Organ:	blood	ZAP70 DEL186-619 #	AATK G600C #	LMTK2 L780M	HER2 P1170A
	Disease:	human acute monocytic leukemia	EPHA3 W924R	FGFR4 G388R #	RON INSnt2465 A->ACCA (R 813 TR)	CSF1R H362R #
			ROR2 T245A	ROR2 V819I	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347) #	TEK V600L #
			TYK2 G363S #	HER3 S1119C #	FLT3 M227T	
SW1088	ATCC® Number:	HTB-12™	ACK1 P725L #	TNK1 INSnt1906 A->AGGT..CCCA (EME..ARP 597-666 EVRSH) #	PYK2 K838T #	TYK2 V362F #
	obtained from:	Roche Bioscience	LMTK2 L780M #	ALK D1529E #	HER2 P1170A #	FGFR1 E633K #
	Organ:	brain	ROR2 T245A	ROR2 V819I	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347) #	ROS T145P #
	Disease:	astrocytoma	ROS K2228Q #	ROS S2229C #	NTRK3 SUB402-410V #	NTRK3 INSnt2288 G->GCTC..TGAG (R 711 RLF..WCE) #
			TYK2 I684S #	FGFR4 G388R #	ROS D2213N #	
U1242	obtained from:	SUGEN	ACK1 P725L #	TNK1 INSnt1906 A->AGGT..CCCA (EME..ARP 597-666 EVRSH)	PYK2 K838T	LMTK2 L780M #
	Organ:	brain	TYRO3 I346N #	HER2 I655V	EPHA3 R914H #	EPHA3 W924R
	Disease:	glioblastoma cells	RON R1335G	ROR2 V819I #	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347) #	ROS T145P #
			RYK N96S	NTRK3 SUB402-410V	NTRK3 INSnt2288 G->GCTC..TGAG (R 711 RLF..WCE) #	VEGFR2 Q472H #
			ALK K1491R #	FGFR4 G388R	ROS S1109L #	
A549	ATCC® Number:	CCL-185™	FAK INSnt2929 C->CCATGGAGGC (L 926 PWRL) #	PYK2 K838T #	FRK G122R #	AATK G600C
	obtained from:	DKFZ Heidelberg	EGFR R521K #	HER2 P1170A #	EPHA10 G749E	FGFR4 G388R #
	Organ:	lung	RON R1335G	RET G691S #	ROR2 V819I	ROS INSnt426 G->GAAT..TGAG (CRE..DGS 76-2347) #
	Disease:	carcinoma	ROS K2228Q #	ROS S2229C #	STYK G204S	NTRK3 SUB402-410V #
			VEGFR3 Q890H #	AATK F1163S	RON INSnt2465 A->ACCA (R 813 TR)	ROS D2213N #
			NTRK3 INSnt2288 G->GCTC..TGAG (R 711 RLF..WCE) #			

2. The biological significance of “known” genetic variations in tyrosine kinases

2.1. The biological significance of genetic alterations in the FES tyrosine kinase

The importance for the goal of finding new possible targets for cancer treatment is not only to know about genetic variations in tyrosine kinase genes, but to understand the biological output, resulting of this alteration in the gene. In this study we tried to address this question by using cellular and molecular assays. Therefore the comparison between wildtype and mutated variants of the FES tyrosine kinase in several experiments was performed.

2.1.1. Generation and expression of mutant variants of the FES tyrosine kinase

To elucidate the biological and physiological consequences of the amino acid exchanges at position 704 and 706 in the FES gene, we produced FES carrying constructs (pCDNA3) which are fused to an HA-tag in frame. Afterwards the respective site was mutated using site directed mutagenesis. Primer pairs were designed which were carrying the triplet for valine (M704V) and another pair was used to mutate arginine into glutamine (R706Q). The sequences were all checked using direct sequencing in a capillary sequencing machine.

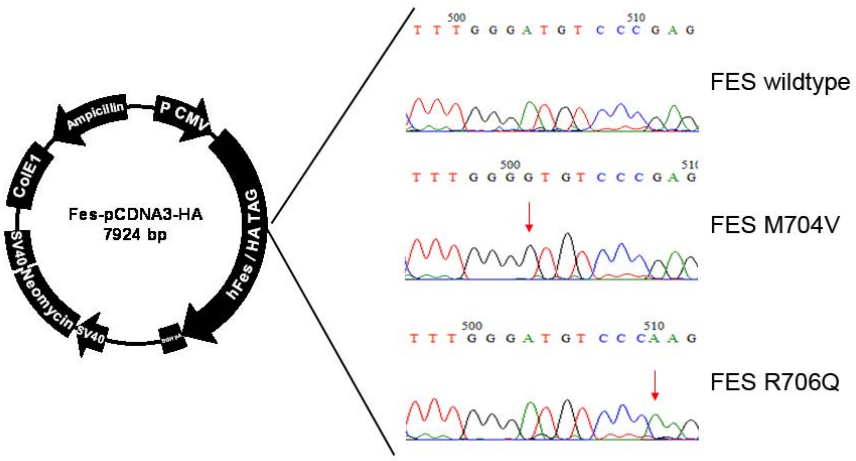


Figure 20: Generation of the different FES constructs. Using site directed mutagenesis the pCDNA3 vector, carrying the FES gene, was mutated to the genetic variants M704V and R706Q, the success was checked by direct sequencing of the vector.

We controlled the expression of the generated constructs via transient transfection into HEK293 cells. These cells are able to be transfected with calcium chloride. As expected we could see an expression of the FES tyrosine kinase and its genetically modified variants, indicated by the bands at the size of 93 kDa. For visualising the proteins we had the choice of taking either the antibody recognising the HA-chain or taking an antibody which is directly binding to the FES tyrosine kinase.

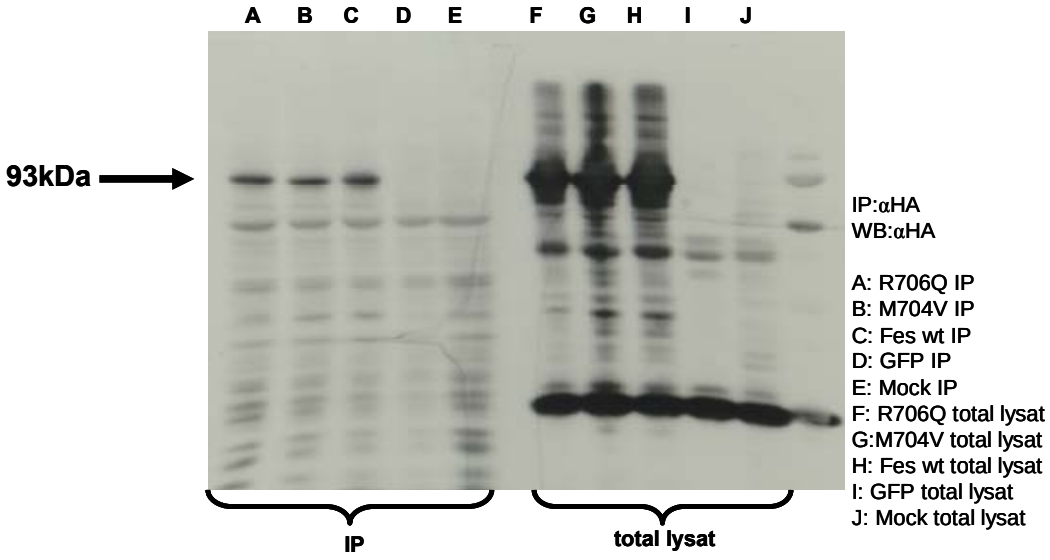


Figure 21: Expression check for the generated FES carrying pCDNA3 constructs. The FES protein was detected using immunoprecipitation with HA antibody and the membrane was incubated with an HA antibody, too. The protein could be visualised in the pull-down as well as in the whole cell lysate.

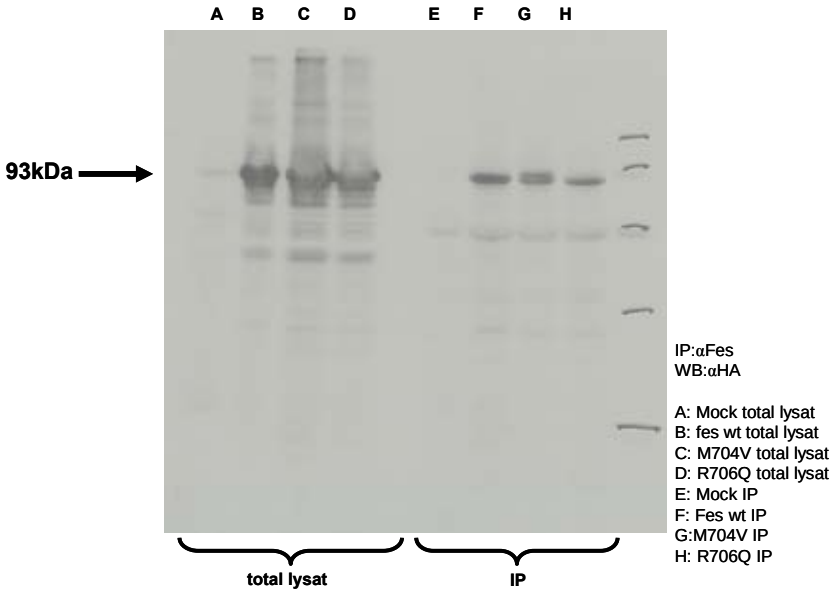


Figure 22: expression check for the generated FES carrying constructs. The FES protein pulled down using immunoprecipitation with the FES antibody and the membrane was incubated with an HA antibody. The protein could be visualised in the pull-down as well as in the whole cell lysate

2.1.2. Stable cell lines expressing the FES tyrosine kinase

To perform follow up experiments with the generated FES constructs we subcloned the fragments into the retroviral pLXSN expression vector. Several reasons made this step necessary. The usage of stable FES expressing clones is of advantage because the transient transfection methodology allows the exogenous insertion of a gene for a limited time period only (in our case the expression lasted 4 to 5 days). For experiments lasting longer than this period this methodology is insufficient. On the other hand, physiological analyses with stable infected NIH3T3 fibroblasts are a well established methodology (Zhang et al., 1999).

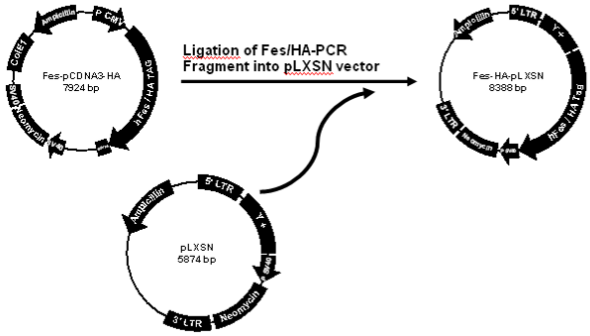
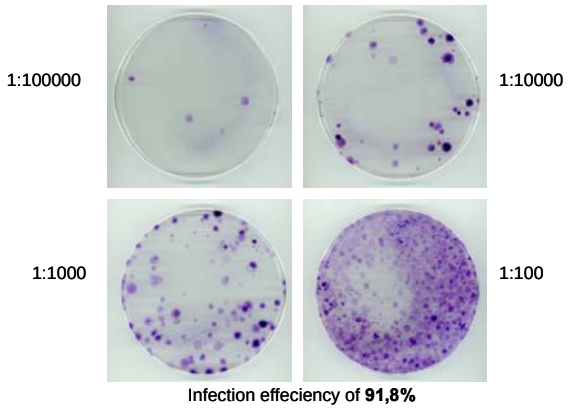
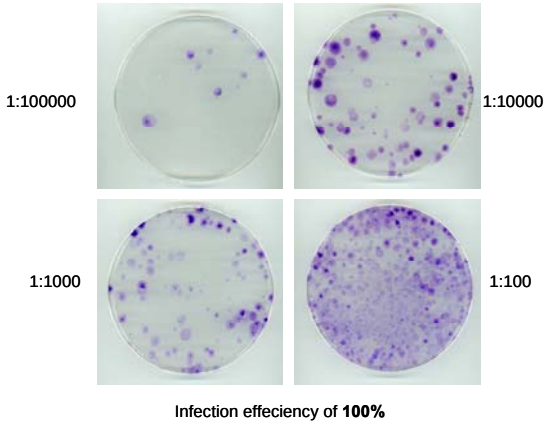


Figure 23: subcloning of the FES gene and ist variants into the pLXSN vector

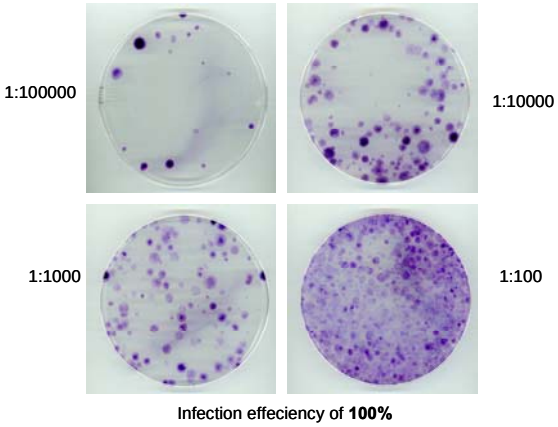
In the search for an applicable workflow we used the retroviral infection with the Phoenix E producer cell line. The individual FES expression level of different NIH3T3 clones can be justified using western blots, but moreover the general efficiency of the infection procedure can be estimated. Therefore we used a dilution series of cells that were seeded on tissue culture plates. They were grown for a specific time period in cell culture medium containing a selection pressure. In our case the lowest infection efficiency was 91,8% for the introduction of the FES wildtype gene and for both the mutated variants the efficiency was about 100%.



Infection of NIH3T3 with FES wildtype



Infection of NIH3T3 with FES-M704V



Infection of NIH3T3 with FES-R706Q

Figure 24: Determination of the infection rate after selection with G418 The infection efficiency was used as a control to determine the applicability of the infection procedure in the functional analysis process. The achieved infection efficiency is indicated under the stainings.

The polyclonal pool of cells was exposed to the selection pressure (in the case of pLXSN the selection is done with G418) for a minimum time period of 14 days and afterwards the expression of the FES protein was checked in western blot analyses. In all the investigated clones we could see a good expression of the FES tyrosine kinase and its modified variants.

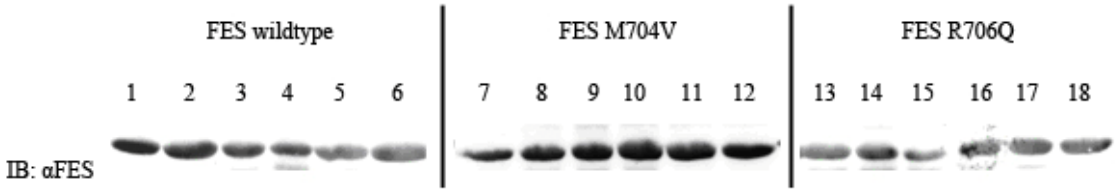


Figure 25: FES expression level in stable NIH3T3 clones After subcloning of the FES constructs into the retroviral pLXSN vector, the generated stable cell lines were analysed for their FES expression on the protein level. The nitrocellulose membrane was incubated with an anti-FES antibody, detecting the FES protein.

2.1.3. Physiological consequences of the mutations

The production and generation of constructs and cell lines that are "ready to use" in appropriate cellular assays is a limiting step in the application of a workflow in high throughput systems. Nevertheless our goal was, to search for candidate alterations that may serve as novel targets in the development of new cancer treatments. In this study we used several cell based experiments that are used to give hints for a transforming potential of a gene-product of interest. A transforming potential e.g. the independence of growth factors, leads to oncogenic properties in a cell. At this point the decision can be made if the investigated target is a promising one, and if it is worth to do the further analyses in respect to the development of disease intervention strategies.

2.1.4. Contact inhibited growth

By means of the focus-formation assay the transforming potential of the gene of interest can be determined. Out of this we could draw conclusions about the oncogenic potential of that gene-product. The normal proliferation of cells stops if they come into contact with other cells and if they are surrounded by cells. The loss of this contact inhibition is one of the characteristics of a transformed cell. The NIH3T3 fibroblasts lose their contact inhibition when they are infected with oncogenes. The result is a multilayer growth of cells at some points which can be visualised with crystal violet (Aaronson, 1991). We used this model system to determine the transforming potential of the FES tyrosine kinase and of the mutated FES kinases. For this purpose we used the stable infected NIH3T3 fibroblasts, expressing the

different FES proteins. As positive controls we used NIH3T3 fibroblasts, expressing transforming HER2 and v-src.

The fibroblasts which are infected with the transforming HER2 and v-src are showing an elevated loss of the contact inhibition and enhanced foci formation, what can be seen as violet dots on the surface of the tissue culture plate. In the case of the fibroblasts which were infected with the empty pLXSN-vector (mock-control) no foci formation was visible which is also true for the fibroblasts expressing the FES wildtype protein. Interestingly the expression of the FES M704V and the R706Q mutants resulted in elevated foci formation compared to the fibroblasts expressing the FES wildtype protein. We could also see a physiological difference of the foci itself. The foci which were visible after FES M704V expression are bigger and the expression of FES R706Q resulted in smaller foci. This is comparable to the differences of the foci after expression of transforming HER2 and v-src.

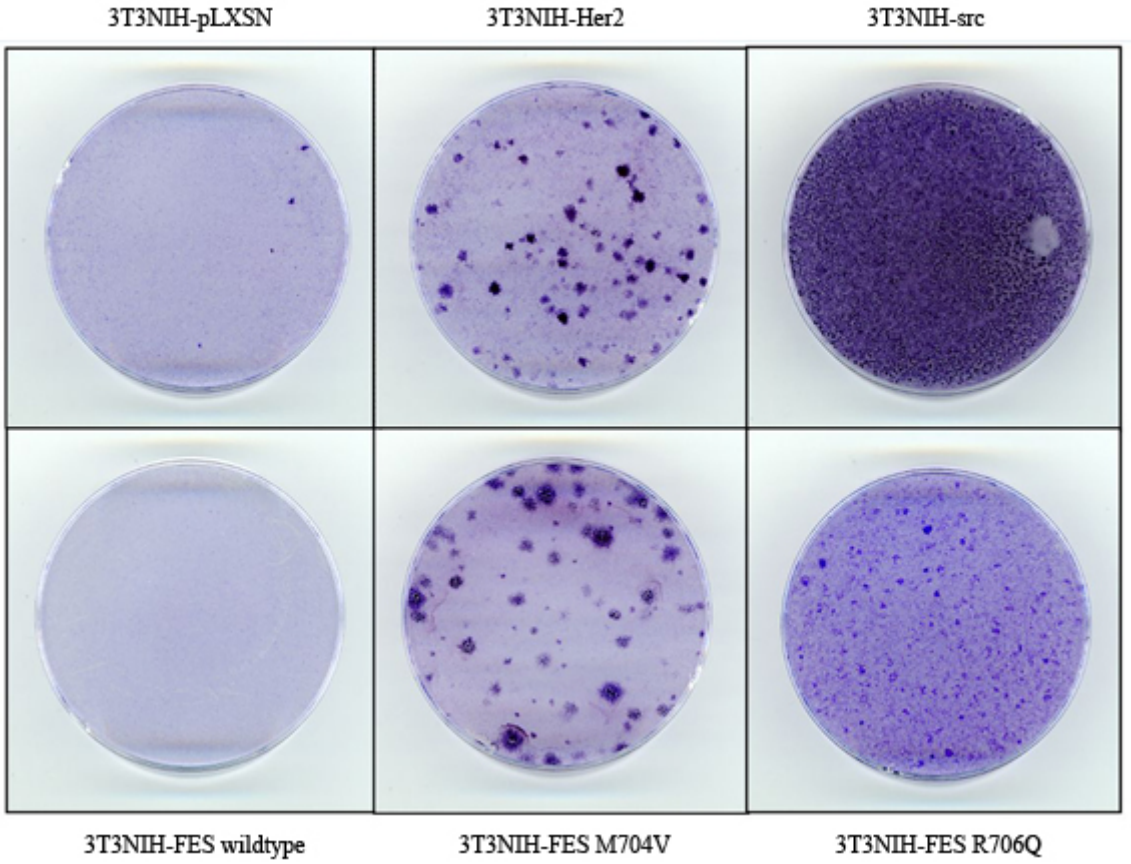


Figure 26: Focus-formation assay of the generated FES expressing NIH3T3 fibroblasts. In this assay the oncogenes HER2 and v-src were used as positive controls. The colonies were stained using crystal violet after a growth period of 21 days.

2.1.5. Adhesion independent growth

Not only the focus formation assay is a tool to estimate oncogenic potential, also the soft-agar colony-formation assay serves as a tool to determine transforming and oncogenic properties of a protein. This assay uses the property of cells to grow on a surface like it is the case with tissue culture plates. Here the cells are plated on an agarose layer supplemented with cell culture medium. The ability of the cells to grow under these conditions and to form colonies within the agarlayer is given, if they are transformed (Pei and Melmed, 1997). After a period of about 21 days the cells are stained with MTT. Again we can conclude transforming capabilities of proteins by the means of this experiment. We used the generated fibroblasts that express the FES tyrosine kinase, their modified variants and several positive controls that are known to induce more or less a cell surface independent growth. A very potent inducer of surface independent growth is the v-src protein. The expression of other control proteins, like colony-stimulating-factor 1 receptor, HER2 and EGFR, resulted in a very slight induction of growth within the agar. For the FES constructs the statement, that can be concluded, is not that clear as it was the case in the focus-formation assay. As the FES wildtype and the FES R706Q expressing fibroblasts formed small and less colonies, comparable to the colonies which are visible for the fms expressing fibroblasts, the FES M704V expressing cells have almost no capability of growing in the agar layer. This is true for the mock-transfected cells, too. These results were reproducible.

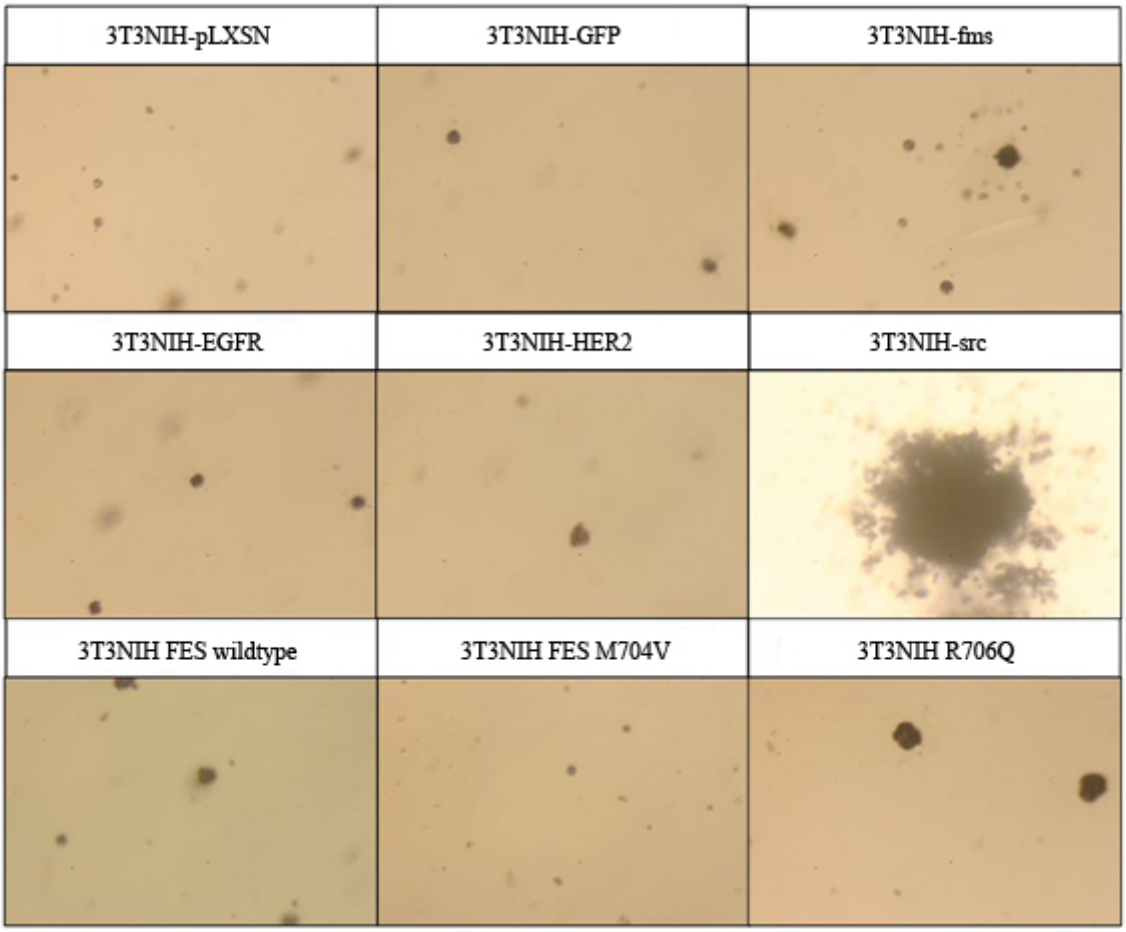


Figure 27: Soft-agar-colony-formation assay of stable NIH3T3 cells. In this assay CSF1R, Her2, EGFR and v-src were used as transforming positive controls. The cells were stained using MTT solution after 21 days and analysed with the microscope. A representative picture of the colony formation was taken.

2.1.6. Migratory effects

Migration of cancer cells is a frequent event in tumour related diseases. To investigate this event under in vitro conditions we used the migration and invasion assay. This assay is performed in Boyden chambers which have a permeable membrane at the bottom, through which cells are able to move. This movement is dependent on transforming properties of cells and can be used to estimate a transforming potential of a cell line. In our case the aim was to get to know, if the mutations in the FES tyrosine kinase gene make the protein a potential oncogene, and if the mutated FES variants transform the cells. Cells in the exponential phase of growth were harvested, washed and seeded in the Boyden chamber under withdrawal of fetal calf serum. These chambers were put into Serum containing medium and after 18 hours the amount of cells that have moved to the outer side of the membrane was quantified. Again several positive controls were used. The following figure demonstrates the results of this

assay, where the migration rate of the mock-infected NIH3T3 cells was set to 100%. As expected the expression of the colony-stimulating-factor 1 receptor results the highest capability of migration, where as the expression of different other controls resulted in 10% increase in cell migration. The same is true for the FES wildtype expressing fibroblasts. By expressing the FES M704V mutant the amount of migrating cells is slightly reduced compared to the NIH3T3-pLXSN cells, which is not significant. Interestingly the cells expressing the FES R706Q mutant showed an equal and even slight higher amount of migrating cells.

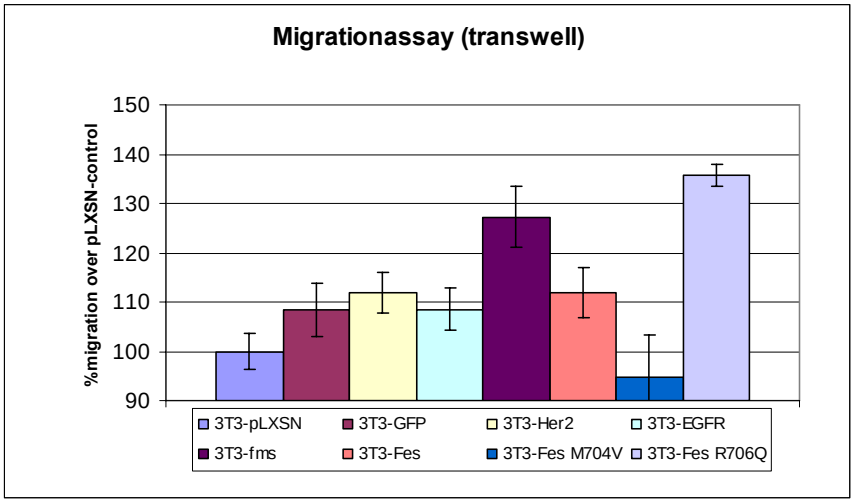


Figure 28: Migratory effects were analysed using a transwell boyden chamber system. In this experiment Her2, CSF1R and EFGR were used as positive controls. The migration of the mock infected cells was set to 100% and the data shown for the other cells is calculated as increase or decrease over the pLXSN control.

2.1.7. Proliferative effects

Uncontrolled cell growth is one criteria of a cancer cell, which is often connected to an enhancement in the rate of proliferation. Enhanced proliferative effects can not always be seen as a consequence of transformation, but together with other oncogenic properties like evasion from the immune system, loss of contact-inhibition and adhesion independent growth, an elevated proliferation leads to an aggressive progression of cancer. We were interested, if the mutations in the FES tyrosine kinase gene are enabling cells, expressing the protein, to proliferate faster. This is another step towards the estimation of te oncogenic relevance of the mutations in the FES tyrosine kinase. Therefore we applied a proliferation assay, where we compared the proliferation rate of cells, expressing the Fes wildtype, FES M704V and FES R706Q proteins.

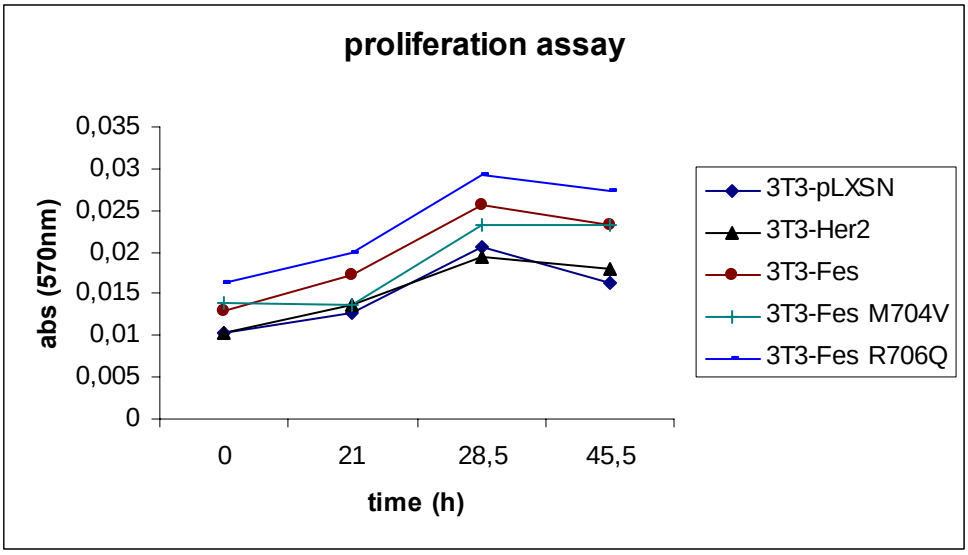


Figure 29: Proliferative effects were analysed using a MTT-proliferation assay. In this experiment Her2, was used as control. The absorbance at 570nm was measured using a micro-plate reader. The incorporation of MTT in viable cells results in a absorbance at 570nm, which is proportional to the amount of cells.

In this assay we could see, that the cells, expressing the wildtype and the M704V variant of the FES protein, are proliferating at comparable rates, which is a bit higher compared to the mock infected cells. In this assay the cells, expressing the R706Q mutant of the FES tyrosine kinase, showed the highest proliferation rate. This fits to the results, that were demonstrated in the soft-agar colony formation and the focus-formation assay.

2.1.8. Modified signal transduction

The analysis of the cellular behaviour that is altered through the introduction of the FES tyrosine kinase and the mutated variants of the FES tyrosine kinase is a critical step in the decision-making process if a gene-product could be of interest for the development of strategies for cancer treatment. In the next part we were interested if the normal signal transduction which is mediated by the FES tyrosine kinase is changed by mutation of FES. Therefore we performed western blot analyses. To determine the autophosphorylation ability we harvested the cells and lysed them. Subsequent, we pulled down the FES protein using the anti-HA antibody and analysed the amount of FES protein that is phosphorylated. In this experiment we used the phosphotyrosine specific antibody 4G10. To distinguish between the basal phosphorylation level and the level of phosphorylated protein after stimulation we

starved the cells for 48 hours and added interleukin 6 as natural ligand for the FES tyrosine kinase. In this experiment we could demonstrate that under starved conditions FES is already phosphorylated, which can be increased by stimulating the cells with interleukin 6. Surprisingly both the mutated variants of the FES tyrosine kinase are not phosphorylated at all. This is true under starved conditions and this does not change after stimulating the cells with interleukin 6. The amount of FES in the whole cell lysate is very high. The mutated variants are also expressed at high levels.

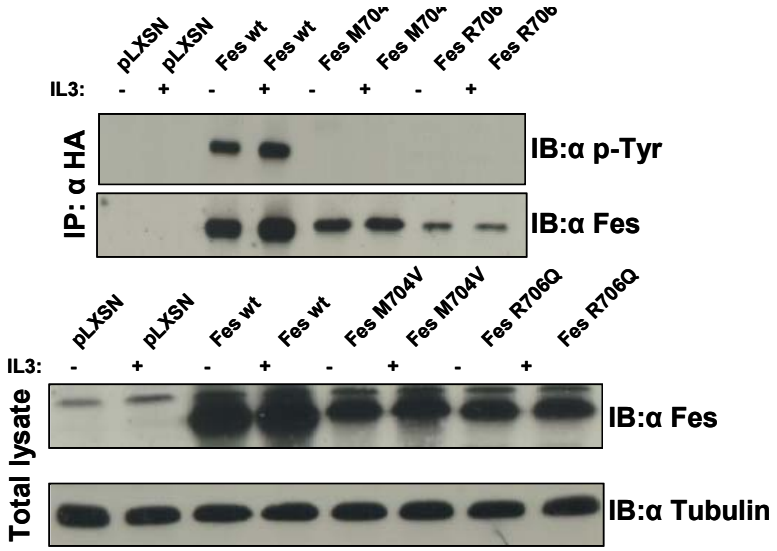


Figure 30: Analysis of the effects of the FES mutations on the signal transduction. The Fes tyrosine kinase (wildtype, M704V, R706Q) expressing cells were starved for 48h. After and without stimulation with interleukin 6 the cells were harvested and lysed. The FES protein was immunoprecipitated using anti-HA antibody and the phosphor-tryrosine content was determined using 4G10 antibody. Tubulin was used as loading control.

3. The biological significance of unknown genetic variations in tyrosine kinases

As already described the search for new candidates for cancer treatment is a growing field in today's research. The Singapore Oncogenome Project resulted in a huge dataset of genetic alterations in the tyrosine kinase transcriptome (Ruhe et al., 2007). These mutations, polymorphisms, insertions or deletions are partly yet unknown, and thus to be seen as possible oncogenic alterations. The workflow that was applied for the investigation of the mutations in the FES tyrosine kinase can be used to screen within this database for genetic alterations that do have an effect on transformation of cells.

3.1. From tyrosine to cysteine – a mutation at position 388 in the fibroblast growth factor receptor 4

There are several mutations and polymorphisms within the fibroblast growth factor receptor family published, which are known to have a clinical impact. Especially the polymorphism G388R in the FGFR4 has been extensively studied in respect to clinical and biochemical parameters (Bange et al., 2002; Streit et al., 2004; Streit et al., 2006). The Singapore Oncogenome Project unravelled a yet unknown mutation in the FGFR4 resulting in an exchange of a tyrosine at position 367 to a cysteine. This mutation occurred in the breast cancer cell line MDA-MB453, and until now this is the only cell line known to harbour this mutation. There is no primary tumour or any other primary tissue sample known that harbours this mutation.

Interestingly parallel to the research in Singapore the investigation of FGFR4 specific mechanisms in the MDA-MB453 cell line in our group was ongoing, and it was noticed that in this cell line the FGFR4 driven activation of the MAPK/ERK pathway is enhanced and independent of any stimulus (unpublished data; A. Roidl).

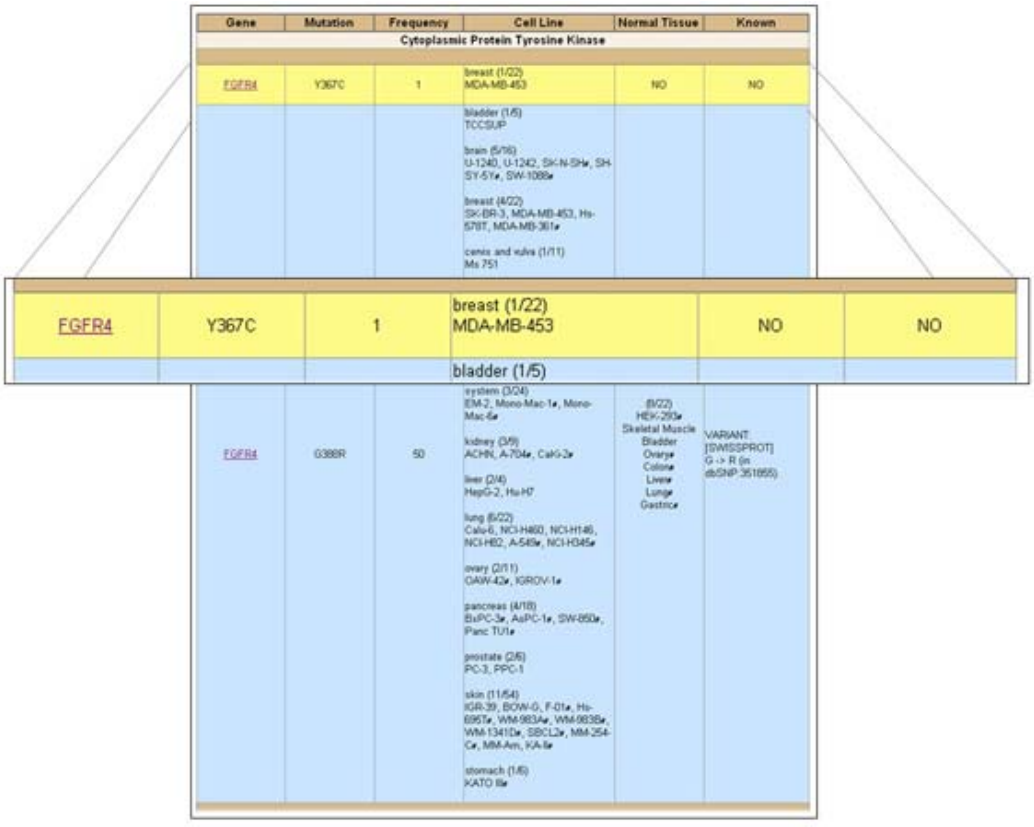


Figure 31: abundance of the FGFR4 Y367C mutation in the breast cancer cell line MDA-MB453 The mutation was discovered within the Singapore Oncogenome Project.

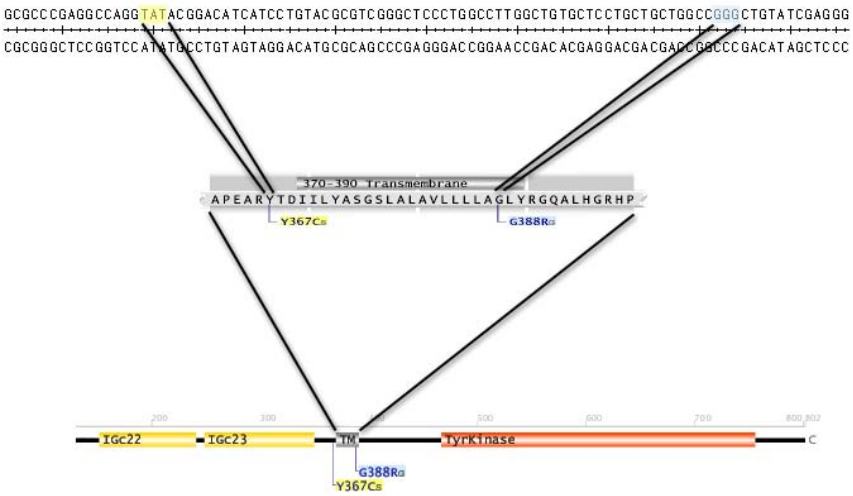


Figure 32: Localisation of the mutation Y367C and the polymorphism G388R within the FGFR4. The Y367C mutation in the FGFR4 gene is located upstream of the region coding for the transmembrane helix. The polymorphism G388R is located in the region coding for the transmembrane helix.

There are mutations in the FGFR2 and FGFR3 leading to a conversion from tyrosine to cysteine ,too, and after comparison of the localisation of the mutations we noticed that in FGFR2, FGFR3 and FGFR4 the amino acid exchange occurs 2 residues in upstrem of the the transmembrane domain. It has been reported that the Y375C mutation in the FGFR2 is a responsible factor for the Beare Stevenson syndrome and that the Y373C mutation in the FGFR3 is a responsible factor for a skeletal disorder called Dwarfism (McGaughran et al., 2006; Ronchetti et al., 2001).

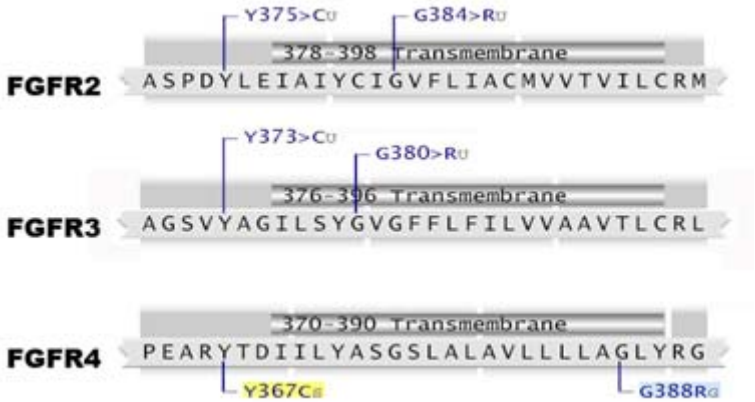


Figure 33: Comparison of the analogue mutations from tyrosine to cysteine in the FGFR family members The mutation leading to the exchange of tyrosine to cysteine is analogue to the mutations in the FGFR2 (Y375C) and FGFR3 (Y373C). It was shown that these 2 mutations are involved in skeletal disorders.

3.2. Expression of the fibroblast growth factor receptor 4 in MDA-MB453

In the MDA-MB453 cell line the fibroblast growth factor receptor 4 is expressed at high levels. As already described data from A. Roidl confirmed that the autophosphorylation of the FGFR4 is ligand and stimulation independent. By adding pervanadate, a chemical phosphorylation agent, the phosphorylation signal could be enhanced. Stimulation with FCS and starvation of the cells did not result in a change of the phosphorylation of the receptor.

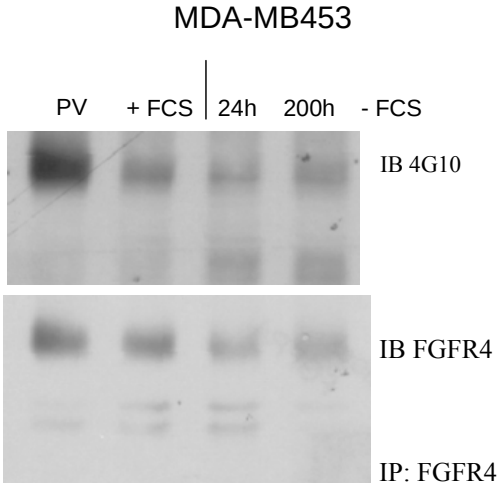


Figure 34: Pervanadate stimulation and starvation of the MDA-MB 453 breast cancer cell line. The FGFR4 was immunoprecipitated using anti-FGFR4 antibody and the phosphorylation signals were detected using the phosphor-tyrosine specific 4G10 antibody.

3.3. Generation of different fibroblast growth factor receptor 4 expressing stable cell lines

To understand the mechanisms that leads to the exceptional behaviour of the FGFR4 in the MDA-MB453 cell line, and to elucidate the consequences of the Y367C mutation in the FGFR4 we created several FGFR4 constructs. To determine the effect of tis mutation also in comparison to the effect of the G388R polymorphism, we made constructs carrying the wildtype FGFR4 gene, the Y367C FGFR4 gene and the G388R FGFR4 gene. Additionally kinase dead variants of the different FGFR4 genes were made. Here we used the methodology of transient transfection of linear vector DNA into HEK293 cells, followed by a subsequent selection to generate stable FGFR4 expressing cells. After the selection period we performed western blot analyses to control the expression of the different fibroblast growth factor receptor 4 proteins.

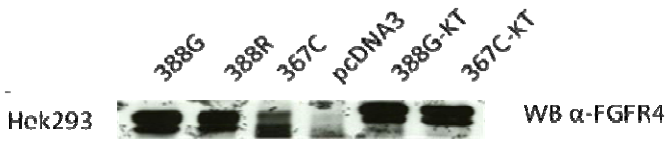


Figure 35: Expression of the exogenous FGFR4 in stable transfected HEK293 cells. The FGFR4 protein was immunoprecipitated using anti-FGFR4 antibody and the immunoblot was done using anti-FGFR4 antibody. The genetically different variants of the FGFR4 are: FGFR4 388R; FGFR4 388G; FGFR4 367C; FGFR4 388G – kinase deas; FGFR4 367C – kinase dead.

3.4. Influence of the cysteine 367 for biological activity of the fibroblast growth factor receptor 4

3.4.1. FGFR4 Y367C activates the MAPK/ERK pathway

After the first results where A. Roidl could show that not only the autophosphorylation of the FGFR4, but also the downstream signalling especially the MAPK/ERK pathway is affected in the MDA-MB453 cell line, it was interesting for us, if this finding is reproducible in another cellular system. Therefore we lysed the generated FGFR4 expressing cells after they were grown under normal conditions in cell culture medium containing 10% fetal calf serum. We analysed the levels of phosphorylated ERK kinase by using western blot analyses. The amount of activated FGFR4 was detected, after the immunoprecipitation of the FGFR4 protein, using the phosphotyrosine specific 4G10 antibody. In Figure 32 we could see that the FGFR4 wildtype and its variant the FGFR4 G388R are inducing a slight activation of the ERK kinase, which is comparable to the phosphorylated level of ERK in the mock transfected cells. On the other hand the FGFR4 carrying the Y367C mutation enhances the activation of the ERK kinase to tremendous levels independent of any stimulus. This is true if the tyrosine 367 is mutated to the cysteine in the wildtype FGFR4 and in the FGFR4 G388R. If another mutation is additionally incorporated, which disables the kinase activity of the FGFR4 the activation of the ERK kinase is completely gone.

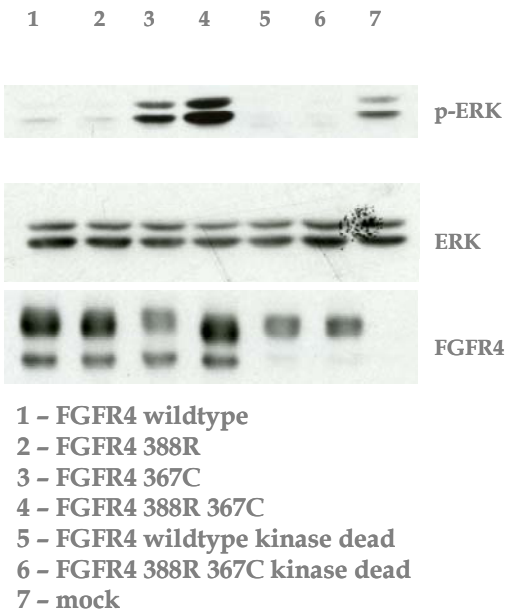


Figure 36: ERK activation in the Hek293 cells expressing different variants of the FGFR4. The phospho-ERK signal was detected using phosphor-ERK specific antibody and the ERK signal using a ERK specific antibody. The FGFR4 was pulled down using a FGFR4 specific antibody and detected with another antibody against the FGFR4. The used cells expressed different variants of the FGFR4: FGFR4 388G (wildtype); FGFR4 388R; FGFR4 367C; FGFR4 388R 367C; FGFR4 388G kinase dead; FGFR4 388R 367C kinase dead.

3.4.2. FGFR4 Y367C enhances the proliferation

After A. Roidl could demonstrate, that the breast cancer cell line MDA-MB453 is characterised by elevated proliferation in comparison to other breast cancer cell lines, we were interested of this fact is due to the mutation in the FGFR4. By applying proliferation experiments we could see that in our exogenous cellular system differences between the individual FGFR4 variants are visible, too. After a time period of 5 days we could clearly demonstrate that the FGFR4 Y367C enhances the proliferation of the cells expressing the receptor under normal conditions, in comparison to the cells expressing the wildtype FGFR4 and to the mock transfected cells.

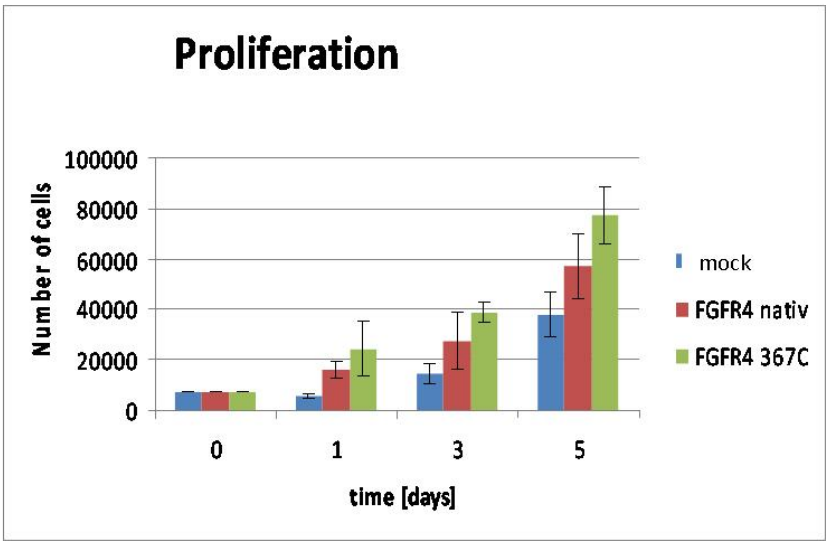


Figure 37: Proliferation rate of the HEK293 cells expressing the wildtype FGFR4 and the HEK293 cells expressing the FGFR4 carrying the cysteine at position 367. Equal number of cells was seeded and the proliferation progress was measured after 1, 3 and 5 days using a Beckman Coulter cell counter.

3.4.3. FGFR4 Y367C potentiates the wound closure process

The previous described results are all empowering the indication, that the mutation Y367C within the fibroblast growth factor receptor 4 transforms cells, expressing the receptor. To elucidate the significance of this statement, we tried to address this question in further cellular assays. The ability of cells to close a wound gives insight into two mechanisms of physiological behaviour. After wounding cells by creating a cell free barrier on a confluent cell layer they may enter this region, on the one hand in a migratory way and on the other hand cells may try to close the wound in a proliferative way. We applied this experiment for our generated cells, expressing the FGFR4 wildtype protein the FGFR4 protein that harbours the cysteine at position 367. With the help of the microscopic images which were taken after a specific time point we quantified the cells that managed to enter the cell free barrier. The quantification resulted that there is a slight increase in the wound closure process for the cells expressing the mutated variant of the FGFR4, compared to the mock transfected cells and to the cells expressing the wildtype FGFR4. This result was reproducible.

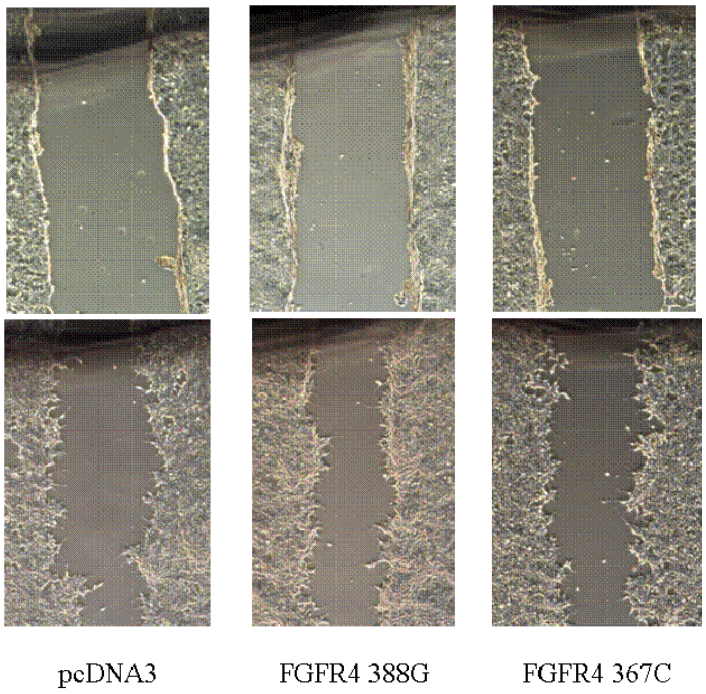
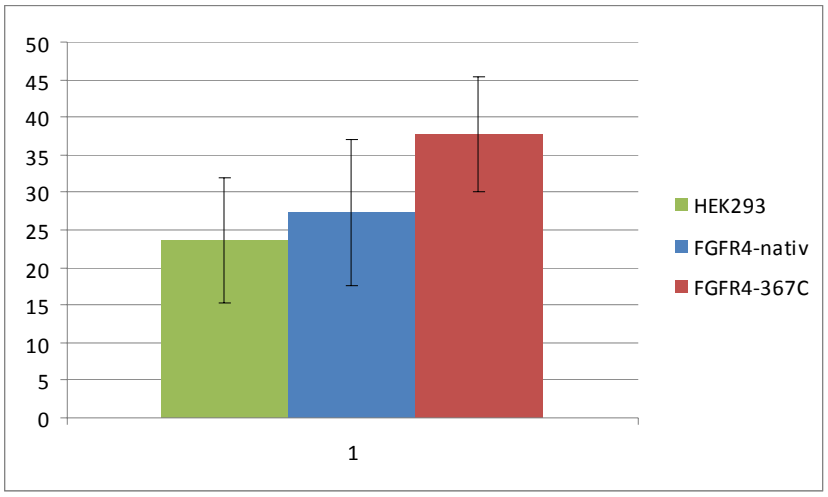


Figure 38: Microscopic images of the wound closure assay. The images were taken after wounding the cells (upper panel) and after 3 days of growth (lower panel). The cells used in this assay expressed the FGFR4 388R or the FGFR4 367C variants.



	Mock	388G	367C
mean	23,6	27,24	37,78
standard-diviation	8,4	9,7	7,6

Figure 39: Quantification of the wound closure assay. The quantification was done using Adobe Photoshop. The size of the cell-free barrier and the number of cells that migrated into the region was measured..

3.4.4. Dimerisation of the FGFR4 Y367C

In this study we could show that cells expressing a mutated variant of the FGFR4 (Y367C) have an unusual behaviour. The ligand and stimulus independent activation of FGFR4 specific mechanisms support the conclusion that the Y367C mutation is a transforming alteration. These findings strengthen the statement that the behaviour of the breast cancer cell line MDA-MB453 is due to the mutation in the FGFR4 resulting in the cysteine at position 367. Now the question arises what the reason for the enhanced activation of the FGFR4, which could be seen in endogenous and exogenous systems, is. The analogue mutation in the FGFR3 has been studied extensively and shows a comparable phenotype if expressed in cells (Ronchetti et al., 2001). Here the mechanism leading to this phenotype was elucidated, and it could be shown that the cysteine mediates a homodimerisation between two FGFR3 molecules. It was demonstrated that this dimerisation is constitutive and that the cysteine forms a disulfide bridge with the other monomer.

For us the very interesting question was, if we are able to see the same effect for the FGFR4. Therefore we applied polyacrylamide gelelectrophoresis and subsequent western blot analyses under non denaturating conditions. In these native gels not only the secondary structure of a protein is maintained but also oligomerisation of proteins can be observed (Harwood, 1996).

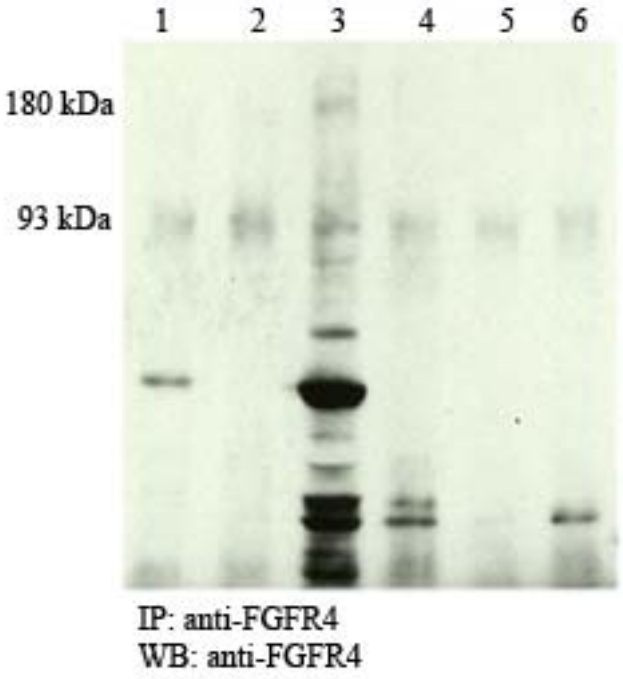


Figure 40: native polyacrylamide gel electrophoresis of different cancer cell lines. The FGFR4 was immunoprecipitated and subsequently the membrane was incubated with a FGFR4 specific antibody; Lanes: 1 – BT474, 2 – MDA-MB361, 3 – MDA-MB453, 4 – ZR75-1, 5 – MDA-MB361, 6 – ZR75-1

In a first experiment we used several cancer cell lines that express the FGFR4 at detectable levels and performed immunoprecipitation after lysis of the cells with RIPA buffer. The precipitates were analysed using non denaturating PAA gel electrophoresis and western blotting. Here we could see that there is a form of the FGFR4 detectable that runs with lower speed through the gel and therefore has to be of bigger size. The size of this protein can be estimated and is about 180 kDa. This would suppose that this protein is a dimer of the FGFR4. This form could not be detected in the other cell lines. From this experiment we could conclude that this dimerisation is due to the mutation Y367C in the FGFR4.

In another experiment we tried to analyse the abundance of a FGFR4 dimer using crosslinker agents to stabilise the possible disulfide bridge between the FGFR4 monomers. After immunoprecipitation, gel electrophoresis and western blotting we could see that in this case there is no FGFR4 dimer detectable. This is due to the fact, that after the crosslinking procedure, there is no FGFR4 at all visible. This method is therefore not suitable for our purpose.

The appearance of the possible FGFR4 dimer in the native gel raised the question, if we are able to reduce this dimer to the momomers, by applying reducing agents. Therefore we took samples of MDA-MB453 lysates as well as BT474 lysates and separated them by native PAA gel elctrophoresis. The lysates were either treated with β-mercaptoethanol and boiled or just

boiled. Additionally samples were taken without any treatment. Here we were able to see that, if one adds β-mercaptoethanol to the sample the dimer is no longer visible. In the samples that were only boiled or not treated at all the dimerised FGFR4 protein is visible. These facts are not true for the BT474 cell line, because here we could only see a FGFR4 monomer in all samples. Again we can conclude that this effect is due to the Y367C mutation in the FGFR4.

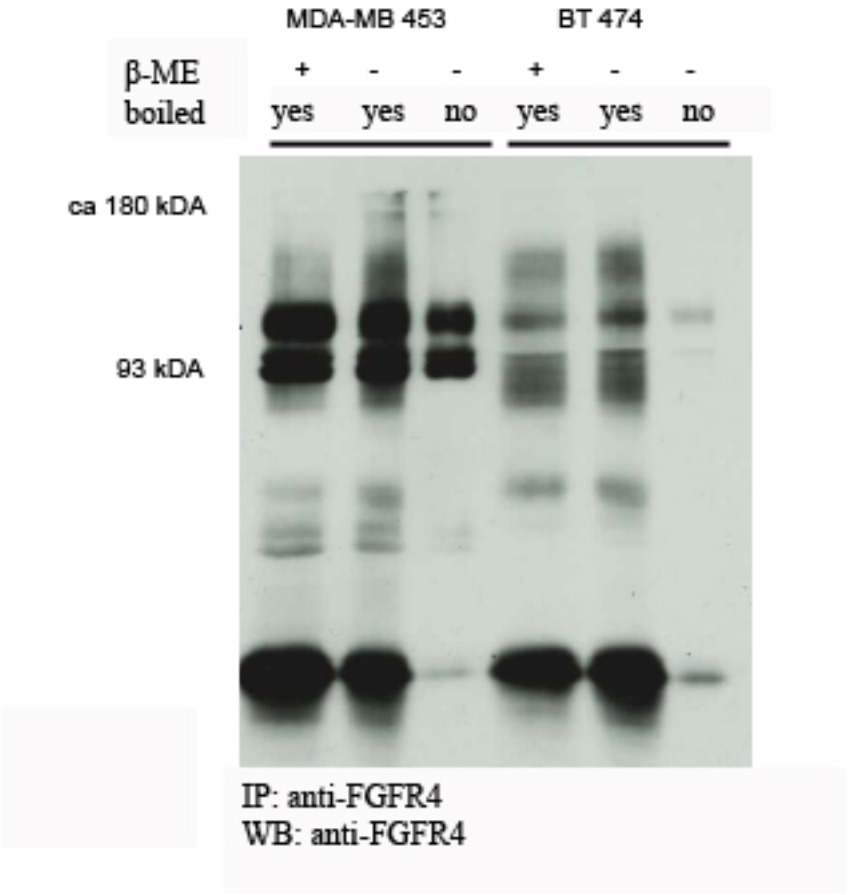


Figure 41: native polyacrylamide gel electrophoresis of differently treated lysates of cancer cell lines. The FGFR4 was immunoprecipitated and the samples were run on a native PAA-gel. The membrane was incubated with a FGFR4 specific antibody. Prior to immunoprecipitation the lysates were either treated with the indicated methods or taken without any treatment.

V. Discussion

1. Large scale screens in the field of cancer genomics

The rapid improvement of methodologies, the laboratory based techniques, facilities, equipments, also the improvement of computational analyses and the growing amount of the published knowledgebase leads to a shift in the field of cancer research. Large scale screens or high throughput methodologies are employed by more and more institutes, and thus the information which is available is growing very rapidly. This new way of dissecting specific scientific goals is in our days used in many different research areas. One growing field is the genomic analysis of whole genomes of species, which peaked with the completion of the human genome project (2004; Hattori, 2005; Venter et al., 2001). In the field of cancer research, the search for candidates that may serve as targets for new intervention strategies is a very promising but also challenging approach. Bardelli and colleagues performed a large scale screen for genetic alterations in tyrosine kinases, tyrosine kinase like and receptor guanylate cyclises (Bardelli et al., 2003). The hope of scientists lies in the difference of the genetic sequence when they compare healthy and cancerous specimens. The optimal course would be to find an altered protein that is responsible for the initiation of a tumour, for the progression of a tumour or for the ability of a cancer cell to migrate into another tissue to form metastases. At this point one would be able to target this altered protein with monoclonal antibodies, like it has been done for the amplification of HER2 in breast cancer patients, or by designing small molecule inhibitors (Fischer et al., 2003). Efforts are also put into the development of siRNA based treatments of tumour related diseases, which is also thinkable as a tool to target an altered gene of interest (Kim et al., 2008; Mohammed and Shervington, 2008; Reagan-Shaw and Ahmad, 2005; Tsui et al., 2005). Although the improvement of methodologies and scientific work is ongoing, this trail of starting a study and ending with a drug is still challenging.

1.1. Differences in using cell lines vs. primary tumours

One major limitation in using primary tumour samples as material for large scale screens is the availability of the material itself. Another limitation is the clinical follow up recording, which is often not sufficient. The advantage of primary tumour samples is the reliability of the results. Here scientists deal with information revealed out of a real cancer situation. On the other hand a good clinical follow up makes it easy to get information about the development or the progress of the disease.

The second way of approaching large scale screens is to take cell lines as basic material. Cell culture methodologies are very sophisticated methodologies that are used since decades (1990). The big advantage of using cell lines is the unlimited availability of the material. The disadvantage in using cell lines is the authenticity (Chabner, 1990). Thus, cell lines are immortalised primary cells they are characterised by an unstable genome which underlies changes the longer cell lines are taken into culture. Nevertheless the results, that are made available by using cell lines, serve as very good indicators of the circumstances that are present in the real disease.

1.2. Challenges of high throughput methodologies in genomics

The amount of data is one of the major challenges when dealing with high throughput methodologies. Thus, computational facilities are very potent in analysing huge sets of information, the factor of the human decision of sense or nonsense is still a necessary step. Other challenges are the follow up studies, where often large scale methodologies are not applicable (Merrell and Camilli, 2002). The one issue is the collection of data which is alone not useful to solve problems scientists are dealing with, and the other point is to validate this data, where manpower is indispensable. Especially in the field of cancer genomics, where the overall goal is defined as a road to the cure of the disease, many problems are arising.

1.3. Identification of germline or somatic alterations

When using cell lines as basis for a screen, like it was performed in the Singapore Oncogenome Project, one has to deal with genetic alterations that are identified. These results then may be of a somatic or a germline origin. To discriminate between these two states one needs to apply other informations too. If mutations and alterations are already published and known to be of somatic or germline origin the choice is clear. Another approach is if a genetic alteration that was identified in a cell line could also be identified in a primary tumour but not in the corresponding healthy tissue material. In this case the alteration can be seen as a event that happened during the genesis of cancer and is thereby a somatic alteration. If there are no additional information available one can not determine if this alteration is of somatic or germline origin (Groves et al., 2002; Wang et al., 1997).

1.4. The search for already known genetic alterations

The goal of our studies was to identify possible genetic alterations that lead to an elevation of a transforming and oncogenic potential and to build a working experimental scheme that serves as a tool in solving this issue.
In 2003 Bardelli et. al published at this time point yet unknown mutations in several tyrosine kinases which were detected in tissue samples of colorectal cancer patients (Bardelli et al., 2003; Bardelli and Velculescu, 2005). These mutations were not further analysed and we wanted to know if the discovered mutations may have an impact on the cancer itself. There are several ways of approaching this issue and one good example is to use cell lines that harbour the mutated gene of interest. Therefore it was important for our study to know whether the investigated mutations, in our case the FES M704V and the FES R706Q mutations, are also present in the cell lines that were available in our laboratories. The screening for this mutation was performed in broad spectrum of cell lines of different origins. As template fopr the amplification of desired DNA Fragments we used cDNA which was prepared in our laboratories. The advantage of using cDNA is that one screens for the actual proteins that are also expressed in the investigated cell line. Using genomic DNA could result in a sequencing of genes, that are not even expressed and that don’t play a role for the

behaviour of a cell. The findings that Bardelli and colleagues made were not reproducible in the set up of our cell lines, which means that we could not detect these mutations. There was no evidence in the analysis of the sequencing data for the existence of the observed alterations. The sequencing data was evaluated with the help of DNA analysis software and additionally all electropherograms have been scanned manually for the existence of heterozygous presence of the mutations. If a mutation in a cell occurs only on one allele the sequencing would result in the original electropherogram plus an additional peak for the mutated base which has not always to be at a ratio of 1:1. This makes it difficult for automated analysis software to detect a heterozygous mutation, what makes the manual evaluation of the data necessary.

2. The oncogenesis of the FES tyrosine kinase

The investigation of the FES tyrosine kinase was performed in an exogene FES expressing cell system. In our study we used this system because there was no appropriate cell system available where the FES kinase is mutated. To estimate the oncogenic potential of the protein we used several assays mimicking conditions that are present in a tumour environment. This is just an estimation of a situation, which is not reproducible in vitro. For us, it was important to get hints if the mutations in the FES tyrosine kinase are transforming. Therefore we used a workflow that consists of a focus-fomation assay, a soft-agar-colony formation assay, migration and proliferation assays. The information that can be concluded out of these assays are serving as hints for transforming potentials that are introduced by proteins.

2.1. Fes as a transforming mediator of cells

With the help of the generated NIH3T3 cells, that do express the FES tyrosine kinase and its modified variants, we could see that there is a loss of contact inhibition. This ongoing growth, despite the embedded layer of neighbouring cells, results in a multilayer growth of the cells, which is a criterion for transformation (Haber et al., 1977). This could only be seen if the mutated variants of the FES tyrosine kinase are introduced in the cells. The expression of the FES wildtype protein did not result in a loss of contact inhibition which is also true for the mock infected fibroblasts. We could also see a difference between the two mutations. This

difference is more of physiological state of the foci itself. Cells expressing the FES M704V mutant are forming fewer foci that are of bigger size compared to cells expressing the FES R706Q mutant. In the R706Q case the foci formation looks more like a grown multilayer which consists of many small colonies. This is comparable to the foci formation that is achieved by expressing the v-src oncogene. To draw a conclusion out of this assay one could say that, here we have the first hint that the mutations in the FES tyrosine kinase donate the protein transforming capabilities.

The second applied transformation assay was the soft-agar colony-formation assay which utilizes the surface dependency for growth of untransformed cells. Cells were resuspended in a medium containing agar solution which is then plated on a bottom agar layer. The cells are now individually spread within the stocking agar and the survival of the cells is dependent on the level of transformation. In this assay the criterion of transformation is the formation of compact and viable colonies which are visualised by staining the plate with MTT (Kanzawa et al., 1987; Lieber and Kovach, 1982; Malmberg et al., 1991). As expected nice colonies were visible if cells are expressing the strong transforming oncogene v-src. In this experiment the transforming properties of EGFR, HER2 and CSF1R were not sufficient to induce a significant growth within the agar. This may also be due to the fact that cells are isolated and not exposed to the paracrine stimulation of neighbouring growing cells. The fibroblasts expressing the FES wildtype protein were not able to grow in this assay which is not surprising, as they did not show a transforming capability in the focus-formation assay too. The same is true for fibroblasts expressing the FES M704V variant, however these cells did show a transformation in the focus-formation test. One can conclude that in this experiment the transformation grade has to be much higher to see any effect. Fibroblasts expressing the FES R706Q protein are showing a slight increase in colony formation, compared to the other two tested FES proteins and compared to the negative control. This matches the result, the v-src expressing cells are showing, because the focus-formation assay as well as the soft-agar colony-formation assay resulted in a similar transforming potential, which is as expected still higher for the v-src oncogene.

The migration and invasion of cells belongs to the hallmarks of cancer. Invasive cells are able to break through the basal membrane and to migrate into the neighbouring tissue. If this happens at the border of blood or lymphatic vessels the cells may enter the blood stream and migrate to other tissues and to form metastases. Migration and Invasion of cells is characterised by the association of actin filaments to membrane standing adaptor proteins like ARP2/3 and WASP followed by an elongation of filopodia, which is mediated by

phosphatidyl inositole phosphate and small GTPases. Integrins come into contact with different ligands of the extracellular matrix which leads to the formation of focal contacts. The proteolysis of the cell membrane is occurring and a, by RHO and ROCK regulated, cell contraction is induced (Kusama et al., 2006). In the last step the cell disconnects from the extracellular matrix and the focal contacts are disassembled. This complex procedure is mimicked in the Boyden chamber where the bottom contains of a filter (Maliakal, 2002). Cells are seeded in this chamber in starvation medium and the whole apparatus is hanging in another well which contains medium with a chemo attractant. In this experiment the criterion of transformation is measured by the amount of cells that were able to move through the filter, and to adhere at the bottom of this filter. In our study we could observe that cells expressing the colony stimulating factor 1 receptor are showing an enhanced migration of the cells into the medium containing the FCS. This is an expected result because it has been shown that the CSF1R is involved in the cytoskeletal remodelling and the spreading and motility of macrophages (Pierce et al., 1990). The other constructs used in this experiment showed no significant increase in cell migration. This is also true for cells expressing the wildtype FES protein and for cells expressing the FES M704V variant. Interestingly the exchange of the arginine at position 706 to glutamine results in an increased migration of the cells expressing this protein. This migration is even comparable to the migratory effects that are mediated by the CSF1R.

3. The fibroblast growth factor receptor 4 as a proto-oncogene?

Compared to the other fibroblast growth factor family members, less is known about the importance of the fibroblast growth factor receptor 4. Especially the importance of the FGFR4 in cancer can still not be evaluated with reasonable certainty. There are many reports that have demonstrated that the FGFR4 plays a role in cancer which makes sense if one looks at the FGFR4 expression in breast, pancreatic and kidney carcinomas (Shah et al., 2002; Wagner et al., 1998). There the expression is often very strong. But other experiments have shown that the overexpression of the FGFR4 has no influence regarding the initiation of pancreatic carcinomas. The conclusion would be that the FGFR4 is not interacting with the initiation of a tumour but with the progress of the disease. Other groups have been demonstrating the involvement of the FGFR4 in the membrane ruffling of breast cancer cells which is not true

for healthy breast epithelial cells (Johnston et al., 1995). This supposes a involvement of the FGFR4 in cell migration.

The single nucleotide polymorphism of the FGFR4 at position 388, where either a glycine or an arginine is present, has been also studied in respect to the ability of playing a role in cancer. Again there is no evidence that the FGFR4 can itself initiate a tumour, but it has been demonstrated for several types of cancer, that the arginine allele often correlates with an aggressive progression of the disease (Bange et al., 2002; Spinola et al., 2005; Stadler et al., 2006; Thussbas et al., 2006). In melanoma patients the arginine allele in the FGFR4 has been linked to the size and invasiveness of the tumour (Streit et al., 2006).

3.1. Tyrosine to cysteine – a transforming conversion

In the Singapore Oncogenome Project a yet unknown mutation in the FGFR4 has been discovered, which leads to the exchange of a tyrosine to a cysteine at position 367. This mutation was found to be present in the breast cancer cell line MDA-MB453 (Ruhe et al., 2007). The fact that the mutation is harboured in a cancer cell line raises the question, if this is a mutation, transforming the FGFR4 from a proto-oncogene to an oncogene. The goal of our studies was to elucidate the consequences of this mutation on cancer specific characteristics. Interestingly within the FGFR family, mutations are known that are analogue to this mutation.

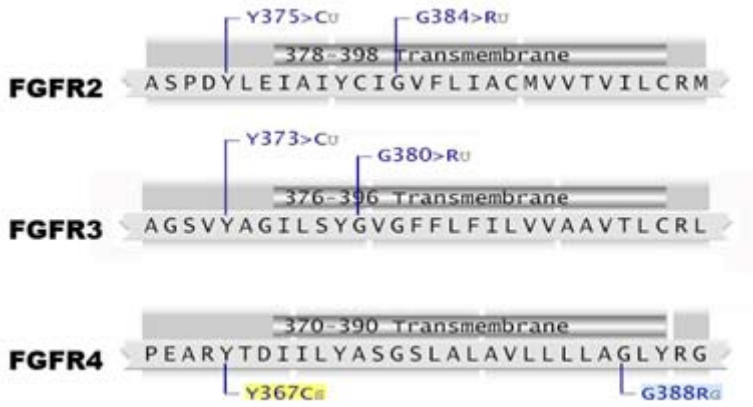


Figure 42: Comparison of the analogue mutations from tyrosine to cysteine in the FGFR family members
The mutation leading to the exchange of tyrosine to cysteine is analogue to the mutations in the FGFR2 (Y375C) and FGFR3 (Y373C). It was shown that these 2 mutations are involved in skeletal disorders.

In FGFR2 the Y375C and in FGFR3 the Y373C mutations are known (Hernandez et al., 2006; McGaughran et al., 2006; Rousseau et al., 1996). It was demonstrated that these mutations are responsible for skeletal disorders in humans like the Beare Stevenson syndrome (FGFR2 Y375C) and the Dwarfism (FGFR3 Y373C). If we looked at the topology of this mutation we were surprised to see, that in all three FGFR family members the mutation is located exactly 2 amino acid residues in front of the transmembrane domain. The big question mark is, if this genetic alteration plays a role in cancer, what would be supported by the fact that the mutation was discovered in a cancer cell line, or if this mutation is not linked to cancer. If this is true the MDA-MB453 cell line could have gained this mutation because of the unstable genome of cancer cells randomly. The homology between the mutations within the FGFR family members would support the assumption that this mutation may be responsible for any other disease, preferably skeletal disorders where until know nobody looked for genetic variation in the FGFR4.

3.2. FGFR4 Y367C in exogenous and endogenous backgrounds

Due to the fact that in our group the breast cancer cell line MDA-MB453 is used for experimental procedures, there were already some results that are able to address the question of the biological significance of this mutation. A. Roidl observed in his work that the MDA-MB453 cell line behaves differently in comparison to other breast cancer cell lines. He could not only show that transformation specific characteristics like proliferation and apoptosis are altered, he was also able to demonstrate that the phosphorylation of the FGFR4 itself and the ERK kinase can not be reduced by starvation. These endogenous experiments made us curious if we are able to reproduce this deliverables in an over expression system.
Therefore we generated HEK293 cell that express several variants of the FGFR4. These cells were used in different assays and western blot analyses.
In this experiment we saw that the cells expressing the FGFR4 388G and the FGFR4 388R protein do not or just very tiny activate the ERK kinase via phosphorylation. This could be seen under normal growth conditions. In the case of the mock transfected HEK293 cells a slight activation of the ERK kinase was visible. If the HEK293 cells are expressing the FGFR4 367C in either the gylcine allelic background or the arginine allelic background we could see a tremendous increase of ERK phosphorylation which is gone if an additional

mutation is inserted that makes the kinase domain inactive. These results are all corresponding to the findings A. Roidl made in the endogenous cell system (MDA-MB453). The ability of rescuing the inactive state of ERK by inactivating the kinase domain shows that this phenomenon is not a kinase independent one. There is a slight difference in the intensity of the phospho-ERK band between the FGFR4 388R 367C and the FGFR4 388G 367C expressing cells what can be explained with the slight lower expression of the FGFR4 388R 367C. Between the two allelic variants of the FGFR4 we could not notice any difference in ERK activation. For the AKT/PI3K pathway we were not able to detect any differences between those cells expressing the wildtype and the 367C FGFR4 (data not shown). Next we looked if the proliferation rate of the cells is dependent on the FGFR4 variant that is expressed. Therefore we applied a standard proliferation test under normal growth conditions. After 5 days the differences in the proliferative behaviour were strong enough to draw conclusions. The cells expressing the wildtype FGFR4 protein showed an enhanced proliferation in comparison to the cells that were transfected with the empty vector construct. In the case for the cells that express the FGFR4 367C we were able to see again an enhancement in the proliferation compared to the cells expressing the wildtype FGFR4 protein. This first physiological result in our cellular system correlates with the findings that were made in the endogenous MDA-MB453 cell system and could be ranked as a first hint that the Y367C mutation in the FGFR4 drives the protein into an oncogenic direction. This fact will be empowered by the next experiment we performed. As it is supposed by findings of others that the FGFR4 may have an impact on cell migration we applied the in vitro wound closure assay to our cell system. Here we compared the ability of the different cells to grow into and thereby close a cell free barrier on a confluent tissue culture plate. In this experiment the differences were not immediately visible when looking at the microscopic images but after quantification with the computer there was a clear trend. The amount of cells expressing the wildtype FGFR4 protein that moved into the cell free barrier (27,24%) was comparable to the amount of cells moving into that part which were transfected with the empty vector (23,6%). If the cells did express the FGFR4 367C protein the number of the cells that made their way into the wound (27,78%) was increased of about 10%. This result is again a physiological one from which one can assume a transforming ability of the FGFR4 367C protein.

3.3. Constitutive dimerisation as a cause for the enhanced biological properties of the FGFR4

In all the previous experiments we could see an enhancement of the normal biological activity of the FGFR4 if the tyrosine at position 367 is mutated to the cysteine. The reason for this hyperactive variant of the FGFR4 was not known to us but there are several hints that may lead to the right answer. These hints can again be found in the analogous mutations in the FGFR2 and FGFR3. Studies have shown that also the FGFR3 carrying the tyrosine-cysteine conversion harbours an elevated biological activity of the receptor and it was supposed that the cysteine may build a covalent disulfide bridge with another FGFR3 molecule. Thus the two monomers are very close to each other as if it is the case after ligand binding and the crosswise autophosphorylation may take place. This statement makes sense because the cysteine is directly in front of the transmembrane domain and thereby has no chance of undergoing great conformational changes (Ronchetti et al., 2001). If this statement would also be true for the FGFR4 the ligand independent activation of the receptor could be explained. Under normal circumstances the ligand facilitates the dimerisation of two receptor monomers by which the signalling cascade is activated. The proximity of two receptor monomers mediated by the covalent disulfide bridge makes a ligand unessential for tha activation of the signalling cascade. To address the question whether the observed facts are due to covalent receptor dimerisation we applied polyacrylamid gel electrophoresis and western blot analysis under native and non reducing conditions. Here we took the endogenous cell system (MDA-MB453) and compared the behaviour of the native proteins in the electrophoresis with other cell lines that express the FGR4 wildtype protein. And indeed after optimising the experimental conditions we were able to see evidences of a covalent dimerisation of the receptor. This dimmer band was only visible if the MDA-MB453 cell lysate was loaded in the gel. We also performed crosslinker methods to enrich the disulfide-bonded monomers but this was not applicable for our purpose. As expected we were able to reduce the dimerised FGFR4 by using reducing conditions in the analysis. This is a good hint that the enhanced biological activity is due to the dimerisation of the protein by disulfide bridging. The best way of proving this statement as a follow up experiment would be to crystallize the dimerised FGFR4 in the MDA-MB453 cell line. An approximation could also be achieved by using in silico computational methodologies like molecular modelling.

VI. Summary

In this study the goal was to find oncogenic genetic alterations in the tyrosine kinome. Therefore a large scale sequencing screen was performed, called the Singapore Oncogenome Project.

In a second part, the aim was to characterise discovered genetic alterations, which may have an impact on cancer, by applying physiological and functional assays. The grade of cellular transformation that is achieved by expressing a mutated tyrosine kinase helps to evaluate, if this mutation is oncogenic.

As a starting point for this thesis we used already published genetic alterations, which were lacking a functional investigation. In a sequencing screen we were not able to detect the mutations in the FES tyrosine kinase in the set up of cell lines we used. Because of that we applied functional assays in an exogenous system. Here we could show that the mutations M704V and R706Q in the FES tyrosine kinase lead to an increased transformation of the cells, expressing the different proteins. Especially we could demonstrate that cells, expressing FES M704V proliferate faster and adhesion independent and that they loose the contact inhibition. Cells, expressing the R706Q mutant are showing an enhanced migration and they loose the contact inhibition, too. Further we could demonstrate that in both the mutated proteins the autophosphorylation capability is lost. This is true under normal and under stimulating conditions.

In a second part the functional investigation of a mutation, which was discovered in the Singapore Oncogenome Project, is described. The genetic alteration Y367C in the FGFR4 was detected in the breast cancer cell line MDA-MB453. In this study we were able to show that this mutation has a very strong effect on the behaviour of a cell expressing the mutated protein. Cells are proliferating faster and are able to accelerate wound closure. Additionally the cells loose their contact inhibition. We could show that these findings are mainly due to the activation of the MAPK/ERK pathway. As the cause for the observed effects, we could show that the cysteine at position 367 leads to a constitutive dimerisation of the receptor, which leads to a ligand independent activation of its biological function, like it has been shown for the FGFR3, too.

VII. Zusammenfassung

Das Ziel dieser Arbeit war oncogene genetische Veränderungen in Tyrosinkinasen zu finden. Dies wurde mit Hilfe von Hochdurchsatz Sequenzierungs Screens im „Singapore Oncogenome Project“ durchgeführt.

Desweiteren sollten die gefundenen Veränderungen der Gene funktionell charakterisiert werden. Hier lag das Hauptaugenmerk darauf, genetische Variationen zu finden, die einen Einfluß auf Krebs haben.

Als Ausgangspunkt wollten wir bereits beschriebe Mutationen analysieren, die auf funktioneller Ebene noch nicht untersucht wurden. Eine groß angelegte Sequenzierung ergab jedoch, dass die zu untersuchenden Mutationen in der FES Tyrosinkinase in den von uns untersuchten Zelllinien nicht vorkommen. Aus diesem Grund führten wie die funktionellen Analysen in einem exogenen System durch. Hier konnte gezeigt werden, dass die Mutationen M704V und R706Q der FES Tyrosinkinase zu einer erhöhten Transformation der Zellen, die das jeweilige Protein exprimieren, führen. Insbesondere konnten wir zeigen, dass Fes M704V exprimierende Zellen schneller und adhäsions-unabhängig wachsen, und dass sie Ihre Kontakt-Inhibition verlieren. Zellen, die die R706Q Variante der FES Tyrosinkinase exprimieren, sind fähig verstärkt Migration durchzuführen, und auch sie verlieren die Kontakt-Inhibition. Weiterhin konnten wir nachweisen, dass beide mutierte Formen der FES Tyrosinkinase unter normalen Bedingungen, sowie nach Stimulation nicht mehr phosphoryliert vorliegen.

In einem weiteren Teil ist die funktionelle Charakterisierung einer Mutation, die in dem „Singapore Oncogenome Project“ gefunden wurde, beschrieben. Die Mutation Y367C im FGFR4 wurde in der Brustkrebs Zelllinie MDA-MB453 gefunden und in dieser Arbeit war es möglich zu zeigen, dass diese Mutation einen starken Effekt auf das Verhalten von Zellen, die das mutierte Protein exprimieren, hat. Diese Zellen wachsen schneller und sind in der Lage Wunden schneller zu schließen. Auch diese Zellen zeigen einen Verlust der Kontakt-Inhibition. Die beobachteten Effekte sind auf die verstärtke Aktivierung des MAPK/ERK Signalweges zurückzuführen, was in dieser Arbeit gezeigt werden konnte. Der Grund für die beobachteten physiologischen und funktionellen Veränderungen liegt in der, durch das zusätzliche Cysten gebildete, Disulfidbrücke mit einem weiteren mutierten Rezeptor. Diese in der Arbeit bewiesene Dimerisierung führt dazu, dass der Rezeptor Ligand unabhängig aktiviert werden kann, was auch am Beispiel des FGFR3 bereits gezeigt wurde.

VIII. References

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IX. Appendix

Abbreviations

Ab	Antibody
AB	Acidic box
Amp	Ampicilline
Amp_r	Ampicilline resistence
APS	Ammoniumpersulfate
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
CADH	Cadherin-like domain
cAMP	Cyclic adenosinmonophosphate
Ca^{2+}	Calcium Ions
CRD	Cysteine-rich domain
DAG	Diacylglycerol
DiscD	discoidin-like domain
DMEM	Dulbecco's modified eagle medium
DN	Dominant negative
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Dooble-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethlendiamintetraacetate
e.g.	Example given
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCH	FPS/FES/FER/CIP4 homology domain
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FER	FES-related kinase
FERT	Testes-specific FER
FES	Feline-sarcoma oncogene
Fig	Figure
Fms	Colony-stimulating-factor-1-receptor
FPS	Fujinami poultry sarcoma
g	Gramm

Gab1	Grb2-associated binder-1
Gab2	Grb2-associated binder-2
GDP	Guanosindiphosphate
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor binding protein 2
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
H_2O_{bidest}	Twice-destilled, deionised Water
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
HER	Human EGFR-related
HNSCC	Head and neck squamous cell carcinoma
IG	Immunglobulin
IP	Immunoprecipitation
IP_3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-ß-thiogalactopyranoside
JM	Juxtamembrane domain
kb	Kilobase
kDa	Kilodalton
KDR	Kinase-domain insert receptor
kit	Hardy-Zuckerman4 feline sarcoma oncogene
KrinD	kringle-like domain
l	Liter
LPA	Lysophosphatydic acid
LRD	Leucine-rich domain
µ	Micro
m	Milli
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
MBP	Myelin basic protein
MEK	MAPK/ERK Kinase
min	Minute
MLK4	Mixed-lineage kinase 4
n	Nano
NRTK	Non-receptor tyrosine kinase
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylenglycole
PI 3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl-fluoride
PNPP	p-Nitrophenyl-phosphate
PTK	Protein-tyrosine kinase
PY	Phospho-tyrosine

Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Natriumdodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	Second
SH2, 3 domain	Src homology 2, 3 domain
SHP-2	SH2-containing PTP-2
src	Homologue to v-src (sarcoma viral oncogene)
TCA	Trichloroacetic acid
TGFα	Transforming growth factor alpha
TEMED	N, N, N‘, N‘-Tetramethyletylendiamine
Tet	Tetracycline
TK	Tyrosine kinase domain
TNFα	Tumor necrosis factor alpha
TM	Transmembrane
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Tween 20	Polyoxyethylensorbitanmonolaureate
U	Enzymatic activity unit
o/n	Overnight
UV	Ultraviolett
V	Volt
VEGFR	Vascular-endothelial-growth factor receptor
Vol	Volume
wt	Wild type

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