universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

"FGFR4 mediated Growth and Survival signals in Colon Carcinoma cells"

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuerin:	Univ.-Prof. Dr. Manuela Baccarini

Acknowledgements

This work was not possible without the support and presence of some people whom I want to express my gratitudes with a few words.

I am mostly thankful to our group leader Prof. Brigitte Marian for her heavenly patience and steady trust in my work – I will give my best to persist that trust forever.

Thanks to Xenia, Christine and Kerstin for the friendly atmosphere they create together and the fun we had that made me smile even at tough times.

Thanks to Nawal for her concern and her friendship; for giving me the opportunity to explain everything in my field I learned.

Special thanks to Paul Breit for rescuing bad films by excellent photography and Irene Herbacek for her professional support in flow cytometry. I thank also Lukas Mager for his support in the development of the invasion assay.

And thanks to my family for their permanent help and encouragements

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AIGF… androgen-induced growth factor

ALT… alternative lengthening of telomeres

APC… adenomatous polyposis coli

ATCC… American type culture collection

BSA… bovine serum albumin

°C… degrees of Celsius

CA… Canada

CRC… colorectal cancer

ctrl… control

DAG… diacylglycerol

DEPC… diethylpyrocarbonat, used for RNase inactivation

DNA… deoxyribonucleic acid

E-Cadherin… epithelial-cadherin

ECM… extracellular matrix

EDTA… ethylenediaminetetraacetic acid

EGF… epithelial growth factor

EMT… epithelial-to-mesenchymal transition, the process when cancer cells get mobile (also important in healthy cells during development of an organism)

et al.… et alumni, common used latin abbreviation for “and others”

EtOH… ethanol, used for sterilization and RNA washing steps

FACS… fluorescent-assorted cell sorting, flow cytometry

FAP... familial adenomatous polyposis

FCS… fetal calf serum, supplement for cell cultivation

FGF... fibroblast growth factor

FGFR… FGF receptor

$FGFR4^{arg}$/$FGFR^{gly}$… arginine or glycine at position 388 in FGFR4 TM domain

FGFBP… FGF binding protein

FHF… FGF homologous factors (FGF11-14)

FRS2… FGF receptor substrate

G… glycine

G-418… commercial name for the antibiotic geneticin

GloboCan… a project of WHO with the aim to “provide contemporary estimates of the incidence of, and mortality from major type of cancers, at national level, for all countries of the world” (from http://globocan.iarc.fr/)

h… hours

hFGF… hormone-like FGF

HNPCC… hereditary non-polyposis colorectal cancer

HIF… hypoxia-inducible factor

HRP… horseradish peroxidase

HSGAG… heparin sulphate glycosaminoglycan

ICR… Institute for Cancer Research

Ig… Immunoglobulin

KD… knock down, down-regulation of a specific mRNA using shRNA or siRNA

MAPK… mitogen activated protein kinase

10% MEM… minimal essential medium containing 10% FCS

MET… mesenchymal-to-epithelial transition, the process when cancer cells settle down in a new tissue (also important in healthy cells during development of an organism)

MMP… matrix metalloproteases, enzymes that degrade ECM

MMR… mismatch repair

mTOR… mammalian target of rapamycin

MUW… Medizinische Universität Wien

NaF and Na_3VO_4… sodium fluoride and sodium orthovanadate, known phosphatase inhibitors (Ser/Thr and acidic phosphatases, the latter Tyr and alkaline phosphatases)

NC membrane… nitrocellulose membrane, an important component of the Western blotting technique on which proteins are transferred to

o/n… overnight, used time span in blotting and antibody incubation

PBS… phosphate buffered saline

PCR… polymerase chain reaction, technique used to amplify

PDGF… platelet derived growth factor

PDK1… phosphoinositide-dependent protein kinase 1, phosphorylates Akt

P/E… PBS/EDTA, phosphate buffered saline/ethylenediaminetetraacetic acid

PI-3K-Akt… phosphatidylinositol-3-kinase-Akt, a distinct pathway

PIP_2… phosphatidylinositol-4,5-biphosphate

PKB… protein kinase B

PKC… protein kinase C, also called Akt

PLCγ... phospholipase C γ, direct target of FGFR4

R… arginine

Rb… Retinoblastoma associated protein

RNA… ribonucleic acids

RNase… ribonuclease, degrades RNA molecules

rpm… revolutions per minute, measure to describe speed of centrifugation

RT… room temperature

RT-PCR… (realtime) reverse transcriptase PCR, method to analyse gene expression on transcriptional level

scr.… scrambled, control used in knockdown experiments

siRNA… short interfering RNA, to reduce mRNA levels

SD… standard deviation

SDS… sodium dodecyl sulfate

SFM… serum-free medium, MEM (usually) with 0.1% BSA used for starving purposes

SH… Src homology domain

TBS… tris(hydroxymethyl)aminomethane buffered saline

TBST0.1%... TBS with 0.1% of the polysorbat detergent Tween20

TF… transcription factor

TM… transmembrane

TGF… transforming growth factor

US… United States

V… Volt

VEGF… vascular endothelial growth factor

WHO… World Health Organisation

1 Introduction

Cancer or malignant neoplasm is a term used for a group of diseases characterised by progressively uncontrolled growth of cells and spread to other organs in the body. A delayed diagnosis makes a treatment difficult so that the disease can become life-threatening.

In general, two types of tumours may be distinguished: benign tumours and malign tumours. Both are abnormal tissue masses whose growth is increased and uncoordinated with the healthy tissue, thus proceeding autonomously. Benign tumours are not classified as cancer because they do not have the ability to migrate and build new tumours in distant organs of the body. But they can still acquire these capabilities which allow them to become malign tumours.

Malignant tumours display three key features: infiltration, destruction and metastasis. Infiltration is the process where the uncontrolled cells invade through the basal membrane in order to reach a blood vessel. For this purpose it destroys nearby cells and matrix material. Metastasis describes the process of migration and attachment to a new environment (other organ) that lead to daughter cells to make new colonies. How the fate of a cell changes to be malignant, is discussed more detailed later on (Schulte-Hermann 2009, Weinberg 2007).

1.1 Epidemiology of Cancer

Malignant neoplasms are the second most common cause of death especially in Western Europe and other developed countries (WHO).

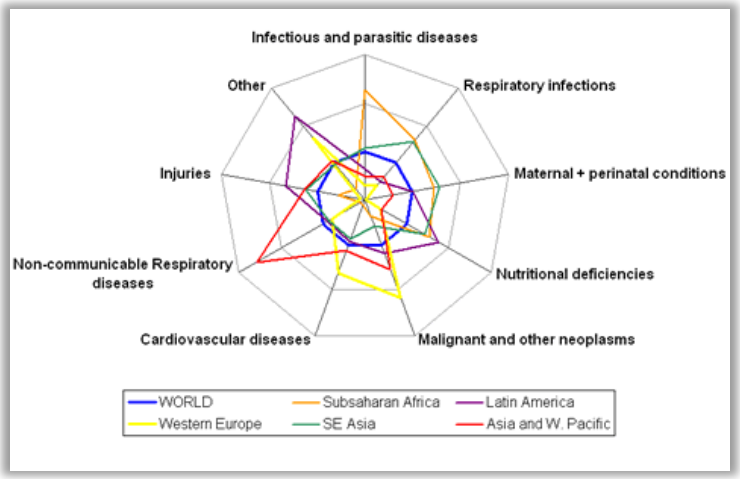


Figure 1-1 distribution of death causes worldwide (source: UCATLAS 2000)

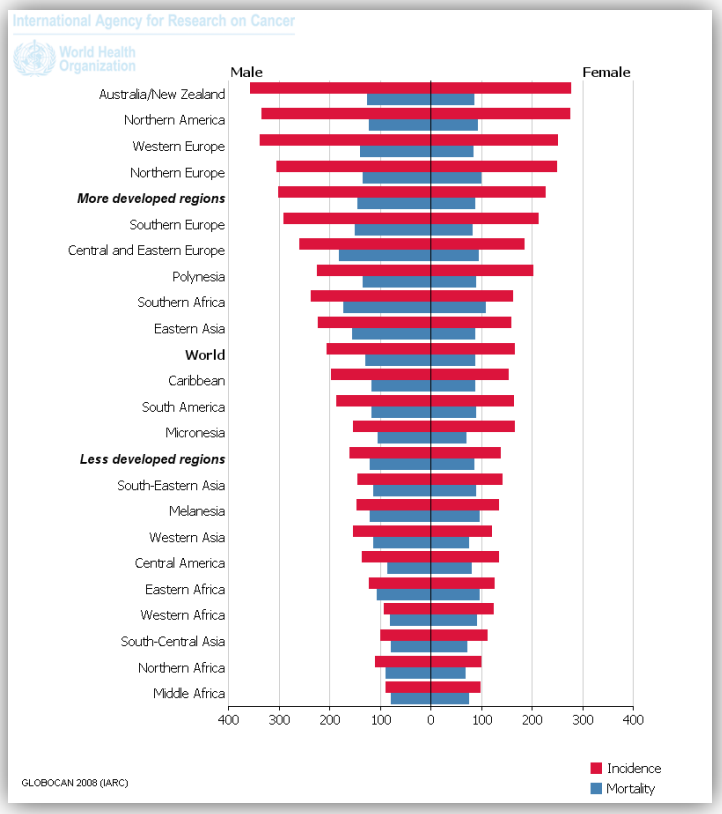
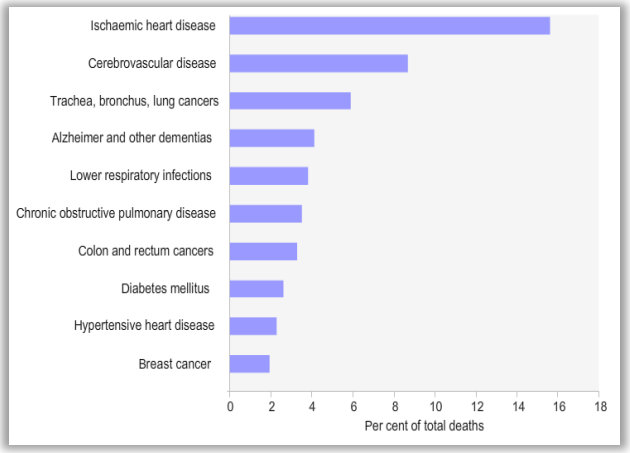


Figure 1-2 incidence and mortality rates of cancer
in different continental regions between men and women (source: WHO)

Incidence and mortality rates are high in New Zealand, Northern America and Western as well as Northern Europe while there African regions have lower incidence rates. (WHO, Dobrucalı 2011)

Every year more than 1.2 million people are diagnosed with cancer and in 2008, cancer accounted for about 7.6 million deaths. According to the WHO, tobacco is a major cancer risk factor together with alcohol use, wrong diet and a lack of physical activity. By the year 2030 the WHO expects cancer deaths to have risen to above 11 million.

Figure 1-3 top 10 causes of death
in high income countries (source: WHO 2008)



In Austria more than one third of deaths were caused by cancer in 2010 (Statistik Austria 2010). The risk of dying from cancer is the lowest for people in their twenties and increases with 30 years of age. According to the data of last year, people in their 50s have the highest risk.

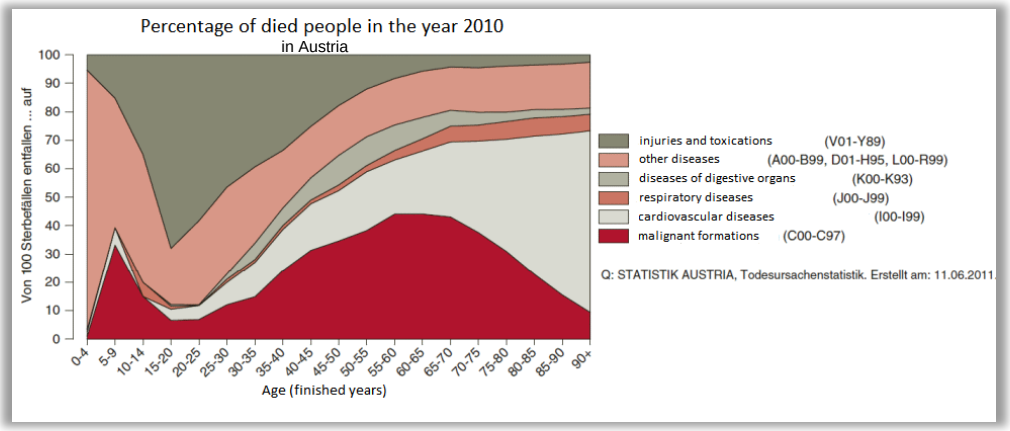
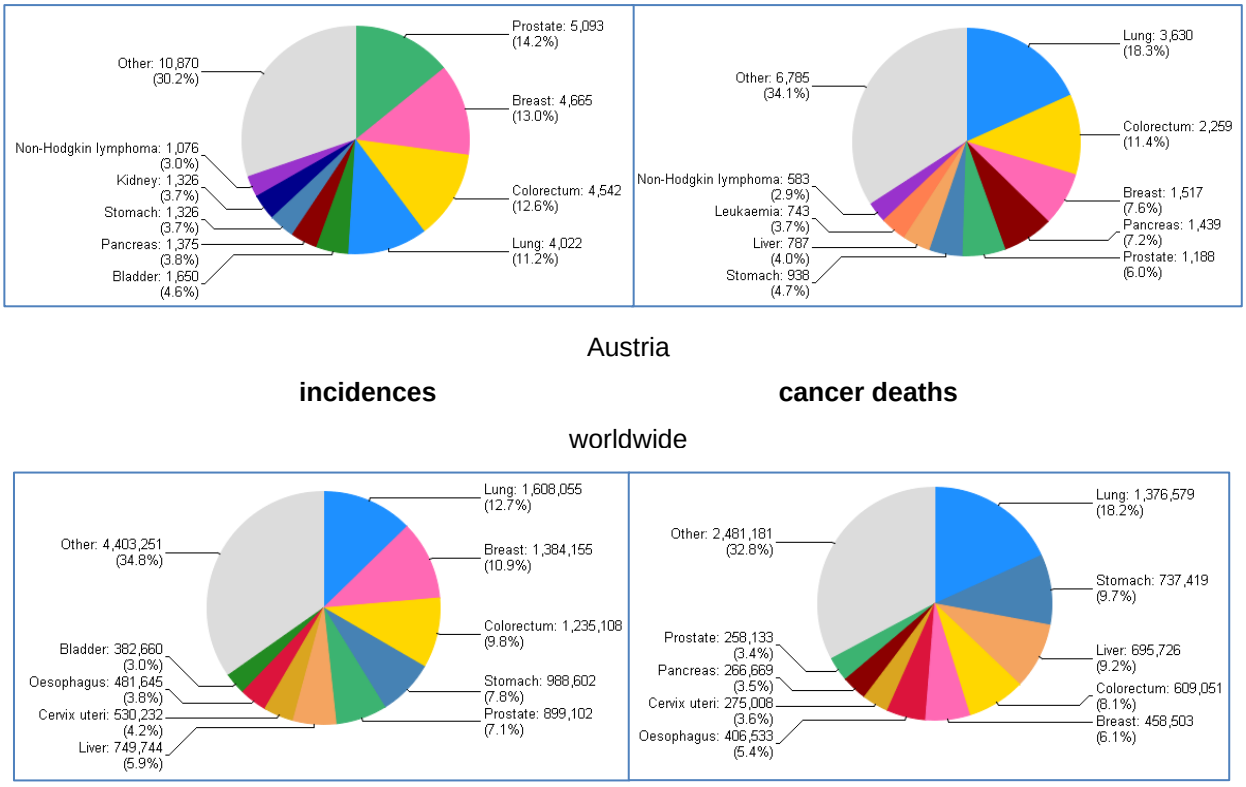


Figure 1-4 percent of death caused by listed reasons in 2010 for Austria (source: Statistik Austria)

1.2 Epidemiology of Colorectal Cancer

Among the different cancer types lung cancer takes the first place worldwide in both incidence and death rates, followed by stomach, liver and colorectal cancer (CRC). Every year more than 1.2 Million people are diagnosed with CRC which represents 9.8% of all cancers. When we compare incidence and mortality rates between male and female, we see prostate cancer with the highest incidence rates for men while for women it is the breast (WHO GloboCan 2008).



Austria

incidences **cancer deaths**

worldwide

Figure 1-5 incidence (left) and death rates (right) of various cancer types in Austria (up) and worldwide (lower graphics) (source: WHO GloboCan 2008)

In Austria colorectal cancer is the second cause of cancer deaths. More than 35,000 people develop cancer annually - 12.6% of them are diagnosed with CRC.

1.3 Risk factors of CRC

CRC development depends on the number of polyps in the colon which can form due to many risk factors including age, family history and immunologic state. In the following, some environmental and hereditary factors will be discussed.

1.3.1 Dietary factors

The frequency of CRC shows differences between people living in distinct geographic regions. In Australia, New Zealand, Northern America and Middle Europe the incidence is high while people living in Asia and Southern America are less likely to get CRC. This can be explained by the different dietary and environmental factors (partly based on the particular culture) of locals.

In 2010, Stefani *et al.* have shown in their factor analysis made in Uruguay (South America) that Western life style ("Western pattern") was associated with an increased risk of colon cancer while people having a prudent diet were inversely associated with rectal cancer. The prudent pattern was the factor that showed "*white meat, dairy foods, desserts, total vegetables, and total fruits*" while they labelled "*high loadings for red meat, total grains, and all tubers*" and "*high negative loadings on white meat and raw vegetables*" as the Western pattern.

This was also proven by the observation that people migrating from low risk to high risk regions are more likely to get CRC. To name one, it is known that Japanese immigrants in U.S. have developed higher risk of CRC within 2 generations and surpassed the risk level of Native Americans than Japanese living in their countries (Flood *et al.* 2000).

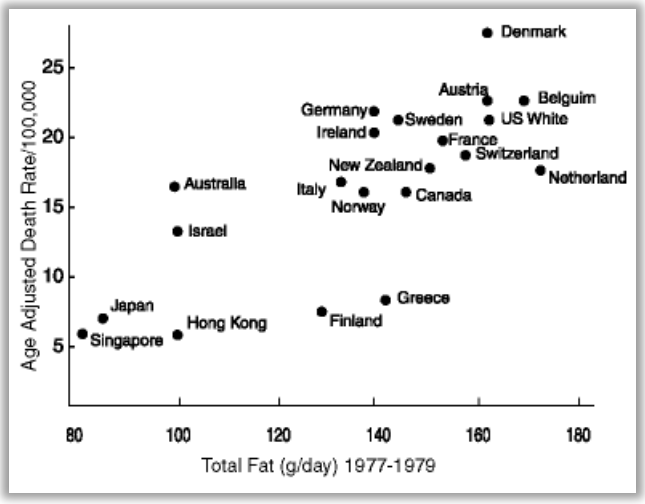


Figure 1-6 fat intake and risk of CRC in various countries between the years 1977-1979

1.3.2 Familial Adenomatous Polyposis

Familial adenomatous polyposis coli (FAP) is a hereditary colon cancer syndrom that is transmitted in an autosomal dominant manner. It results from a mutation or deletion in the *APC* gene lying on the long arm of the fifth chromosome (5q). The frequency is about one per 10,000 and leads to adenomas in teenage years (Menko 1993). At least 100 polyps are formed in the colon at young ages - one of which will surely develop into a carcinoma.

APC encodes for a tumour-suppressor protein and is mostly known for its role in the degradation of a protein called β-catenin (Wnt-pathway, detailed description in chapter 1.7.1). Loss of APC results in increased levels of free β-catenin leading to uncontrolled cell expansion. APC also interacts with the microtubules, so that in its absence results in defects of the mitotic spindle arise that can lead to chromosome abnormalities.

1.3.3 Hereditary Non-Polyposis Colorectal Cancer

Abbreviated as HNPCC, this type of hereditary colon cancer results from the loss of mismatch repair genes (MMR genes) and leads to an accelerated cancer development (in 1-2 years). The MMR system is a group of proteins that monitor any mistakes made during DNA replication. Recognition of mistakes, the affected replicon is removed and remade. When these proteins are missing or mutated, mutations accumulate much more rapidly and pass on to the next generation of cells during mitosis.

1.4 How does cancer develop?

Back in the 40's, the concept of initiation and promotion emerged. It was based on an experiment where a tumorigenic substance was applied exogenously on the skin of mice. This substance is known as an *initiator* of cancer and fails to cause tumours unless the dosage is high enough. Additional administration of agents triggering inflammation acts synergistically by creating a growth advantage for initiated cells, thus *promoting* tumour development. The sequence and interval of administration was quite important: mice did not develop any cancer when cancer initiating agents were applied after the promoters or when intervals between promoter applications were too long. However, even when considerable time had passed after initiation, appliance of promoting substances still led to cancer. This means that initiation is an irreversible step, affecting the genes, while promotion is just a trigger.

Tumour development happens in many steps (Figure 1-7) and begins with a slight change in one single cell which can be seen as the initiation step described above. Daughter cells gain proliferation advantage and accumulation of genetic alterations results in hyperplasia. In the next cell division, a dysplasia with a proliferative tendency is born. With more mutations the cells speed up their cell-cycle which gives new daughter cells the chance to escape from home through invading basal layer. This is the first step of invasiveness and allows the cells to penetrate into blood vessels to transport daughter cells. The process is called epithelial-to-mesenchymal transition (EMT) where the cell decreases production of adherence proteins and boosts expression of proteins that facilitate free movement (Schulte-Hermann).

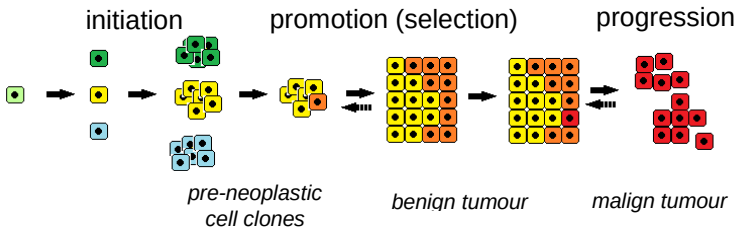


Figure 1-7 concept of initation and promotion
(own drawing, concept from Prof. Schulte-Hermann)

1.5 Cancer on the cellular level

Eukaryotic cells of multicellular organisms are regulated by numerous intra- and extracellular signalling pathways. Within a normal tissue in an adult organism multiple anti-proliferative signals are produced to maintain the cells in a quiescent state thus maintaining tissue homeostasis. Specific mitogenic growth signals are required to induce cell proliferation. Proliferation is limited by an intrinsic cell-autonomous program, and -after a defined number of divisions- cells stop growing and become senescent.

Also tumour cells progressively lose their sensitivity for induction of apoptosis. Each cell has sensors (death-receptors of TNF-receptor protein family and regulators of mitochondrial-permeability) that monitor the extracellular and intracellular environment. In case of damage, like a double strand break in the DNA, they can activate effector molecules which initiate apoptotic mechanisms and lead to cell death. The immune system has specialised cells which can promote apoptosis and remove the dead cells by phagocytosis.

These intra- and intercellular control mechanisms have to be overcome to produce a cancer cell so it can survive and proliferate.

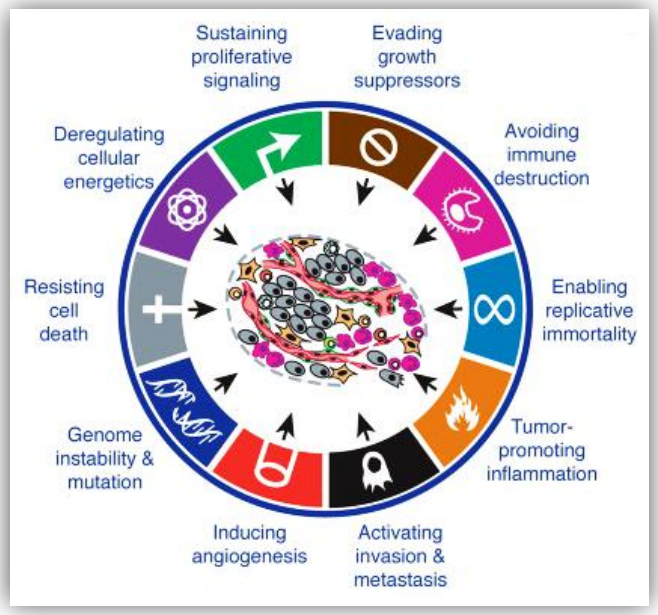


Figure 1-8 nine characteristics of a cancer cell
(source: Hanahan and Weinberg, 2011)

Douglas Hanahan and Robert Weinberg have postulated nine characteristics of malignant cells (Figure 1-8) which will be shortly reviewed and important actors highlighted in the following passages.

1.5.1 Production of growth signals

As described earlier normal cells are kept in a steady homeostatic state between growth signals and anti-growth signals by other cells in the environment. The former are called growth factors and include the family of epithelial growth factors (EGF), the platelet-derived growth factors (PDGF) and the fibroblast growth factor family (FGF) as well as the tumour growth factors (TGFs) and vascular endothelial growth factors (VEGF and numerous others). These extracellular proteins act through binding to receptors on the surface of a cell which activate intracellular proteins by phosphorylation in a signalling cascade and lead to the expression of different target genes.

With time, cancer cells can acquire the ability to produce the growth factors to stimulate their own proliferation (autocrine stimulation). Alternatively, they may induce other cells in their environment to produce growth factors. Other strategies can also be mutation(s) in the receptor molecule that fires permanently and independently from ligand binding. It is known that B-Raf has an activating-mutation (V600E) in 40% of the human melanomas (Maurer *et al.* 2011, Davies and Samuels 2010, Baccarini 2005). Evidence of alterations downstream of the MAPK pathway having wildtype B-Raf (and N-Ras) also exist (Curtin *et al.* 2005). Although rare, chromosomal translocations that lead to fusion genes with B-Raf or C-Raf have been described to have a pro-proliferative role and support migration in prostate cancer, gastric cancer and melanoma (Maurer *et al.* 2011, Baccarini 2005).

1.5.2 Loosing sensitivity to anti-growth signals

It is not sufficient to up-regulate growth promoting factors because there are counteracting mechanisms involving tumour suppressor proteins. Therefore cancer cells deactivate these proteins by different mechanisms. The retinoblastoma-associated protein (Rb) is one example.

Transcription factors (TFs) that are important during the G1 phase of the cell cycle have to bind to DNA in order to activate transcription. These are bound to the Rb-protein and phosphorylation of this protein is required to release the TFs and

permit their binding to respective DNA sequences and start mitosis. Any critical mutation of Rb leading to a release of TFs provides the cancer cell to proceed the cell cycle. The Rb gene is inactivated in all retinoblastomas, in 60% of osteosarcomas and 30% of breast cancers as well as bladder carcinomas. (Hanahan and Weinberg 2011)

1.5.3 Replication ad infinitum

Healthy cells have an intrinsic switch that tells them when to stop dividing. When the so-called “Hayflick limit” is reached, cells undergo senescence – a state growth arrest that ultimately leads to cell death.

Shortly reviewed, telomeres are the chromosome ends with various proteins that form the telomeric loop in order to protect these from degradation. They consist of many repeats of a hexanucleotide with a length of 10kb (in humans). Because no RNA-primer can attach for the synthesis of the Okazaki fragments at the chromosome ends, part of the continuous strand remains unreplicated, shortening the chromosome with every replication cycle until they are too short for binding of the protective proteins. If this happens, the open DNA end is recognised as DNA-damage and the cell cycle is arrested. This problem is solved by an enzyme called telomerase which can produce new telomere repeats from an RNA template. However, in normal adult cells telomerase is not expressed enough so that aging occurs.

Progressed neoplastic cells can upregulate their telomerase expression or lengthen their telomeres by different mechanisms. A well-known method is called the alternative lengthening of the telomeres (ALT) where parts of other chromosomes are broken to fuse by recombination (breakage-fusion-cycles).

1.5.4 Apoptosis in cancer

Active cell death is needed during development of an organism and throughout its whole life to remove abnormal and damaged cells that cannot be repaired in order to protect the whole body.

On the molecular level, apoptosis is induced by typical signalling pathways: signals bind to receptors which trigger activation of intracellular effectors that lead to cell death. An apoptotic cell changes its morphology and typically shrinks. A condensation occurs and membrane-enclosed fragments (apoptotic bodies) form.

This is recognized by nearby cells as well as phagocytes that engulf the cell and prevent leakage of toxic contents that could otherwise harm the environment.
In tumour cells this mechanism is a great barrier for growth and survival. A well-known strategy is mutation or loss of the tumour suppressor gene p53 (see chapter 1.7.3) but also a constitutive activation of the PI-3K-Akt pathway is helpful to prevent apoptosis (Hanahan and Weinberg, 2000).

1.5.5 Neo-Angiogenesis

Angiogenesis is the process where new blood vessels are built from existent vessels. This normally happens during the development and wound healing.
However, neoplastic cells need external resources to survive and therefore start the neo-angiogenetic program. They do so by upregulating angiogenesis promoting factors including vascular endothelial growth factor (VEGF) (Ferrara *et al.*, 2003), hypoxia inducible factor-1 (HIF-1) (Pugh and Radcliffe, 2003) but also matrix degrading proteins and proteins from the fibroblast growth factor family (FGF-2, FGF-1) that can stimulate angiogenesis-supporting cell types.
As is in the name, VEGF stimulates (especially vascular) endothelial cells and prevents apoptosis through the PI-3K-Akt pathway (discussed in chapter 1.7.2.2). The hypoxia inducible factor (HIF) gets stabilized when tissue parts are short on oxygen levels. From there it can enter the nucleus to activate target genes like VEGF (Ziello *et al*., 2007).

1.5.6 Ability to metastasize other organs

Metastasis is the process that enables malignant cells to invade tissues and colonize new organs. For that purpose, the cell undergoes an epithelial-to-mesenchymal transition (EMT) and exploits a gene transcription program that is usually activated during development to conduct the progenitor-cells to their right places. Decreased cell-cell contacts and increased expression of proteins aiding in the flexibility are characteristics of this expression pattern.
Thus, alterations affect mostly proteins involved in cell-cell and cell-ECM (extracellular matrix) adhesion. Classic example is the E-cadherin expression which is important for sustaining the quiescent state of epithelial cell sheets through cell. In numerous studies, either decreased or aberrant expression of E-Cadherin is found in most cancer types.

1.5.7 New characteristics

The already described hallmarks are still not the whole story. Additional characteristics emerge as cancer research progresses. Genome instability supports tumour progression together with a steady inflammation that harbours many types of cells capable of supporting tumour tissue with all the discussed characteristics (e.g. Morbus Crohn). It is also new that tumour cells change their energy metabolism, (even under aerobic conditions) preferably to glycolysis.

1.6 Colon and Rectum – a short anatomic overview

Before taking a look at colorectal carcinogenesis, it is necessary to shortly examine the digestive system and architecture of the colon.

When soluble and chewed food is transported through the channel called oesophagus (Figure 1-9 left) to the stomach it is formed to a mash and enriched with gastric acid. Pancreatic juice and bile are added to this cocktail in the small intestine. Here, nutrients are extracted and sugar and amino acids delivered to the blood while lipids go through the lymphatic vessels for further processing (degradation). The last station is the colon where mainly the resorption of water takes place.

The colon is part of the large intestine. With about 1.5 – 2 m length and 2.5 - 5 cm diameter it ascends on the right side, proceeds crossing to the left side, descends and warps to the back to form the sigmoid colon (Figure 1-9 right). This extended part is called rectum and is about 12 cm long. Here the indigestive remains are formed to a tube to excrete them through the anus.

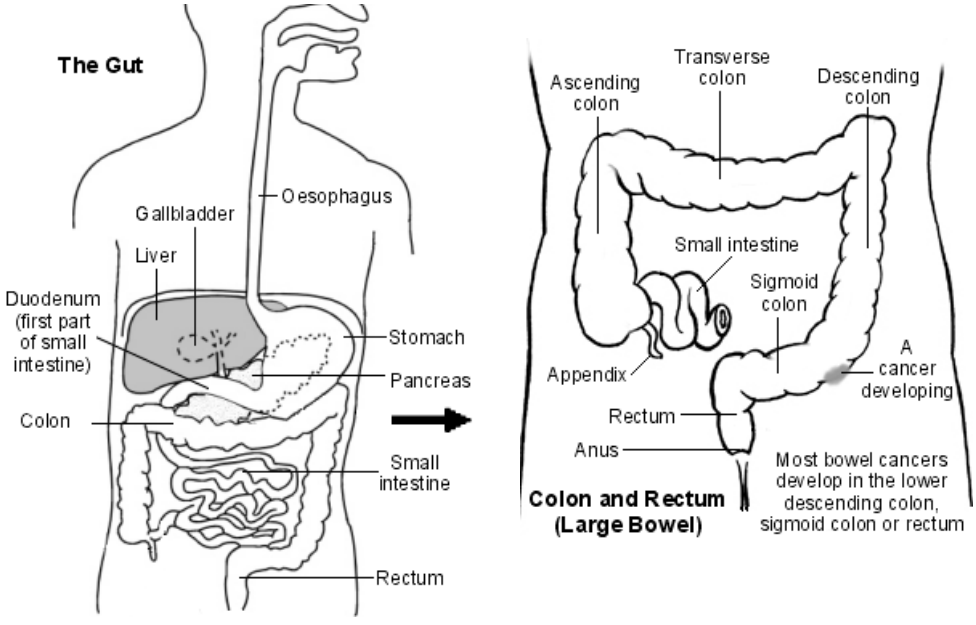


Figure 1-9 left anatomical position of colon and rectum in the body, right an image of the large bowel (source: patient.co.uk)

A single-layer epithelium and an interlayer of connective tissue together make up the intestinal mucosa. An outer muscle layer with circular and longitudinal muscles provides contraction with the latter expanding and the former constricting. This ensures transport of the food mash along this channel system (Brockhaus 2011).

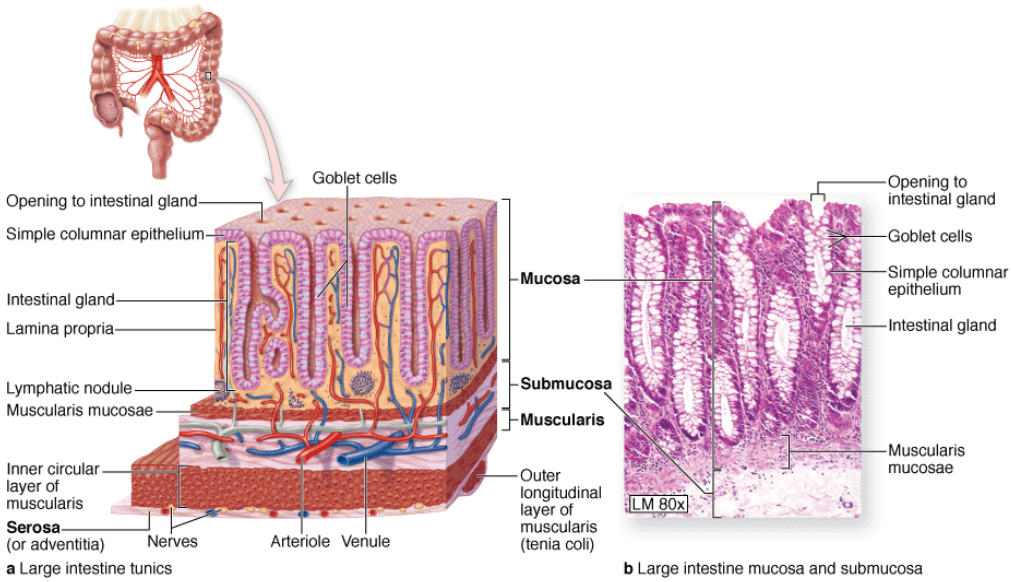


Figure 1-10 composition of the colon wall with 4 layers (written in bold)
(source: accessmedicine.com – Junqueira's Basic Histology 12th ed.)

Resorption is accomplished through different transport mechanisms by cells called enterocytes that lie along the columnar epithelium. Goblet cells secrete mucus to keep the intestinal lumen wet. In this way the remaining material is passed on to the rectum.

Renewal of the cells takes place every 4 - 5 days from a pool of stem cells (v. d. Flier and Clevers 2009) that lie at the bottom of the 0.2 - 0.4 mm deep tubular cavities called crypts. From an asymmetric division transit-amplifying cells arise and proliferate every 12 hours until they turn into non-proliferating differentiated daughter cells. The stem cells can be regarded as progenitors of the different cells in the colon as they will differentiate to absorptive cells, goblet cells, hormone-secreting cells and Paneth cells.

The cell organisation in the colon is so that new cells push old ones upwards and the higher a cell is in the crypt, the more differentiated it is. Terminally differentiated cells at the top undergo apoptosis.

Any malfunctioning in the stem cell renewal system can lead to a growth advantage and uncontrolled cell division which may initiate tumour development (Pinto and Clevers 2005).

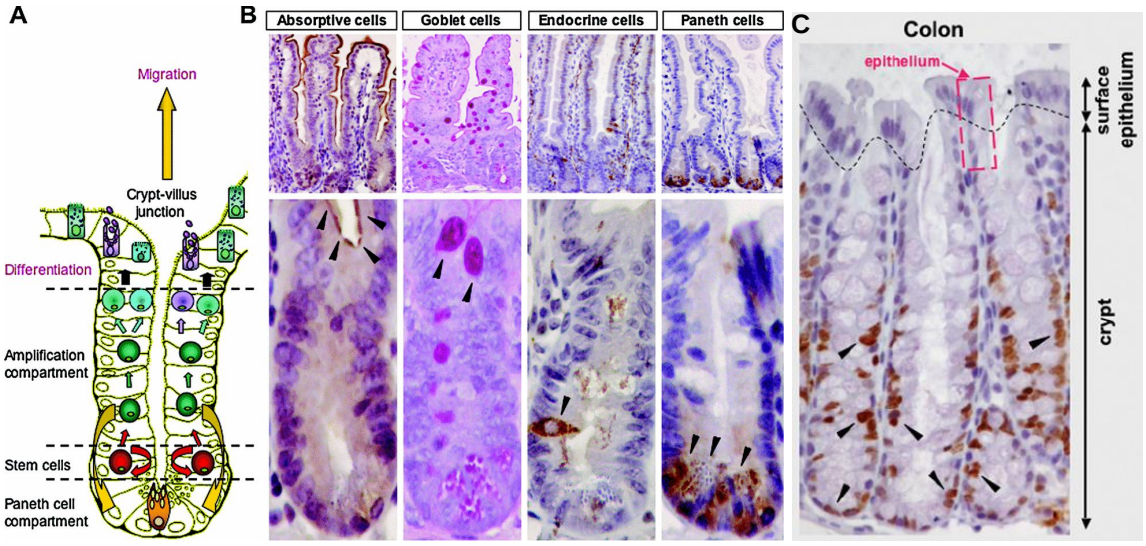


Figure 1-11 cells in the gastrointestinal tract
(A) cellular organisation in the small intestine (similar in the colon (without villus junction),
(B) histologic pictures of the small intestine with arrowheads showing cell types,
(C) Ki-67 staining of the colon to show proliferating cells (arrows)
(source: Pinto and Clevers, 2005)

1.7 Colorectal Carcinogenesis

Normal colon epithelium is renewed every 4 - 5 days by stem cells in the bottom of the crypts (v. d. Flier and Clevers 2009). Any mutation leading to abnormal growth can cause development of colonic lesions called aberrant crypt foci. Further growth and proliferation leads to the formation of adenomatous polyps.

When existent adenomatous polyps or intramucosal lesions progress by penetrating the submucosa, we can speak of an invasive cancer. This step will be then followed by a local expansion and later involve lymph nodes and the blood vessels. The tumour may also infiltrate the serosa and metastasise to other organs – most commonly the liver and lungs. Since the rectum is lacking the serosa, metastasis occurs easily.

Fearon and Vogelstein have proposed a genetic model to answer the question, by which mutations CRC development is triggered (Figure 1-12). Based on the published data and the observations, their idea was that CRC develops stepwise

through the accumulation of distinct genetic alterations - the first being a mutation or complete loss of the *APC* gene on the short arm of chromosome 5, that confers growth advantage to the epithelial cells. Eventually the increased growth produces adenomas that can progress by acquiring mutations in the proto-oncogene K-Ras and the gene protector p53. In addition, loss of various tumour suppressor genes on the long arm of chromosome 18 accounts for tumour progression.

The role of these gene products in cancer and colorectal cancer will be described in the following sections.

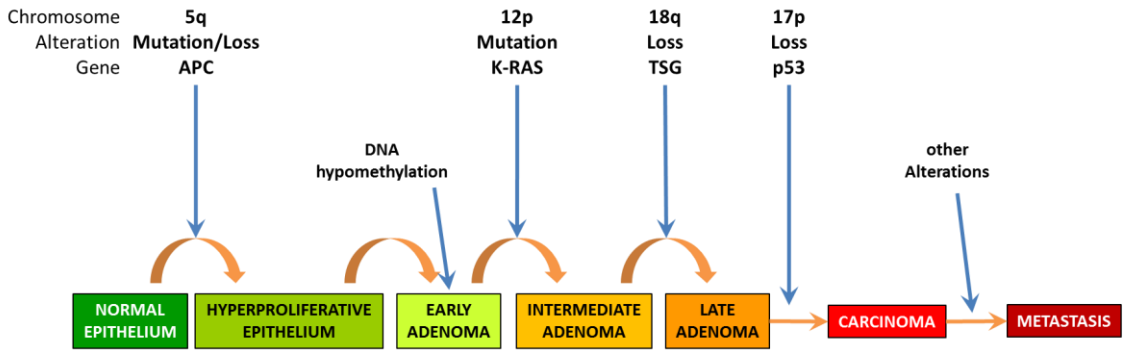


Figure 1-12 genetic model for colorectal carcinogenesis proposed by Fearon and Vogelstein (1990; own drawing)

1.7.1 Wnt-pathway

The Wnt pathway is named after the Wnt proteins that were discovered as a key player in the wing development in Drosophila. With distinct and often overlapping functions, the 19 human Wnt proteins are equipped with a fatty acid chain on their N-terminal site and can activate at least 3 different intracellular pathways – one of them being the Wnt/β-catenin pathway (often termed canonical Wnt pathway).

Wnt binds to a seven-pass transmembrane receptor called Frizzled and the co-receptor LDL-receptor-related protein (LRP). Recruitment of the scaffold protein Dvl (Dishevelled) to the cytoplasm inhibits the axin/GSK-3β/APC complex which cannot phosphorylate β-catenin for degradation any more. The resulting stabilisation leads to translocation of β-catenin to the nucleus and associates with transcription factors (TCF/LEF) that begin transcription of target genes like the proto-oncogene Myc, Cyclin D1 (one of the cell cycle regulators), MMP7 (matrix-

metallo-proteinase-7) and PPARδ (a nuclear hormone receptor to regulate peroxisome number and size).

The canonical Wnt pathway plays an important role in developmental processes, especially the axis determination in the body and during limb development.

Because it regulates expression of proteins involved in growth, the cell cycle and degradation of the ECM, the Wnt pathway is an important key pathway to promote cancer development (Alberts *et al*. 2008).

Mutations in the APC gene lead to hyperproliferative epithelium and are one of the triggering events for CRC development. Germline mutations often cause the FAP syndrome (discussed earlier in chapter 1.3.2) which most probably lead to cancer formation. (Fearon and Vogelstein 1990) In about 80% of CRC cases both copies of the APC gene is inactivated (Alberts *et al*. 2008).

1.7.2 Ras: two pathways to activate

Ras proteins are small GTPases and play important roles in cell differentiation, proliferation and migration as well as adhesion. The family consists of many members, amongst them the proto-oncogenes N-Ras, H-Ras and K-Ras.

Ras is a protein controlling more than one pathway and correct functioning is crucial for cell homeostasis. In about 30% of colorectal cancers, mutated K-Ras is found and supports abnormal growth. (Calcagno *et al.* 2008)

1.7.2.1 MAPK signalling pathway

The MAPK signalling cascade is a prototypic signal transduction pathway, which transmits signals from outside of the cell to its interior and can be activated through various signals including mitogens, osmotic stress and pro-inflammatory cytokines.

Upon receptor tyrosine kinase stimulation by extracellular ligands, the receptors dimerise and recruit adaptor protein complex GRB2-SOS. SOS is a guanine nucleotide exchange factor (GEF), and induces the release of GDP from Ras, which is then free to bind GTP. Activated Ras recruits the protein kinase Raf to the membrane and enhances its activity, thereby triggering a phosphorylation cascade going through Mek and resulting in Erk phosphorylation and activation. Activated Erk phosphorylates its targets in the nucleus, cytoplasm, cytoskeleton and mitochondria. This has an effect on crucial cell properties, like proliferative potential, survival and motility (Baccarini 2005).

1.7.2.2 PI-3K-Akt pathway

Another pathway that Ras activates is the phosphatidyl inositol-3-kinase-Akt (PI-3K-Akt) pathway that is important for growth and survival. It does so by directly activating PI 3-kinase that phosphorylates membrane-bound $PI(4,5)P_2$ to generate $PI(3,4,5)P_3$. This serves as a docking site for two other protein kinases – the protein kinase B, also called Akt, and PDK1 (phosphoinositide-dependent protein kinase 1). A kinase that is able to phosphorylate Akt on a serine (usually mammalian target of rapamycin or mTOR) is needed in order to be activated by PDK1. This phosphorylation leads to a conformational change of Akt so that a threonine is exposed and can be phosphorylated by PDK1. Active Akt dissociates and phosphorylates target proteins. A protein called Bad, for example, promotes apoptosis by inhibiting an apoptosis-inhibitory protein. Akt can release this protein by phosphorylation of Bad and indirectly prevent apoptosis (Alberts *et al.* 2008).

1.7.3 Tumour suppressor p53

Named after its molecular weight, p53 plays an important role in protecting the DNA from damage and together with p63 and p73 belongs to the p53 protein family. All three members can be expressed in various isoforms – two of them differing in their N-terminal domain: the TA-isoform and the partly dominant-negative ΔN-isoform. (Levine *et al.* 2011)

In a healthy cell p53 watches over the chromosomes and prevents mitosis when DNA is damaged. For this purpose increasing p53 concentrations arrest the cell in the G1 phase through upregulation of p21 that inhibits cdk2/4/6 complexes. Consequently, these kinases cannot phosphorylate and release Rb protein, until DNA repair proteins fix the problem and p53 is degraded to allow mitosis. If this is not possible, the cell is immediately sent to apoptosis to prevent any transfer of genetic alterations to daughter cells. Any malfunction of p53 enhances tumour formation.

In about 80% of sporadic cancers a defect is found in p53. 40-50% of CRC have p53 mutations while in adenomas this occasion is rare. This demonstrates that p53 mutations are important at later stages of CRC development (Iacopetta, 2003).

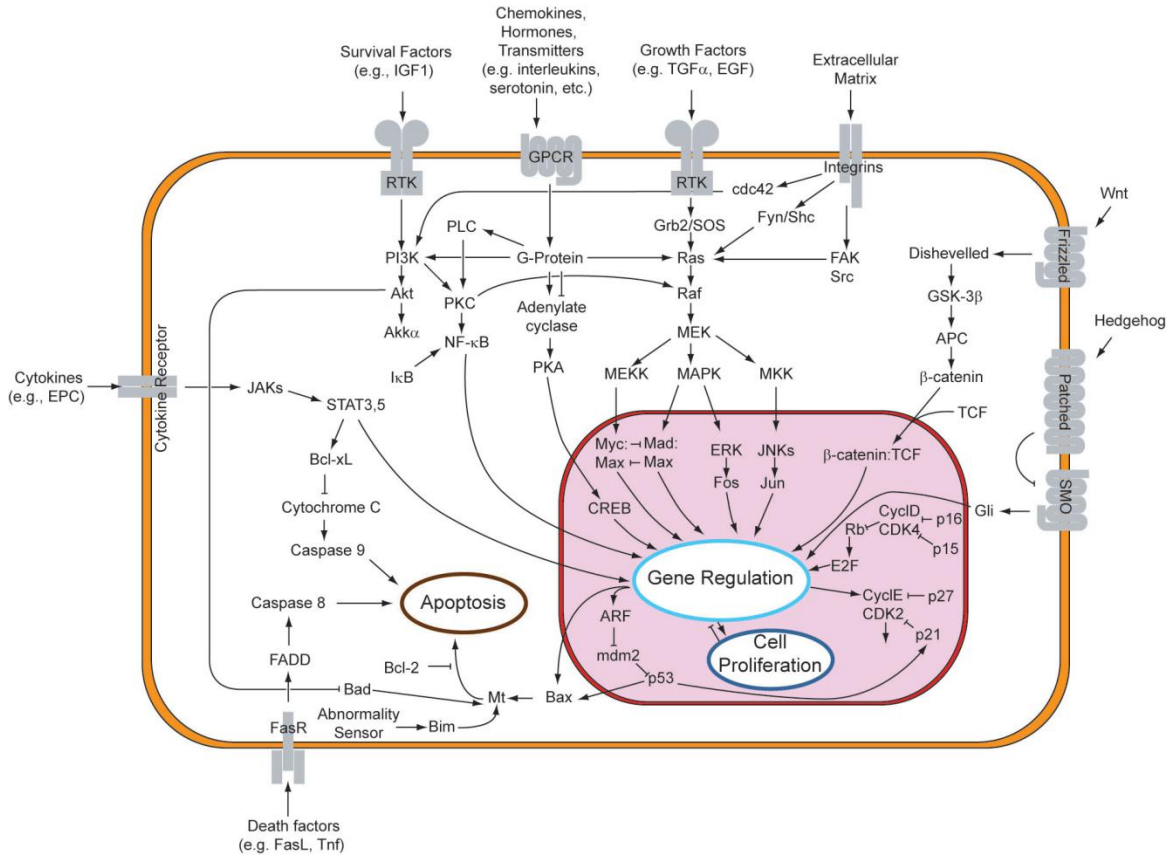


Figure 1-13 summary of signal transduction pathways

1.8 Fibroblast Growth Factors and their receptors

The fibroblast growth factor family consists of 4 FGF homologous factors (FHFs) and 18 FGFR ligands which are essential for cell growth, morphogenesis, wound healing and angiogenesis. In most tumour types the expression of FGF and their receptors is deregulated. Based on their sequence and general function, they are divided into three groups:

- FGF homologous factors FGF11-14, also called intracellular FGFs
- canonical subfamily consisting of the subfamilies 1, 4, 7, 8 and 9
- hormone-like FGFs (hFGFs): FGF19 (FGF15 in mice), 21 and 23

1.8.1 FGF structure

FGFs consist of 12 antiparallel β-strands (120-130 amino acid long, core region) with different N- and C-termini. In the core region a binding site for heparin sulphate glycosaminoglycan (HSGAG) can be found that is positively charged in paracrine FGFs. In the hFGFs there is a structural difference that reduces HSGAG binding and leads to an endocrine secretion. This work focuses on FGFs 18 and 19.

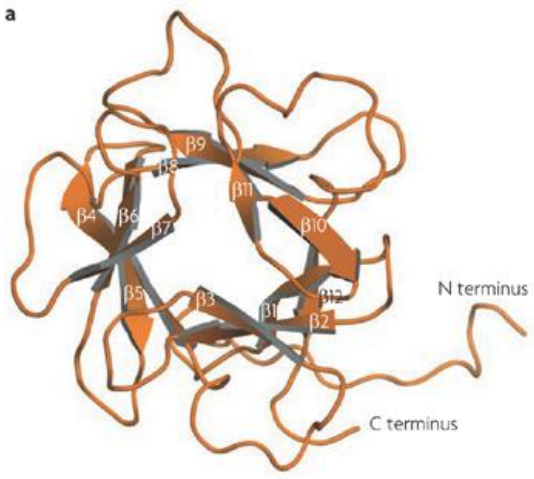


Figure 1-14 FGF structure
(source: Beenken and Mohammadi, 2009)

1.8.1.1 FGF8 subfamily

The FGF8 subfamily belongs to the canonical FGF subfamily and consists of FGF8, FGF17 and FGF18. All members have a signal sequence and act in a paracrine way preferably through binding to FGFR c isoforms (Beenken and Mohammadi 2009, Zhang *et al.* 2006).

The FGF8 gene can be found on the long arm of the chromosome 10 and was first identified as androgen-induced growth factor (AIGF) found in the conditioned medium of the androgen-dependent mouse mammary carcinoma. It preferably binds to FGFR3c but was also found associating with FGFR4. FGF8 plays an important role in forebrain patterning and in the development of brain, limbs, ears and eyes. Mice lacking FGF8 cannot gastrulate and defect FGF8 has been seen to cause reduction in limb bud size and the Kallmann syndrome (a pathologic condition with olfunction deficiencies and decreased gonad function) (Yun *et al.* 2010, Beenken and Mohammadi 2009, Zhang *et al.* 2006, Powers *et al.* 2000).

FGF17 is found on the short arm of chromosome 8 and has affinity for FGFR3c but can also bind to FGFR4, FGFR2c, FGFR1c and FGFR3b. Development of cerebral and cerebellar structure is impaired in FGF17 knockout mice (Powers *et al.* 2000, Yun *et al.* 2010, Zhang *et al.* 2006).

The long arm of the chromosome 5 harbours the gene coding for the 207 amino acid long protein, called FGF18. FGF18 was found to be primarily expressed in the lungs and kidneys but also in the heart, testes, spleen, skeletal muscle and brain. It binds preferentially to FGFR3-IIIc but also has affinities for FGFR4 and weak affinity for FGFR2c (Yun *et al.* 2010, Hu *et al.*1998).

FGF18 stimulates NIH3T3 cells, osteoblasts, chondrocytes and glial cells and when secreted, can influence tumour cells and cells of the connective tissue in the micro environment. It is an essential mitogen in the embryonic development of the organs as well as the cartilage and bones. Loss of FGF18 leads to severe skeletal diseases (Zhang *et al.* 2006).

Together with FGF16, FGF20 and the controller protein sprouty4, FGF18 is a direct target gene of the β-catenin/Tcf4 complex (Katoh and Katoh 2006*, Shimokawa *et al.* 2003). Constitutive activation of the Wnt-pathway (see chapter 1.7.1) – like in most intestinal cancers – leads to an up-regulation of its expression. It was found upregulated in many adenomas and adenocarcinomas of the colon and was survival-supporting *in vitro* under starving conditions, thus exerting

autocrine effects. Also FGF18 treatment of colonic fibroblasts lacking serum is known to restore migration. FGF18 knockdown in the colorectal adenocarcinoma cell line via siRNA led to decreased cell proliferation and reduced colony formation. (Sonvilla *et al.* 2008, Shimokawa *et al.* 2003)

1.8.1.2 Hormone-like FGF subfamily

Endocrine secretion of the hFGFs (FGF19, FGF21 and FGF23) arises from the weak binding affinity of HSGAGs that leads to an increased diffusion. Instead of HSGAGs, the protein klotho augments binding to FGFRs. Klotho consists of a single-pass transmembrane domain and an extracellular domain with two internal repeats called KL1 and KL2 having β-glycosidase similar sequence. It is found membrane bound and is secreted into the blood and cerebrospinal fluid. It can associate with K^+, Na^+ ATPases to regulate calcium homeostasis. (Beenken and Mohammadi 2009, UniProtKB KLOT_HUMAN, Imura *et al.* 2004, Kurosu and Kuro-o 2009)

The FGF19 gene can be found on the long arm of chromosome 13 and is expressed in adult gall bladder epithelium and in different fetal tissues like skin, retina and the small intestine. FGF19 is the human orthologue of FGF15 in the mouse and plays an important role in the heart development and the brain development during the embryogenesis. Mice lacking FGF15 display increased bile excretion and have cardiac defects, thus not viable. It binds highly specifically to FGFR4 and regulates bile acid metabolism in the liver (Itoh 2010, Inagaki *et al.* 2005, Ornitz and Itoh 2001, Xie *et al.* 1999).

FGF21 is involved in the regulation of the energy metabolism. Its expression is induced under fasting conditions in adipose tissue and the liver through PPARγ and PPARα. Expression was also found in thymus, in pancreatic islet β-cells and in skeletal muscle via activation of Akt. FGF21 needs the co-factor β-klotho to bind FGFR4 and activate FGF signalling. Weak interaction with the c isoforms of FGFRs1-3 could also be detected (Beenken and Mohammadi 2009, Kurosu and Kuro-o 2009, Zhang *et al.* 2006).

FGF23 is important for the regulation of the phosphate homeostasis and when mutated, leads to hypophosphataemic rickets, symptoms like growth retardation, bone softness and decreased phosphate levels in the blood. It is highly expressed in the bone. FGF23 acts in the kidney as a vitamin D regulator via down-regulation of metabolizing enzymes and inhibits parathyroid hormone secretion

which leads to decreased phosphate uptake from the bone. FGF23 can bind to FGFR2c, 3c and FGFR4 but is mostly effective when bound to the FGFR1c-Klotho complex. Like mice having mutated Klotho, FGF23$^{-/-}$ mice display increased expression of the enzyme 1α-hydroxylase that catalyses inactive vitamin D to active calcitriol (1,25$(OH)_2D_3$) (Beenken and Mohammadi 2009, Kurosu and Kuro-o 2009) .

subfamily	members	bind to…[a]	plays important role in…[b]
FGF1	FGF1 FGF2	all FGFRs FGFR1c, 3c > 2c, 1b, 4Δ	not established; loss of vascular tone and slight loss of cortex neurons in FGF2$^{-/-}$ mice
FGF4	FGF4 FGF5 FGF6	FGFR1c, 2c > 3c, 4Δ	Limb development, cardiac valve formation hair growth cycle regulation myogenesis
FGF7	FGF3 FGF7 FGF10 FGF22	FGFR2b > 1b	inner ear development branching morphogenesis " presynaptic neural organizer
FGF8	FGF8 FGF17 FGF18	FGFR3c > 4Δ > 2c > 1c >> 3b	development of brain/eye/ear/limb cerebral/cerebellar development bone development
FGF9	FGF9 FGF16 FGF20	FGFR3c > 2c > 1c, 3b >> 4Δ	gonadal development, organogenesis cardiac development neurotrophic factor
FGF19*	FGF19 FGF21 FGF23	*FGFR1c, 2c, 3c*, 4Δ (*weak activity*)	bile acid homeostasis, lipolysis, gall bladder fasting response, glucose homeostasis, lipolysis and -genesis phosphate and vitamine D homeostasis

Table 1-1 information about canonical FGFs and *hFGFs, KO = knockout

(source: [a] Zhang *et al.* 2006; [b] Beenken and Mohammadi, 2009)

1.8.2 FGFR structure

Fibroblast growth factor receptors belong to the family of receptor tyrosine kinases of the immunoglobulin (Ig) superfamily which are a class of enzyme-coupled receptors (Alberts *et al*. 2008, Grose and Dickson 2005).

There are seven main FGFRs that are encoded from four FGFR genes. They consist of a cytoplasmic tyrosine kinase domain with an activation loop, a single-pass transmembrane domain and three loop-shaped Ig ectodomains which are termed D1-D3. These have an acid box containing an acidic, serine-rich sequence between D1 and D2 that may be important for receptor autoinhibition. The ligand binding and specificity is regulated in the D2-D3 fragment.

FGF receptors 1-3 can be transcribed through exon skipping to create isoforms that lack the D1 domain and/or the acid box. Isoforms b and c are produced by

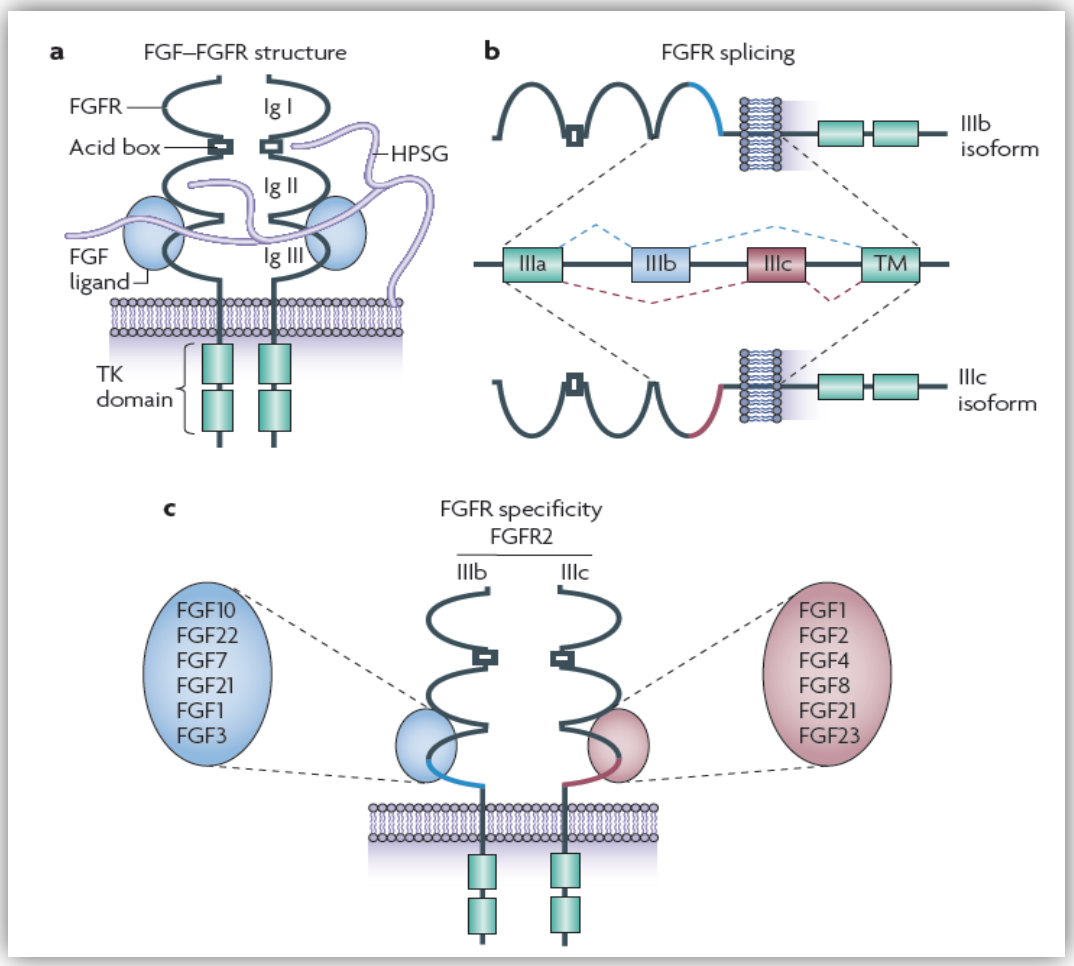


Figure 1-15 a structure of FGFRs, **b** alternative splicing creates isoforms, **c** specifity difference between the two isoforms shown in the example of FGFR2 (source: Turner and Grose, 2010)

alternative splicing of the D3 domain and display distinct FGF binding affinities. While the b isoform is mostly found in epithelial tissue, expression of the c isoform happens usually in mesenchymal tissue and gets activated by a ligand produced in the opposite tissue. This allows signalling and thus control of epithelial and mesenchymal tissue. An exception is the FGF1 that can bind to both b and c isoforms (Beenken and Mohammadi 2009, Grose and Dickson 2005).

1.8.3 FGF-FGFR binding and signalling

ECM-bound FGFs are released by a carrier protein called FGF-binding protein (FGFBP) and bind to HSGAGs. This complex then interacts with the FGFR and activates a signal transduction cascade. For this purpose, FGFRs dimerise and get activated via transphosphorylation on their A loop. Further phosphorylations serve as binding and interaction sites for different target proteins (Beenken and Mohammadi, 2009).

FGFR substrate 2 (FRS2) is a protein directly associating with the intracellular domain of the FGFR. When phosphorylated by the receptor, it can activate two distinct pathways. Association with the adaptor proteins SOS and Grb2 leads to the activation of the MAPK pathway (described earlier in chapter 1.7.2.1). Grb2 can also recruit Grb2 associated protein (Gab1) that starts the PI-3K-Akt pathway (Turner and Grose 2010).

Phospholipase C γ (PLCγ) is another protein which, through its Src homology 2 (SH2) domain, can bind directly to the intracellular phoshotyrosine residue near the carboxyl terminus. This starts an enzymatic reaction were phosphatidylinositol-4,5-biphosphate (PIP_2) is hydrolysed to IP_3 and diacylglycerol (DAG). These two products can now serve for different functions: while IP_3 induces Ca^{2+} from the smooth endoplasmic reticulum, DAG induces translocation and activation of protein kinase C (PKC). PKC can now phosphorylate Raf, thus activating MAPK signalling which supports not only proliferation but was also found to be important in the migration of cells for the primitive streak during chicken development. Also activation of STAT and ribosomal protein S6 kinase 2 is known to happen - depending on cellular context. (Hardy *et al.* 2011, Turner and Grose 2010)

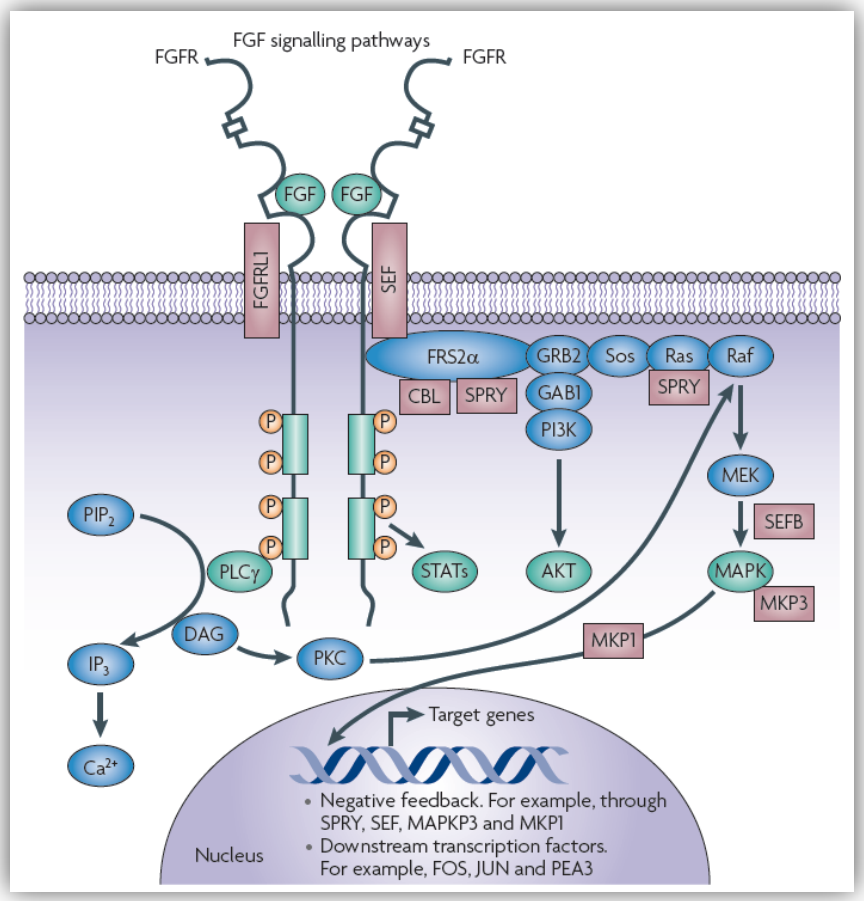


Figure 1-16 pathways activated through FGF ligand binding
(source: Turner and Grose, 2010)

1.9 FGF-FGFR in cancer

All receptor-ligand systems are suitable as therapeutic targets for cancer cells since control of these is essential for survival and progression.

They can benefit from activating mutations of FGFR that leads to dimerization and constitutive activation of the receptor independent from ligand binding. Mutations in the extracellular (EC) domain of FGFR2 are known causes for pathologic conditions like the Crouzon's syndrome (changes in the growth pattern of the skull (craniosynostosis) and other facial symptoms) through autonomous covalent dimerization of the receptor. FGFR3 mutations in the transmembrane domain were found in nearly all cases of achondroplasia (hereditary short stature) (Turner and Grose 2010, Beenken and Mohammadi 2009).

Translocations combining the N-terminus of a transcription factor with the receptor kinase domain are also known to drive intracellular signalling. Constitutive dimerization of the kinase domain of FGFR1 is an example and leads to the 8p11 myeloproliferative syndrome. Mutations that lead to deficient auto-inhibition of the FGFR2 kinase domain are another example and cause skeletal deformities but were also seen in endometrial cancers (Turner and Grose 2010, Beenken and Mohammadi 2009, Katoh 2009).

Also gene amplifications, a known mechanism of cancer cells, can result in sustained signalling through overexpression of the receptor. FGFR specificity can also be changed through abnormal splicing. Development of other craniosynostical syndromes may happen through gain-of-function mutations in the ectodomain of FGFR2c that facilitate binding of FGFR2b binding ligands.

Elevation of FGF release from the ECM but also the stimulation of stromal cells to secrete more FGFs, are known mechanisms of deregulation. Amplification of FGF3 was found in about 15-20% of human breast cancers and correlated with increased invasiveness in node-negative breast carcinoma (Wesche *et al.* 2011).

This process can also lead to metastasis since the ligands can act on endothelial cells and initiate angiogenesis (Turner and Grose 2010, Beenken and Mohammadi 2009).

The autocrine acting of the ligands may also inure to the benefit of cancer progression: increased production of FGF ligands can be used to self-stimulate cancer cells (Turner and Grose 2010).

However, also evolutional changes like polymorphisms may contribute to the progression of pathologic conditions, especially during cancer development. Short nucleotide polymorphisms in the intron 2 of the FGFR2 gene were associated with breast cancers carrying mutations in the BRCA2 gene (Turner and Grose 2010).

1.9.1 FGFR4 and the G388R polymorphism in cancer

FGFR4 plays an important role in the liver and regulates systemic cholesterol and bile acid metabolism as well as lipid metabolism. Disruption was found to affect organs like gall bladder and lead to increased cholesterol metabolism. Unlike FGFR1-3, it is expressed as a single isoform but displays paralogy to FGFR3c (Beenken and Mohammadi 2009).

In cancers of the prostate and breast as well as in rhabdomyosarcoma, gynaecological and gastric cancers, FGFR4 was found to be upregulated (Ye *et al.* 2011, Taylor *et al.* 2009, Roidl *et al.* 2009, Wang *et al.* 2008, Sahadevan *et al.* 2007, Jaakkola *et al.* 1993).

As described earlier, FGF19 binding to FGFR4 is highly specific and antibodies blocking this interaction were found to inhibit growth of colon carcinoma xenografts (Desnoyers *et al.* 2008).

Using biochemical and genetic methods, activation of FGFR4 was found to inhibit NFκB signalling and negatively affect proapoptotic signalling (Drafahl *et al.* 2010).

Recently, an FGFR4 polymorphism is gaining importance in the cancer research. A single point mutation in the transmembrane domain at position 388 causes replacement of glycine to an arginine. About 45% of all white population examined were hetero- or homozygous for the R388 allele (Wang *et al.* 2008).

Association between this polymorphism and cancer prognosis was investigated by Frullanti *et al.* in a meta-analysis including cancers affecting brain, breast, colorectal, head and neck, larynx, lung, melanoma, prostate and sarcomas. They showed that FGFR4-R388 led to an increased risk of poor survival than people homozygous for FGFR4-G388. FGFR4-R388 homozygosity was also significantly associated with nodal involvement (Frullanti *et al.* 2011).

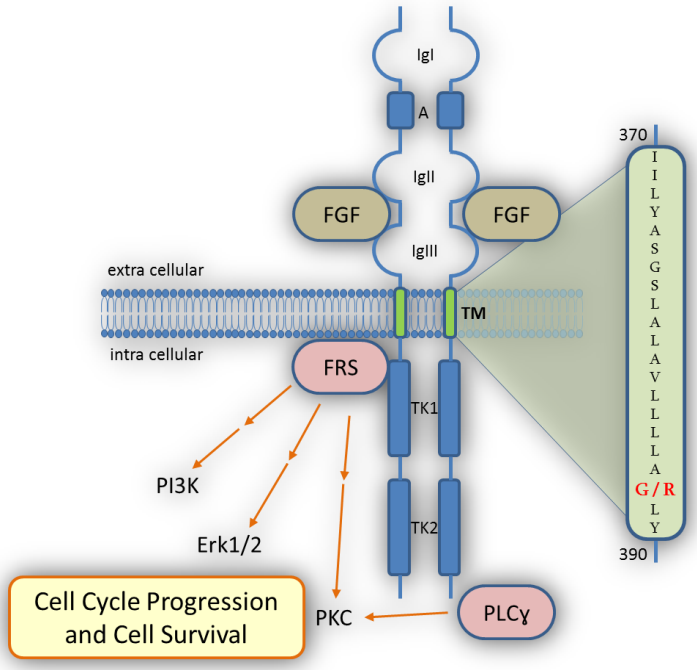


Figure 1-17 FGFR4 and location of the G388R polymorphism

(source: C. Heinzle)

Table 1-2 information about different pathologies involving FGFRs

(source: Turner and Grose 2010)

receptor	amplification	mutation	translocation
FGFR1	Breast, ovarian, bladder cancers and rhabdomyosarcoma	melanoma	Myeloproliferative syndrome chronic myeloid leukemia
FGFR2	Gastric and breast cancers	Endometrial and gastric cancer Germline SNP in the 2nd intron: increased breast cancer incidence	
FGFR3	Bladder, salivary adenoid cystic cancers	bladder cancer (mostly non-muscle invasive), cervical cancer, myeloma, spermatocytic seminoma	Myeloma Peripheral T cell lymphoma
FGFR4		G388R SNP on the TM domain: better progression of cancers of the breast, prostate, neck and lung	

2 Aim of the thesis

The exact role of the FGFR4 G388R polymorphism in CRC is not yet sufficiently understood.

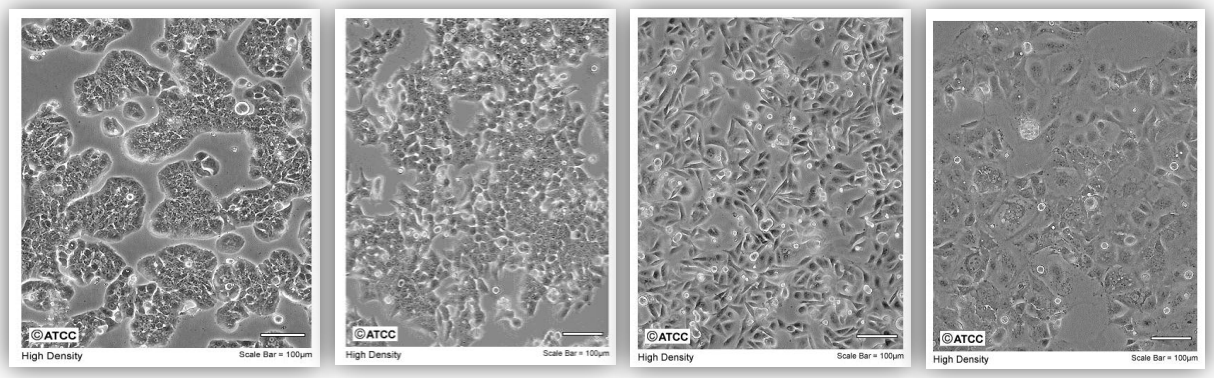
The aim of the thesis was to investigate this in CRC cell lines. Using FGFR4 overexpressing colorectal carcinoma cell lines, the main focus of attention was to examine effects on survival, growth and different malignant characteristics like migration, invasion and attachment as well as the EMT state. Whether FGFR4 overexpression alters intracellular behaviour and if so, through which signalling pathways this happens, was another goal to be achieved.

3 Materials and Methods

3.1 Cell lines

Cell lines that were obtained from the American Type Culture Collection include ATCC® numbers (Table 3-1).

Table 3-1 appearance of cell lines obtained from ATCC



HT29	HCT116	SW480	Caco-2
HTB-38™	CCL-247™	CCL-228™	HTB-37™

3.1.1 HT29

HT29 is a colorectal adenocarcinoma cell line with an epithelial morphology and adherent growth. The cells were first isolated in 1964 from a 44 year old Caucasian woman. They express c-myc, K-ras, H-ras, N-ras, fos and overexpress mutated p53.

Cells were cultured in 10%MEM and grown to 80% confluence prior to splitting (1:5 - 1:10). Renewal of the medium occurred every two days and 0.45 mg/ml G-418 was added to transfected cells.

3.1.2 HCT116

The growth of HCT116 cells is adherent and their morphology epithelial. They are colorectal carcinoma cells and were derived from an adult male. Among TGFβ1 and TGFβ2 they also express mutated ras.

Passaging was done 1:10 - 1:20 after full confluence in 10%MEM while renewing medium once in a week. Transfected cells received 0.5 mg/ml G-418 for selection purposes.

3.1.3 SW480

Dividing every 24 hours SW480 cells are primary colorectal adenocarcinoma cells at an early invasive tumour stage and originate from a 50 year old Caucasian male. With a positive expression profile of c-myc, mutated Ki-ras, fos and mutated p53 they grow adherent and display an epithelial morphology.

New medium was added once a week and passaged 1:10 - 1:20 at a confluency of 90 - 100%. Cells containing vector got 0.3 mg/ml G-418 additionally.

3.1.4 Caco-2

Obtained from a 72 year old Caucasian male, Caco-2 cells display epithelial morphology and grow adherent with a doubling time of about 62 hours. They are derived from colorectal adenocarcinoma and, upon confluence, differentiate into enterocytes.

This cell type is cultured in 20%MEM and grown to a confluency of 90-100%. Medium was exchanged twice a week.

cell line	origin	description
LT97-1	ICR MUW	colon microadenoma cell line
LT97-2	ICR MUW	later passage of LT97-1
T84	72 year old male (ATCC)	CRC derived lung metastasis
SW620	51 year old male (ATCC)	colorectal adenocarcinoma at Dukes' type C (lymphatic invasion)
AKH4	Caucasian male (ICR MUW)	CRC derived liver metastasis
AKH14	Caucasian male (ICR MUW)	Different passage of AKH4 cells, less differentiated

Table 3-2 information about other used cell lines

3.2 Cell culture

Cells were cultured either in 10 cm or in 6 cm petri dishes depending on their usage under default conditions: 37°C 5% CO_2. 10% MEM refers to Eagle's minimal essential medium (Sigma-Aldrich) with 10% of fetal calf serum (PAA). After washing with 10mM PBS/EDTA (Merck), cells were detached with trypsin/PBS (PAA). Counting of cells occurred with a counting chamber using a microscope.

3.3 Transfection

3.3.1 Stable FGFR4 expressing cell lines

To create the cell lines overexpressing FGFR4^{arg} or FGFR4^{gly} allele, we transfected HT29, HCT116 and SW480 cells using lipofection.

This method uses liposomes to introduce vectors of interest into a cell. The phospholipid bilayer of these liposomes allows a cell fusion, thereby releasing the DNA-cargo into the cytoplasm.

We obtained TransFectin™ Lipid Reagent (Bio-Rad, Philadelphia, PA, US) and seeded 3x10^5 cells per well of a 6-well plate. After 24 – 48h we renewed the medium and prepared transfection reagent. 10µl TransFectin was added to a total volume of 250µl SFM. 2µg plasmid was prepared in an extra tube also in 250µl SFM. The plasmids were kindly provided by Axel Ullrich (Max-Planck-Institut, Germany) and contained an FGFR4^{gly} or FGFR4^{arg} gene at the multiple cloning site of a A3 vector (Figure 3-1). An ampicillin resistance marker for selection in E. coli and a neomycin resistance for selection of stably expressing higher cell clones were also on the plasmid. The DNA and Transfectin solutions were mixed and after 20' of incubation, 1.5ml was added to each well of the plate. The medium was exchanged 6h later to minimize toxicity on cells.

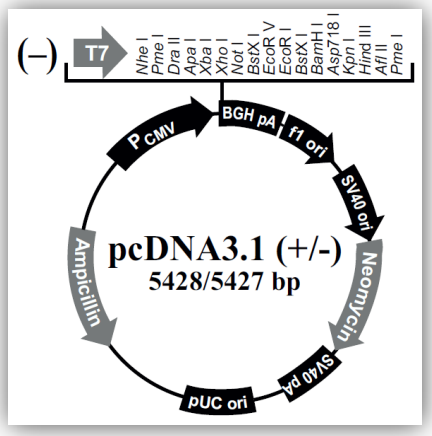


Figure 3-1 map of the used vector

The cells were transferred to 10cm petri dishes after 24h, and 48h later the aminoglycoside antibiotic Geneticin (G-418) was added to select cells stably expressing the vector. For each cell line different concentrations were used:

- 1.0 mg/ml for HCT116
- 0.6 mg/ml for SW480
- 0.9 mg/ml for HT29 cells

3.3.2 FGF18 knockdown

24 hours after setting 5x10^5 cells to 6cm PDs the cells were transfected with FGF18 siRNA oligos. For this purpose, 9µl silentFect reagent was diluted with serumfree medium containing 0.1% BSA to a volume of 750µl and added to equal volume of 40nM siRNA (scrambled siRNA served as control). Cell medium was removed and 1.5ml fresh medium was added. After 20 minutes incubation of the transfection solution, 1.5ml was added to each plate for a total volume of 3ml.
24 hours later medium was changed to SFM 0.1% BSA to starve cells and protein was isolated for western blot analysis.

3.4 Viability

3.4.1 Neutralred assay

1 – 2 x10^3 cells were seeded in 24-well plates for a total volume of 500µl.
For stimulation experiments the cells were starved in SFM for another 24hours and then stimulated with 10ng/ml either FGF18 or FGF19 (human, recombinant, P10052, P9122 from Biovision) in SFM 0.1%BSA. Medium without factor served as a negative control.
At the time point given in the figures (24 hours for standard experiments, daily for growth curves) medium was changed to freshly made neutralred (Merck) solution (in 0.05 mg/ml serum free medium containing 0.1% BSA) and incubated at 37°C for 2 hours. The cells were then washed with PBS and cell bound neutral red was extracted by neutralred-fix solution.

Neutralred-fix solution	
1%	acetic acid
70%	ethanol
	bidist. water

Table 3-3 neutralred-fix solution

This assay is based on the reaction in the lysosome of the cells that results in a colour change to pink. The colour intensity correlates with the concentration of living cells and is measured at the wavelength 620nm (and 562nm as reference).

3.5 Migration in vitro

Analysis of migration behaviour of cells was done by using the following assays described below.

3.5.1 Migration Assay – Transwell assay

This assay works with microporous filters of 8µm pore size obtained from BD. Triplicates for each approach in a 24-well plate were used and each well was filled with 800µl of the appropriate medium. 10^4 cells in 200µl medium were seeded into the inserts. Depending on the cell line used, the cells in the well were fixed with methanol after 3 – 4 days, stained with crystal violet and counted by visual means.

3.5.2 Scratch Assay

For this assay we seeded 1 - 1.5 x10^6 cells into each well of a 6-well plate and a scratch was made using sterile white tips after adhesion phase (24 - 48h). Washing at least once with P/E removed detached swimming cell clusters and dead cells. Medium was changed to either 10% MEM for positive control, starving medium for negative control or starving medium with FGF18 or FGF19 in appropriate concentrations. Photos were taken at 4x magnification during the following day up to 24h. For each approach three wells were used and scratch width was measured from four different positions of each well using Photoshop. The percentage of original scratch width was calculated ($100 - \frac{current\ width}{original\ width}$).

3.5.3 Invasion assay

Cell culture inserts (for 24-well plates, obtained from BD Biosciences) were coated with 28µl collagen (from Sigma, 0.01% diluted 1:12.25 in 1xPBS) and incubated in the appropriate plate at 37°C o/n. The next day 4x10^4 cells were seeded in 200µl 10% MEM into the inserts with 800µl 20% MEM in the wells. Filters were removed after 3-4 days. Cells were fixed with methanol after several days of growth. Crystal violet was used for staining.

Testing approaches were done with different coating reagents (Table 3-4). Matrigel™ was obtained from BD Biosciences.

Table 3-4 dilutions of used coating reagents

12/24µl Matrigel	12/56µl Collagen
2	24.5
1	12.25
No dilution	6.125

3.6 Attachment assays

3.6.1 Clonogenicity assay

100 cells were plated into each well of a 6-well plate, using triplicates for each experimental group. This simulates the metastasis where small numbers of tumour cells have to settle down to a new environment and grow autonomously without any contact to other cells. Therefore this method is also known as “colony formation assay”.

To select cells attached within 24h, we exchanged medium to 10%MEM after 24h to stimulate cell growth. After colony formation (usually five days later), we fixed the cells with methanol and stained with crystal violet. The cell colonies were counted by visual means.

3.6.2 Adhesion assay

100 cells were applied for each well of a 96-well plate, using 6 wells for every approach. 24 hours later the wells were washed and fixed with methanol. After staining with crystal violet the number of cells was determined using microscopy at 10-fold magnification.

3.7 Gene expression methods

3.7.1 Standard Realtime-PCR

3.7.1.1 RNA isolation

Cells were seeded in 10cm plates at a concentration of $2x10^6$ and RNA isolated using Trizol (peqGold Trifast, peqLab). After 5 minute incubation in 1ml Trizol the cells were scraped and transferred into eppendorf tubes. The tubes were vortexed with chloroform and left for phase separation for ten minutes at room temperature. They were centrifuged at 15,000 rpm for 15 minutes at 4°C and upper phase was transferred to new tubes. RNA was precipitated with isopropanol

for ten minutes and centrifuged at 12,200 rpm at 4°C. The pellets were washed with EtOH (70% in bidest. water treated with DEPC for RNase free working) and centrifuged again for 15 minutes. After resuspension in 30µl water (DEPC treated) RNA was denaturated at 65 - 70°C for 10 minutes.

3.7.1.2 Complementary DNA synthesis

RNA concentration was calculated using nanoDrop spectrophotometer (peqlab) (µg/µl= $\frac{\text{OD 260nm x dilution factor x 40}}{1000}$) and 1 - 5µg of RNA in 10µl was used for cDNA synthesis. 100µM Random Hexa-Primer (Fermentas) were added and incubated for 5 minutes at 70°C. Mastermix was prepared containing buffer, dNTP Mix and RNase Inhibitor (both Fermentas) and then added and incubated at 25°C for 5 minutes. Finally reverse transcriptase (Revert Aid™ M-MuLV, Fermentas) was added to start complementary DNA synthesis. The following program was then applied:

- 10 minutes at 25°C
- 60 minutes at 42°C
- 10 minutes at 70°C

80µl DEPC treated bidistilled water was added and stored at -20°C.

3.7.1.3 RT-PCR

Polymerase chain reaction is a method to amplify DNA sequences. The main steps are denaturation of the double stranded DNA, annealing of the primers and elongation by using dNTPs. Repeating these steps, results in an exponential increase in the amount of the DNA sequence to be amplified.

Realtime-PCR uses labelled primers for realtime measurement of DNA amount. The outcome is the Ct value which is the lowest cycle number in the linear increase of the signal-cycles curve.

For the actual PCR, 96-well plates (MicroAmp® Fast Optical 96-Well Reaction Plate) were used and mixed solutions (Table 3-5) were pipetted to a total volume of 10µl for each well.

4.5 µl cDNA-Mix	5.5 µl TaqMan®-Mix
1.0 µl cDNA 3.5 µl NCF water	0.5 µl TaqMan® (ABI) 5.0 µl TaqMan® Supermix

Table 3-5 used amounts for RT-PCR

Taqman probes contain the dye 6-carboxyfluorescein (FAM) and the appropriate primers. Every analysis was made in triplicates to exclude mistakes made during pipetting. The amplification and quantification was done on an ABI Prism 7000 instrument which reads out the intensity of the signal. Non-template controls served as negative controls without any cDNA.

Table 3-6 used cycling conditions for realtime-PCR and used probes for analyses

step	condition
initiation	10' at 95°C
denaturation	15'' at 95°C
annealing/elongation	1' at 60°C
40 cycles	

TaqMan probes
FGFR4 (Hs00242558_m1)
FGF18 (Hs00818572_m1)
FGF19 (Hs00391591_m1)

3.7.2 Allelic Discrimination

In order to make sure our generated cell lines were overexpressing the correct FGFR4 alleles a different type of RT-PCR protocol was used with appropriate primers for the two alleles. This analysis was done on an ABI Prism 7500 device. For this purpose a calibration curve made by Christine Heinzle was used to read out the $FGFR4^{arg}$/$FGFR4^{gly}$ ratio in the cells (Table 3-8, see thesis C. Heinzle 2011).

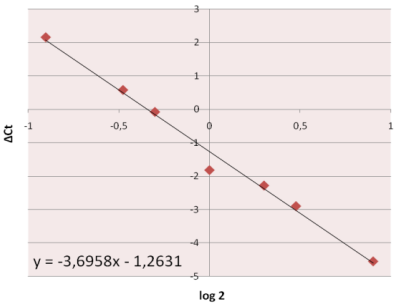
Reagent	[stock] µM	[final]	µl / reaction
Genotyping MM	2x	1x	5.00
Forward primer	100	900	0.09
Reverse primer	100	900	0.09
Probe Arg388 (FAM)	100	100	0.01
Probe Gly388 (VIC)	100	100	0.01
NCF water			2.80
cDNA			2.00
		Total	10.00

step	condition
initiation	2' at 50°C
denaturation	10'' at 95°C
annealing	15'' at 95°C
elongation	1' at 60 °C
40 cycles	

Table 3-7 RT-PCR protocol and cycling conditions for allelic discrimination

Table 3-8 calibration curve for calculation of Arg/Gly ratio in a sample (kindly provided by Christine Heinzle)

2.687	Arg
0.888	08:01
0.440	03:01
0.274	02:01
0.149	01:01
-0.323	01:02
-0.501	01:03
-0.927	01:08
-2.269	Gly



3.7.3 Immunological detection of proteins

3.7.3.1 Protein isolation

About $5x10^5$ cells were used for 6cm or $2x10^6$ for 10cm petri dishes and medium was changed for starving medium. Then cells were stimulated for 5 and 15 minutes with 10ng/ml either FGF18 or FGF19. For analysis of phosphorylated proteins, we added the phosphatase inhibitors 1M sodium fluoride (NaF, 1µl/ml) and 1M sodium orthovanadate (Na_3VO_4, 10µl/ml) immediately after the incubation period. These reagents inhibit Ser/Thr and acidic phosphatases as well as Tyr and alkaline phosphatases. The plates were washed with ice cold 1xTBS containing NaF and Na_3VO_4 (pH 7.6) and proteins were isolated by scraping under cold conditions. After a short centrifugation step the pellet was resuspended and incubated in 40µl lysis buffer at 4°C for 30 minutes. To aid in cell lysing, the protein lysates were vortexed at least three times in between. Ultrasonic treatment was done and centrifuged at 15,000rpm for five minutes to remove insoluble fragment.

lysis buffer	
1M	Hepes
1M	NaCl
0.5M	EDTA
1M	NaF
1M	$NaVO_4$
1M	$MgCl_2$
	complete
	Igepal
	Bidist. water

Table 3-9 lysis buffer contents

The supernatants containing the proteins were transferred to new tubes and protein concentration was measured using equal amounts of Bio-Rad Coomassie's Protein Assay Dye Reagent. Known BSA concentrations were used for calibration curve and absorption was measured at 590nm.

Table 3-10 pipetted amounts for determination of protein concentration

[µg/µl] BSA	0	1	2	3	4	5	6
Aqua bidest [µl]	9	8	7	6	5	4	3
Lysis buffer [µl]	1	1	1	1	1	1	1
BSA [µl]	0	1	2	3	4	5	6
	Total 10µl						

3.7.3.2 SDS-PAGel electrophoresis

Protein concentrations were calculated and the right amount for 50µg protein was prepared with half of 4x sample buffer.

Table 3-11 sample buffer and TBS contents

4x sample buffer	1x TBS (1L)
4% SDS	0.02 M Tris
20% Glycerol	1.40 M NaCl
10% 2-mercaptoethanol	Bidist.water
0.125M Tris/HCl pH 6.8	HCl to pH 7.6
Bromphenol blue	
Bidist. water	

For protein separation 12% separating gel and 4% stacking gel was made and incubated at RT, each for one hour to polymerize. Protein lysates containing 50µg protein were denaturated for five minutes at 80°C and adjacently loaded to the gel. We used 5µl PageRuler™ Prestaind Protein Ladder (Fermentas) as a marker.

Table 3-12 contents of used buffer

10x Electrophoresis buffer	10x Blotting buffer
14.4% Glycin	14.4% Glycin
3.0% Tris	3.0% Tris
1.0% SDS	0.2% SDS

The gel was run in 1x electrophoresis buffer at 60V for 15 minutes to concentrate proteins and then the voltage was increased to 125V for another 1¾ hours to separate proteins according to their molecular weight. Used device was purchased from Bio-Rad.

3.7.3.3 Blotting on membrane

For each gel six pieces of filter paper and a piece of Polyvinylidene Fluoride (PVDF) membrane was cut to the appropriate size and soaked in blotting buffer (1:10 diluted from 10x concentrated buffer and 20% methanol added). The membrane was activated in methanol prior to sandwich assembly and then blotted o/n at constant 25V using PerfectBlue™ Tank Electro Blotter (peqlab).

Table 3-13 assembly of the blotting sandwich

black/(-) pole
thick pad
2x filter paper
gel
membrane
2x filter paper
thick pad
red/(+) pole

The membrane side containing proteins was labelled at one edge and flushed with bidistilled water. To test the blotting success we incubated the membrane in a solution of Ponceau Red stain (Sigma).

The membrane was then washed in TBST 0.1% and blocked with 5% BSA to exclude unspecific binding of antibody molecules. After a repeated washing step the first antibody (Table 3-14) was incubated o/n.

The blots were then washed again and incubated with an HRP conjugated secondary antibody (goat anti-rabbit IgG-h+l in 3% BSA TBST0.1%, BETHYL laboratories and goat anti-mouse, Thermo Scientific) for an hour. Before detection, the blots were washed again and reagents prepared. The detection was done by incubating the blots for at least 5 minutes with a mix containing equal amounts of luminol solution and peroxide solution (Amersham ECL™ Prime Western blotting detection reagent, GE Healthcare). The blots were incubated in stripping buffer for 30-45’ to remove unwanted antibodies and blocked anew. Methanol was applied before storage at 4°C.

Table 3-14 information about used antibodies

origin	antibody	company	… dilution …
rabbit	pErk1/2 (Thr202/Tyr204)	Cell signalling (US)	1:2000 in 3% BSA TBST 0.05%
rabbit	total Erk1/2	upstate (US)	1:5000 in 5% BSA TBST 0.05%
rabbit	pS6 (Ser235)	Signalway Antibody (US)	1:1000 in 3% BSA TBST 0.1%
rabbit	total S6	Cell signalling (US)	1:2000 in 3% BSA TBST 0.1%
rabbit	pGSK3β	Cell signalling (US)	1: 1000 in 3% BSA TBST 0.05%
rabbit	total GSK3β	Cell signalling (US)	1: 1000 in 3% BSA TBST 0.05%
rabbit	pPLC-γ1 (Tyr783)	Cell signalling (US)	1:1000 in 3% BSA TBST 0.05%
rabbit	total PLC-γ1	Cell signalling (US)	1:1000 in 3% BSA TBST 0.05%
rabbit	pSrc (Tyr418)	ABM (CA)	1:1000 in 3% BSA TBST 0.1%
rabbit	total Src	ABM (CA)	1:1000 in 3% BSA TBST 0.1%
mouse	β-actin	Sigma-Aldrich (US)	1:500 in 1% milk PBST 0.05%

Stripping buffer	
0.10 M	β-mercaptoethanol
0.05 M	Tris pH 6.8
2 %	SDS

Table 3-15 stripping buffer contents

3.7.4 FACS

10^6 Cells were trypsinized and pelleted at 1,100 rpm for 5 minutes. After washing with PBS, the pellet was resuspended in 200µl and divided equally to two distinct tubes to distinguish positively and negatively labelled probes. 30µl FCS was added for blocking purposes and incubated for 10 minutes on ice.

In the meantime PE labelled antibody (anti-human CD334, Biolegend) was prepared in the right concentration to a total volume of 13µl. For the control tube an anti-mouse control antibody was used.

The tubes were wrapped in aluminium foil to assure a dark environment and incubated for 45 minutes in a box. After washing with PBS the supernatant was discarded and pellets resuspended in 500µl PBS. The solution was transferred to new FACS tubes and submitted to Irene Herbacek for FACS analysis.

3.7.5 Immunological staining

For this method 2.2 x 10^4 cells were prepared in 100µl. Autoclaved flexible cell culture chambers (contain 12 wells, FlexiPERM®) were set onto sterile glass slides and coated with 100µl collagen. 10 minutes later the collagen solution was removed and cell suspension was pipetted into the wells. Depending on the cell line this construct was incubated for 24h or 48h at 37°C to ensure cell adhesion and standard protocol was implemented. Pictures were taken at 60x magnification.

step	reagent with concentration	time span and cond.
Fixation	4% formaldehyde	30‘at -20°C
	3:1 methanol/aceton	2‘-5‘ at -20°C
Washing	cold 1x PBS	5‘ at RT
Clamping into slide rack (Shandon “Sequenza“)		
Inactivation	NH_4Cl solution	5‘ at RT
Washing	cold 1x PBS	5‘ at RT
Permeabilisation	0.5% Triton X in 1x PBS	5‘ at RT
Washing	cold 1x PBS	5‘ at RT
Blocking	0.2% fish gelatine in 1x PBS	30‘ at RT
1st antibody	1:100 in fish gelatine sol.	60‘ at RT
Washing	3x with cold 1x PBS	3x5‘ at RT
Continued in the dark		
2nd antibody	1:1,000 in fish gelatine sol. with 1:100,000 To-Pro®-3 Iodide (#A-11001, invitrogen)	45‘ at RT
Washing	3x with cold 1x PBS bidistilled water	3x5‘ 5‘ at RT
Covering	cold Mowiol® (polyvinyl alcohol 4-88 Fluka)	

Table 3-16 detailed protocol of immunostaining

3.7.6 Enzyme-linked Immunosorbent Assay

An ELISA kit for pFRS2 was obtained from R&D Systems (KCB5126) and enclosed protocol applied.

10^4 cells were seeded in each well of a 96-well plate and incubated two days at 37°C. Every approach was set four times including one for the negative control. 4% formaldehyde was used to fix the cells followed by three times washing. Quenching buffer (0.6% H_2O_2 in washing buffer) was added and incubated at RT for 20'. After washing, the cells were blocked to prevent unspecific binding of the antibody and washed again. The primary antibody-mix was prepared, 100µl added to each well and incubated o/n at 4°C. The same amount of blocking buffer was used for negative controls.

The next day cells were washed and treated with the secondary antibody-mix for 2h at RT. After washing with buffer and 1xPBS, the 1^{st} substrate was added to each well followed by the 2^{nd} substrate addition (without aspiration) with a 40' incubation time for each at RT. The plate was read at an excitation wavelength of 540nm and emission wavelength of 600nm for pFRS2 (Y436) using a fluorimeter. For FRS2 the plate was read again at 360/450nm.

4 Results

4.1 Expression results

4.1.1 FGFR4 ligand screening in various colon cancer cell lines

Expression level of the FGFR4 ligands FGF18 and FGF19 was checked in different colon cancer cell lines. Figure 4-1 shows results normalized on SW480 levels. In the adenocarcinoma cell line HT29 there is hardly any FGF18 while FGF19 is expressed at a similar level as in SW480's. Caco-2 cells do not seem to express either of the ligands. In SW620, a colorectal adenocarcinoma at lympho-invasive stage and in the CRC cell line HCT116, high FGF19 levels could be detected while FGF18 was rather low in the latter. The microadenoma cell lines LT97 and in cell lines derived from metastastic tissues (T84, AKH4 and AKH14) low FGF19 and low FGF18 expression was determined. Interestingly LT97-2 cells which were derived from later passages of LT97-1 and AKH14 that are less differentiated but derived from the same tissue of AKH4, had moderate levels of FGF19.

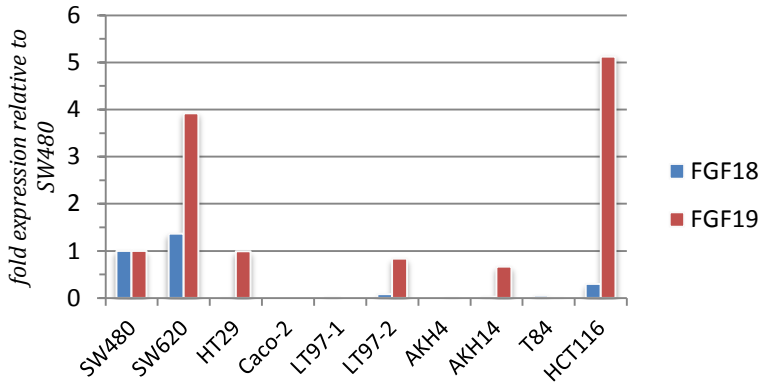


Figure 4-1 FGF18 and FGF19 expression results in various colon cancer cell lines, in x-fold expression levels (SW480 taken as 1)

4.1.2 FGFR4 ligand screening in transfected cell lines

Expression of the FGFR4 ligands FGF18 and FGF19 was also investigated in Arg, Gly and the control pcDNA transfectants. As Figure 4-2 shows, both over-expressing cell lines – Arg and Gly – showed minimal FGF19 expression. In Arg-transfectants, FGF18 is expressed less than in the control while FGFR4^{gly} over-expression led to high FGF18 mRNA.

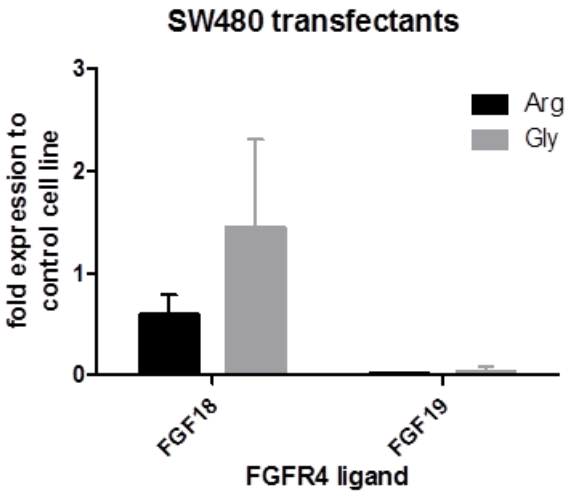


Figure 4-2 FGF18 and FGF19 mRNA expression in transfected SW480 cells

4.1.3 FGFR4 expression in transfected cells

In Figure 4-4, FACS analysis results are presented that demonstrate high FGFR4 expression levels in both HT29 Gly and HT29 Arg cells compared to the control. SW480 cells transfected with the $FGFR4^{arg}$ vector have more FGFR4 on their cell surface than $FGFR4^{gly}$ transfectants. When compared to the control cell line they both overexpress FGFR4. FACS analysis confirmed the RT-PCR results of SW480 cells (Figure 4-4, C. Heinzle thesis 2011).

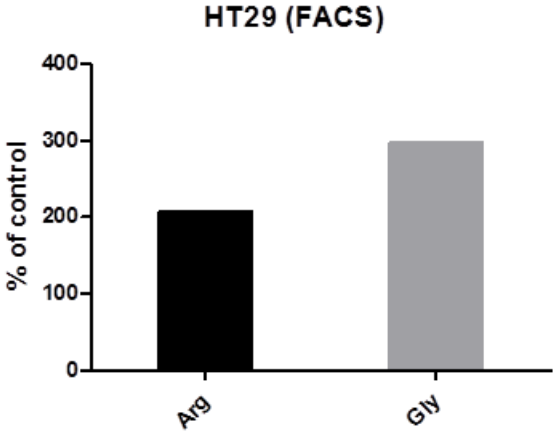
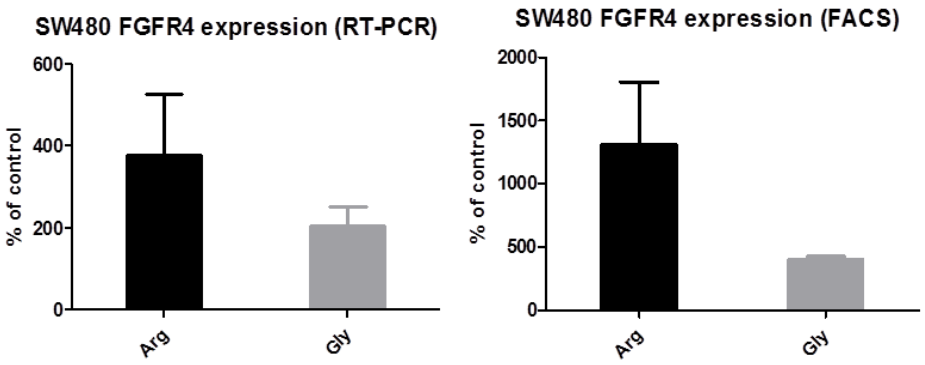


Figure 4-3 FGFR4 expression results in transfected HT29 cells

Figure 4-4 SW480 cells **(lower panel)** using different methods



4.1.4 FGFR4 allelic determination in transfected cells

The expression of the specific alleles was also checked by RT-PCR.

Table 4-1 shows that in HT29 cells transfected with the FGFR4^{arg} vector the expression is HT29^{arg} only while HT29^{gly} cells express threefold more FGFR4^{gly} than FGFR4^{arg}. The control cell line (depicted as pcDNA in the table) has three times more Arg expression than Gly (see Materials and Methods).

HT29-	primer	mean	Arg - Gly	R:G ratio
Arg	fgfr4_a	29,54	29,54	Arg only
	fgfr4_g	0,00		
Gly	fgfr4_a	35,34	1,26	1: 4
	fgfr4_g	34,09		
pcDNA	fgfr4_a	34,17	-3,84	4: 1
	fgfr4_g	38,01		

2,687	Arg only
0,888	08:01
0,44	03:01
0,274	02:01
0,149	01:01
-0,323	01:02
-0,501	01:03
-0,927	01:08
-2,269	Gly only

Arg:Gly ratio

Table 4-1 left calculation of FGFR4^{arg}:FGFR4^{gly} ratio in transfected HT29 cell lines, note the colour coding described in the **right** panel

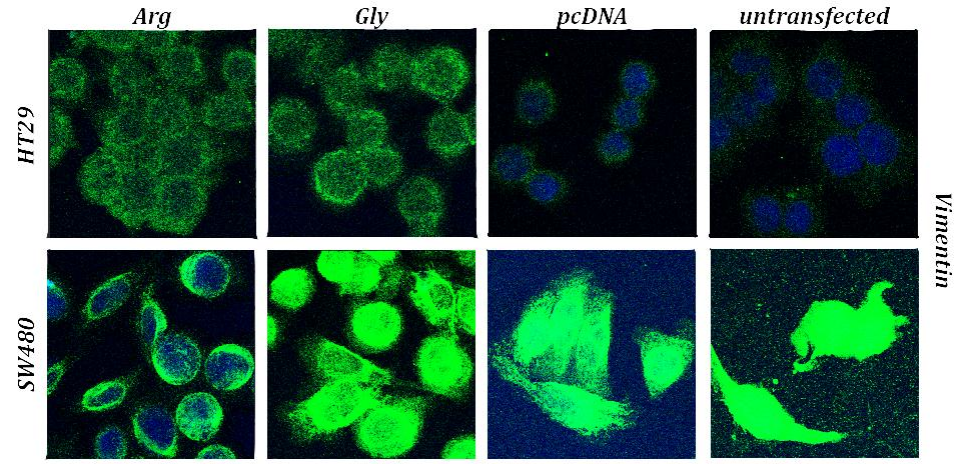
4.1.5 Influence of FGFR4 on the expression of EMT markers

Expression of EMT markers Vimentin and Fibronectin were checked using fluorescein-labelled antibodies. Vimentin expression results are depicted in Figure 4-5. In HT29 FGFR4 overexpressing cells there was more fluorescent stain than in the control cell line pcDNA and untransfected HT29 cells. In SW480 cells the pictures show low expression in the Arg cells, more concentrated on the plasma

membrane. SW480^{gly} cells were approximately the same level as the control cells and the untransfected SW480's.

In Figure 4-6 transfected HT29 and SW480 cells are compared with untransfected cells. An elevated fibronectin expression could be seen in FGFR4 overexpressing HT29 cells, concentrated on the plasma membrane. In SW480 transfectants the expression decreases in the following order: Gly – untransfected – pcDNA – Arg.

Figure 4-5 Vimentin expression of transfected and untransfected HT29 (**upper** panel) and SW480 (**lower** panel) cells



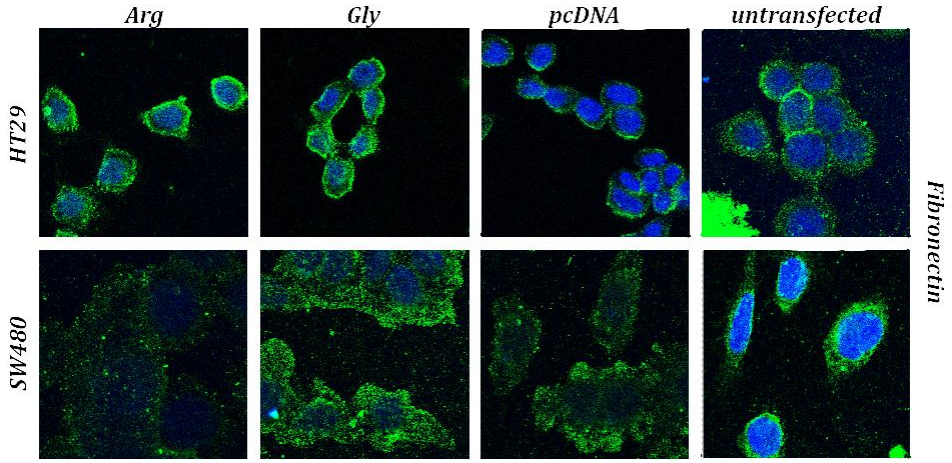


Figure 4-6 Fibronectin expression of transfected and untransfected HT29 (**upper** panel) and SW480 (**lower** panel) cells

4.2 Viability

Growth of the cell lines HT29, SW480 and HCT116 was recorded over a period of six days. Values depicted in Figure 4-7 values were normalized to viability on the first day and percentages were plotted. The plots of HT29 and SW480 cells show high statistical variations, thus precluding significant differences. Transfected HCT116 cell lines grew similarly and did not show any big differences.

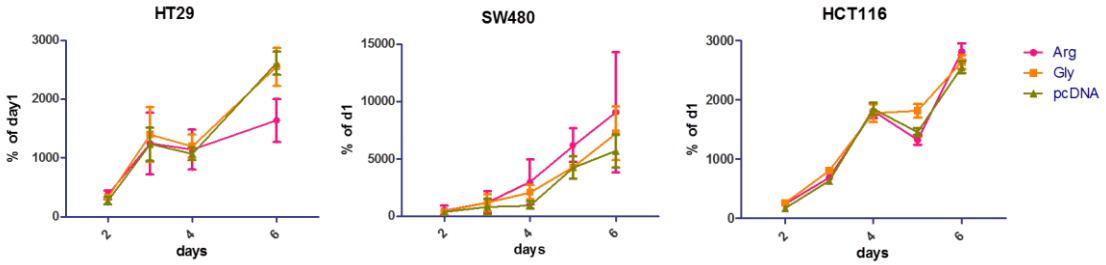


Figure 4-7 growth curves of different transfected cell lines as labelled in the titles of the graphs

Growth of the transfected cells was investigated over five days with stimulation of the FGFR4 ligands FGF18 and FGF19 (10ng/ml). As was described in chapters 4.1.1 and 4.1.2, HT29 cells expressed little FGF18 while the transfected SW480 cells displayed moderate FGF18 but hardly any FGF19 production. Because of this, FGF18 was selected for stimulation of HT29, and FGF19 for SW480 cells.

In general, SW480 cells were growing faster than HT29 cells (Figure 4-7 and Figure 4-8). When the transfected cell lines were compared with each other, we could see no differences in the HT29 derived cell lines (Figure 4-8 upper panel).
In SW480s (Figure 4-8 lower panel), $FGFR4^{arg}$ transfected cells were growing faster than the cells overexpressing $FGFR4^{gly}$ or the empty vector pcDNA3.1.
When we analysed FGFR4 ligand effects we could see some differences in the Gly cell line (Figure 4-8 upper panel): there were slightly more untreated cells on day 4 than cells which were factor treated. However, the standard deviations were too high, precluding any significance.

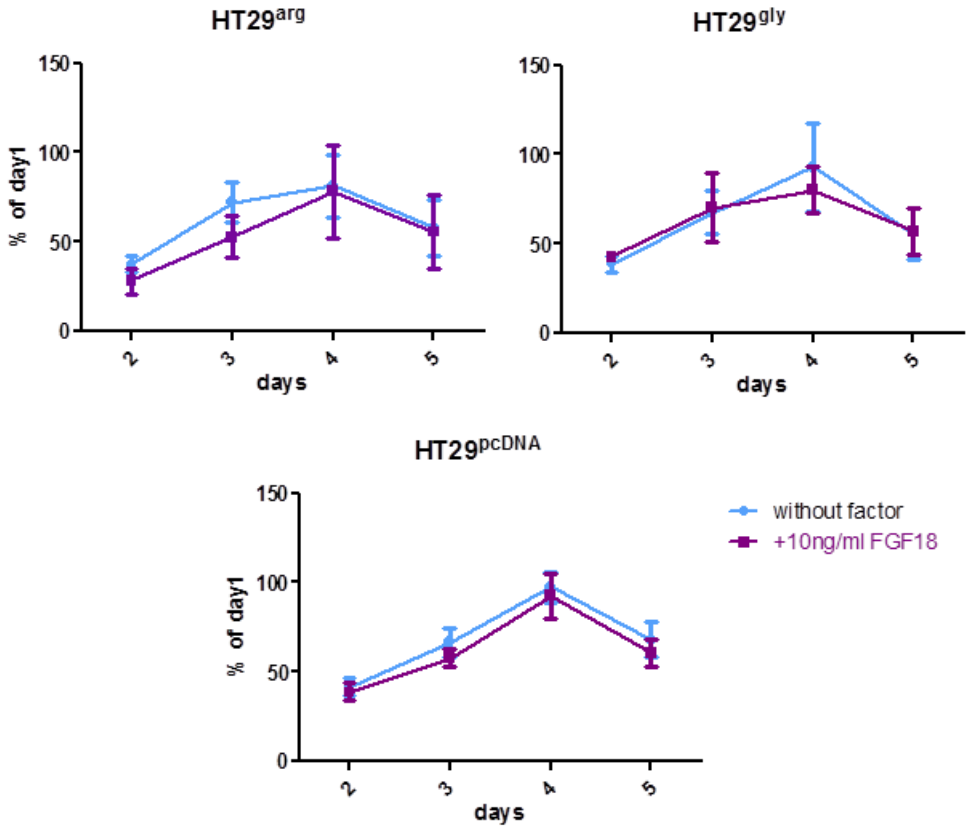
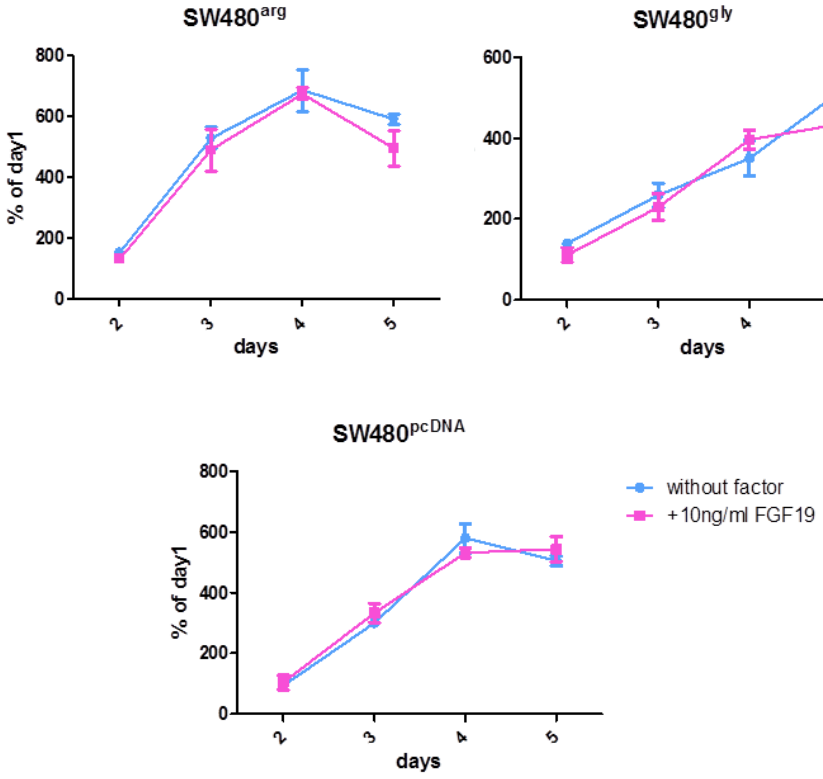


Figure 4-8 transfected HT29 (**upper panel**) and SW480 (**lower panel**) cell growth recorded over five days, 1st day taken as baseline; note the different y-axis in the plots; error bars represent ±SD



4.3 *Adhesion and colony formation in FGFR4 transfectants*

To determine effects of the FGFR4 G388R polymorphism on the attachment and colony formation ability of cells in the absence of any cell-cell contacts, clonogenicity experiments were performed in the presence of FGFR4 ligands FGF18 and FGF19. In Figure 4-9 the results of FGF18 stimulation are shown. Different FGF18 concentrations do not seem to affect the clonogenic ability. However, as expected from our previous studies (see C. Heinzle thesis 2011), an effect of the FGFR4 alleles could be detected (Figure 4-9 and Figure 4-10: +control): cells transfected with FGFR4^{gly} achieved better attachment and colony formation than the FGFR4^{arg} overexpressing cells.

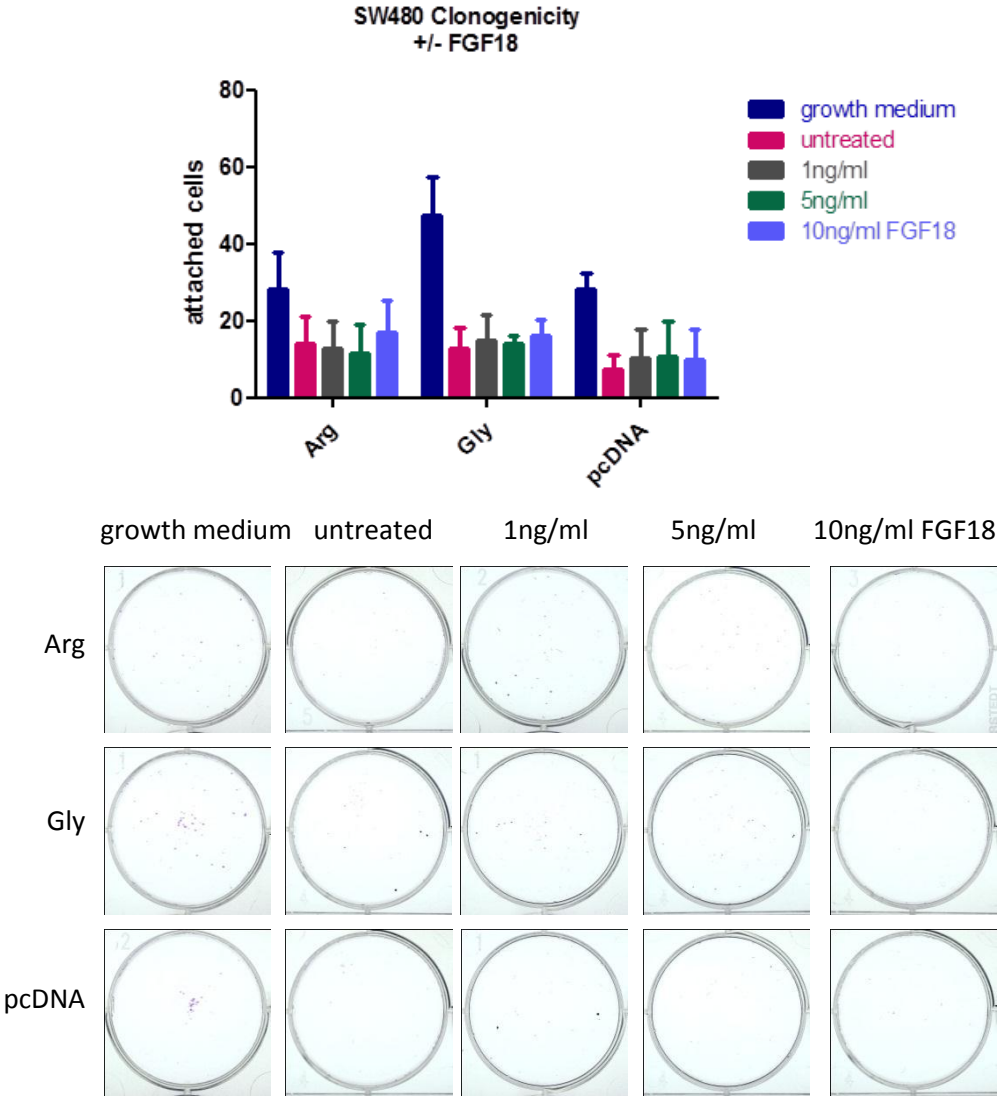
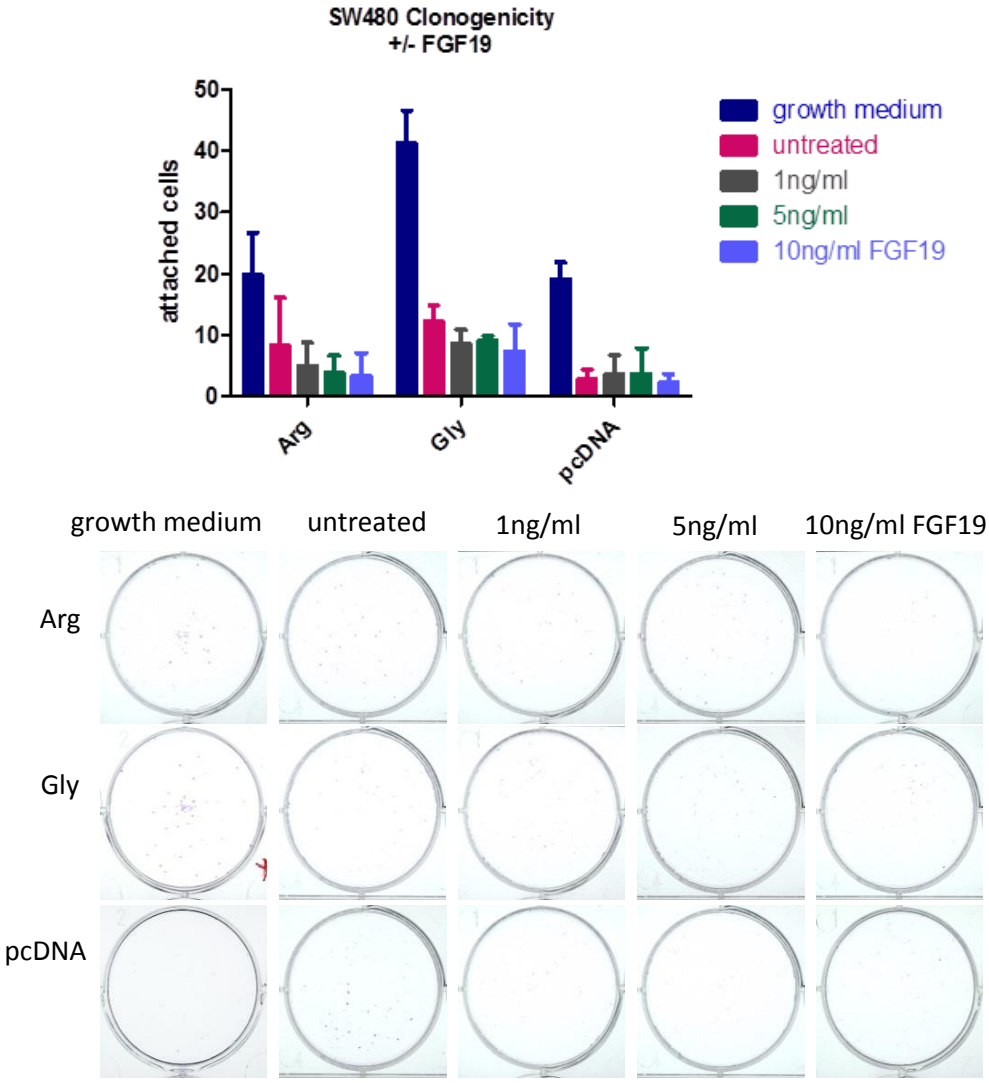


Figure 4-9 clonogenicity assay to test cell adhesion and colony formation at low cell density: transfected SW480 cells without and with FGF18 treatment; bars represent ±SD

Figure 4-10 FGFR4^{arg} or FGFR4^{gly} overexpressing SW480 tested on their clonogenic ability with or without FGF19 stimulation; bars represent ±SD



The same experimental approach was used for different concentrations of FGF19 (Figure 4-10) and the same enhancing effect of FGFR4^{gly} on attachment and colony formation was observed. Apart from this, in FGFR4 overexpressing cells, especially the Arg allele, FGF19 led to a decrease of the cell colony number.

4.3.1 Adhesion assay

To investigate the adhesion capability of cells independent of their growth potential, we used standard adhesion assay on our transfected cells with stimulation of FGF19. Results are shown in Figure 4-11. The positive controls grown in conventional growth medium attached as expected and confirmed the results of the clonogenicity experiments (chapter 4.3): $SW480^{gly}$ cells showed better adhesion than cells overexpressing $FGFR4^{arg}$. Also in the negative control, $SW480^{gly}$ cells attached slightly better than $SW480^{arg}$ cells.

When different concentrations of FGF19 were added, both transfectants attached better and a characteristic pattern could be seen: while in $FGFR4^{arg}$ overexpressing cells the adhesion remained constant, in $SW480^{gly}$ we saw that 1ng/ml was the optimal concentration for attachment and increasing concentrations of FGF19 led to reduced adherence.

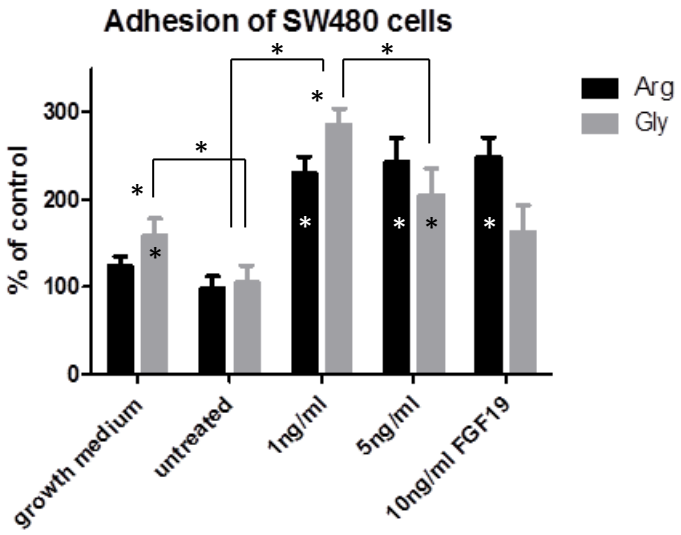


Figure 4-11 tested adhesion experiment on transfected SW480 cells; * p < 0.5;
Unconnected asterisks above bars represent significance between Arg and Glydata;
asterisks on columns represent significance to control

4.4 Migration to close the scratch

4.4.1 Development of a scratch assay protocol

In order to develop a protocol for scratch assay experiments, scratches were made on cell monolayers at different cell densities and photos taken after several time points to find the optimal time span for migration.

To stimulate the cells for migration a nearly 100% confluency was used. For this purpose two experimental groups were used with 700,000 and a million cells per 6-well. The scratch was made with yellow tips which led to swimming cell clusters. For that reason, white tips were used to make the scratches and photos were taken to record original width. 24 hours later the new width was determined using microscope at 4x magnification.

As Figure 4-12 shows scratch closure was the same in both densities.

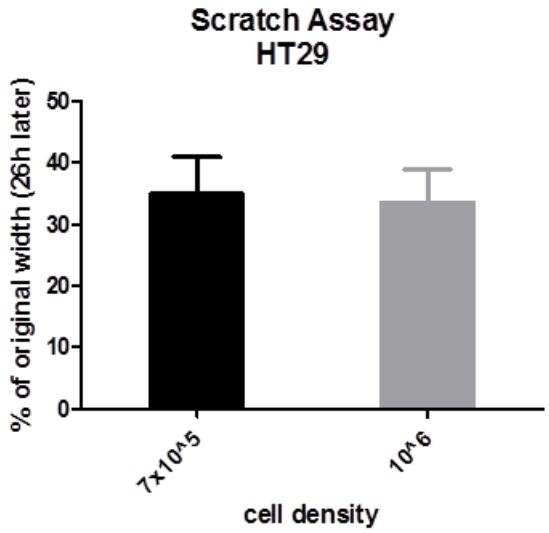


Figure 4-12 scratch closure tested in HT29 cells in two different cell densities

Further, photos of the scratch were taken at different time points (3-6-24-27-30-48 hours) and analysed to find out the optimal time period of migration (Figure 4-13). There was no change observed in the first six hours but a day later, up to 40% of the scratch was closed in HT29 cultures and nearly 70% in the case of HCT116 cells. Later time points did not show any big migration activities and hence were excluded for further experiments.

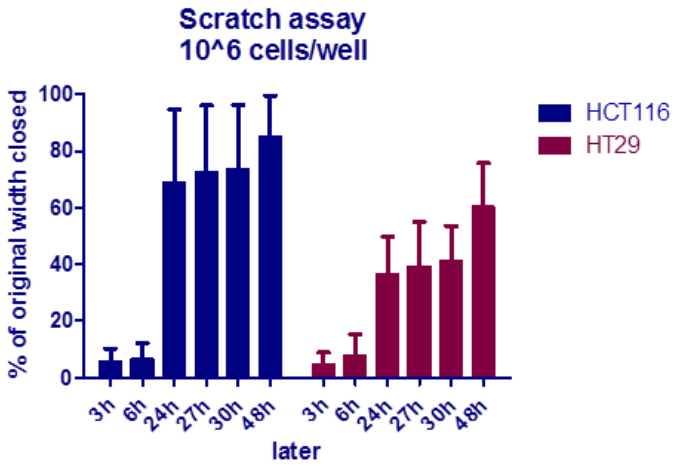


Figure 4-13 percentage of scratch closure after several hours in HCT116 and HT29 cells

4.4.2 Migration behaviour of cells to close the scratch

Based on the established protocol, scratch assays were performed with different approaches.

Migration of the cell lines HCT116 and HT29 into the “wound” is depicted in Figure 4-14 with corresponding photos. The photos show a significant narrowing in the case of HCT116 while HT29 cells seem to be slower in migrating. Calculation of the ratio to the original width (Figure 4-14 right) shows a difference of about 20-30% in 24 hours between the two cell lines.

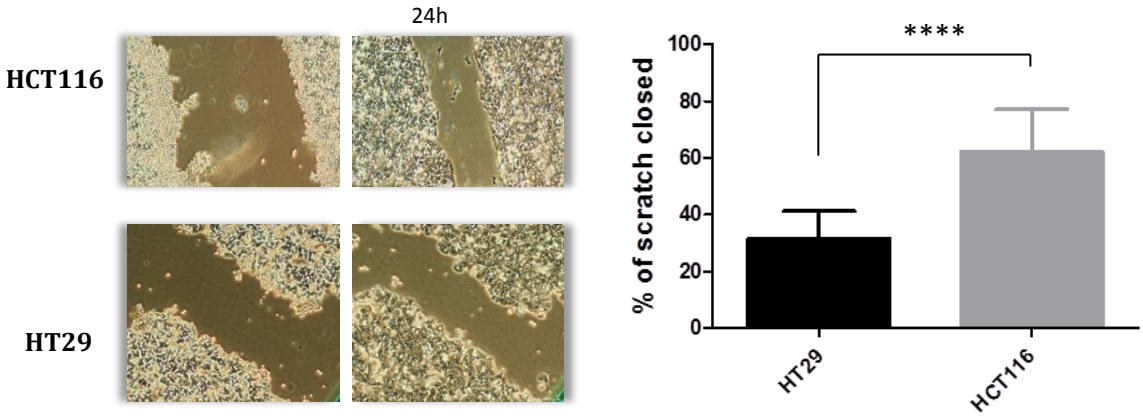


Figure 4-14 scratch results for the cell lines HCT116 and HT29: **Left**, photos of the original scratch and 24 hours later taken at 4x magnification; **Right**, plot of measured and calculated width closure

****$p < 10^{-17}$

Transfected cell lines were observed (Figure 4-15) and data normalized to pcDNA3-controls. It emerged that the FGFR4^{gly} cells were less able to close the scratch at the given time point than cells overexpressing the Arg allele, but still above 100% and thus better than the controls. Notably, the HT29 Gly cell line was about the same level or slightly lower than the control cell line (taken here as 100%) pointing out a possible role of FGFR4^{gly}.

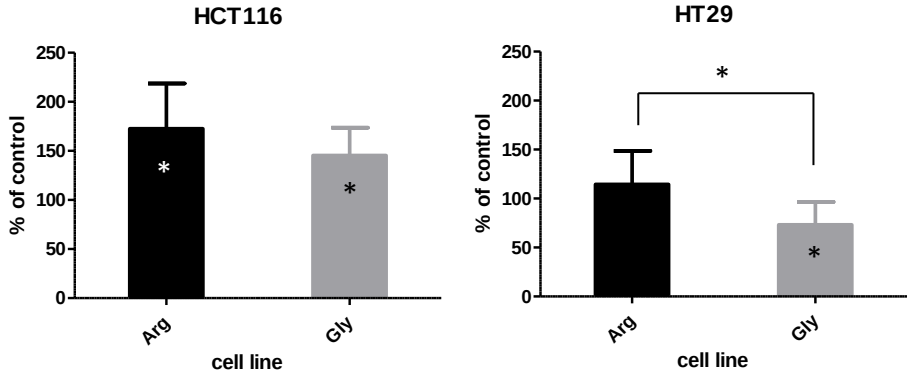


Figure 4-15 scratch assay results of **left** HCT116 and **right** HT29 cells stably overexpressing either the Arg or Gly allele of FGFR4
bars represent ±SD; asterisks on columns represent significance to control; *p < 1.0

4.4.3 FGFR4 ligand stimulated migration

Treatment of HT29 cells with different concentrations of FGF18 and FGF19 led to results shown in Figure 4-16. FGF18 did not change migration when compared to control.

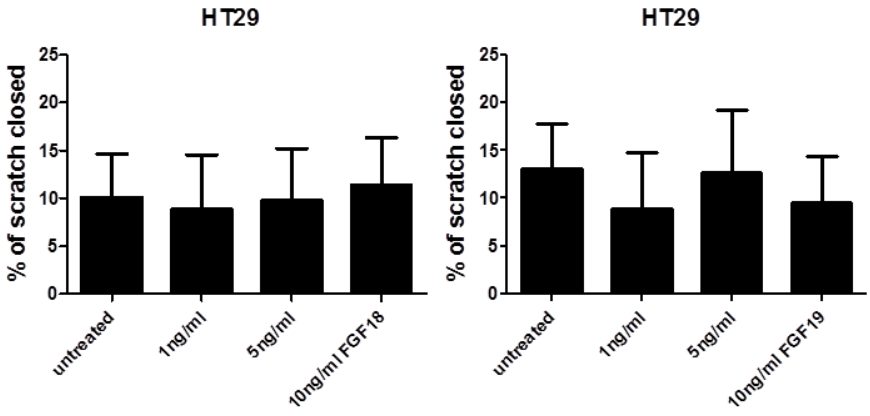


Figure 4-16 percentage of scratch closure at different concentrations of factor treatment in HT29 cells; **Left** FGF18 and **right** FGF19

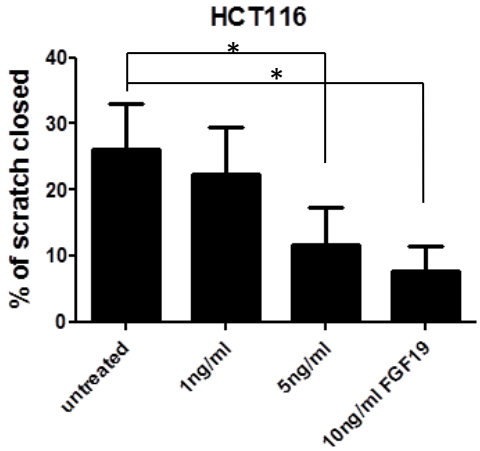


Figure 4-17 effect of exogenous FGF19 to the scratch closing ability (migration) on HCT116 cells; bars represent ±SD; *p <1.0

By contrast, we could see an effect of FGF19 on the migration of untransfected HCT116 cells: as Figure 4-17 shows, increasing concentrations of FGF19 impaired the “wound healing” ability.

The same approach was used for FGFR4 overexpressing HT29 and HCT116 cells. Figure 4-18 shows results for the scratch closing ability of HCT116 transfectants with FGF18 and FGF19 treatment.

FGF19 significantly inhibited migration of FGFR4 overexpressing HCTs when compared to untreated controls (Figure 4-18). As in HT29 cells FGF18 caused hardly any difference, while FGF19 inhibited migration (Figure 4-19).

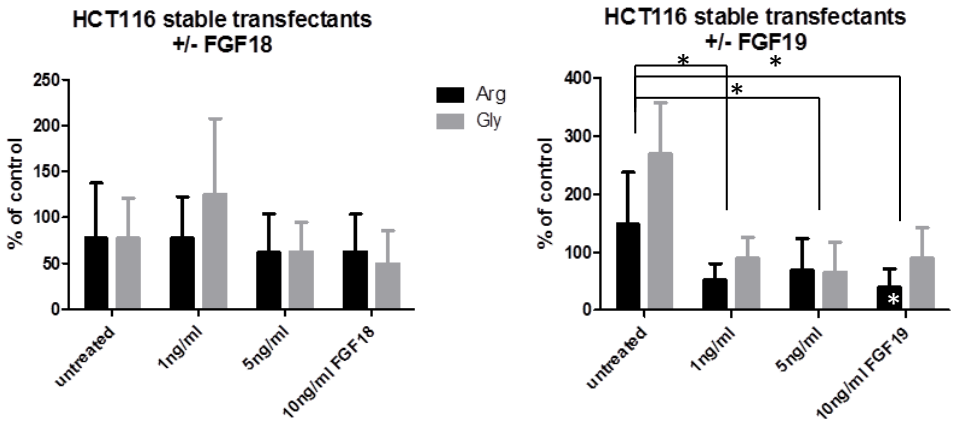
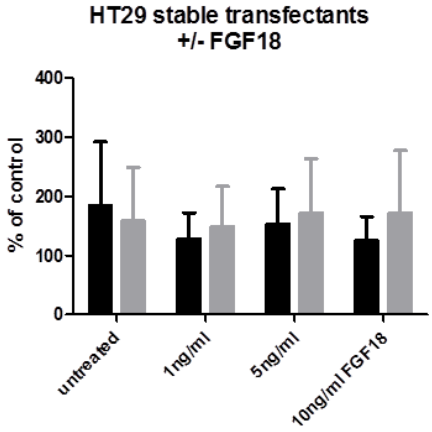


Figure 4-18 scratch closing results of stably FGFR4^{arg} or FGFR4^{gly} overexpressing HCT116 when stimulated with different concentrations of **left** FGF18 and **right** FGF19; asterisk on column represents significance to control, *p < 1.0

Figure 4-19 scratch closing results of stably FGFR4^{arg} or FGFR4^{gly} overexpressing HT29 when stimulated with different concentrations of FGF18



4.5 Invasive properties of cells

4.5.1 Development of a protocol for invasion assay

To determine the invasive properties of the cells, a new protocol was established using migration filters coated with ECM protein. HCT cells were chosen for optimisation experiments since they had the highest migration potential (see chapter 4.4.2). Matrigel and collagen were tested in different concentrations for coating of migration filters (see Materials and Methods, chapter 3.5.3). Figure 4-20 shows photos of wells containing the cells that reached the lower chamber. Cells that had to invade Matrigel™ seem to have migrated as clusters and thicker Matrigel gels inhibited migration. When the reagent was diluted in twice the amount of PBS, significant numbers of cells reached the lower chamber (Figure 4-20 right picture set, lower panel 1:1 and pure). In the filters coated with collagen, cells were detected in the bottom of all wells, increasing with the collagen concentration. Collagen was therefore chosen for further experiments.

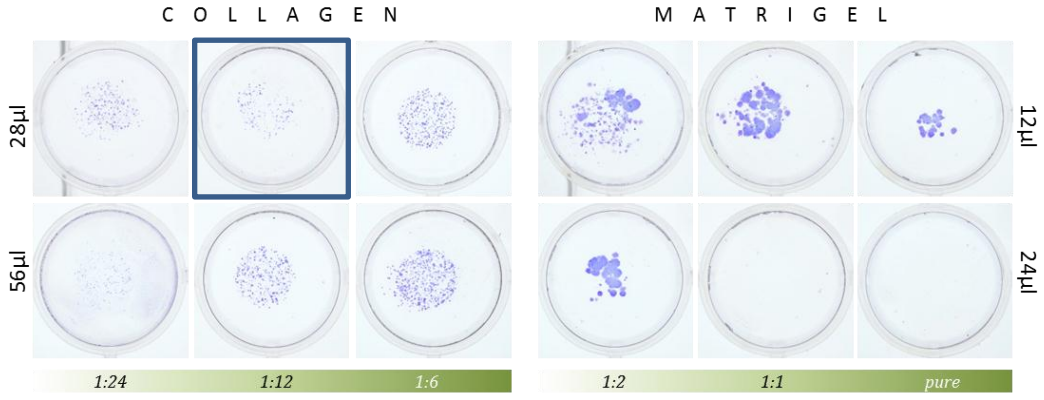


Figure 4-20 results of the invasion assay test approach; used cells were HCT116

4.5.2 Invasion ability of FGFR4 overexpressing cells

The invasive potential of FGFR4 overexpressing cell lines was tested for SW480 and HCT116 transfectants. Either allele of FGFR4 improved the invasive ability of both cell lines (Figure 4-21). In both cell lines (HCT116 and SW480), FGFR4^{arg} transfectants showed increased invasion: compared to control and Gly cells there is nearly 100% difference (Table 4-2).

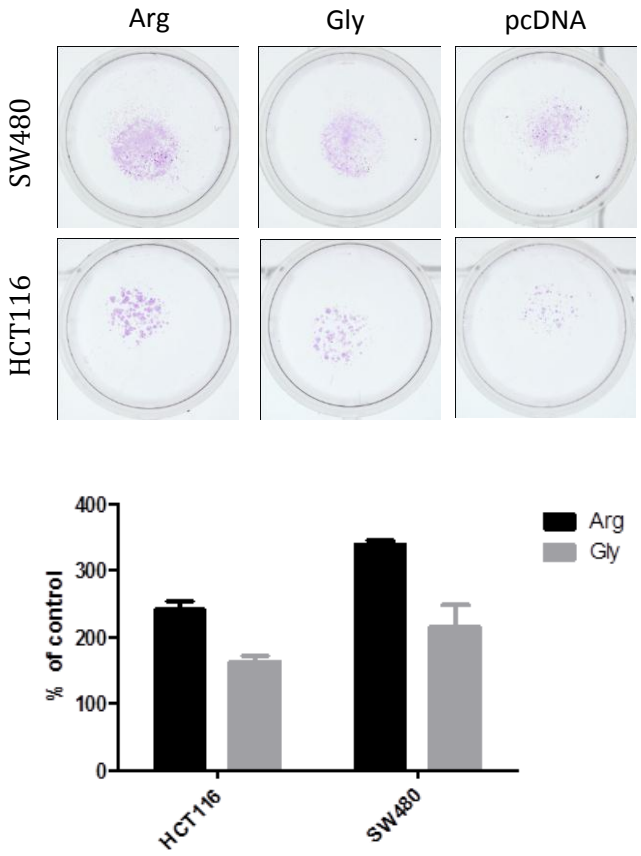


Figure 4-21 in-well pictures of stained SW480 and HCT116 FGFR4 overexpressing cells migrated through collagen layer (**upper panel**)
plot of calculated percentage in the **lower** panel, values shown in **Table 4-2**

	% of control ±SD	
	FGFR4^{arg}	FGFR4^{gly}
HCT116	240.6 ± 5.5%	162.5 ± 5.4%
SW480	338.1 ± 2.0%	214.2 ± 15.7%

Table 4-2 invasion values normalized to mean of pcDNA ±SD

4.6 Signalling

The state of the direct FGFR target FRS2 was checked using ELISA for three different cell lines. Two different antibody incubations were performed for phosphorylated and total FRS2 respectively (see chapter 3.7.6), and the fluorescence measured at appropriate wavelengths. Relative fluorescence units were defined and ratios of phospho/total FRS2 calculated. Finally, values were normalized to the control cell line.

FRS phosphorylation in SW480 cells was equal to or slightly above pcDNA-controls while HT29 overexpressors displayed lower phosphorylation states. FGFR4 overexpressing HCT116 cells showed nearly 1.5 fold increase of FRS2 phosphorylation.

When comparing the two FGFR4 alleles, SW480gly cells had slightly more phosphorylated FRS2 than SW480^{arg} while for HT29 and HCT116 there were no significant differences.

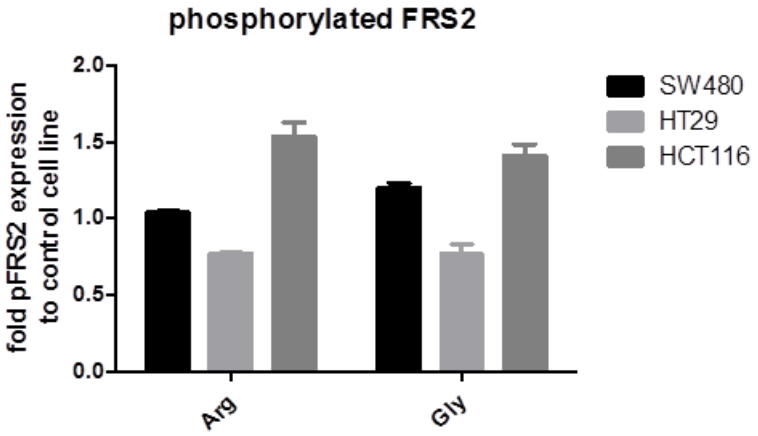


Figure 4-22 phosphorylation ratio in FGFR4 overexpressing SW480, HT29 and HCT116 cells, bars represent ±SD

4.6.1 Downregulation of FGF18

Using siRNA oligonucleotides, FGF18 was downregulated in the transfected SW480 cell lines. Efficiency was checked with RT-PCR analysis. RNA-nucleotides (si-scrambled) were used for control. Figure 4-23 shows efficient downregulation of FGF18 mRNA with at least 80% less FGF18 mRNA than in scrambled controls.

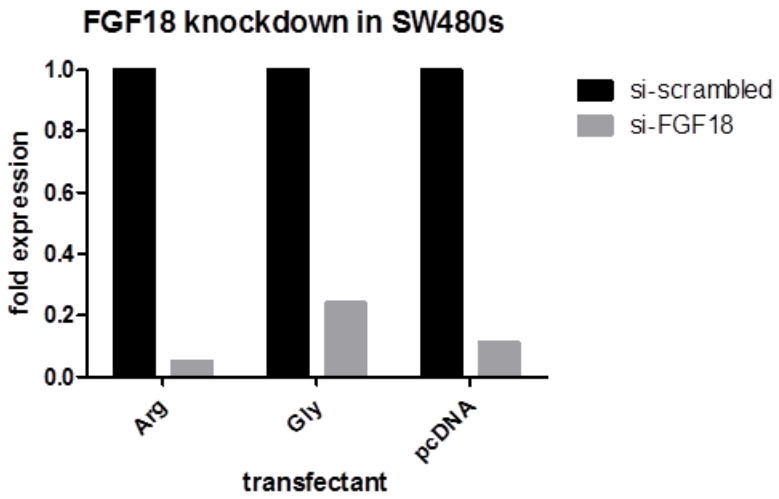


Figure 4-23 siRNA transfection of FGFR4 overexpressing SW480 cells

Protein was isolated from the FGFR4 overexpressing and FGF18 downregulated SW480 cells (further referred as KD) which were either stimulated with FGF18 for 5'/15' (5'/15' KD) or not stimulated (unstim. or FGF18-). Phosphorylation of direct (PLCγ) and indirect downstream targets (ERK, S6, Src, GSK3β) of FGFR4 were analysed using Western Blotting. Bands were semi-quantified and analysed with Photoshop. Quotient of total protein to the loading control (β-actin) of the control cell line was calculated. Phosphorylation ratio was normalized to that of the control cell line.

FGF18 knockdown increased total PLCγ levels in Gly cells which could be partly reversed when treated with factor for 5'. FGFR4^{arg} overexpressing cells did not display any significant change in the FRS2 levels with FGF18 downregulation. Phosphorylation of the direct FGFR4 target, PLCγ, was significantly decreased with FGF18 knockdown in FGFR4^{gly} overexpressing cells. A rescue could be seen after 5 minutes of factor treatment which nevertheless fell back 10' later. A similar effect was observed in controls with mostly endogenous FGF18. FGFR4^{arg} transfectants were not affected significantly.

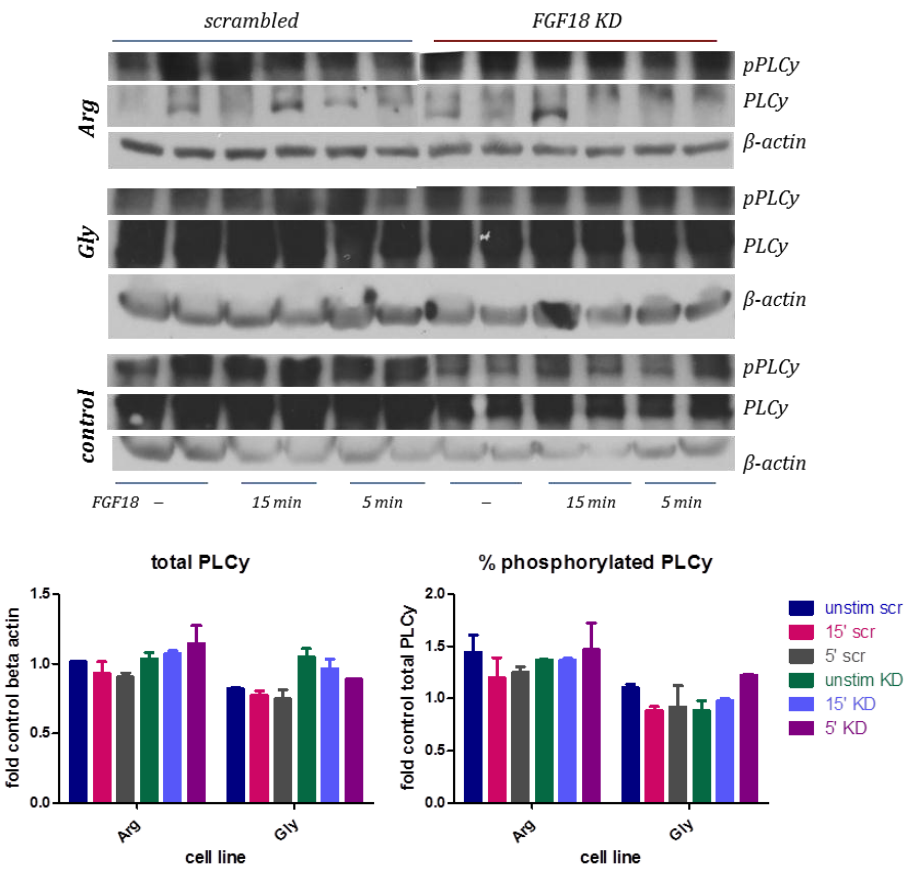


Figure 4-24 upper panel pPLCγ/PLCγ bands for FGFR4^{arg} or FGFR4^{gly} overexpressing SW480 cells with knockdown (KD) and/or addition of FGF18 in a given time; scr refers to non-knockdown
lower panel plots of quantified bands normalized to that of control cell line, bars represent ±SD

FGF18 knockdown did not significantly affect total Src levels – neither in FGFR4^{arg} nor in FGFR4^{gly} overexpressing cells.

However, Src activation via phosphorylation was reduced in FGF18 downregulated Gly cells which could be rescued by FGF18 treatment. There were no changes detected in total Src levels of Arg cells.

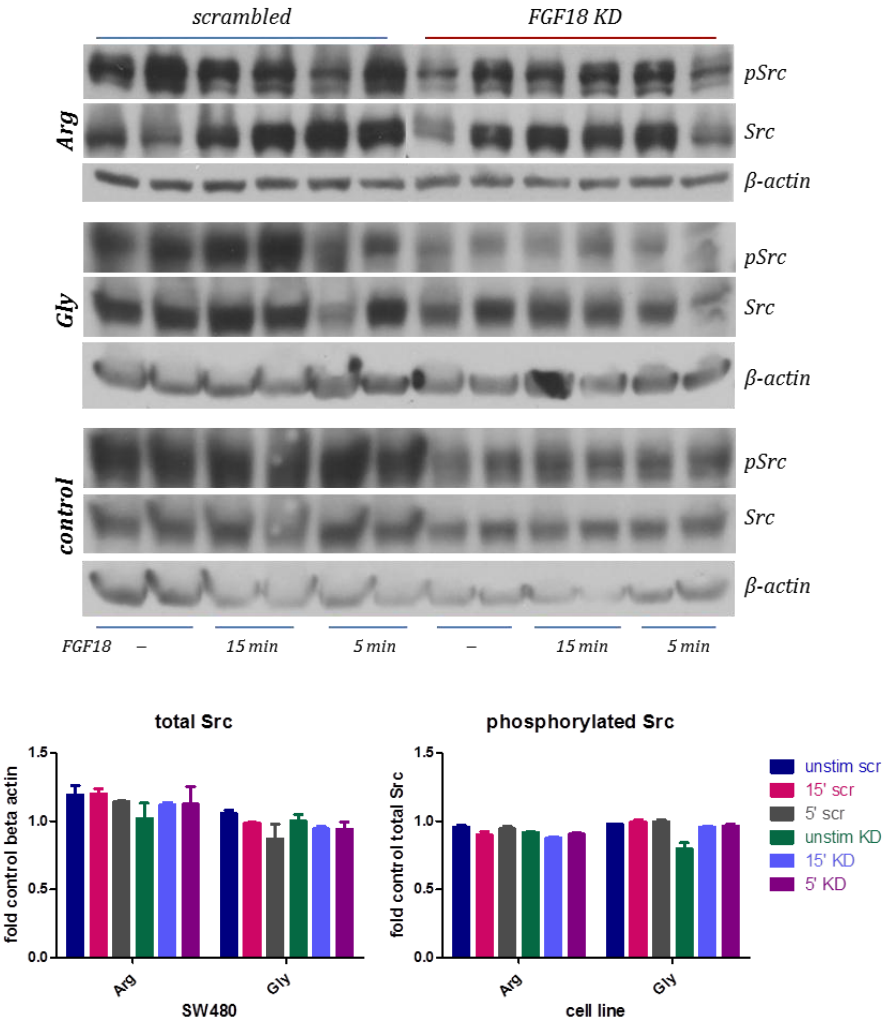


Figure 4-25 upper panel pSrc/Src bands for FGFR4arg or FGFR4gly overexpressing SW480 cells with knockdown (KD) and/or addition of FGF18 in a given time; scr refers to non-knockdown **lower panel** plots of quantified bands normalized to that of control cell line, bars represent ±SD

Ratio of total ERK protein to the pcDNA control group revealed a downregulation of ERK with FGF18 knockdown in both FGFR4^{arg} and FGFR4^{gly} overexpressing cells. Exogenous factor treatment for 5' partly rescued this phenotype.

ERK protein phosphorylation was slightly increased by FGF18 downregulation in SW480^{arg} cells which were reversed by addition of exogenous FGF18. In non-knockdown controls, factor treatment for 5' lowered phosphorylation of ERK.

ERK activation of FGFR^{gly} overexpressing cells was not affected by FGF18 mRNA reduction.

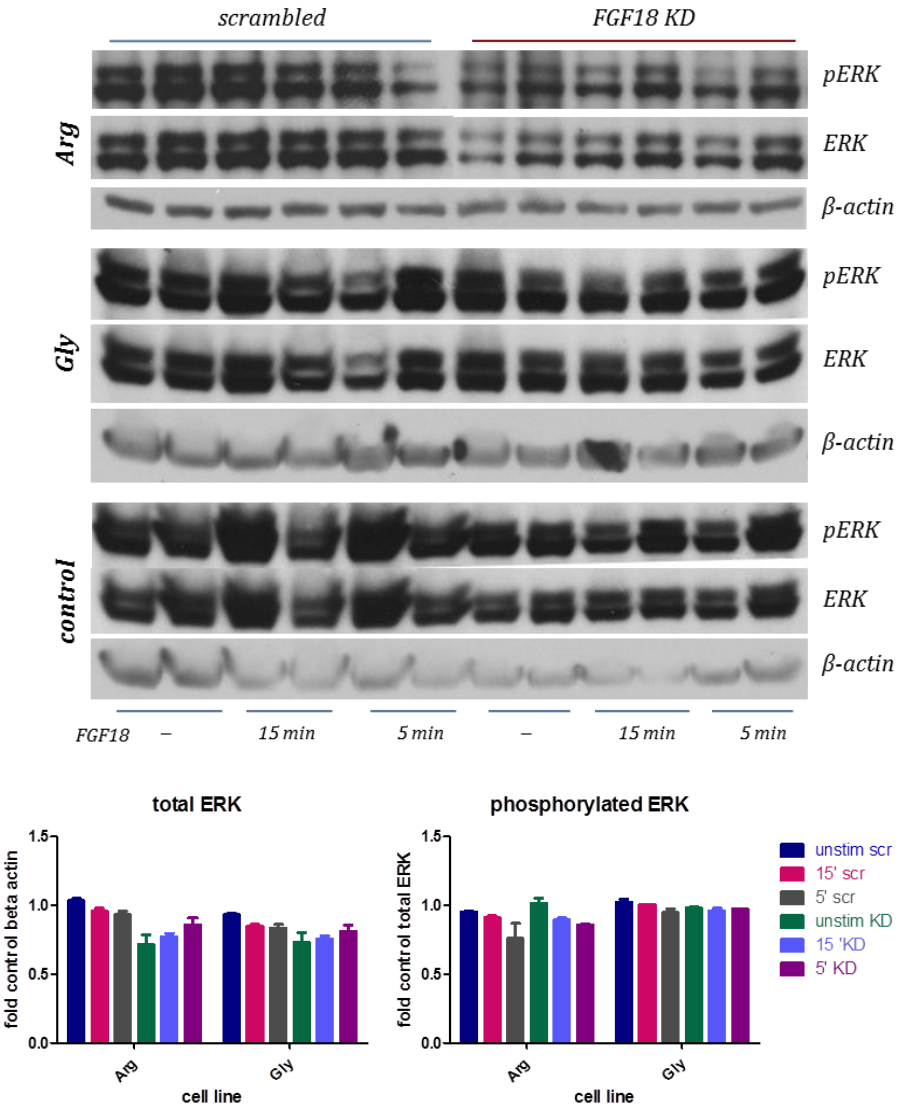


Figure 4-26 upper panel pERK/ERK bands for FGFR4^{arg} or FGFR4^{gly} overexpressing SW480 cells with knockdown (KD) and/or addition of FGF18 in a given time; scr refers to non-knockdown **lower panel** plots of quantified bands normalized to that of control cell line, bars represent ±SD

FGF18 downregulation led to reduced total GSK levels in both FGFR4 overexpressors but could be rescued by factor addition and incubation for 5 minutes in Arg overexpressors. 15' stimulation with factor decreased GSK levels further and a similar effect could be observed in non-knockdown cells treated for 5'.

GSK phosphorylation levels were slightly downregulated in Arg KD cells. In contrast, $FGFR^{gly}$ overexpressing cells displayed an increase in the GSK phosphorylation level with FGF18 knockdown. This could be partly rescued with treatment of exogenous factor for 15 minutes.

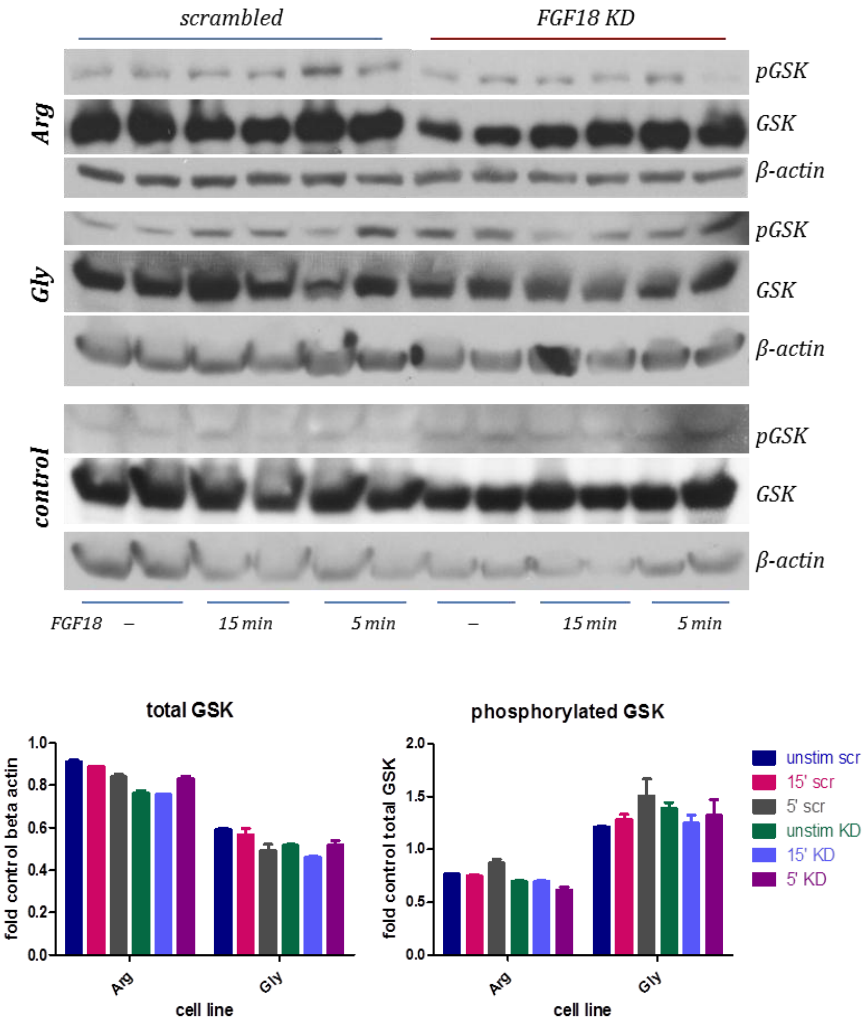


Figure 4-27 upper panel pGSK/GSK bands for FGFR4arg or FGFR4gly overexpressing SW480 cells with knockdown (KD) and/or addition of FGF18 in a given time; scr refers to non-knockdown
lower panel plots of quantified bands normalized to that of control cell line, bars represent ±SD

Total S6 levels were strongly reduced by knockdown of FGF18 in FGFR4^{arg} overexpressing cells and a response to factor addition was not seen. In Gly cells a slight decrease was observed which was stronger when FGF18 was administered.

S6 activation was reduced with FGF18 knockdown in both FGFR4 overexpressing cells. Additional FGF18 did not rescue this and even lowered levels in scrambled controls when treated for 5'.

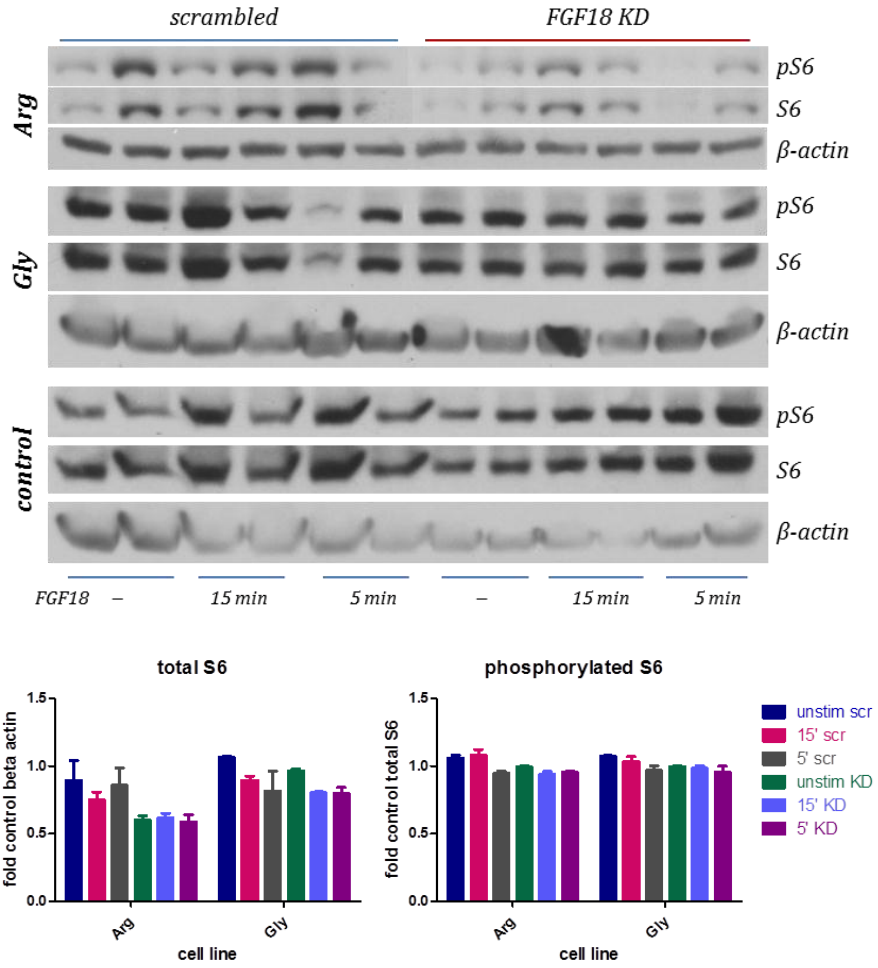


Figure 4-28 upper panel pS6/S6 bands for FGFR4arg or FGFR4gly overexpressing SW480 cells with knockdown (KD) and/or addition of FGF18 in a given time; scr refers to non-knockdown
lower panel plots of quantified bands normalized to that of control cell line, bars represent ±SD

5 Discussion

FGFR4 is a member of the fibroblast growth receptor family and plays an essential role in proliferation, differentiation and survival. In cancers of the breast, prostate and muscles (rhabdomyosarcoma) a pro-oncogenic role has been described (Ye *et al.* 2011, Taylor *et al.* 2009, Roidl *et al.* 2009, Wang *et al.* 2008, Sahadevan *et al.* 2007, Jaakkola *et al.* 1993).

Especially the G388R polymorphism was shown to increase tumour risk and decrease survival rate in many cancer types (Frullanti *et al.* 2011). Wang and his colleagues (2008) suggested a mechanism that increases FGFR4 stability and leads to prolonged signalling.

How this polymorphism acts, which intracellular signalling pathways are involved and what role it plays in CRC development *in vivo* and *in vitro* is not elucidated.

Here, we observed malignant characteristics like growth, migration and adhesion in FGFR4 overexpressing CRC cell lines and tried to determine intracellular alterations with or without the FGFR4 ligand FGF18 which was defined as a pro-tumorigenic and pro-metastatic factor (Sonvilla *et al.* 2008).

5.1 FGFR4 ligand expression in colon cancer cell lines and in FGFR4 overexpressing cells

Expression of the FGFR4 ligands FGF18 and FGF19 was analysed because both ligands could cause autocrine stimulation loops with FGFR4 (Zhang *et al.* 2006).

FGF19 levels were high in two cell lines at progressed tumour stages (HCT116 and SW620), suggesting a possible increase of FGF19 expression with the tumour stage. Also in the cell line Colo-201 FGF19 expression was about 6-fold to that of SW480 (not shown, C. Heinzle found 16-fold expression). This also fits with the work of Desnoyers *et al.* (2008) who described autocrine stimulation of CRC cells by FGF19. These 3 cell lines were found to express more FGFR4 than SW480 do, with HCT116 and SW620 being $FGFR4^{gly}$ homozygous and Colo-201 cells expressing both alleles (C. Heinzle thesis 2011). Desnoyers *et al.* (2008) used a specific anti-FGF19 monoclonal antibody and achieved growth inhibition of colon tumour xenografts *in vivo*. FGF19 could specifically act on $FGFR4^{gly}$ and mediate growth.

FGF18 level was the highest in the SW620 cells (a cell line obtained from a tumour tissue at lympho-invasive stage), while adenoma cells showed no detectable expression, correlating with the data of Sonvilla *et al.* (2008).

C. Heinzle showed highest FGF18 expression in SW480 cells, followed by Caco-2, SW620 and LT97-2 (thesis 2011). Transfection with FGFR4 expression vectors seems to have a negative effect on FGF18 levels when compared to pcDNA3-control. There was no FGF19 mRNA expression detected in the transfectants suggesting a negative feedback loop.

5.2 FGFR4 overexpression in colon cancer cell lines

The cell lines HT29 and SW480 were transfected with FGFR4 overexpressing vectors and expression was checked. Overall, FGFR4 overexpression could be determined on high levels in both cell types.

On the transcriptional and translational levels, two to four fold expression above the control could be achieved in SW480s. $FGFR4^{arg}$ overexpressing SW480 cells showed higher expression levels than $FGFR4^{gly}$ transfectants in both RNA and protein, suggesting a higher stability and stronger signalling of the Arg variant.

In heterozygous HT29s, the Gly transfectants showed higher expression than Arg cells.

Allelotypic analysis of HT29 transfectants revealed that the control cells showed mostly $FGFR4^{arg}$ overexpression with an Arg/Gly ratio of 6:1. In $HT29^{arg}$ the Arg allele was clearly the majority of all expressed FGFR4 RNA molecules while in $HT29^{gly}$ cells the Arg allele was still expressed, associating with the dominance of the Arg allele, but the 3-fold amount of RNA from the transfected Gly allele was also detected. Similar results were obtained by C. Heinzle in FGFR4 overexpressing HCT116 and SW480 cells (see C. Heinzle thesis 2011).

5.3 EMT markers Fibronectin and Vimentin

Expression of the two EMT markers fibronectin and vimentin was checked in HT29 and SW480 cells using fluorescent antibody. Fibronectin is a mesenchymal marker and can be found in the extracellular matrix. High fibronectin expression was correlated with progressed tumour stage (Saito *et al.* 2008) and node-positive CRC formation (Meeh *et al.* 2009). Vimentin is a cytoskeletal protein (intermediate filament) that is expressed in mesenchymal cells.

In HT29 cells baseline levels for both markers were low and FGFR4 overexpression increased immunodetectable protein in both cases. For the Gly allele this effect was stronger than for the Arg allele. By contrast, SW480 untransfected and control cells expressed high levels of both fibronectin and vimentin. FGFR4 overexpression in these cells had little impact on the mesenchymal markers. Only $FGFR4^{gly}$ transfectants had slightly increased marker staining.
This suggests that $FGFR4^{arg}$ and $FGFR4^{gly}$ have different influence on the expression of these EMT markers. These results need to be reconciled with the observation that $FGFR4^{arg}$ transfectants display higher migration and metastatic activity which is usually related to EMT (Thiery 2002).

5.4 $FGFR4^{arg}$ improves viability

Cell growth was not particularly affected through FGFR4 but a slight improvement was detected in $FGFR4^{arg}$ overexpressing SW480 cells cultured in starvation medium without factors. We could not detect any growth supporting effects by FGF19 on SW480 cells or FGF18 on HT29 cells. This contradicts reports from Pai *et al.* (2008) who used FGF19 to promote of HT29 and Colo-201 cells through activation of the Wnt signalling pathway (increased activation of β-catenin). However, they used five- to ten-fold more FGF19 than we did. The difference between these results and our own data need to be further explored.

5.5 Effects on migration, invasion and adherence

The FGFR4 polymorphism affected migration and adhesion in HT29 (data not shown), HCT116 and SW480 cells: cells overexpressing the Gly allele adhered better and were able to form more colonies while the Arg allele rather contributed to migration and invasion. This was also confirmed by C. Heinzle's work (see thesis 2011) and is in line with the findings of Bange *et al.* (2002) associating the $FGFR4^{arg}$ allele with early lymph node and advanced tumour-node metastasis. $FGFR4^{arg}$ was shown to interact and stabilise membrane-type 1 matrix metalloproteinase (MT-MMP-1) and support collagen invasion (Sugiyama *et al.* 2010*, Sugiyama *et al.* 2010).
FGF19 administration improved attachment of FGFR4 overexpressing SW480 cells but impaired migration in HCT116 – both transfected and untransfected. How this relates to the results described by Pai and his colleagues (2008) is not

yet understood because the role of Wnt-signalling on the migration and adhesion of CRC cells is still unknown.

SW480 cells express more FGF18 than FGF19 (see chapter 4.1.1) which could act negatively on FGFR4^{arg} and impair attachment that is then restored by FGF19 administration.

In HCT116 cells, FGF18 expression is lower when compared to the SW480s but FGF5 expression levels were similar to FGF19 levels (C. Heinzle thesis 2011). Therefore, FGF19 may inhibit FGF5 activity through blocking the receptors und thus impair its effects on migration. FGF5 is a key player in the hair follicle growth cycle and takes part in myogenesis (Beenken and Mohammadi 2009). Whether it is involved in migration processes of CRC (or in general cancer) cells would be a new discovery. Thus, these findings infer a novel role for FGF19 (and maybe FGF5) in malignant behaviour and need further investigation.

5.6 Development of the Invasion assay and obtained results

An invasion assay protocol was developed using collagen and Matrigel™ to coat membrane filters. This provides not only an obstacle for invasion but also ECM contacts that are usually absent from the 2D in vitro assays we routinely used in this study. Surprisingly, the more collagen was deposited on the filter membrane, the more cells were able to invade, indicating that interaction with the matrix protein stimulates invasion. In a recent paper, Spivey *et al.* (2011) could positively link collagen (type XXIII) to metastasis through cell-cell and cell-ECM adhesion. Cell-matrix interaction was even more obvious when using Matrigel™ as this hanged cell behaviour. The tumour cells formed clusters and increasing concentrations of Matrigel™ inhibited invasion.

5.7 Signalling effects and influence of FGF18 downregulation

FRS2 levels were analysed in three different CRC cell lines, overexpressing either Arg or Gly allele of FGFR4 and compared to the control cell line (pcDNA). No significant differences were found between the two alleles. Compared to control, phosphorylation of FRS2 was high in HCT116, equal in SW480 but lower in HT29 cells. This shows that FGFR4 acts differently depending on the cell line and seems to have more influence on the signalling of progressed colorectal tumour cell lines. It is consistent with better migration of HCT116 cells which seems to be mediated by endogenous FGF19 interaction, probably through FRS2 and Src

(Lieu and Kopetz 2010). FGF19 was inhibition was previously described to reduce FRS2 and ERK2 phosphorylation along with active β-catenin levels (Pai *et al.* 2008).

	PLCγ	Src	ERK	GSK3β	S6
FGFR4^{arg}	↔phospho ↔total	↔phospho ↓total	↔phospho **↓total**	↔phospho **↓total**	↔phospho **↓total**
FGFR4^{gly}	↓phospho **↑total**	**↓phospho** ↔total	↔phospho **↓total**	↑phospho ↓total	↔phospho ↓total

Table 5-1 intracellular effect of FGF18 knockdown on FGFR4 overexpressing SW480 cells
↑ up- and ↓ downregulation or ↔ unchanged, strong effects in **bold**

	t	PLCγ	Src	ERK	GSK3β	S6
FGFR4^{arg}	5' 15'	↔	↑p ↑total	**↓p ↑total**	**↑p ↓total** ↔	↔
FGFR4^{gly}	5' 15'	**↑p ↓total** ↔	**↑p**	**↑total** ↔	↔ **↓p ↓total**	**↓total**

Table 5-2 intracellular effect of exogenous FGF18 on FGFR4 overexpressing cells
t refers to incubation time, p to phosphorylated protein
↑ up- and ↓ downregulation or ↔ unchanged, strong effects in **bold**

FGF18 knockdown downregulated phosphorylation of PLCγ and Src, while it upregulated phosphorylation of GSK3ß in SW480^{gly} cells, but did not affect phosphorylation status of signalling proteins in SW480^{arg} (Table 5-1). Addition of exogenous FGF18 reversed the KD-effects in SW480^{gly}. In SW480^{arg} cells it increased phosphorylation of Src and GSK3ß, but downregulated pERK. Interestingly, PLCγ was only affected in Gly but not in Arg-transfectants.

PLCγ is a direct target of FGFR4 and through PKC activation via DAG, is involved in cell growth, differentiation and migration (Suh *et al.* 2008). Stable silencing of PLCγ was reported to have anti-tumorigenic effects on CRC cells (Tan *et al.* 2007). FGF18 downregulation slightly decreased phosphorylated but strongly increased total PLCγ in SW480^{gly} cells but did not affect FGFR4^{arg} overexpressing cells. Treatment with factor for 5' reversed these effects. FGFR4 G388 was reported to inhibit motility and invasion of a type of breast cancer cells by suppression of specific genes involved in these processes (Stadler *et al.* 2006). Knockdown resulted in enhanced invasion through MT1-MMP (Sugiyama *et al.*

2010*). PLC signalling has been shown to modulate PI-3K dependent cell motility (Kolsch *et al.* 2008). Whether general FGF dependent modulation of PLC activity plays a role in this context needs to be determined.

Src is a kinase and interacts with PLCγ in tumour cells (Suh *et al.* 2008, Tvorogov *et al.* 2005). It is crucial for activation and termination of FGFR signalling (Sandilands *et al.* 2007). It can affect survival through regulation of PI-3K-Akt pathway, proliferation through the MAPK pathway, angiogenesis by STAT3/5b and motility as well as invasion (Lieu and Kopetz 2010). N-Cam was found to induce FGFR1 stabilisation and recycling leading to Src mediated cell migration (Francavilla *et al.* 2009). Phosphorylation was decreased in FGF18 knocked down SW480^{gly} protein lysates while in SW480^{arg} cells Src protein level was slightly downregulated. FGF18 administration rescued both effects. This gives FGF18 a role in the mobility of cells through the Gly allele while the FGFR4^{arg} may facilitate independence from the FGF18 and thus from Src signalling.

ERK is a downstream component of the MAPK signalling pathway (chapter 1.7.2.1) and was found to be downregulated with FGF18 knockdown in SW480^{arg} as well as in SW480^{gly} cells. While factor addition rescued these phenotypes in SW480^{arg} cells and even down-regulated ERK phosphorylation, this effect was only transient in SW480^{gly} cells. Sonvilla *et al.* (2008) already reported of the pro-tumorigenic effect of FGF18. Here, the Arg allele may render the receptor more sensitive to FGF18, leading to a long-lasting pro-proliferative effect through activation of the MAPK pathway.

Glycogen synthase kinase 3β protein level was also decreased in SW480^{arg} and SW480^{gly} cells treated with si-FGF18 with an increase of pGSK3β level in the latter. Downregulation of total GSK3β was with the consequence of factor addition for 5’ in SW480^{arg} cells. Interestingly SW480^{gly} cells responded 10’ later with an upregulation of the pGSK3β level while total protein was further decreased. As discussed earlier, this kinase plays a role in the degradation of β-catenin. Since FGF18 is a target gene of β-catenin (Katoh and Katoh 2006*, Shimokawa *et al.* 2003), it could be crucial to suppress inhibition of GSK3β, resulting in an auto-inhibition. Response to FGF18 treatment in the later time point and rescue indicates that the FGFR4^{gly} may act through a different pathway in the absence of FGF18.

Not phosphorylated, active GSK3β negatively regulates SNAIL activity which then cannot repress E-cadherin transcription (Katoh and Katoh 2006). In our immunostaining experiment, we showed upregulation of the mesenchymal markers fibronectin and vimentin in FGFR4^{gly} overexpressing SW480 cells. FGF18 seems to decrease GSK3β activity through decreasing total GSK3β and/or increasing deactivation through phosphorylation and further be responsible for the mesenchymal polarisation of especially the SW480^{gly} cells.

S6 or S6 kinase (S6K) is a component of the ribosomal subunit 40S and known to play a role in the regulation of proliferation, protein translation, survival and tumour growth. Its activation is regulated through mTORC1 and PDK1 that lies downstream of the PI-3K-Akt pathway (Fenton and Gout 2011). Knockdown of FGF18 decreased total S6 levels in SW480^{arg} cells and also in FGFR4^{gly} overexpressing cells. FGF18 administration did not change anything in SW480^{arg} cells but had a further negative effect on the FGFR4^{gly} transfectants.

5.8 Conclusions

Our results indicate a role for FGFR4 G388R polymorphism in tumour progression and motility of cells. FGFR4 seems to be an oncogene with the Gly allele responsible for attachment, colony formation *in vitro* and malignant growth *in vivo* (C. Heinzle thesis 2011) while Arg stimulates invasiveness and acts strongly pro-metastatic (also in tissues – C.Heinzle diss. 2011).

Structural alterations have to exist since intracellular signal transduction differed depending on the overexpression of FGFR4 alleles and the ligand. It seems that FGFR4 R388 variant shifts intracellular signalling to mainly the MAPK pathway. Further investigations are required to resolve remaining inconsistencies regarding results from ligand stimulated cells. Studies will be pursued also in 3D culturing to simulate *in vivo* environment.

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7 Abstract

The FGF-FGFR system consists of 22 ligands and various receptors expressed from four FGFR genes that play important roles in survival, migration and neo-angiogenesis. Cancer cells deregulate and exploit this system by various mechanisms including overexpression of FGFs and FGFRs and autocrine stimulation of tumour cells. This leads to sustained signalling resulting in tumour cell growth and cancer progression. Specifically FGFR4 is involved in tumour aggressiveness in several tumour types. A single nucleotide polymorphism (G388R) in the transmembrane domain was described with the presence of FGFR4 Arg allele leading to enhanced metastasis.

We investigated (1) the biological role of the FGFR4 polymorphism in colorectal cancer cells and (2) the differential activation of the polymorphic alleles by the ligands FGF18 and FGF19. The results of this study demonstrate higher aggressiveness of $FGFR4^{arg}$ cells which could also be confirmed through investigation of the invasion activity especially in HCT116 cells. Cells expressing mostly $FGFR4^{gly}$ displayed an increased attachment and colony formation ability. In contrast, expression of mesenchymal markers was quite low in $FGFR4^{arg}$ overexpressing SW480 cells and also the $FGFR4^{arg}$ expressing HT29 cells while overexpression of the Gly allele enhanced mesenchymal markers.

Addition of FGF18 and FGF19 to cell culture medium did not stimulate growth and clonogenicity. FGF19 inhibited cell migration and increased cell attachment independent of FGFR4 allele.

FGF18 knockdown downregulated phosphorylation of PLCγ and Src, while it upregulated phosphorylation of GSK3ß in $SW480^{gly}$ cells, but did not affect phosphorylation status of signalling proteins in $SW480^{arg}$. Addition of exogenous FGF18 reversed the KD-effects in $SW480^{gly}$. In $SW480^{arg}$ cells it increased phosphorylation of Src and GSK3ß, but downregulated pERK. These observations indicate FGF18 signalling FGFR4, especially the Gly allele. Mechanistic details still need to be elucidated.

In summary, our observations suggest that autocrine stimulation by FGF18 and FGF19 via FGFR4 is a player in tumour growth and progression that needs to be further elucidated.

8 Zusammenfassung

Das FGF-FGFR System besteht aus 22 Liganden und 4 Rezeptoren die eine wichtige Rolle für das Überleben, die Migration und Neo-Angiogenese spielen. In Krebszellen ist dieses System u. A. durch Überexpression von FGFs und deren Rezeptoren dereguliert. Das führt durch autokrine Stimulation zu einer anhaltenden Signalaktivität, die das Tumorzellwachstum fördert und zur Krebsprogression beiträgt. Speziell FGFR4 wurde mit Tumoraggressivität in verschiedenen Tumortypen assoziiert. Ein Polymorphismus wurde beschrieben, der das Gly an der Stelle 388 der FGFR4 Transmembrandomäne durch ein Arg ersetzt was die Metastasierung fördert.

Wir untersuchten (1) die biologische Rolle dieses Polymorphismus in kolorektalen Karzinom-Zellen und (2) die unterschiedliche Aktivierung der Genprodukte der Allel durch die Liganden FGF18 und FGF19. Die Ergebnisse dieser Studie zeigen höhere Aggressivität von $FGFR4^{arg}$ Zellen, die auch durch Invasionsdaten besonders in HCT116 Zellen bestätigt wurden. Zellen, die hauptsächlich $FGFR4^{gly}$ exprimieren, wiesen erhöhte Anheftungsfähigkeit und verbesserte Kolonienbildung auf. Expression von mesenchymalen Markern hingegen, war niedrig in SW480 $FGFR4^{arg}$ Transfektanten wie auch in den $FGFR4^{arg}$ heterozygoten HT29 Zellen, während Überexpression eines Gly Allels die Expression der mesenchymalen Marker förderte.

Zugabe von FGF18 und FGF19 in das Medium hatte keinen Effekt auf das Wachstum und die Kolonienbildung. FGF19 inhibierte Zellmigration und erhöhte Zellanheftung unabhängig vom FGFR4 Allel.

Herunterregulation von endogenem FGF18 wirkte sich negativ auf die Phosphorylierung von PLCγ und Src aus, erhöhte aber GSK3ß Phosphorylierung in $SW480^{gly}$ Zellen während in $SW480^{arg}$ Zellen der Phosphorylierungsstatus von Signalproteinen unberührt blieb. Zugabe von exogenem FGF18 konnte die Knockdown-Effekte in $SW480^{gly}$ aufheben. In $SW480^{arg}$ Zellen erhöhte FGF18-Zugabe Phosphorylierung von Src und GSK3ß aber pERK Level wurde erniedrigt. Diese Beobachtungen deuten auf eine FGF18 Signalwirkung durch FGFR4, besonders dem Gly Allel.

Unsere Studie weist auf die Möglichkeit einer autokrinen Stimulation von Tumorzellen durch FGF18 und FGF19 hin, die durch den FGF Rezeptor 4 vermittelt wird.

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