universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Impact of Fibroblast Growth Factor Receptor 4 on the pro-metastatic behaviour of HCC model cell lines

verfasst von

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer. nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuer:	Ao. Univ. Prof. Dr. Christian Seiser

Danksagung

An er erster Stelle möchte ich von Herzen meiner ganzen Familie, insbesondere meinen Eltern, und meinem Freund danken. Ihr habt mich durch die Höhen und Tiefen dieses Studiums begleitet, seid immer für mich da gewesen und hattet für jedes Problem ein offenes Ohr. Außerdem habe ich erst durch eure bedingungslose Unterstützung und euren unerschütterlichen Glauben an mich das nötige Selbstvertrauen in mich und meine Fähigkeiten gewonnen um so weit zu kommen.

Des Weiteren gilt mein Dank Ao. Univ.-Prof. Dr. Bettina Grasl-Kraupp, die mir die Möglichkeit gab, meine Diplomarbeit in ihrem Labor durchzuführen und mich in allen Belangen unterstützt und beraten hat.
Bei Ao. Univ. Prof. Dr. Christian Seiser bedanke ich mich für die Betreuung meiner Diplomarbeit.

Ebenso danke ich Ao. Univ. Prof. Dr. Walter Berger, Ass. Prof. Dr. Michael Grusch, Ao. Univ. Prof. Dr. Klaus Holzmann und Ao. Univ. Prof. Dr. Brigitte Marian und den restlichen Mitgliedern der FGF-Runde für die anregenden Besprechungen und die gute Zusammenarbeit.
Birgit Mir-Karner, Helga Koudelka und Krystyna Bukowska danke ich für die technische Unterstützung und ihre Hilfsbereitschaft im Labor.

Ein besonderer Dank gilt auch meinen Labor- und ArbeitskollegInnen Walli, Meli, Mario, Dani, Chrisi, Salma, Teresa, Jakob, Christine, Karin, Alex, Andi und Robert nicht nur für das sensationelle Arbeitsklima, die inspirierenden Diskussionen und die Unterstützung im Labor, sondern auch für Geburtstagsfrühstücke und Pizzasessions nach langen Arbeitstagen, für die Kaffeepausen und die vielen Plaudereien und die auch über die Arbeit hinausreichende Freundschaft.

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1. Abstract / Zusammenfassung

1.1. Abstract

Hepatocellular carcinoma (HCC) is one of the most aggressive cancer types. Despite medical progress, the majority of cases are diagnosed at an advanced stage, implicating rather limited treatment options and a poor prognosis. Precedent studies on HCC tissue samples revealed deregulation of fibroblast growth factor receptor 4 (FGFR4) pointing towards an involvement of this growth factor receptor in late stages of the disease. Recently, a Gly388Arg single nucleotide polymorphism in the transmembrane domain of the receptor has been described. Thereby, FGFR4/Arg has been associated with an increased malignancy of HCC.

The aim of the present study was to investigate the significance of FGFR4 for pro-metastatic processes such as cell-matrix and cell-cell adhesion as well as the interaction of tumour cells with endothelial cells. For our mechanistic studies, two HCC/hepatoma model cell lines were used. The interaction of tumour cells with endothelial cells was investigated by using a 3-D co-culture in vitro assay, which was adapted to mimic tumour-cell mediated gap formation in blood or lymphatic vessels. Cell-cell and cell-matrix adhesion were examined by using a hanging drop or adhesion assay, respectively.

First, we examined the impact of bile acids and specific inhibitors on the gap forming capacity of HCC/hepatoma cells in order to gain insights into mediators affecting epithelial-mesenchymal interactions in advanced hepatocarcinogenesis. Second, FGFR4/Gly and FGFR4/Arg were transiently or stably overexpressed. In a third approach, FGFR4-mediated signaling was impaired by infecting cells with adenoviral constructs coding for a dominant negative FGFR4 or for the soluble part of FGFR4, which blocks the ligand binding part of this receptor.

Spheroid induced gap formation of HCC/hepatoma cell lines in endothelial cell monolayers was impaired by the use of the specific inhibitors BAY 11-7082, GM6001 and Baicalein, indicating that NF-κB, MMPs and lipoxygenases might be involved in endothelial cell retraction. On the other hand, bile acid had no effect on gap forming capabilities.

Transient or stable overexpression of mutant or wildtype version of FGFR4 in the HCC/hepatoma model cell lines decreased cell-matrix adhesion, with FGFR4/Arg showing more pronounced effects

than FGFR4/Gly. Cell aggregation capabilities and spheroid induced gap formation was increased due to FGFR4 overexpression. Blockade of FGFR4 signalling through adenoviral infection impaired cell-matrix adhesion and intercellular adhesion as well as gap formation in blood and lymphatic endothelial cell monolayers.

To conclude, FGFR4 might affect the pro-metastatic behaviour of HCC cells due to its influence on cell-matrix adhesion, cell aggregation and spheroid-induced gap formation. Further studies are necessary to elucidate the underlying mechanisms.

1.2. Zusammenfassung

Das hepatozelluläre Karzinom (HCC) ist eine der aggressivsten Krebsarten und trotz medizinischer Fortschritte wird der Großteil der Fälle in einem bereits fortgeschrittenen Stadium diagnostiziert. In diesem Stadium sind die Behandlungsmöglichkeiten limitiert, was eine schlechte Prognose zur Folge hat. In vorangehenden Studien mit HCC Gewebeproben wurde eine Fehlregulierung des Fibroblasten Wachstumsfaktor Rezeptor 4 (FGFR4) festgestellt, welche darauf hindeutet, dass der Rezeptor eine Rolle in späten Phasen des Krankheitsverlaufs spielt. Vor kurzem wurde ein Gly388Arg Einzelnukleotid-Polymorphismus in der Transmembrandomäne des Rezeptors beschrieben, wobei FGFR4/Arg mit einer erhöhten Bösartigkeit des Tumors assoziiert wurde.

Das Ziel der vorliegenden Studie war die Bedeutung von FGFR4 in pro-metastatischen Prozessen, wie der Zell-Matrix und Zell-Zell Adhäsion sowie der Interaktion von Krebszellen mit Blut- oder Lymphendothelzellen, zu ermitteln. Für unsere mechanistischen Studien wurden zwei HCC/Hepatom-Zelllinien verwendet. Die Interaktion von Tumorzellen mit Endothelzellen wurde mit Hilfe eines dreidimensionalen Ko-Kulturmodells untersucht, welches adaptiert wurde um die von Leberkrebszellen verursachte Lückenbildung in Blut- oder Lymphgefäßen zu imitieren. Zell-Zell und Zell-Matrix Adhäsion wurden durch den Einsatz eines „Hanging Drop Assay“ bzw. eines „Adhesion Assay“ untersucht.

Zuerst wurde die die Auswirkung von Gallensäuren und spezifischen Inhibitoren auf die Fähigkeit von HCC/Hepatomzellen zur Lückenbildung überprüft um einen Einblick zu gewinnen, wie verschiedene Mediatoren die Interaktion zwischen Epithel- und Mesenchymzellen in fortgeschrittenen Stadien der Hepatokarzinogenese beeinflussen. Des Weiteren wurden FGFR4/Gly und FGFR4/Arg transient und stabil überexprimiert. In einem dritten Ansatz wurde die FGFR4 Signalübertragung durch Infektion

der Zellen mit adenoviralen Konstrukten, die für ein dominant negatives FGFR4 oder den löslichen Teil von FGFR4, welcher die Liganden bindende Domäne des Rezeptor blockiert, inhibiert.

Die Spheroid-induzierte Lückenbildung von HCC/Hepatom-Zelllinien in Endothelzell-Monolayern wurde durch den Einsatz der spezifischen Inhibitoren BAY 11-7082, GM6001 und Baicalein verringert, was darauf hinweist, dass NF-κB, MMPs und Lipoxygenasen an dem Vorgang beteiligt sein könnten. Andererseits hatten Gallensäuren keine Auswirkung auf die Lückenbildung.

Transiente wie auch stabile Überexpression der mutierten oder normalen Form von FGFR4 in HCC/Hepatom-Zelllinien verringerte die Zell-Matrix Adhäsion, wobei der Effekt von FGFR4/Arg stärker war als der von FGFR4/Gly. Die Fähigkeit zur Zellaggregation und zur Spheroid-induzierten Lückenbildung wurde aufgrund der FGFR4 Überexpression erhöht werden. Blockade der FGFR4 Signalübertragung durch adenovirale Infektion beeinträchtigte die Zell-Matrix Adhäsion, die interzelluläre Adhäsion wie auch die Induktion von Lücken in einer Blut- oder Lymphendothelzellschicht.

Zusammenfassend könnte der FGFR4 das pro-metastatische Verhalten von HCC Zellen, aufgrund der Wirkung auf die Zell-Matrix Adhäsion, Zellaggregation und die Spheroid-induzierte Lückenbildung, beeinflussen. Weiterführende Studien sind notwendig um die zugrunde liegenden Mechanismen aufzuklären.

2. Introduction

2.1. Hepatocellular carcinoma

2.1.1. Epidemiology of HCC

Hepatocellular carcinoma accounts for 85%-90% of all liver cancer cases and is therefore the most dominant form of primary hepatic malignancy outranging other types like cholangiocarcinomas and hepatoblastomas (McKillop, 2006; El-Serag, 2007). Generally, primary liver cancer is the fifth most prevalent neoplasm in men and the seventh in women (El-Serag, 2011). Due to its high mortality, nearly 700.000 deaths from liver cancer were estimated in 2008, ranking it to the third leading cause of cancer-related mortality worldwide (Ferlay, 2010).

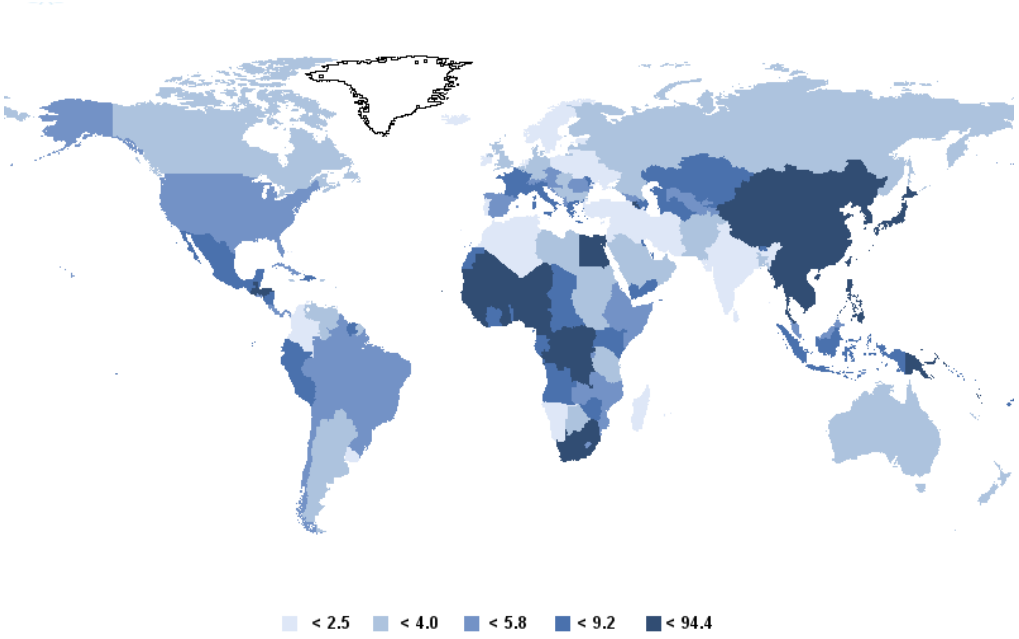

Figure 1: Estimated age-standardised incidence rate of liver cancer per 100,000 (Globocan 2008).

The incidence of HCC is characterized by distinct differences in the distribution throughout the world depending on the prevalence of the major risk factors (**Figure 1**). Almost 85% of HCC cases occur in developing countries in sub-Saharan Africa, Southeast Asia and East Asia where chronic infections with hepatitis B virus (HBV) and exposure to aflatoxin B1 are endemic (Ferlay, 2010; El-Serag, 2011). By contrast, incidence is much lower in Europe, North America, Central and South America, Australia and New Zealand with hepatitis C virus (HCV) as the major risk factor (Ferlay, 2010; El-Serag, 2011). In the past decades, HCC gained major interest in Europe and the USA as an upward trend was reported, whereas it decreased in developing countries (McGlynn, 2001). For example, incidence

rates tripled in the United States from 1975 to 2005, which is believed to be driven by the growing prevalence of HCV infections between 1960 and 1990 (Altekruse, 2009; El-Serag, 2011).

Typically, the risk for HCC occurs to be particularly higher in men, reflected by an overall sex ratio male: female of 2:1 and 4:1 (Parkin, 2005). This variance may be explained by sex-specific differences in exposure to various risk factors like alcohol, smoking or HBV and HCV infections (El-Serag, 2007). However, experiments support the hypothesis that androgens influence the progression of HCC in men rather than the sex-specific exposure to risk factors (El-Serag, 2007). But also non-environmental endogenous factors, including body mass index and increased androgen receptor signaling may affect the men's risk to develop HCC (El-Serag, 2007).

In Austria, the incidence of HCC is comparatively rare, but because of its poor prognosis it belongs to the ten most prevalent cancer deaths (Statistics Austria, 2012). Annually, approximately six of 100,000 persons come down with malignant growths in the liver and for five of 100,000 persons the disease is mortal (Statistic Austria, 2012). The age-adjusted incidence and mortality rates are respectively 2.8 and 2.9 higher in men than in women (Statistic Austria, 2012). Data from the Statistics Austria present an increase in incidence and mortality over the period of 1986-2010 (**Figure 2**; Statistics Austria, 2012). The average, age-adjusted rates of HCC in men increased from 7 per 100,000 during 1986-1989 to 11 per 100,000 during 1999-2004 (Statistic Austria, 2012). Since then, incidence rates decreased to 8-9 per 100,000 persons (Statistic Austria, 2012). In women, incidence rates increased from 2 per 100,000 in the mid 1980s to approximately 4 per 100,000 in 2003 and finally settled down to 3 per 100,000 in 2010 (Statistic Austria, 2012).

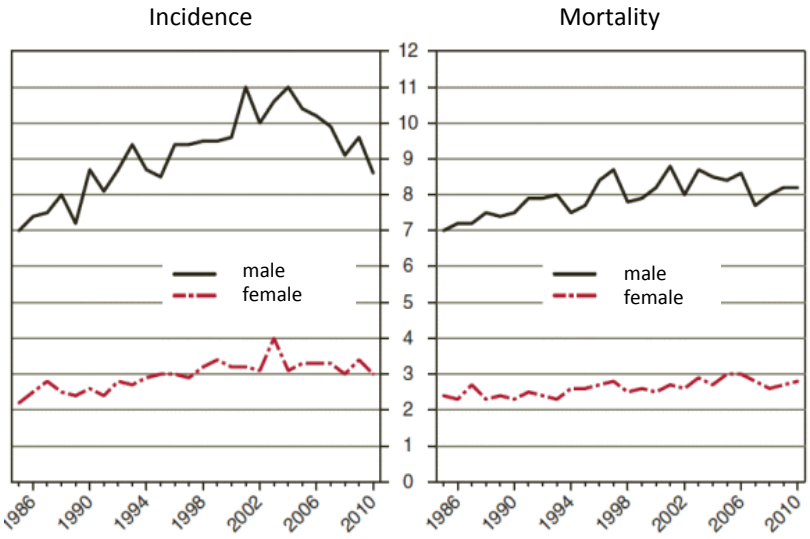

Figure 2: Incidence and mortality rates of liver cancer from 1986 to 2010 in Austria. *Age-adjusted rates per 100,000 persons (Statistics Austria, 2012).*

2.1.2. Risk factors and prevention

Unlike many other cancer entities, already much is known about the development and the risk factors of HCC. In 80%-90% of all detected [HCC] cases, chronic liver diseases and cirrhosis are the predisposing factors for the development of hepatic neoplasms (Oyagbemi, 2010; El-Serag, 2011). However, the main causal factors affecting HCC vary by geographic location (Bosch, 2004). Chronic Infections with HBV and HCV as well as alcohol consumption and dietary aflatoxin B1 intoxication have been identified as major environmental risk factors for HCC (El-Serag, 2011; Yang, 2010).

Frequently, HCC develops in the course of a long-term infection with either HBV or HCV. More than 50% of all HCC cases are attributed to chronic infection with HBV and particularly applies to areas where HBV is endemic (Parkin, 2006; Gurtsevitch, 2008; Sherman, 2010; El-Serag, 2011). In these areas, HBV infections most often (~70%-80%) cause a chronic liver disease, which increases the risk of carriers to develop HCC about 100-fold (Szabo, 2004). Areas with high incidence of HCC and endemic HBV infection coincide with areas with high dietary exposure to aflatoxin B1 (AFB1), which is produced by a fungus of the genus *Aspergillus* (Monalto, 2002; Gomaa, 2008). AFB1 intake is suggested to increase the risk for HCC in HBV infected patients (Groopman, 1996; Monalto, 2002). HBV vaccination is the most effective measure to reduce the global burden of HCC (Kensler, 2003). A nationwide immunization of infants between 1984 and 1986 in Taiwan is representative for the evidence that HCC is preventable as the incidence of HCC declined dramatically from 15% to 1% in children aged 6-14 (Chang, 1997).

Accounting for 20% of all HCC cases, chronic infection with HCV is the second most common cause of HCC (Bosch, 2004). It increases the risk about 15-20 fold (Sun, 2003; El-Serag, 2011) and is the leading cause for chronic liver disease and HCC in most Western countries (Di Bisceglie, 2003). Prolonged, heavy use of alcohol is often found to be an important cofactor in patients with HCV infections, additionally increasing the risk for developing HCC (Hassan, 2002; Donato, 2002).
Prevention of HCV infections are challenging as a vaccination is not available (Forns, 2002). Most of the strategy in decreasing the incidence of HCV induced HCC relies on preventing transmission by transfusion of blood products (Llovet, 2003). The risk for development of HCC in patients with existing HCV infection may be decreased by antiviral strategies, such as pegylated interferon and rivabirin, which are thought to prevent the progression to advanced fibrosis or cirrhosis (Llovet, 2003).

Besides chronic infections with HBV and HCV, an increasing body mass index and diabetes resulting in the development of non-alcoholic steatohepatitis (NASH) represent significant causes for the disease (Blonski; 2010). Especially NASH is an emerging risk factor in developed countries as it is tightly linked to obesity, which increased to epidemic proportions over the last three decades (Marrero 2002; Blonski, 2010).

2.1.3. Molecular pathology of hepatocarcinogenesis

Cirrhosis is the major risk factor for developing hepatocarcinogenesis, associated with almost 80% of all HCC cases worldwide (El-Serag, 2011). Cirrhosis most often develops after long latencies (20-40 years) of chronic liver disease in the context of constant inflammation, cell proliferation and fibrosis, secondary to viral and chemical inputs (**Figure 3**; El-Serag, 2007). This results in the development of preneoplastic lesions such as macroregenerative nodules, low-grade and high grade dysplastic (Leong, 2005). In all preneoplastic conditions, hepatocytes accumulate different molecular and genetic alterations finally leading to the malignant transformation of the cells (Thorgeirsson, 2002). Thus, cancer formation is believed to occur through a pathway of accelerated proliferation and development of monoclonal hepatocyte populations by upregulation of mitogenic pathways (Levrero, 2006). HBV and HCV infections are crucial initiators of genetic damage. In patients with chronic HCV, an increase in TGF-α and IGF-2 contribute to accelerated hepatocyte proliferation (Llovet, 2008). Simultaneously, integration of HBV DNA may result in promoter activation of oncogenes and growth factors, inactivation of tumour suppressor genes, DNA rearrangement and chromosomal instability (Leong, 2005).

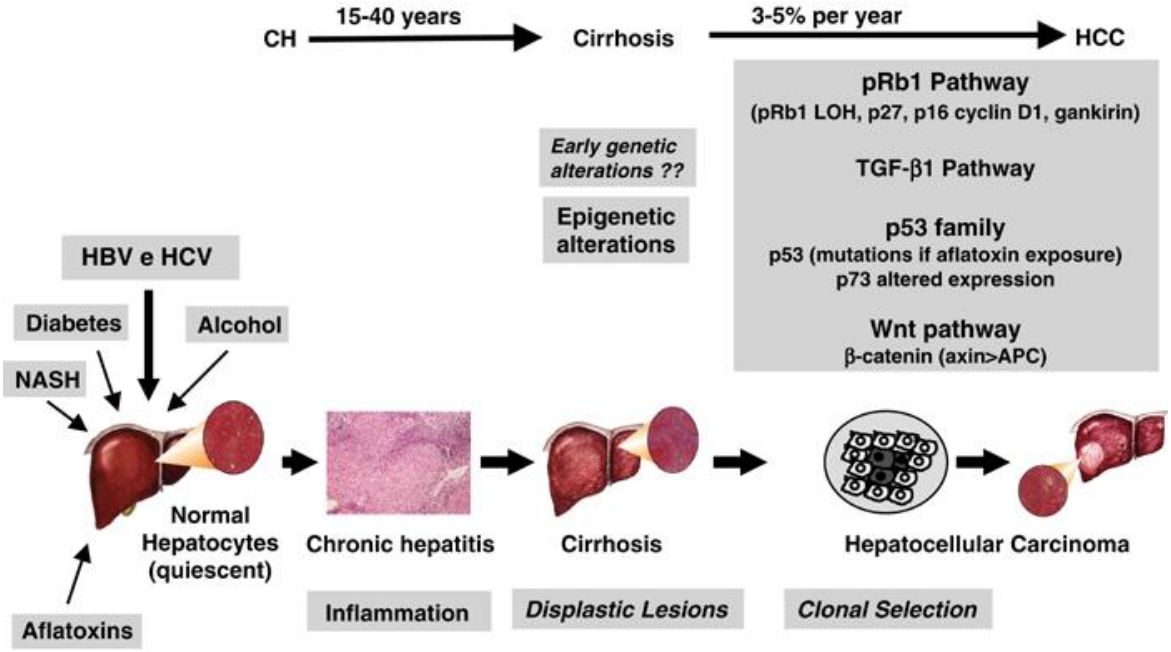

Figure 3: Pathogenesis of human hepatocellular carcinoma (HCC; Levrero, 2006).

Hepatocellular tumours display a high degree of genetic heterogeneity since multiple molecular pathways can be deregulated by both genomic and epigenetic alterations (Walther, 2011). According to Hanahan and Weinberg certain pathways need to be deregulated for cancer development: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tumour invasion and metastases (Hanahan, 2000). These concepts can be integrated into the molecular pathogenesis of HCC. In half of the cases, tumour suppressor genes involved in cell cycle regulation, such as p53, p16, Rb or cyclin D1, are affected (El-Serag, 2007). Through the abrogation of cell cycle checkpoints, genetically altered hepatocytes gain selective advantages allowing their clonal expansion (El-Serag, 2007). Moreover, aberrant angiogenesis occurs already in early hepatocarcinogenesis due to autocrine/paracrine secretion of VEGF, PDGF or angiopoietin-2 (Kensler, 2003). Finally, cells are able to evade apoptosis by deregulation of pro-apoptotic pathways and to ensure limitless replicative potential by reactivation of TERT (Kensler, 2003). However, cell proliferation seems to be most important in liver cancer progression. Various pathways have been described to be involved in HCC i.e. Wnt, RAS/MAPKK, IGF, c-met or Akt/mTOR signaling (Kensler, 2003).

Structural genomic alterations develop rarely in the early preneoplastic phase, but increase distinctly in dysplastic hepatocytes and HCCs (Thorgeirsson, 2002). In general, a variety of chromosomal aberrations can be found in this cancer entity due to a high level of chromosomal instability (Walther, 2011). Allelic deletions as well as gains in chromosomes, contributing to the activation or inactivation of oncogenes or tumour suppressor genes have been frequently reported (Thorgeirsson, 2002).

2.1.4. Therapeutic options for HCC

The applicability of treatments is strongly dependent on the patient's performance status, number and size of nodules, cancer symptoms and liver function described by the Barcelona Clinic Liver Cancer (BCLC) staging system (Forner, 2010; El-Serag, 2011). In the western world, only 30%-40% of patients are eligible for curative treatments since they are most effective at an early stage (Bruix, 2002; Llovet, 2003). Diagnosis of HCC at an early stage is currently difficult since discrimination between single, asymptotic but malignant lesions from cirrhotic nodules is demanding (El-Serag, 2002). Furthermore in most cases vascular or distant metastasis has already occurred (El-Serag, 2002).

In a very early or early stage of HCC, surgical resection, liver transplantation or percutaneous local ablative therapy achieve the best outcome in well-selected patients represented by a 5-year survival

rates of 50%-70% (Llovet, 2003). Patients with advanced stage HCC, identified by mild cancer-related symptoms, vascular invasion or extrahepatic spread, are not suitable for radical therapies (El-Serag, 2011). However, transaterial chemoembolization (TACE) was shown to improve the survival among patients with preserved liver function and asymptotic multinodular tumours without vascular invasion or extrahepatic spread (El-Serag, 2011). At intermediate stages, treatment may increases the median survival rate of patients from 16 month to 20 month only (Llovet, 2008).

For patients with advanced HCC, systemic therapies with conventional chemotherapeutic agents were shown to be consistently inconclusive as most HCCs have an inherent high level of chemoresistance or have poor tolerance due to underlying liver dysfunction (Yang 2010). The identification of several important molecules and pathways in liver carcinogenesis may provide new, putative targets for selective molecular therapies (Llovet 2008). Sorafenib, a multikinase inhibitor targeting RAF kinase, VEGFR and PDGFR signaling pathways, has presented promising results in clinical trials with advanced-stage patients as it increases the median overall survival rate by 37% (Llovet, 2008). Results from studies with Sorafenib encourage the search for additional molecular agents to further improve the patient's survival.

2.2. The FGF signalling system

2.2.1. FGFs and FGFRs

Since the first isolation of prototypic fibroblast growth factors (FGFs) as mitogens from bovine brain tissue in the 1970s, a multitude of physiological functions controlled by the FGF family have been discovered (Gospodarowicz, 1978). FGFs and their receptors were implicated in the regulation of developmental processes from mesoderm patterning in the early embryo to the development of multiple organ systems (Kimelman, 1987; De Moerlooze, 2000). In adult organisms, FGF signalling contributes to tissue homeostasis as well as tissue repair, angiogenesis and inflammation (Dillon, 2004; Turner, 2010; Haugsten, 2010).

The mammalian FGF family comprises >20 members (FGF1-23) and despite of their diverse function, FGFs share a conserved core of ~120 amino acids with 30%-60% identity (**Figure 4**; Itoh, 2004; Ornitz, 2001). Due to their mechanisms of action, they can be classified as intracellular, canonical and hormone-like FGFs (Itoh, 2011). The mouse FGF15 is the only member, which has no human equivalent (Itoh, 2011). Similarly, the human FGF19 has no equivalent in mice. Therefore it is assumed that FGF19 and FGF15 are orthologous genes in vertebrates (Itoh, 2011). FGFs generally bind to heparan sulphate proteoglycans (HSPGs), which are low-affinity receptors, present in the

extracellular matrix and on the cell surface of most cells (Wesche, 2011). By binding FGFs, HSPGs protect the ligands from degradation and stabilize the FGF-FGFR interaction, thus forcing the dimerization of a ternary complex consisting of FGF, FGFR and HSPG (Harmer, 2004; Mohammadi, 2005).

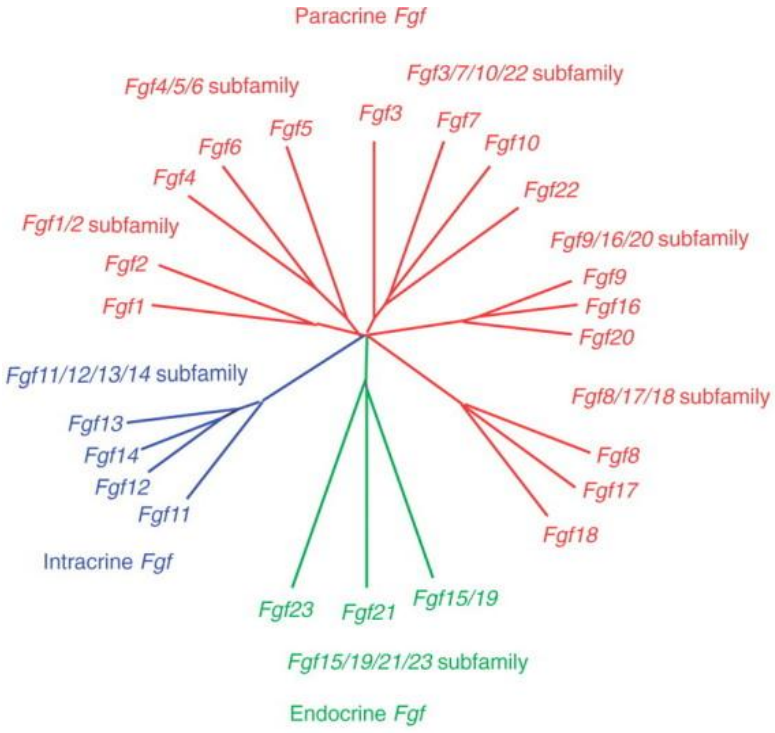

Figure 4: Phylogenetic analysis suggests the arrangement of 22 FGF genes into seven subfamilies. *Branch lengths are proportional to the evolutionary distance between each gene (Itoh, 2010).*

Most of the FGFs are secreted glycoproteins and act as local paracrine signalling molecules by activating the cell surface tyrosine kinase FGFRs (Turner, 2010; Ahmad, 2012). Subsequent to the release of FGFs in the extracellular matrix by heparinases, proteases or specific FGF-binding proteins, the affinity of those FGFs to HSPGs causes them to bind to cell surface HSPGs near the source of their expression (Beenken, 2009).

FGF11-14, also known as FGF homologous factors 1-4 (FHF1-4), are not secreted and appear to bind directly to intracellular targets independent of FGFRs (Olsen, 2003; Ahmad, 2012). Intracellular FGFs present functional differences when compared to other FGFs as they interact with intracellular domains of voltage gated sodium channels and with a neuronal MAPK scaffold protein, thereby regulating the electrical excitability of neurons and possibly other cell types (Goldfarb, 2007; Xiao, 2007; Shakkottai, 2009).

FGF19, 21 and 23 comprise an exception from the ligands' actions in a classic autocrine/paracrine manner, as they were identified to function over long distances as endocrine hormones (Kharitonenkov, 2009; Itoh, 2010). However, biological responses are thought to be mediated by activation of FGFRs (Itoh, 2011). The ability of endocrine FGFs to diffuse from the source of expression is caused by the decreased affinity to HSPGs but accompanied by this, signalling depends on the presence of the co-receptors α-Klotho and β-Klotho in the respective target tissue (Itoh, 2011).

FGFs trigger their cellular response by activation of a family of four receptor tyrosine kinases, designated the high-affinity receptors FGFR1-4 (Johnson, 1993). FGFRs are members of the immunoglobulin (Ig) superfamily, and like other RTKs, they consist of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular split tyrosine kinase domain (**Figure 5**; Johnson, 1993; Schlessinger, 2000; Hunter, 2000). The extracellular domain of FGFRs is composed of three immunoglobulin (Ig) like domains (domain I-III), a positively charged heparin-binding sequence in domain II and an acidic, serine-rich region between domains I and II, termed as acidic box (Schlessinger, 2000). The first Ig-like domain together with the acidic box is reported to play a role in the receptor's autoinhibition (Olsen, 2004), whereas domain II and III provide the FGF ligand binding site (Ornitz, 1996).

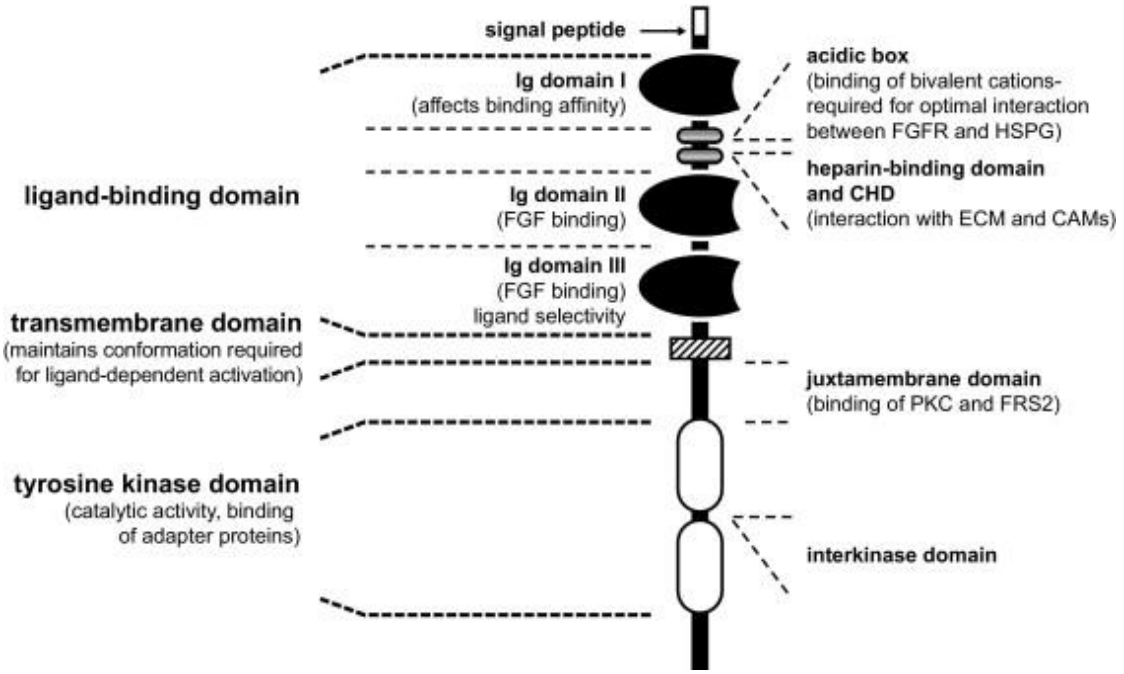

Figure 5: Structure of FGFR proteins. *FGFRs possess three extracellular ligand-binding domains (Ig domain I-III), a single transmembrane domain and a cytoplasmic tyrosine kinase domain (Iyer, 2012).*

FGFs basically differ in their preference to certain FGFRs, but alternative splicing in domain III profoundly alters ligand-binding specificity, thus providing enhanced functional diversity (**Table 1**; Ornitz, 1996). The different isoforms also include FGFRs with truncated COOH-terminal domain, soluble and secreted FGFRs as well as FGFRs with either two or three Ig-like domains (Haugsten, 2010). Alternative splicing occurs in FGFR1-3, but not in FGFR4 and specifies the C-terminal halt of domain III, resulting in either the IIIb or IIIc isoform of the receptor (Eswarakumar, 2005). Thus, control of FGF-FGFR specificity is mediated by the expression of particular ligands and different FGFR isoforms in a tissue specific manner, e.g. the IIIc isoform is usually expressed in mesenchymal tissue whereas the IIIb isoform is mostly found in epithelial cells, particularly during developmental stages (Ahmad, 2012).

Table 1: Ligand specificities of FGFR isoforms (Eswarakumar, 2005).

FGFR isoform	Ligand specificity
FGFR1b	FGF1, -2, -3 and -10
FGFR1c	FGF1, -2, -4, -5 and -6
FGFR2b	FGF1, -3, -7, -10 and -22
FGFR2c	FGF1, -2, -4, -6, -9, -17 and -18
FGFR3b	FGF1 and -9
FGFR3c	FGF1, -2, -4, -8, -9, -17, -18 and -23
FGFR4	FGF1, -2, -4, -6, -8, -9, -16, -17, -18 and -19

2.2.2. FGF/FGFR downstream signalling and negative regulation

Binding of FGFs to domain II and III induces receptor dimerization and leads to a conformational shift in the structure of the receptor. This activates the intracellular kinase domain, enabling the intermolecular trans-phosphorylation of tyrosine residues of the kinase domain at the C-terminus (Beenken, 2009). Phosphorylated tyrosine residues become available as docking sites for various adaptor proteins, which in turn may be directly phosphorylated by FGFR, leading to the activation of several signal transduction pathways (**Figure 6**; Eswarakumar, 2005).

FGFR substrate 2 (FRS2) is a key adaptor protein and particularly specific for FGFRs (Gotoh, 2008). It constitutively binds to highly conserved regions in the juxtamembrane domain of FGFRs through its phosphotyrosine binding (PBT) domain (Gotoh, 2008). Tyrosine phosphorylation of FRS2 is followed by the recruitment of growth factor receptor bound 2 (GRB2) and son of sevenless (SOS) complexes resulting in the activation of RAS and the downstream RAF and MAPK pathway (Kouhara, 1997).

Additionally, GRB2 is associated with the constitutive binding of GRB2-associated binding protein 1 (GAB1) to its C-terminal SH3 domain. Phosphorylation of GAB1 results in the recruitment of signaling proteins like PI-3 kinase (PI3K) and the subsequent activation of an AKT-dependent anti-apoptotic pathway (Altomare, 2005).

Independently of FRS2, PLC-γ binds to phosphotyrosine residues on the C-terminus of FGFRs via its Src homology 2 (SH2) domains (Eswarakumar, 2005). PLC-γ activation leads to the stimulation of phosphatidylinositol-4, 5-bisphospate (PIP_2) hydrolysis and the generation of the second messenger phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) and diacylglycerol (DAG) (Turner, 2010; Ahmad, 2012). Ca^{2+} release in response to the second messengers activates protein kinase C (PKC), which reinforces the MAPK pathway via phosphorylation of RAF (Turner, 2010; Ahmad, 2012). Other pathways activated by FGF signalling include the p38 MAPK, Jun N-terminal kinase, signal transducer and activator of transcription (STAT) and ribosomal protein S6 kinase 2 (RSK2) pathway (Hart, 2000; Turner, 2010).

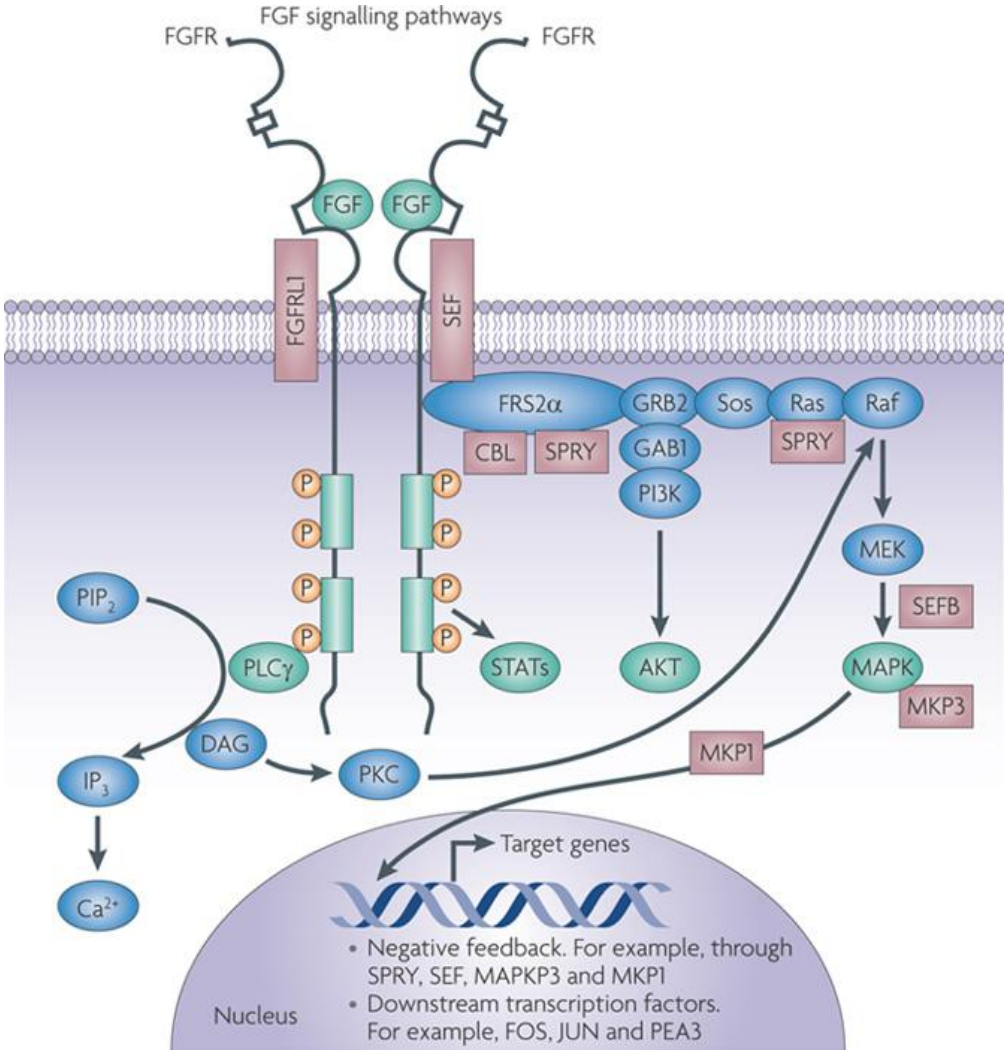

Figure 6: FGF signalling network. *Activation of FGFR through binding of FGFs leads to the activation of four key downstream pathways: RAS-RAF-MAPK, PI3K-AKT, STAT and PLCγ (green). Negative regulation of signalling occurs by receptor internalization or the induction of negative regulators, including FGFRL1, SEF, SPRY, CLB, MKP1 and MKP3 (brown), which modulate ligand binding (FGFRL1 and SEF) or interfere with intracellular signalling through modulation of the MAPK pathway (Turner, 2010).*

Negative regulation of FGF signalling mainly occurs by internalization and subsequent degradation or recycling of FGFRs, which is partially controlled by CBL mediated monoubiquitylation (Thien, 2001), but also several regulator proteins such as MAPK phosphatase 3 (MKP3), Sprouty (SPRY) and SEF family members attenuate downstream signalling at several points of the cascade (Turner, 2010; Ahmad, 2012).

2.2.3. Deregulation of FGF signalling in cancer

Deregulation of the FGF/FGFR signalling system has been implicated in the pathogenesis of many cancer entities (Ahmad, 2012). The mechanisms behind are largely tumour specific and can be grouped into genomic FGFR alterations driving ligand-independent receptor signaling and alterations mediating ligand-dependent activation (**Figure 7**; Turner, 2010).

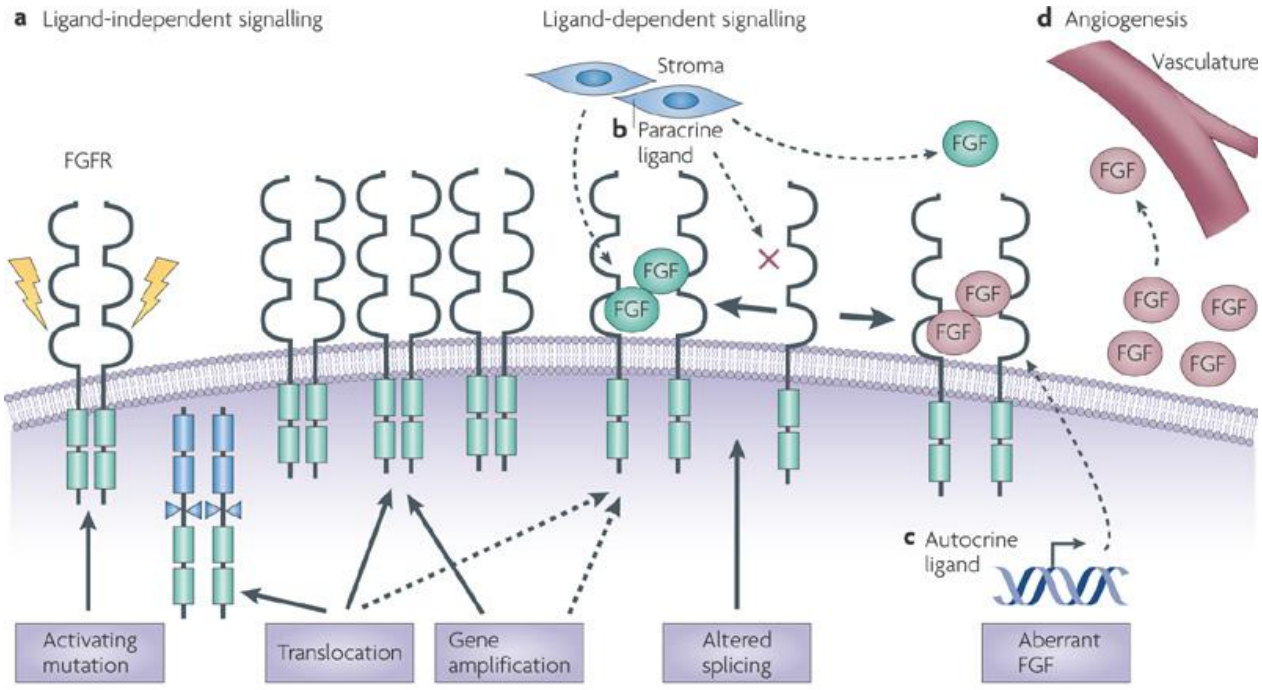

Figure 7: Mechanisms of deregulated FGF signalling in cancer cells. *Alterations of FGFs and FGFRs in cancer can be arranged in 4 groups. (A) Genomic alterations of FGFR lead to ligand-independent signalling. (B) Establishment of a paracrine loop. (C) Establishment of an autocrine loop. (D) Angiogenesis as an effect of stromal FGF expression (Turner, 2010).*

Haematological malignancies provide the strongest evidence for involvement of FGF signalling in cancer (Ahmad, 2012). Here, the N-terminus of a transcription factor is fused to a FGFR kinase domain through chromosomal translocation, resulting in constitutive activation of the receptor (Ahmad, 2012). For example, in 15%-20% of multiple myeloma (MM) cases FGFR3 is overexpressed because of a t (4; 14) translocation, which brings FGFR3 under the control of a strong IgH enhancer region (Keats, 2006).

FGFR overexpression or chromosomal amplification have been found in a number of cancers such as prostate, breast, lung, brain, gastric, sarcoma, head and neck and MM (Ahmad, 2012). Recent studies demonstrated the amplification of FGFR1 in 3% of lung adenocarcinomas and 21% of squamous cell carcinomas (Murphy, 2010). FGFR1 is frequently upregulated in prostate cancer, but the mechanism is still uncertain (Murphy, 2010). Similarly, FGFR1 is amplified in 10% of breast cancer cases, which are additionally oestrogen receptor positive (Chin, 2006). In 10% of gastric cancers, FGFR2 is amplified, which is often associated with a poor prognosis (Jang, 2001).

Somatic mutations affecting the FGF signalling pathway, particularly FGFRs, are numerous in cancers of the prostate, bladder, breast, brain, lung, uterus, stomach, head and neck, colon and MM (Ahmad, 2012). FGFR3 mutations have been described in cervical cancer, MM, prostate cancer, as well as benign skin disorders (Ahmad, 2012). Activating mutations of FGFR2, identical to such found in skeletal disorders (Apert syndrome and Crouzon syndrome) are found in approximately 10% of endometrial/uterine cancers (Dutt, 2008). Mutations of FGFR4, associated with advanced stages and poor prognosis, have been identified in 7-8% of rhabdomyosarcomas (RMS) (Taylor, 2009). Additionally, FGFR4 G388R mutations correlate with higher resistance to chemotherapy in breast cancer patients and promotion of cancer progression and metastasis in mammary carcinoma models (Bange, 2002; Thussbas, 2006). Mutations in negative regulators of FGF signalling, impairing an appropriate termination, also contribute to tumorigenesis (Ahmad, 2012).

Ligand-dependent signaling is also likely to have an important role in the pathogenesis of cancer, either through autocrine production of ligand in cancer cells or paracrine production of ligands by surrounding stroma at physiological levels or in response to abnormal cancer cells at supra-physiological levels (Turner, 2010). For example, a FGF2-FGFR1 autocrine loop has been found to promote the development of melanomas (Wang, 1997). Amplification of FGF1 has been described in ovarian cancer, and it is suggested that aberrantly expressed FGF1 works in a paracrine manner to promote of angiogenesis (Birrer, 2007). FGF19 was detected to be overexpressed in parts of the liver, colonic and lung squamous carcinomas (Ahmad, 2012).

In a screen of 210 cancers it was found, that the FGF signalling pathway is the most commonly mutated system amongst the somatic mutations detected (Greenman, 2007). Thus, the potential role of FGF signalling in human carcinogenesis has to be further elucidated to allow a specific targeting of FGFs/FGFRs in therapeutic approaches. Several tyrosine kinase inhibitors are currently in clinical trials, inhibiting FGFRs and VEGFRs at the same time (Turner, 2010). The benefit of targeting both angiogenesis and proliferation of tumour cells has to be seen cautiously as many of those TKIs are less potent to inhibit FGFRs (Turner, 2010). Additionally, targeting multiple RTKs at once increases the side effects and limits the delivery of the drug at doses necessary for FGFR inhibition (Turner, 2010).

2.3. Fibroblast growth factor receptor 4

2.3.1. Characteristics of FGFR4

FGFR4 possesses structural components being characteristic for FGFR proteins, e.g. three Ig-like domains in the extracellular space und a split tyrosine kinase domain (Johnson, 1993; Schlessinger, 2000; Hunter, 2000). The amino acid sequence shows approximately 55% homology to FGFR1 and FGFR2 (Dionne, 1990). In contrast to other FGFRs, the FGFR4 harbour only 18 instead of 19 exons and since the missing exon is responsible for the IIIb splice variant; there is no alternative splicing in the IgIII loop (Eswarakumar, 2005). However, other isoforms of the receptor have been described, e.g. a soluble non-transmembrane-type isoform was detected in human gastrointestinal epithelial and pancreas cells in tumours (Takaishi, 2000). Furthermore, a truncated soluble FGFR isoform was found in a breast cancer cell line, which only contains domain I and the acidic box (Ezzat, 2001).

Generally, FGFR4 seems to impact adult organ homeostasis rather than development. Weinstein et al. showed that genetic defects of FGFR4 in mice have no obvious effects on developmental processes (Weinstein, 1998). In contrast, other studies indicate an involvement in muscle differentiation and the maturation of bone cells in the skull (Marics, 2002; Yu, 2004). However, FGFR4 knockout does not affect muscle development but the induction of muscle regenerations appeared to be impaired (Zhao, 2006). Cornish et al. suggest that FGFR4 is involved in the maintenance of foveal cones in the retina and in alveogenesis of the murine lung in direct cooperation with FGFR3 (Weinstein, 1998; Cornish, 2004). In adult organisms, FGFR4 is expressed in various tissues but it is the only FGFR kinase significantly expressed in mature hepatocytes (Hughes, 1997).

FGFR4 has also been implicated in the negative regulation of bile metabolism and glucose tolerance as FGFR4 is the predominant receptor for mediating the liver-specific effects of FGF19 (Beenken, 2009). Overexpression of FGF19 in transgenic mice increased hepatocyte proliferation, which may ultimately lead to HCC formation (Nicholes, 2002). It is suggested that activation of FGFR4 is the mechanism whereby FGF19 induces hepatic proliferation, but contradicting evidence also propose a protective role of FGFR4 in suppressing tumour progression (Nicholes, 2002; Huang, 2009; Wu, 2010).

Studies on the bile acid metabolism in FGFR4 deficient mice revealed the receptor's critical physiological function in the liver. Disruption of FGFR4 showed reduced FGF19 activity resulting in elevated CYP7A1 activity, increased bile acid pools and decreased gall bladder volume (Yu, 2000). Similar phenotypes have been detected in mice deficient in βKlotho expression indicating that the Klotho protein is an essential co-factor for activation of FGF signalling (Kurosu, 2006). Surprisingly, Huang et al. reported an inhibition of the development of fatty liver in FGFR4 -/- mice under high fat diet (Huang, 2009).

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Although FGFR4 shows a lower mitogenic potential than FGFR1-3, deregulation of FGFR4 has been reported in various cancer entities (Dailey, 2005). Several mutations in the kinase domain of FGFR4 have been identified in 7-8% of the childhood sarcoma RMS (rhabdomyosarcoma), which were shown to be associated with advanced stage and poor survival (Taylor, 2009). In human breast cancer cell lines, ectopic FGFR4 expression has been linked to enhanced resistance to chemotherapy (Roidl, 2009). Somatic mutations of FGFR4 have also been described in lung adenocarcinomas (Takanami, 1996). Additionally, FGFR4 is overexpressed in hepatocellular carcinoma (Ho, 2009; Gauglhofer, 2011), renal cell carcinoma (Takahashi, 1999), malignant melanoma (Streit, 2006), pancreatic cancer (Motoda, 2011), gastric cancer (Ye, 2011) and prostate cancer (Wang, 2008).

2.3.2. FGFR4 Gly388Arg single nucleotide polymorphism

Single nucleotide polymorphisms of FGFRs have been identified in various human cancers (Ahmad, 2012). In 2002, Bange et al. described a sequence polymorphism in the transmembrane domain of the human FGFR4 gene in MDA-MB-453 mammary carcinoma cells in which glycine is substituted by arginine due to a G→A conversion at codon 388 (Bange, 2002). In contrast to similar mutations in the transmembrane domains of FGFR2 and 3, which are rare in the general population, the Arg^{388} variant is present in about 50% of the population (Bange, 2002).

A meta-analysis by Xu et al. reported a significant relationship between the FGFR4 Arg388Gly polymorphism and the risk of cancer (Xu, 2010). Apparently, the prevalence of FGFR4-Arg varies substantially between different racial or ethnic populations (Wang, 2004). A highly significant difference was especially found among controls of Asians (37.2%), Europeans (29.5%) and African-Americans (11.7%; Xu, 2010). Additionally, the FGFR4 SNP was linked to an increased susceptibility to breast and prostate cancer (Xu, 2010).

Several studies are dealing with the association of the occurrence of the Arg^{388} allele and the predisposition for the development of cancer. In breast cancer patients, the presence of the Arg^{388} variant was associated with an about 5-fold increased disease progression and metastasis than in Gly^{388} homozygotes due to increased motility and invasiveness of the tumour cells (Bange, 2002). However, no association with the incidence of the disease was reported (Bange, 2002). In colorectal cancer, the Arg^{388} allele is associated with increased lymph node metastasis (Bange, 2002). Morimoto et al. found a correlation between FGFR4-Arg and a poor prognosis in soft tissue sarcoma (Morimoto, 2003). In squamous cell carcinomas of the head and neck, high expression of the Arg^{388} allele is associated with reduced survival whereas expression of the Gly^{388} allele does not influence cancer progression (Streit, 2004). A study on prostate carcinoma found a contribution of FGFR4-Arg to the incidence and aggressiveness of the malignancy possibly due to the increased motility of tumour cells (Wang, 2008). Importantly, the presence of a R388 does not indicate a predisposition to the development of a malignancy, but rather to a more aggressive progression and a poor prognosis once cancer has been developed (Stadler, 2006). Studies by Stadler et al. proposed, that FGFR4-Gly suppresses motility of breast cancer cells and that the negative effect of the Arg^{388} allele on the prognosis rather originates in the loss of the suppressive functions of the Gly^{388} allele than on the action of FGFR4-Arg (Stadler, 2006). In malignant gliomas, no association of the polymorphism with the course of the disease was found whereas overexpression of FGFR4-Arg in melanoma is correlated with a poor prognosis (Mawrin, 2005; Streit, 2006). The polymorphism has relevance for lung adenocarcinomas and it is correlated with advanced disease stage, decreased survival and earlier age at tumour onset (Spinola, 2005). Contradictory, Matakidou et al. found no association in small-cell and non-small-cell lung cancer (Matakidou, 2007). In hepatocellular carcinoma, appearance of the Arg^{388} allele correlates with increased AFP secretion, which is linked to disease progression and tumour aggressiveness (Ho, 2009). In agreement with this result, Gauglhofer et al. also found an association of FGFR4-Arg with tumour progression and shortened survival in hepatocellular carcinomas (Gauglhofer, 2010).

2.3.3. FGFR4 in HCC – Preliminary Results

Overexpression of FGFR4 in HCC has already been shown in various studies indicating a possible role of FGFR4 in the development or progression of HCC (Tsou, 1998; Ho, 2009). Investigations done by Dr. Christine Gauglhofer confirm those findings as the receptor was found to be upregulated in almost 50% (15/34) of HCC cases investigated (Gauglhofer, 2010). Furthermore, presence of the Arg^{388} allele was shown to be associated with an increased risk to develop a liver malignancy (Gauglhofer, 2010).

Since the overall expression of FGFR4 in liver cells is high and appears to be upregulated in a subset of HCC samples, mechanistic studies were performed to determine the role of FGFR4 and the G388R polymorphism in the aggressive behaviour of HCC cells (Gauglhofer, 2010). The cell lines used for those studies were previously characterized as they differ profoundly in their specific behaviour (**Table 2**; Gauglhofer, 2010).

Table 2: Characteristics of HCC model cell lines (Gauglhofer, 2010).

	CCL-13	HCC-1.2	HepG2
FGFR4 expression level	+	+++	++++
FGFR4 genotype	Gly/Gly	Gly/Gly	Arg/Arg
Migration	++	+/-	-
Population doubling	+++	+(+)	+
Colony formation	+++	+	+
Clone formation in soft agar	++	-	+
Tumorigenicity in SCID mouse	++	-	++ (50% Matrigel)

Native HepG2 cells per se were shown to induce rapidly growing tumours in SCID mice (Gauglhofer, 2010). Thus, overexpression of mutant or wildtype FGFR4 has only minor effects on the tumorigenicity of HepG2 cells (Gauglhofer, 2010). In contrast, HCC-1.2 cells, which are not tumorigenic in SCID mice, rendered tumorigenic by overexpression of FGFR4-Gly^{388} (Gauglhofer, 2010). Interestingly, overexpression of FGFR4-Arg^{388} had no effect on the tumorigenicity of HCC-1.2 cells in SCID mice (Gauglhofer, 2010).

The impact of FGFR4 and the G388R polymorphism on the behaviour of hepatocarcinoma cells was investigated with stable and transient overexpression as well as with siRNA-mediated knockdown and signaling interference with dominant negative FGFR4 variants (**Table 3**).

Table 3: Summary of preliminary results (Gauglhofer, 2010; Naegelen, 2010; Schrottmaier, 2012).

	CCL-13		HCC-1.2		HepG2	
Transient overexpression	Gly	Arg	Gly	Arg	Gly	Arg
Colony formation	-	+	-	+	-	+
Clone formation in soft agar	-	+	-	+	-	+
Adhesion	No effect	No effect	No effect	No effect	No effect	No effect

	CCL-13		HCC-1.2		HepG2	
Stable overexpression	Gly	Arg	Gly	Arg	Gly	Arg
Colony formation	Not done	Not done	++	++	+	+
Clone formation in soft agar	Not done	Not done	++	++	+	++
Adhesion	Not done	Not done	--	--	No effect	No effect

	CCL-13	HCC-1.2	HepG2
siRNA-mediated knockdown			
Colony formation	-	-	-
Clone formation in soft agar	--	Not done	-
Adhesion	No effect	No effect	No effect

	CCL-13		HCC-1.2		HepG2	
Adenoviral blockade	solFGFR4	kdFGFR4	solFGFR4	kdFGFR4	solFGFR4	kdFGFR4
Colony formation	-	-	--	--	--	--
Clone formation in soft agar	--	-	Not done	Not done	--	--
Adhesion	Not done	Not done	Not done	Not done	Not done	Not done

+ increased ++ significantly increased - decreased -- significantly decreased

Stable overexpression of wildtype or mutant FGFR4 in HCC-1.2 cells strongly enhanced clone formation of cells at low density with both allele variants (Gauglhofer, 2010). In HepG2 clones, only a marginal increase in clonogenicity was detected (Gauglhofer, 2010). Transient overexpression of FGFR4/Arg increased clone formation (Schrottmaier, 2012). Interestingly, transient overexpression of FGFR/Gly moderately decreased clonogenic growth (Schrottmaier, 2012). Knock-down of FGFR4 mediated by siRNA as well as blockade of FGFR4 signalling by application of dominant-negative FGFR4 isoforms reduced clonogenicity of HCC-1.2 and HepG2 cells (Gauglhofer, 2010; Naegelen,

2010). Effects were similar in CCL-13 cells but appeared to be less pronounced (Gauglhofer, 2010; Naegelen, 2010).

Stable overexpression of FGFR4-Gly^{388} increased clone formation of HCC-1.2 and HepG2 cells in soft agar, which was even more pronounced when cells expressed FGFR4-Arg^{388} (Gauglhofer, 2010). Transient overexpression of FGFR4/Arg increased clone formation in soft agar, whereas enhanced FGFR4/Gly expression reduced the clonogenic growth (Schrottmaier, 2012). Both, siRNA-mediated knock-down and blockade of FGFR4 signalling reduced clone formation of HCC-1.2 and HepG2 cells in soft agar (Gauglhofer, 2010; Naegelen, 2010).

Adhesive capabilities of HCC-1.2 clones to untreated culture vessels and collagen coated surfaces appeared to be significantly diminished, whereas HepG2 clones did not show alterations in cell adhesion (Naegelen, 2010). Transient overexpression and siRNA-mediated knockdown of FGFR4 in CCL-13, HCC-1.2 and HepG2 cells had no effect on cell-matrix adhesion to polystyrene or fibronectin (Naegelen, 2010; Schrottmaier, 2012).

In summary, previous studies showed that FGFR4 clearly contributes to clonogenic growth at low density and anchorage-independency. Moreover, overexpression of FGFR4 seems to reduce cell adhesion and increase the tumorigenicity of hepatocarcinoma cells in SCID mice. Since these results may point to a role of FGFR4 for tumour progression and pro-metastatic events in HCC, further investigations are needed to clarify the importance of FGFR4 for the metastatic process.

2.4. FGFR4 in metastasis and invasion

2.4.1. Initial steps in metastasis

In general several events have been defined as common to cancer cells including self-sufficiency in proliferation signals, insensitivity to growth inhibitory signals, evasion from cell death, limitless replicative potential, aberrant angiogenesis, as well as tissue invasion and metastasis (Hanahan, 2000). In 90% of all cancer deaths, tumorigenesis is associated with metastasis, thus metastasis represents one of the primary causes of human mortality (Sporn, 1996). Hence, prevention of cancer dissemination and formation of secondary tumour must be a major goal of cancer therapy.

The metastatic cascade is described as a multi-step process whereby some epithelial cells in primary tumours (1) locally invade into the host stroma through the surrounding ECM, (2) enter the circulation through penetration of blood or lymphatic vessels, (3) survive in the circulation, (4)

adhere either to capillary endothelial cells or to subendothelial basement membranes, (5) extravasate into distant tissues, (6) survive within the organ to form micrometastases and (7) re-initiate proliferative programs at metastatic sites generating clinical detectable neoplastic growths (**Figure 8**; Fidler, 2003).

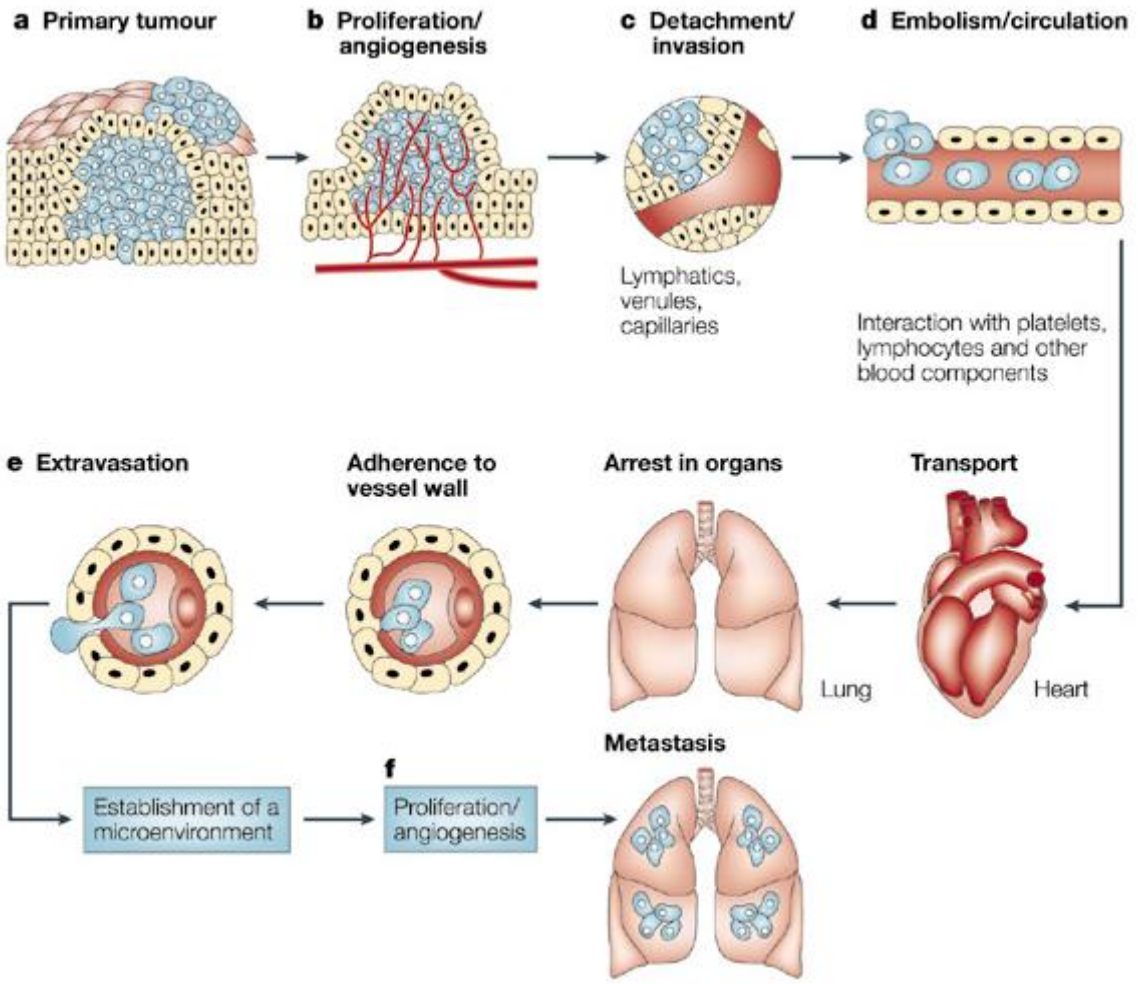


Figure 8: Main steps in the metastatic process (Fidler 2003).

According to Stephen Paget's "seed and soil" theory, cancer cell (the "seed") growth is dependent on the competence of the distal organ (the "soil), proposing that metastasis occurs in an organ-specific pattern (Paget, 1889). This has been approved for various cancer types, e.g. breast carcinomas metastasize preferentially into the bone, the lung and the brain, whereas colorectal and pancreatic cancers show preferences for the liver and the lung (Van Zijl, 2011). Anyhow, some cancers show only proximal metastasis although they are highly invasive. In particular, HCC most frequently show intrahepatic colonization favoured through the dense hepatic vasculature in the liver microenvironment (Van Zijl, 2009).

Vascular connection of the tumour and the acquisition of migratory and invasive capabilities are prerequisite for cells to evade from organized tissue structures and invade into more distal locations (Gotzmann, 2004). In the course of this initial step in tumour cell evasion, a phenotypical conversion of cells occurs, which is collectively designated as epithelial to mesenchymal transition (EMT; Gotzmann. 2004). In general, EMT is a central event during embryogenesis and in pathophysiological situations like wound healing, inflammation and carcinoma progression (Kalluri, 2009). During the last decades, EMT has been intensively investigated and increasingly recognized as a crucial process in cancer progression and metastasis. So far, it has been described in the malignant progression of breast tumour, colorectal cancer, lung cancer, prostate carcinoma, pancreatic cancer cells and HCC (Van Zijl, 2011).

Loss of cell-cell and cell-ECM adhesion, which allows epithelial cells to acquire motile features, is basically the main characteristic of EMT (Gotzmann, 2004). In contrast to developmental processes, cancer cells undergoing EMT are usually capable of self-sufficient autocrine loops of growth factors and mechanisms to evade apoptosis and elicit angiogenesis for independent nutrient supply (Gotzmann, 2004). The autonomy of cancer cells due to the gain of these abilities, allows them to spread in whole organism (Gotzmann, 2004).

Evasion of cells from their epithelial entities is caused by dissociation of intercellular adhesions through disintegration of junctional protein complexes (Gotzmann, 2004). This is most often accompanied by the downregulation or even loss of E-cadherin, which is an important component of adherence junctions and stabilizes cell-cell contacts through interaction with the extracellular domain of E-cadherins on neighbouring cells (Van Zijl, 2011). Concomitantly, N-cadherin is upregulated, leading to a rearrangement of the cytoskeleton and increased motility of EMT-transformed cells (Christofori, 2006). Thus, carcinoma cells lose their epithelial organization and gain the ability to detach from cell clusters to move as single cells (Van Zijl, 2011). Several factors have been identified to induce EMT, including integrins, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (Thiery, 2009). Recently, novel factors such as chromodomain helicase/ATPase DNA binding protein 1-like gene and connective tissue growth factor have been identified as inducers of EMT in hepatocarcinogenesis (Chen, 2010; Mazzocca, 2010).

It is thought that in the course of the metastatic cascade of epithelial cancers such as breast, endometrial and colorectal cancers, carcinoma cells start to invade collectively (Christiansen, 2006). The collective cell invasion is characterized by intact cell-cell interactions during movement and

multicellular coordination of polarity and cytoskeletal activity, which generates traction forces (Friedl, 2010). Additionally, remodelling of ECM and rearrangement of the basement membrane through secretion of MT1-MMPs are essential in initiating track formation in both single cell and collective tumour invasion (Friedl, 2010).

The initial evasion of carcinoma cells is followed by incomplete or complete EMT, which is reversed by process referred to as mesenchymal to epithelial transition (MET; Christiansen, 2006; Kalluri, 2009). Thereby cells regain the epithelial cell-cell junctions for metastatic colonization at distal sites (Thiery, 2009).

Local invasion is followed by intravasation, which involves invasive carcinoma cells entering into the lumina of blood or lymphatic vessels (Valastyan, 2011). The blood circulation is considered the main route of metastatic spread although invasion of carcinoma cells into the lymphatic system is also routinely observed in human tumours (Kerjaschki, 2004). Interestingly, recent studies provide evidence that mammary carcinoma cells spontaneously form cohesive groups, which directly penetrate lymphatic vessel walls through large discontinuities (Kerjaschki, 2004). As the level of lymph node metastasis is a clinical predictor for distant organ metastasis and patient outcome, understanding of early steps of intravasation is crucial for the development of treatments preventing metastatic processes (Carlson, 2009). In 1993, Offner et al. developed a 3-dimensional in vitro assay simulating the sequential adhesive interaction of tumour cells with vascular endothelium during tumour extravasation (Offner, 1993). To gain insight into the mechanism underlying lymphatic bulk invasion, Kerjaschki et al. modified the assay established by Offner et al. to mimicking some features of the in vivo situation. Thereby, tumour cell spheroids are formed in vitro, which are placed on monolayers of endothelial cells (Kerjaschki, 2004). The interaction of spheroids with the endothelial cells serves to stimulate the invasion of tumour aggregates into intra-metastatic lymphatic vessels (Kerjaschki, 2004). In MCF-7 spheroids, several genes were found to be specifically induced when compared with monolayers, including the hypoxia-inducible enzyme ALOX15 (Kerjaschki, 2004). ALOX15 generates 12(S)-HETE from arachidonic acid (AA), which has been shown to increase the malignant behaviour of various tumour entities as well as endothelial cell retraction (Kerjaschki, 2004). Lymphatic endothelial cells, exposed to 12(S)-HETE in vitro, transiently destabilize VE-cadherin resulting in focal disruption of inter-endothelial adhesions and induction of migration of these cells (Kerjaschki, 2004). This leads to the formation of “circular chemorepellent-induced defects” (CCIDs) and the loss of the integrity of the lymphatic endothelial cell layer (Kerjaschki, 2004). Otherwise, 15(S)-HETE, the alternative product of AA metabolism, is not able to induce CCIDs (Kerjaschki, 2004).

2.4.2. Effects of FGFR4 on adhesion and invasion

Research over the past decades has made substantial progress in the understanding of the molecular mechanisms of tumour cell interaction with vascular endothelium and extracellular matrix. So far, most of the cell adhesion molecules (CAMs) reported, including integrins, cadherins, selectins, immunoglobulins and proteoglycans, have been implicated to be critical in various stages of tumour progression and metastasis (Cavallaro, 2004). In addition to participating in tumour invasion and metastasis, adhesion molecules have been described to have a variety of cellular functions including signal transduction, cellular communication, inflammatory responses, cell motility, cell growth and apoptosis (Cohen, 1997). Conversely, signalling molecules can directly change cell-cell and cell-matrix interaction through affection of adhesion molecules (Cavallaro, 2004).

In the majority of epithelial tumours, E-cadherin expression was found to be absent or dysfunctional concomitant with the gain of expression of mesenchymal cadherin (N-cadherin; Cavallaro, 2004). This "cadherin switch" is suggested to be required for tumour cells to gain invasive capabilities and is also known as a hallmark of epithelial-to-mesenchymal transition (EMT; Cavallaro, 2004; Thiery, 2003).

Deregulation of FGF signalling has been shown to coincidence with EMT in adult tissue, e.g. switch of epithelial FGFR2b to mesenchymal FGFR2c has been proposed to be an appearance of EMT (Avecedo, 2009). In addition to changes in isoforms, downstream molecules of FGF signalling may drive EMT during cancer progression. FGFR1 may promote the expression and stability of Snail, which is a potential transcriptional repressor of E-cadherin, through MAPK and Akt pathways in collaboration with TGF-β (Avecedo, 2009). Still, the number of molecular mechanisms involved in EMT is growing. Identification and functional characterization of molecules regulating EMT is crucial to facilitate the development of novel strategies to efficiently inhibit tumour progression.

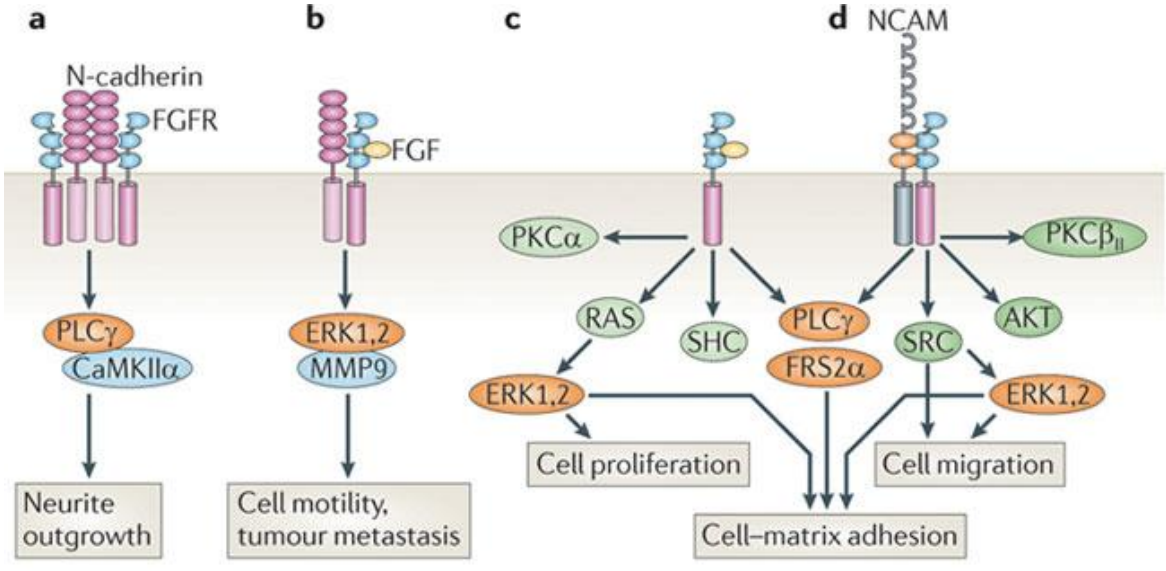


Figure 9: Crosstalk between cell adhesion molecules and the FGF-FGFR signaling system. *(A)* cis-*dimerization of N-cadherin promotes ligand-independent activation of FGFR signaling. (B)Interaction of N-cadherin with FGFR prevents receptor internalization and leads to sustained FGFR activation. (C) Signaling pathways activated by the canonical ligand FGF. (D) Interaction of FGFR with NCAM activates various signaling pathways ultimately leading to cell migration (Cavallaro, 2001).*

Several reports showed that FGFR1 can be activated by cell adhesion molecules like N-cadherin, L1 and N-CAM via special binding motifs, which are conserved in FGFR1, FGFR2 and FGFR4 (Doherty, 1996). Cavallaro et al. described a connection between N-cadherin and FGFR family members in various non-transformed and tumour cell types (Cavallaro, 2001). Subsequently, it has been proposed that N-cadherin facilitates FGFR dimerization leading to FGF independent signalling (Williams, 2001). In parallel, N-cadherin is also involved in growth factor-dependent FGFR activation (**Figure 9 A**; Williams, 2001). Interactions of N-cadherin with FGFR prevent receptor internalization, which results in increased levels of the receptor on the cell surface and sustained activation by FGFs (**Figure 9 B**; Cavallaro, 2001). Generally, crosstalk between N-cadherin and the FGF-FGFR system seems to influences the migratory and invasive properties of single cells (Suyama, 2002).

In contrast to N-cadherin, interaction of NCAM with FGFR has negative effects of FGF signalling and represses cell proliferation and matrix adhesion (**Figure 9 C and D**; Cavallaro, 2001; Francavilla, 2007). Several reports showed a direct interaction between NCAM and FGFR presenting NCAM as a non canonical ligand for this receptor family (Kiselyov, 2003; Christensen, 2006; Francavilla, 2009). Studies by Doherty et al. highlighted an association of NCAM with FGFR signalling regulating cell migration and invasion, as has been shown to occur in neurons during neurite outgrowth (Doherty, 1996). In pancreatic tumour cells, N-cadherin physically interacts with FGFR4 in the presence of NCAM, subsequently affecting β_1-integrin mediated cell-matrix adhesion (Cavallaro, 2004). Deregulation of the NCAM-FGFR4 signalling complex may therefore correlate with malignancy (Cavallaro, 2004). Interestingly, reduction of N-CAM expression was linked to increased malignancy and poor prognosis in less differentiated colorectal, pancreatic and gastric cancers (Fogar, 1997). Moreover, crossing of N-CAM knockout mice with Rip1Tag2 transgenic mice, which develop β-cell tumours in the pancreatic islets of Langerhans, resulted in a distinct increase in tumour metastases mainly in the draining lymph nodes (Hanahan, 1985; Perl, 1999). Hence it is suggested that the N-CAM/FGFR4 complex has the potential to prevent dissemination of metastatic tumours (Cavallaro, 2004). Other than cell-matrix adhesion, cell-cell interactions and FGFR signalling are individual and independent activities of NCAM (Cavallaro, 2001; Francavilla, 2009).

Bange et al. observed that Cos7 and PC12 cells are able to adhere to cell culture dishes coated with the extracellular domain of FGFR4 (Bange, 2002). In this regard, they suggested that FGFR4 can act either as ligand or as receptor and interact with other membrane-anchored proteins on the same or neighbouring cells (Bange, 2002). In the effort to demonstrate differences between the Arg^{388} and Gly^{388} form of FGFR4, it was reported that MDA-MB-231 cells expressing FGFR4-Gly showed decreased motility, which appears to be caused by increased adhesion to either the substratum or neighbouring cells (Bange, 2002). Studying patients with colon cancer, expression of the FGFR4 Arg^{388} allele was associated with an increased number of lymph node metastases (Bange, 2002). Concluding, Bange et al. argued that the differences in adhesive and migratory capabilities of cells expressing either the wildtype of the mutant version of FGFR4 and the link between the FGFR4 Arg^{388} allele and the poor clinical outcome of patients might be caused by a disruption of N-CAM/FGFR4 complexes (Bange, 2002).

Studies in pituitary tumours done by Ezzat et al. demonstrated remarkable differences in the capability of the wildtype FGFR4 and the truncated FGFR4 isoform ptd-FGFR4 to maintain cell adhesion (Ezzat, 2004). Thereby, expression of ptd-FGFR4 reduced matrix adhesion through reduced interaction with NCAMs in vitro, which corresponds to increased invasion in vivo (Ezzat, 2004). Additionally, ptd-FGFR4 expression was shown to diminish cytoplasmic N-cadherin expression associated with reduced cell adhesion in vitro and increased invasion in vivo (Ezzat, 2004).

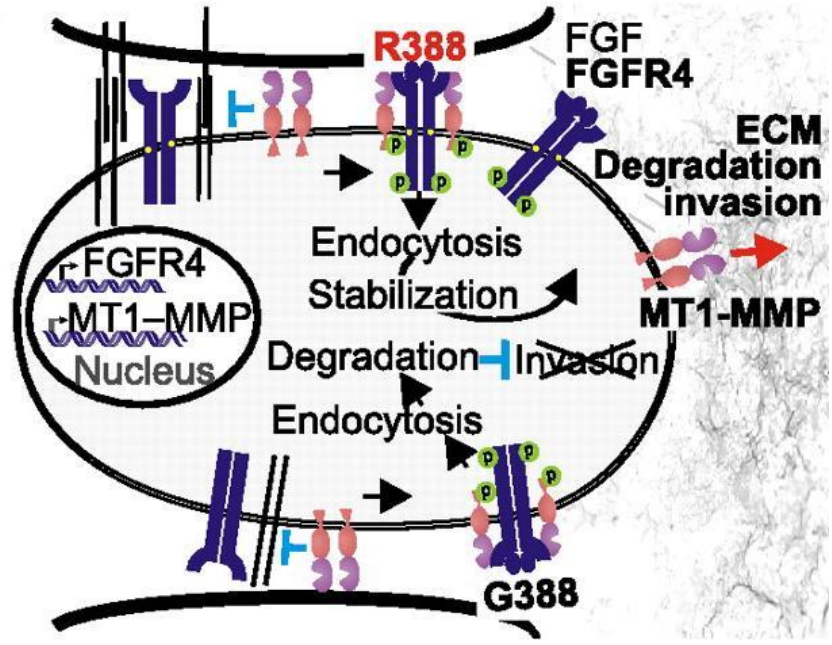


Figure 10: Model for the function of the FGFR4/MT1-MMP complex (Sugiyama, 2010/1).

In many tumour entities MT1-MMPs were identified to be crucial for the metastatic process (Rowe, 2009). Recently, Sugiyama et al. found that both FGFR4 variants form complexes with MT1-MMPs and induce MT1-MMP phosphorylation (Sugiyama 2010/1). However, the polymorphism seems to have opposite effects on the MT1-MMP level (Sugiyama 2010/1). In detail, FGFR4-R388 stabilizes MT1-MMP whereas FGFR4-G388 down-regulates the protein. It is suggested that the increased stability of the Arg^{388} variant is responsible for the differences rather than the interaction with MT1-MMP itself (Sugyiama, 2010/1). Therefore it is most likely, that the FGFR4-Arg^{388} alters the interaction with vesicular sorting proteins resulting in enhanced transport of MT1-MMP/FGFR4 complexes to recycling instead of lysosomal degradation (**Figure 10**; Sugyiama, 2010/1). On the other side, interaction of MT1-MMPs with FGFR4-Gly^{388} leads to the degradation of both proteins (Sugyiama, 2010/1).

Co-expression of MT1-MMP and FGFR4 was selectively found at the human tumour-stroma borders and tumour invasion fronts in poorly differentiated breast adenocarcinomas and prostate carcinomas (Sugiyama, 2010/2). The MT1-MMT/FGFR4-Arg^{388} complex was localized to cell-cell junctions in MDA-MB-453 cells, consistent with a model in which cell surface MT1-MMP promote the dissociation of cell-cell interactions and FGFR4 internalization (Sugiyama, 2010/2). Studies with prostate carcinoma cell lines indicate that FGFR4-Arg^{388} increases MT1-MMP dependent collagen invasion *in vitro*, whereas knock-down of either of the two proteins results in blocked tumour cell invasion and growth in collagen (Sugiyama, 2010/2). Simultaneously, phosphorylation of FRS2 and Src was impaired, E-cadherin was up-regulated and N-cadherin was suppressed, which implicated an association of $FGFR4^{388}$ and EMT (Sugiyama, 2010/2). In contrast, knock-down of FGFR4-Gly^{388} increased invasive tumour cell growth *in vivo* and *in vitro* (Sugiyama, 2010/2). Results suggest a different role for FGFR4-Arg^{388} in collective and single cell invasion (Sugiyama, 2010/2). On the one hand, FGFR4-Arg^{388} sustains cell-cell adhesion and promote MT1-MMP dependent collective invasion (Sugiyama, 2010/2). On the other hand the allele reduces cell-cell adhesion resulting in single cell invasion (Sugiyama, 2010/2).

2.4.3. The impact of bile acids on metastatic processes

Bile acids (BAs) are steroid metabolites of cholesterol and the main components of bile together with cholesterol, phospholipids and bilirubin (Baptissart, 2012). The primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver via two different metabolic pathways and are secreted in the bile mainly as glycine or taurine conjugates (**Figure 11**; Russell, 2003). During the entero-hepatic cycle, 85% of excreted bile acids are reabsorbed in the ileum and colon and recycled to the liver and the gall bladder (Ridlon, 2006; Caballeria, 2008). The remaining 15% reach the colon,

where they are deconjugated and enzymatically modified by the intestinal microflora to form the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA; Ridlon, 2006; Caballeria, 2008). Tertiary bile acids, such as ursodeoxycholic acid (UDCA) and sulpho-LCA (SLCA) are subsequently formed through epimerization of CDCA or sulphation of LCA (Debruyne, 2001).

Besides their mechanical role in digestion and fat solubilisation, BAs have been described as signalling molecules regulating many physiological functions such as glucose and energy metabolism by activation of the nuclear receptor Farnesol-X-receptor (FXRa, NR1H4) and G-protein-coupled receptor TGR5 (GPBAR1, G-protein-coupled bile acid receptor; Baptissart, 2012).

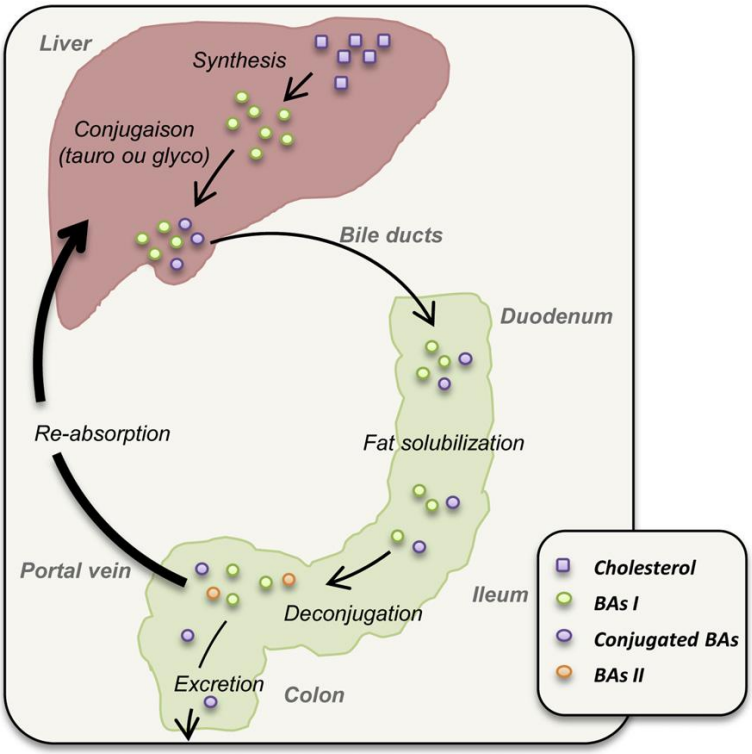


Figure 11: The enterohepatic circulation of BAs. *Cholesterol is metabolized to primary BAs in the liver and conjugated with glycine or taurine. The resulting bile salts are stored in the gallbladder until they are excreted into the duodenum and participate in the digestion of fat. In the ileum, secondary BAs are formed through deconjugation and modification of primary BAs. The majority of BAs is reabsorbed in the ileum and colon and transported to the liver (Baptissart, 2013).*

BAs were first implicated in carcinogenesis in 1940 by Cook et al., confirmed by the observation that DCA induced tumours in mice when injected subcutaneously (Cook, 1940). Since then, a number of experiments were done to elucidate the role of bile acids in carcinogenesis. Bernstein et al. showed that the bile acids LCA, taurodeoxycholic (TDCA), DCA as well as CA promoted colon tumour formation in rats (Bernstein, 2005). Furthermore, Earnest el al. reported the absence of invasive tumours in a rat model of colon carcinogenesis upon feeding with UDCA (Earnest, 1994). As a consequence one might speculate that UDCA has a chemopreventive effect and is be a useful in the treatment of colorectal cancer.

Several studies also show an association of bile acids with liver tumorigenesis. Indeed, in vivo experiments on rats showed that DCA is a relatively strong tumour promoter resulting in the appearance of preneoplastic lesions (Cameron, 1982). Furthermore, a report by Tsuda et al. indicated that elevated bile acid levels in pathological conditions might act as endogenous promoter of hepatocarcinogenesis in rats (Tsuda, 1984). In vitro, there is evidence that hydrophobic bile acids such as DCA, glucuro-CDCA or tauro-CDCA affect hepatocytes directly by induction of ROS production leading to DNA damage and apoptosis (Yerushalmi, 2001; Tan, 2007).

There are two possible reasons for the impact of BAs on the neoplastic transformation of intestinal epithelial cells. First, BAs are detergents and thus highly toxic for cell membranes. High concentrations of DCA and LCA in particular may cause unspecific cell damage resulting in focal destruction of the intestinal epithelium (Peterlik, 2008). Subsequently, repair mechanisms, inflammatory responses and hyperproliferation of undifferentiated cells would increase the risk for transition to a cancerous state (Peterlik, 2008). Second, CA, DCA and LCA have the ability to induce pre-neoplastic lesions in the colonic epithelium, referred as aberrant crypt foci (ACF) (Peterlik, 2008).

In humans, several reports indicate an association between high levels of BAs and increased incidence of cancer of the laryngopharngeal tract, oesophagus, stomach, pancreas, the small intestine and the colon (Bernstein, 2005). Regardless, the impact of BAs in normal and neoplastic growth of epithelial cell is still controversial as both beneficial and undesirable effects were previously reported (Nagengast, 1995; Zimber, 2000). Findings by Debruyne et al. showed that the bile acids CDCA, LCA, CA and DCA but not UDCA or conjugated forms of CDCA or LCA stimulate invasion of HCT-8/E11 and PCmsrc human colorectal cancer cells into collagen type I gels (Debruyne, 2002). Cell rounding, loss of filopodia, and enhanced haptotaxis were observed as consequences of BA treatment implicating an invasive phenotype. In contrast, no differences in cell-cell or cell-matrix adhesion, gelatinase activity, wound healing motility or E-cadherin dependent compaction were found (Debruyne, 2002). Others demonstrated that treatment of human colorectal cancer Caco-2 cells by LCA but not SLCA resulted in enhanced secretion of gelatinase A or MMP-2 (Halvorsen, 2000).

3. Aims

Previous studies on the role of FGFR4 in hepatocarcinogenesis revealed a significant overexpression of the receptor in subgroups of human HCC, implicating an involvement in late stages and progression of this disease. Additionally, the FGFR4-Arg^{388} allele was linked to a predisposition to develop HCC and a more aggressive phenotype. In vitro studies on human cells showed that both variants of FGFR4 enhance clonogenic growth at low density, anchorage independent growth and tumorigenicity in SCID mice. Interestingly, FGFR4 was also implicated in the regulation of cell-matrix adhesion which agrees with several reports.

The general aim of this study was to get further insight into the significance of FGFR4 for tumour progression and to investigate in detail the impact of the receptor on pro-metastatic processes.
The present study is composed of two parts, as follows:

Part I: Pro-metastatic behaviour of HCC model cell lines

All three hepatoma cell lines used for previous studies have already been characterized by Dr. Christine Gauglhofer regarding to their genotype, FGFR4 expression, clonogenicity, anchorage-independent growth, viability, proliferation, migration and tumorigenicity in SCID mice.

Therefore, the purpose of the first part of this study was to characterize the cell's capability to adhere, to form aggregates and to interact with BECs or LECs.
Furthermore, we were interested whether bile acids, MMPs, 12-lipoxygenase, and the phosphorylation of IκBα affect the gap forming capacities of the cell lines.

Part II: Impact of FGFR4 on pro-metastatic behaviour of HCC model cell lines

Preliminary studies showed that transient and stable overexpression of FGFR4 enhances the aggressive behaviour of HCC model cell lines, whereas siRNA mediated knock-down and blockade of FGFR4 signalling exerted opposite effects. Hence, the second part of the study aimed to define whether enhanced or inmpaired FGFR4-mediated signalling affects cell aggregation, cell-matrix adhesion and gap formation in monolayers of BECs and LECs.

4. Materials and Methods

4.1. Cell culture

4.1.1. Cell lines

The human epithelial cells lines CCL-13 (ATCC® CCL-13™) and HepG2 (ATCC HB-8065) were purchased from ATCC (Manassas, USA). The CCL-13 cell line was originally thought to be derived from normal liver tissue but was subsequently found to have been established by HeLa cell contamination. CCL-13 cell are generally characterized by growing into epithelial monolayers and a fibroblastoid-like morphology. Additionally, CCL-13 cells produce a C type retrovirus and are positive for keratin.
The HepG2 cell line is derived from a hepatocellular carcinoma of a young, male patient. HepG2 cells do not form a typical cell monolayer instead they grow in three-dimensional aggregates due to the strong intracellular adhesion.
The epithelial cell lines HCC-1.2 and HCC-3 were established at the Institute of Cancer Research (Medical University, Vienna) from patient tumour tissue provided by the General Hospital of Vienna. Recently, both cell lines were characterized as described by Dr. Sandra Sagmeister (Sagmeister, 2008).
HCC-1.2 clones stably overexpressing either wildtype FGFR4-G388 (FGFR4/Gly7), mutant FGFR4-R388 (FGFR4/Arg5) or the empty host vector pcDNA-3.1 (FGFR4/LV8) were established by Dr. Christine Gauglhofer at the Institute of Cancer Research, Vienna (Gauglhofer, 2010). Both FGFR4 expressing constructs harbour the full length version of the receptor, which is fused to a VSV-tag on the C-terminus for immunological detection.
Human blood (BECs) and lymphatic (LECs) endothelial cells were generously provided by the group of Priv. Doz. Dr. Michael Grusch. Both, BECs and LECs are stably transfected with telomerase cDNA to achieve immortalized growth.

4.1.2. Standard cultivation of cell lines

CCL-13, HCC-1.2 and HCC-3 cells were cultivated in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% FCS (fetal calf serum) (RPMI10). HepG2 cells were grown in MEME (Minimum Essential Medium Eagle) medium supplemented with 1 mM sodium pyruvate, 1% non-essential amino acids and 10% FCS (MNP10). Stable FGFR4 overexpressing HCC-1.2 clones were maintained in RPMI10 medium supplemented with the selective antibiotic G418 (1.4 mg/ml, PAA laboratories) and 1% penicillin/streptomycin (100x, PAA laboratories). BECs and LECs were grown in

EGM2 MV medium (EBM2-based medium CC3156 and supplement CC4147; Lonza, Walkersville, MD, USA).
All cells types were cultured under standard growth conditions, meaning in T25 (25 cm^2 flasks, Corning) or T75 tissue culture flasks (75 cm^2 flasks, Corning) in a humidified incubator (New Brunswick Scientific, Innova CO-170) at 37°C and 5% CO_2.

For splitting, medium was aspirated and cells were washed once with trypsin/EDTA to remove the remaining medium. Detachment of cells from the tissue flask surface was achieved by adding trypsin/EDTA (T/E) for an appropriate time at 37°C. Trypsin activity was stopped by addition of FCS-containing medium. Cells were collected and centrifuged for 5 minutes at room temperature at 800 rpm. The supernatant was discarded and the cell pellet was resuspended in the respective standard culture medium. According to the splitting proportion designated, an appropriate aliquot of cells was taken and added to standard culture medium in a new tissue culture flask. Cell concentrations were determined using a Neubauer counting chamber.

4.1.3. Freezing of cells

Cells were grown to a confluence of about 80% in T75 tissue culture flask (75 cm^2 flasks, Corning). As in standard cell culture, cells were trypsinized and centrifuged for 5 minutes at room temperature at 800 rpm. Cells were then resuspended in the appropriate standard culture medium containing 5% sterile dimethyl sulfoxide (DMSO, Fluka/Sigma). Aliquots with a volume of 1ml were transferred to cryotubes, which were wrapped in tissue papers to allow slow freezing. Samples were frozen over night at -70°C before they were stored in liquid nitrogen. Approximately two weeks after nitrogen storage, one aliquot was thawed and tested for contaminations.

4.1.4. Thawing of cells

Cryotubes stored in liquid nitrogen were slewed in lukewarm water until only a small ice-clot was left over. Tubes were then slewed in air to thaw the remaining ice. Cells were gently resuspended in cold standard culture medium and centrifuged for 5 minutes at 4°C at 800 rpm. The supernatant was discarded and the cell pellet was resuspended in 7 ml standard culture medium and seeded in T25 tissue culture flasks (25 cm^2 flasks, Corning). To remove remnants of DMSO, the medium was changed 24 hours after seeding.

4.1.5. Adhesion assay

The capability of cells to adhere was examined on uncoated polystyrene tissue culture plates or on plates coated with collagen or fibronectin. For coating, 96-well plates (BD Biosciences) were

incubated with rat collagen or human fibronectin over night at 37°C. Thereby, the collagen solution evaporated completely, whereas remaining fibronectin solution was collected and reused.
Cells were seeded at concentrations of $1x10^4$ cells per well in uncoated or coated 96-well plates (BD Biosciences) and allowed to attach to the surfaces for different time intervals. After 1, 2, 3 and 4 hours of incubation, medium with non-adherent cells was removed and attached cells were washed twice with 1x PBS to eliminate weakly adherent cells. The number of cells attached to the tissue culture plate was determined via MTT assay.

4.1.6. MTT assay

The MTT assay is a quantitative, colorimetric assay measuring the cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes (Berridge, 1996) . In the process, the yellow tetrazole MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) is reduced to water-insoluble formazan, giving an intense colour reflecting the relative amount of living cells (**Figure 12**; EZ4U manual, Biomedica).

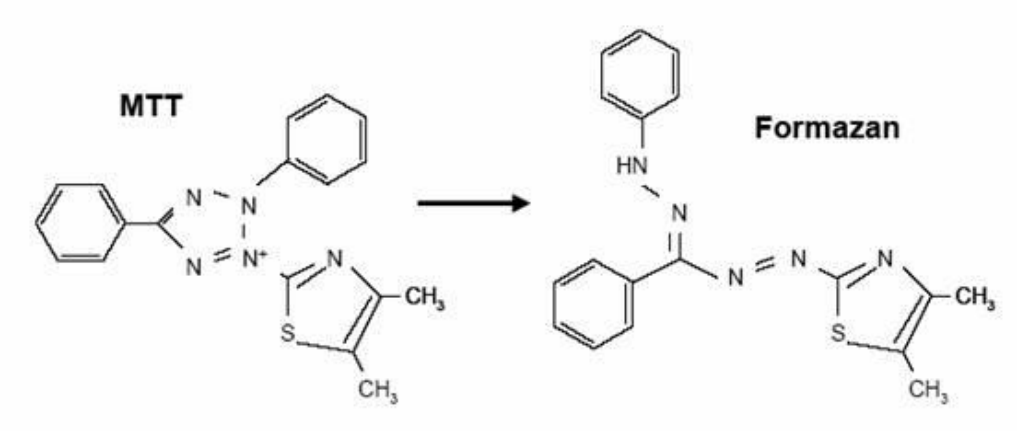


Figure 12: Reduction of MTT to formazan. *The yellow coloured tetrazolium MTT is reduced to intensely purple coloured formazan through the metabolic activity of viable cells (Fame Pharmaceuticals).*

In this study, EZ4U reagent (Biomedica) was used to determine the number of viable and adherent cells. Activated reagent in a final concentration of 10% in serum-free medium was added to the cells and incubated for two to five hours at 37°C until a significant increase in colour intensity was yielded. After incubation, plates were gently mixed by tipping on the sides to disperse precipitated formazan. Using an Asus Expert Plus plate reader, absorbance was measured at 450 nm with 620 nm as a reference.

4.1.7. Hanging Drop Assay

To investigate cell aggregation capabilities, a new assay was established following the procedure for production of embryoid bodies (Kurosawa, 2007; Wang, 2008).

A cell suspension with a concentration of $5x10^5$ cells per ml was prepared. Drops of 20 µl, containing approximately 10,000 cells, were placed on the inner site of the lid of a tissue culture plate. The lid was then placed on the culture plate, which generated hanging drops. The drops were incubated for different time spans under standard culture conditions. After 72 hours, pictures of cell aggregates formed in the hanging drops were taken with an Axiovert (Zeiss, Jena, Germany) fluorescence microscope. The mean top view aggregate area (TVAA) was evaluated by measuring the TVAA of all aggregates developed in the medium drop by ImageJ software (Wayne Rasband, NIH).

4.1.8. Gap Assay

An in vitro co-culture system was used to analyze the mechanisms of tumour cell-mediated disruption of blood or lymphatic vessels. In principle, spheroids made out of HCC cells were placed on the top of endothelial cell monolayers, which were used as surrogates for intra-metastatic blood or lymphatic vessels. As a result, spheroids caused the formation of gaps in the monolayers precisely underneath the HCC spheroids.

For spheroid generation, 1.2 g of autoclaved methyl-cellulose (M-0512, Sigma Aldrich) was resuspended in 100 ml pre-warmed RPMI10 medium to get a final stock concentration of 1.2%. The solution was stirred over night at 4°C until it turned clear and centrifuged for 2 hours at 4,700 rpm to pellet undesired debris. HCC cells were diluted in EGM2-MV containing 0.24% methyl cellulose (final concentration) to a concentration of $1x10^3$ cells per 150 µl. A total of 150 µl of this cell suspension was transferred to round bottom microtitre plates (BD Biosciences) to allow cell aggregation and spheroid formation within the following 48 hours.

BECs and LECs were seeded in EGM2-MV medium in 24-well plates (BD Biosciences) and grown for 48 hours until confluence was reached. Cell monolayers were then stained with cytotracker green (2µg/ml, Invitrogen) for one hour at 37°C and washed three times with a PBS/EGM2-MV solution (1:1). Afterwards, HCC spheroids were carefully transferred on the top of BEC or LEC monolayers and incubated for 2-5 hours. Experiments were performed in triplicates with at least 10 spheroids per well.

After 2-5 hours of co-culturing, spheroids and gaps in BEC or LEC monolayers underneath were pictured using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope. Visible light was used for detection of spheroids and a FITC filter for visualization of endothelial cells stained with cytotracker green (**Figure 13**). The top view spheroid areas (TVSA) and gap areas underneath the spheroids were measured by ImageJ software (Wayne Rasband, NIH). Since just the size of spheroids seemed to

influence the gap formation, spheroids deviating too much from the mean TVSA were excluded from the evaluation process.

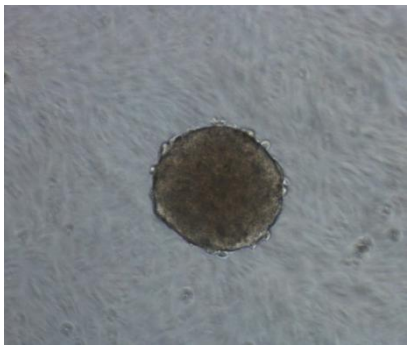
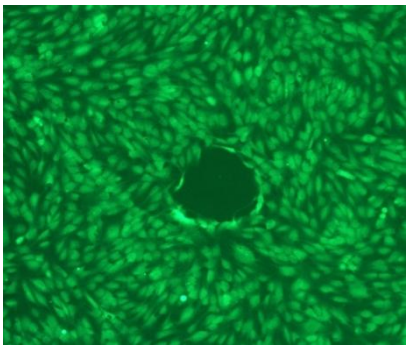



Figure 13: Principles of gap evaluation. *Pictures of HCC spheroids and gap areas underneath were taken with light and fluorescent microscopy, respectively.*

The impact of MMPs, 12-lipoxygenase, the NF-κB pathway and bile acids on the gap forming ability of HCC spheroids was investigated by application of various inhibitors and bile acids. All inhibitors used are listed in **Table 4**. Bile acids were kindly provided by Univ.-Prof. Dr. Michael Trauner (Medical University of Vienna).
For experiments, spheroids were incubated with inhibitors or bile acids for 1 hour at the concentrations indicated before spheroids were transferred on endothelial cell monolayers.

Table 4: Inhibitors and bile acids applied on HCC spheroids.

Inhibitor	Company	Target
BAY 11-7082	Merck	TNF-α-inducible phosphorylation of IκBα
GM6001	Merck	MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9
Baicalein	Merck	12-lipoxygenase

4.2. Transfection/Infection of cells

4.2.1. Plasmid transfection

Transient overexpression of FGFR4 was accomplished through transfection of CCL-13 and HCC-1.2 cells with plasmids encoding either the wild type FGFR4-G388 (FGFR4/Gly) or the mutant FGFR4-R388 (FGFR4/Arg). Both plasmids harbour a full length version of the receptor fused to a VSV-tag on its C-terminal end. Transfection with the empty host vector pcDNA-3.1 (LV) served as negative control. Transfection with a plasmid expressing eGFP was used to determine the transfection

efficiency. All plasmids were kindly provided by Axel Ulrich (Max Planck Institute, Martinsried, Germany).

Transient transfection of CCL-13 and HCC-1.2 cells was performed with Nanofectin (PAA) reagent, consisting of a positively charged polymer with DNA binding capacity, which is embedded into a porous nanoparticle. The DNA-nanoparticle complex protects the DNA from degradation by nucleases and is readily taken up by the cells due to its unique size (Nanofectin Kit manual, PAA).

Cells were seeded at a concentration of $2.3x10^5$ cells per 6-well (BD Falcon) or $11.2x10^5$ cells per PD100 dish (Corning) and incubated over night at 37°C. Separately, plasmid DNA and Nanofectin were diluted in appropriate volumes of 150 mM NaCl according to **Table 5** and mixed gently by vortexing. Subsequently, the Nanofectin solution was added to the plasmid DNA solution all at once, mixed immediately by tapping or vortexing and incubated for 25 minutes at room temperature. The DNA/Nanofectin complexes formed were added drop-wise to fresh culture medium to the final volume indicated in **Table 5** and homogenized by gently swirling the plate/dish. The transfection process was stopped after 24 hours by changing the culture medium.

Table 5: Complex preparation for different cell culture formats.

Culture vessel	Plasmid DNA [µg]	Diluent [µl]	Nanofectin [µl]	Diluent [µl]	Final volume [µl]	Final volume of medium per well/dish [ml]
6-well	1	100	4	100	200	2
PD100	5	500	20	500	1000	10

4.2.2. Adenoviral infection

Transient knock-out of FGFR4 was achieved by infection of CCL-13 and HCC-1.2 cells with two dominant-negative adenoviral pAD-CMV-5 vectors, referred to as kinase-dead FGFR4 (kdFGFR4) and soluble FGFR4 (solFGFR4). Infection of cells with a construct expressing the antisense RNA of COX2 (COX2 AS) was used as negative control. Infection with a virus coding for eGFP was used as expression control. All viruses were cloned and kindly provided by Prof. Dr. Walter Berger (Institute of Cancer Research, Vienna).

The kdFGFR4 construct comprises a protein, in which the kinase domain is substituted by an eCFP-tag at the C-terminus. The expressed protein is still able to form homo-dimers with the native receptor thus inhibiting auto-phosphorylation and the subsequent signal transduction into the intracellular space. The soluble form of FGFR4 only consists of the extracellular Ig I loop, which is able to bind to

the FGF binding motif on the endogenous receptor. Hence, solFGFR4 is thought to inhibit the FGFR4 signaling cascade by competing with FGFs for the binding to the receptor (Ezzat, 2001).

Cells were seeded at a concentration of $1x10^5$ (CCL-13), respectively $1.5x10^5$ cells (HCC-1.2) per 6-well (BD Falcon) or $8x10^5$ cells per PD100 (Corning) and incubated for approximately 8 hours at 37°C until most of the cells have attached to the cell culture dish. Culture medium was changed to remove non-adherent cells and diluted adenoviruses were added drop-wise to the cells according to the multiplicity of infection given in **Table 6**. The MOI is defined as the ration of the number of infectious virus particles to the number of target cells present in a defined space. Culture medium was changed 24 hours after infection.

Table 6: Multiplicity of infection (MOI) used for transient adenoviral transfection.

	eGFP	COX2 AS	kdFGFR4	solFGFR4
CCL-13	2	2	30	5
HCC-1.2	2	2	30	5

4.3. RNA analysis

4.3.1. RNA Isolation

RNA was harvested 48 hours after transfection/infection either from cells grown in monolayers or cells in suspension using the Trifast reagent. The reagent includes phenol and guanidinium thiocyanate in a monophasic solution allowing RNA isolation in a single step liquid phase separation.

For cells in monolayers, culture medium was aspired and cells were washed once with 1x PBS. After addition of Trifast reagent (Peqlab) at volumes of 300 µl per 6-well (BD Falcon) and 1 ml per PD100 (Corning), cells were scratched off from the tissue culture surface and transferred into microcentrifuge tubes. Cells in suspension were pelleted by centrifugation for 5 minutes at room temperature at 800 rpm and washed once with 1x PBS. After anew pelleting 300 µl Trifast reagent was added. After addition of chloroform (-20°C, Merck) at a ⅕ volume Trifast , samples were shaken vigorously by hand for 15 seconds and kept for 10 minutes at room temperature. Samples were then centrifuged for 10 minutes at 4°C at 12,000 g to yield a phase separation. The upper colourless aqueous phase contains the RNA, the interphase the DNA and the lower red organic phase the protein. The aqueous phase was transferred into a new microcentrifuge tube without destroying the other phases to avoid contaminations of the RNA with DNA or protein. RNA was precipitated by adding isopropanol (-20°C, Merck) at ½ volume Trifast. Samples were mixed and incubated at -20°C

over night. Precipitated RNA was pelleted by centrifugation for 10 minutes at 4°C at 12,000 g. The supernatant was discarded carefully and the RNA pellet was washed once with 70% EtOH (Merck). After centrifugation for 8 minutes at 4°C at 14,000 g, the supernatant was decanted and the pellet was air-dried before resuspension in 20-50 µl diethylpyrocarbonat (DEPC) treated water. For long time storage, RNA samples were kept on -80°C.

4.3.2. Determination of RNA concentration

RNA samples were readily checked for concentration and quality using the NanoDrop 1000 Spectrophotometer. Before samples were measured, a DEPC treated water sample was loaded to ensure cleanness of the instrument. After initial blank measurements with DEPC treated water, the absorbance of nucleic acids was measured at 260 nm and 280 nm. Thereby, the ratio 260/280 estimates the purity of the isolated RNA as a ratio lower than ~2.0 indicates the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Additionally, the ratio 260/230 is determined, which also reflects the purity of nucleic acids. Values usually lie in the range of 1.8-2.2. Lower values indicate the presence of co-purified contaminants (NanoDrop 1000 Spectrophotometer V3.7 manual).

4.3.3. Synthesis of complementary DNA by reverse transcription

For further experiments, RNA was transcribed into complementary DNA (cDNA). A recombinant m-MuLV reverse transcriptase (RT) was used offering an RNA-dependent and DNA-dependent polymerase activity and a RNase H activity specific to RNA in RNA-DNA hybrids (RevertAid RT, Fermentas, manual).

Table 7: Mastermix for synthesis of cDNA.

1xmastermix	**volume [µl]**
5xReaction buffer (Fermentas)	5.0
dNTP mix, 10mM each (Fermentas)	1.56
RiboLock RNase Inhibitor (40U/µl, Fermentas)	0.625
DEPC H_2O	1.19
RevertAid Reverse Transcriptase (200U/µl, Fermentas)	1.0
Total volume	9.37

For synthesis of cDNA, an appropriate volume for 1 µg RNA was diluted with DEPC treated water to a final volume of 10 µl. 0.6 µl of random hexamer primers (100 µM, Fermentas) and 5 µl DEPC treated water were added to each sample and probes were incubated for 2 minutes at 70°C to allow random

primer annealing. The mastermix was prepared as indicated in **Table 7** and added to each sample. cDNA first strand synthesis was performed by incubation of the samples for one hour at 42°C. The synthesis reaction was stopped by incubation for 5 minutes at 94°C. Afterwards, the samples were filled up with DEPC treated water to a final volume of 75 µl and stored at -20°C.

4.3.4. Real-time quantitative reverse transcriptase-PCR (qRT-PCR)

Real-time qRT-PCR is an advanced technology for amplification and simultaneous quantification of DNA sequences based on the principles of standard PCR. In this study, sequence-specific DNA probes labelled with a fluorescent reporter were used, which allowed detection only after hybridization with the complementary sequence.

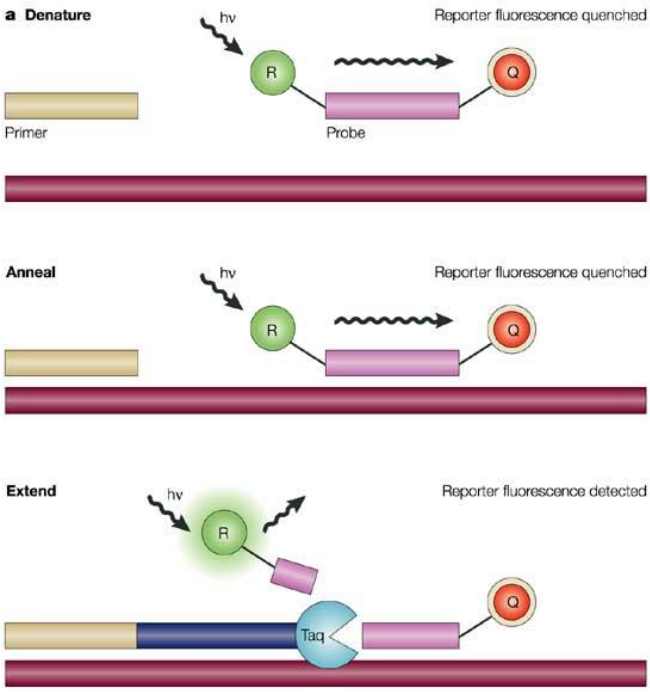


Figure 14: Principle of TaqMan probe hydrolysis. *Probes are cleaved during the extension step of the polymerase chain reaction (PCR) by the 5'-3' exonuclease activity of the Taq polymerase. The detectable reporter flourophore (R) is separated from the quencher (Q), whereby fluorescence is emitted when excited by an external light source (hv). Emitted fluorescence is thereby proportional to the amount of the product (Koch, 2004).*

TaqMan gene expression assays (Applied Biosystems) are nonextendible hybridization probes with a fluorescent dye on the 5' end of the probe and a quencher at the 3' terminus. Usually, FAM (i.e., 6-carboxyfluorescein) serves as fluorophore and TAMRA (i.e., 6-carboxy-tetramethylrhodamin) as quencher. On an intact probe, the two dyes are in close proximity resulting in fluorescent resonance

energy transfer (FRET), meaning that the fluorescence emission of the reporter dye is absorbed by the quencher. In course of the extension step during the PCR cycle, a sequence specific primer hybridizes with the complementary sequence and is extended by the Taq polymerase. When the enzyme reaches the probe downstream from the primer sites, its 5'-3' exonuclease activity degrades the bound probes whereby the reporter dye is released from the quencher and fluorescent emission at 518 nm can be detected (**Figure 14**). The fluorescence emitted at each PCR cycle is proportional to the amount of product formed (Heid, 1996).

As shown in **Table 8**, genes of interest were analyzed with TaqMan gene expression assays with exception of the housekeeping gene human β-2-microglobulin (hβ2m), for which individually designed primer probes were used.

For qRT-PCR, gene-specific mastermixes were prepared as indicated in **Table 9** and mixed with the sample cDNA or H_2O (negative control). Experiments were performed in duplicates. Samples were transferred into 96-well qRT plates (MicroAmp Fast Optical 96-well reaction plates with barcode, 0.1 mL, Applied Biosystems), which were covered with adhesive film (MicroAmp Optical Adhesive Film, Applied Biosystems). Samples were analyzed on an ABI-Prism/7500 Fast Real-Time PCR System (Applied Biosystems) applying the thermal program shown in **Table 10** and the 7500 Fast Sequence Detection System Software (Applied Biosystems).

Quantification of mRNA relies on plotting the fluorescence against the number of cycles on a logarithmic scale. Thereby, C_T is defined as the number of cycles at which the fluorescence crosses the threshold set slightly above background. To determine gene expression, C_T values from the gene of interest are normalized to the expression levels of the housekeeping gene human β2m, commonly called $\Delta\Delta C_T$-method.

Table 8: TaqMan Gene Expression Assays.

Gene	TaqMan Gene Expression Assay ID (Applied Biosystems)
Human FGFR4	Hs00608751_g1
Human klotho β	Hs00545621_m1
Human FGF19	Hs00192780_m1

Table 9: Mastermixes for qRT-PCR.

1xMastermix (human β-2-microglobulin)	**Volume [µl]**
Primer forward	1.15
5'-TGA CTT TGT CAC AGC CCA AGA TA-3' (Eurogentec)	
Primer reverse	1.15
5'-AAT GCG GCA TCT TCA AAC CT-3 (Eurogentec)	
Probe	0.3125
5'-FAM-TAA GTG GGA TCG AGA CAT GTA AGC AGC ATC A-TAMRA-3' (VBC Biotech)	
TaqMan Gene Expression MasterMix (Applied Biosystems)	6.25
DEPC H_2O	1.6375
cDNA	2.0
Total volume	12.5
1xMastermix (TaqMan Gene Expression Assays)	**Volume [µl]**
TaqMan Gene Expression Assay (Applied Biosystems)	0.625
TaqMan Gene Expression MasterMix (Applied Biosystems)	6.25
DEPC H_2O	2.625
cDNA	3.0
Total volume	12.5

Table 10: Thermal cycling program for qRT-PCR

Cycles	**Temperature**	**Time**
1x	50°C	2 min
1x	95°C	10 min
40-50x	95°C	15 sec
	60°C	1 min
1x	95°C	15 sec
(dissociation control)	60°C	1 min
	95°C	15 sec

4.4. Protein analysis

4.4.1. Protein isolation

Protein was harvested 48 hours after transfection/infection either from cells grown in monolayers or cells in suspension. For cells seeded in plates, culture medium was removed and cells were washed once with 1x PBS. After addition of lysis buffer at volumes of 45 µl per 6-well (BD Falcon), cells were scratched off from the tissue culture surface and transferred into microcentrifuge tubes. Cells in suspension were pelleted by centrifugation for 5 minutes at room temperature at 800 rpm and washed once with 1x PBS. After anew pelleting 100 µl lysis buffer were added to the samples.

Cells were disrupted with ultrasound sonification (1 second at 70% power, Bandelin Sonopuls HD2070) and cell debris was pelleted by centrifugation for 5 minutes at 4°C at 14,000 rpm. For long time storage, samples were kept at -20°C.

4.4.2. Determination of protein concentration

Concentrations of protein samples were determined by the Bradford Protein Assay (5x dye concentrate, BioRad). The method is based on a shift of the absorbance maximum from 465nm to 595nm of an acidic CoomassieBrilliant Blue G-250 dye solution when binding to protein. Thereby, the amount of bound dye is proportional to the protein concentration (BioRad Protein Assay, manual).

For determination of protein concentration, 1 µl of the protein sample was diluted in 9 µl H_2O in 96-well plates (BD Falcon). Simultaneously, protein standards containing 0-6 ng bovine serum albumin (BSA) in 9 µl H_2O and were prepared. 1 µl lysis buffer was added to each standard and protein sample solution. Standards and protein solutions were analyzed in duplicates. 100 µl of the 1x Bradford solution were added to each well and mixed with the samples by pipetting. Absorbance was then measured at 595nm with a Tecan Infinite plate reader.

4.4.3. Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE)

The SDS-PAGE technique is widely used to separate proteins according to their electrophoretic mobility, which is dependent on the length of a polypeptide chain and its charge. For analysis, the anionic detergent sodium dodecyl sulfate (SDS) is applied to protein samples, which is able to denature secondary and non-disulfid-linked tertiary structures. Further, SDS adds a negative charge to the proteins directly proportional to the mass. This enables a fractionation by approximate size in an electric field.

Protein samples were diluted in lysis buffer to concentrations of 20 µg protein in a volume of 15 µl. 2 µl of 6x Laemmli red loading dye was added and samples were heated for 5 minutes at 95°C. According to the size of the protein, polyacrylamid (PAA) gels with an appropriate concentration of PAA were prepared as listed in **Table 11**. After samples were loaded on the gel, proteins were compressed in the stacking gel at 60 V (30 minutes) and separated in the separating gel at 130 V (90 minutes). Protein weights were determined by co-separation of a PageRuler^{TM} Prestained Protein Ladder (Fermentas, #SM0671).

Table 11: Ingredients for 10% PAA gels.

Separating gel (10% PAA)	**Volume [ml]**	**Stacking gel (4% PAA)**	**Volume [ml]**
Acrylamid/Bisacrylamid 40% (Biorad)	3	Acrylamid/Bisacrylamid 40% (Biorad)	0.5
1.5 M Tris buffer pH 8.8	3	1.5 M Tris buffer pH 8.8	0.625
10% SDS (Sigma)	0.12	10% SDS (Sigma)	0.05
ddH_2O	5.8	ddH_2O	3.8
10% APS (Sigma)	0.12	10% APS (Sigma)	0.05
TEMED (Sigma)	0.012	TEMED (Sigma)	0.01

4.4.4. Western blot

Western blot is a technique in which proteins separated by gel electrophoresis are transferred to a membrane, typically nitrocellulose or PVDF and subsequently visualized with antibodies binding specifically to the protein of interest. In this study, the transfer of the proteins to the membrane was done by wet blotting. For this purpose, an activated membrane was placed on top of the gel, stacked between buffer-soaked filter papers and sponges on both sides. The entire stack was placed in a blotting apparatus (BioRad) and blotting was performed in 1x transfer buffer at 4°C over night. For long time storage and reuse, membranes were dried and kept at 4°C.

To prevent unspecific interactions between the membrane and the antibodies used for detection of a protein, membranes were blocked in 1x PBST with 3% skim milk powder (Fluka) for two hours. After blocking, membranes were incubated with dilute solutions of primary antibodies listed in **Table 12** over night at 4°C with gentle agitation. In order to remove unbound primary antibody, membranes were rinsed three times with 1x PBST for 15 minutes each and then incubated with the secondary antibody (**Table 12**) for 2 hours at room temperature. Antibodies used were linked with a horseradish peroxidase, which cleaves a chemiluminescent agent and produces luminescence proportional to the amount of the protein. Before detecting the luminescence, membranes were

washed 3 times with 1x PBST for 15 minutes each. A detection reagent (Amersham ECL Plus western blotting detection system, GE Healthcare, RPN2132) was applied to the membranes for 5 minutes, before a sensitive sheet of photographic film (CL-Xposure Film, Thermo Scientific) was places against the membrane, whereby the light from the product of the chemiluminescent reaction created an image of the antibodies bound to the membrane.

Table 12: Primary and secondary antibodies.

Primary antibody	**Company**	**Dilution**	**Mol. weight [kDa]**
Anti-FGFR4, rabbit polyclonal IgG against the extracelllar domain	sc-9006, Santa Cruz Biotechnology, Inc.	1: 200	95, 125
Anti-VSV-G tag, rabbit polyclonal IgG	ZSK2339, eBioscience	1: 2000	

Secondary antibody	**Company**	**Dilution**
Goat-anti-rabbit-HRP	Santa Cruz Biotechnology, Inc.	1: 10000

4.5. Buffers and solutions

Lysis buffer

2mL Hepes 500mM pH 7.5
3mL NaCl 1M
2mL Glycerol 100%
40µL EDTA 500mM pH 8.2
2mL NaF 100mM
100µL NaVO4 100mM
200µL Triton X-100
30µL MgCl2 1M
Ad 20mL ddH2O
2 tablets Complete Mini → Mix and aliquot on ice, store at -20°C

DEPC water

1mL DEPC (diethyl pyrocarbonate, Sigma)
1L ddH2O → stir for 7 hours, autoclave

10% SDS

10g Sodium dodecyl sulfate

100mL ddH2O

1.5M Tris pH 8.8

90.75g Trizma base

Ad 500mL ddH2O →adjust to pH 8.8

1.0M Tris pH 6.8

60g Trizma base

Ad 500mL ddH2O →adjust to pH 6.8

10% APS

5g Ammonium persulfate

50mL ddH2O

6×Laemmle Red loading dye

300mM Tris/HCl pH 6.8

60% Glycerol

10% SDS

0.025% Bromphenol blue

7% β-mercaptoethanol →Mix and aliquot on ice, store at -20°C

10×Laemmli running buffer

30g Trizma base

144g Glycine

10g SDS

Ad 1L ddH2O →autoclave

50kb DNA ladder

40µL GeneRuler 50bp DNA ladder (0.5µg/µL, Fermentas)

32µL 6×Loading Dye (Fermentas)

128µL DEPC H2O →aliquot

10× PBS

14.4g $Na_2HPO_4 \cdot 2H_2O$

26.2g $NaH_2PO_4 \cdot H_2O$

58.6g NaCl

Ad 1L ddH_2O → adjust to pH 7.4, autoclave

1×PBSt (0.05% Tween)

1L 1×PBS (pH 7.4)

500µL Tween-20

2.5×Transfer buffer

15g Trizma base

72g Glycine

316mL Methanol (=250g)

Ad 2L ddH_2O

10x Ponceau S solution

2% Ponceau S (Sigma)

10% acetic acid (Merck)

in 1x PBS

5. Results

5.1. Part I: Pro-metastatic behaviour of HCC model cell lines

Cellular motility and invasive abilities are fundamental in the progression of cancer and define the ability of cancer cells to leave the primary tumour and disseminate to distant anatomical sites. For this purpose, cells have to alter their cell-cell and cell-matrix adhesion in order to detach from the primary tumour (Valastyan, 2011). Since this is also true for metastatic processes in HCC, cell lines representative for human HCC were characterized and compared with regard to their basic ability to aggregate and to interact with matrix components and lymphatic or blood endothelial cells.

5.1.1. Aggregation capabilities and matrix adhesion of HCC model cell lines

Loss of intercellular interactions and simultaneous gain of migratory and invasive abilities is crucial for dissemination of carcinoma cells (Friedl, 2003). For this reason, we applied an assay, referred to as hanging drop assay, with the interest to study cell-cell interaction and aggregation behaviour. Basically, the assay followed a procedure used to produce embryoid bodies published, as by Kurosawa et al. Thereby cells were allowed to aggregate for 72 hours in absence of any contact to a matrix in a medium drop.

As presented in **Figure 15 A** cells aggregated in the time course of incubation. However, a relatively high number of single cells remained. Evaluation of the data proved to be challenging as an automatic measurement of the size of individual cell aggregates was not realizable. In the following, we tried to disperse the hanging drops manually by shaking to separate single cells from cell aggregate. Cells were subsequently fixed and stained with Hoechst, but because of too much unspecific staining and high background, the approach was abandoned. Alternatively, we decided to determine the mean top view aggregate area (TVAA) by measuring the TVAA of all aggregates found in a drop and to exclude remaining single cells. We hypothesized that the volume of cells of the different lines is similar and that this area is direct proportional to the number of cells arranged in an aggregate.

By the help of the hanging drop assay we tried to investigate the ability of HCC cells to aggregate. A comparison of all HCC cell lines investigated showed that CCL-13 cells produced significantly smaller cell aggregates with more remaining single cells after 72 hours of incubation than HCC-1.2 and HepG2 cells (**Figure 15 B**). On the other hand HepG2 cells showed bigger cell aggregates than HCC-1.2 cells.

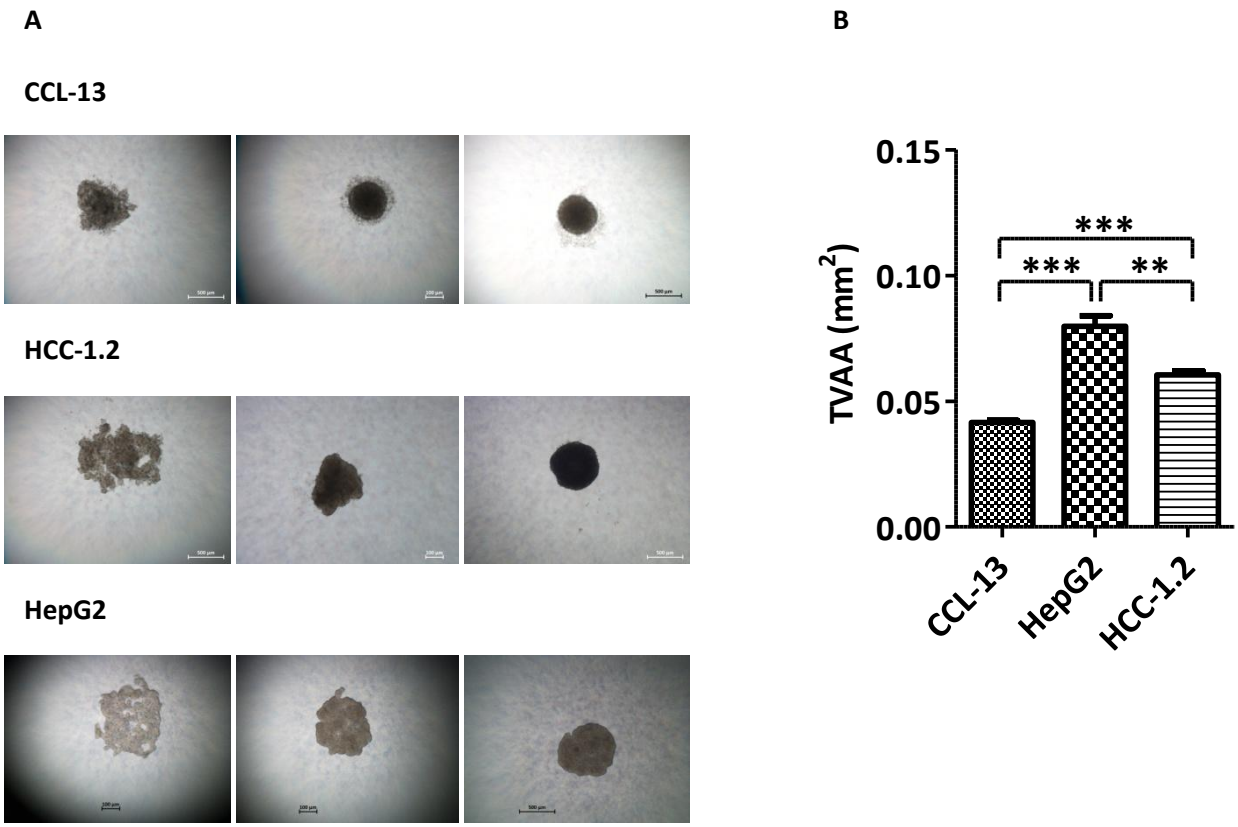


Figure 15: Cell aggregation of HCC model cell lines. *CCL-13, HCC-1.2 and HepG2 cells were suspended at a concentration of 1x10^4 in a volume of 20µl.A drop of 20 µl was placed on the inner site of the lid of a cell culture plate. The lid was then placed on the culture plate, which generated a hanging drop .In (A) cell aggregate formation of HCC model cell lines was followed by taking pictures after 24, 48 and 72 hours. In (B) hanging drops were incubated for 72 hours and pictures of cell aggregates formed were taken. The mean top view aggregate area (TVAA) was evaluated by measuring the TVAA of all cell aggregates found in a drop by ImageJ software. Data are given as mean ± SD of four independent experiments. Significant differences between cell lines are indicated as follows: unpaired t-test, *p<0.05, **p<0.01, ***p<0.001.*

So far, multiple reports associate deregulated cell-matrix adhesion with various stages of tumour progression and metastasis. Thus we were interested to test whether the HCC cell lines used exhibit altered cell-matrix adhesion by means of an adhesion assay carried out on uncoated tissue culture plates composed of polystyrene.

Overall, CCL-13 cells adhered more efficiently to the polystyrene surface than any other cell line investigated (**Figure 16**). HepG2 cells showed marginally less adherent cells than HCC-1.2 cells. Additionally, the amount of cells attached to the tissue culture plate increased clearly with CCL-13 cells over the period of incubation whereas an increase was hardly observable with HCC-1.2 and HepG2 cells.

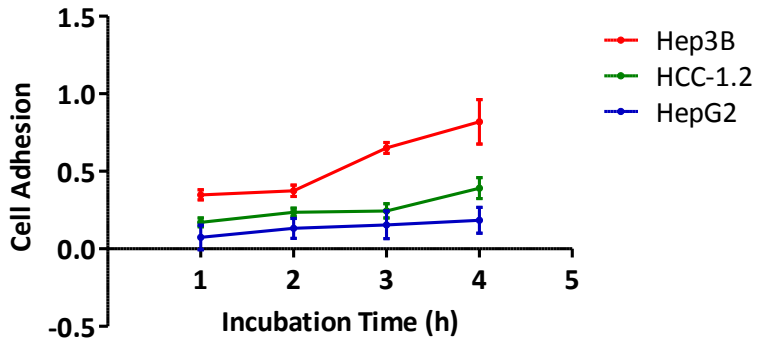


Figure 16: Matrix adhesion of HCC model cell lines. *1x10^4 CCL-13, HepG2 and HCC-1.2 cells were seeded onto 96-well plates composed of polystyrene. At timepoints indicated, medium was discarded and cells were washed twice with PBS to remove non attached cells. The number of adherent cells was determined by MTT assay. Data are given as mean ± SD of four independent experiments.*

5.1.2. Interaction of HCC model cell lines with endothelial cells

In 2011, Kerjaschki et al. used a 3-dimensional bulk invasion assay to analyze the mechanism of tumour cell-mediated disruption of lymphatic vessels and recapitulate the mechanism of metastasis (Kerjaschki, 2011). For this, MCF-7 mammary carcinoma cells were used to form spheroids, which were placed on telomerase immortalised human LECs grown to confluent monolayers. LEC monolayers were pre-labelled with cytotracker (green) to visualize the presence or absence of LECs underneath tumour spheroids. Co-cultivation of spheroids on LECs resulted in the formation of circular discontinuities in the monolayers precisely beneath the spheroids, termed as circular chemorepellent-induced defects (CCID). Furthermore, centrifugal migration (retraction) of LECs instead of apoptosis was defined as the mechanism for gap formation (Kerjaschki, 2011).

Here, we adopted the system used by Kerjaschki et al. for investigation of the interactions between HCC cells and BECs or LECs. First, several HCC cell lines were characterized for their ability to form spheroids. Generally, all cell lines used were able to form spheroids, but showed differences in shape and compactness. Optically, HepG2 spheroids appeared to be more granular based on less interaction between the cells (**Figure 17 A**). Interactions seemed to be more pronounced with CCL-13 cells showing more compact but still granular spheroids, whereas the surface of HCC-1.2 and HCC-3 spheroids appeared to be smooth and well defined. Evaluation of the mean top view spheroid area (TVSA) showed that HepG2 cells formed larger and hence less compact spheroids, whereas CCL-13, HCC-1.2 and HCC-3 spheroid areas appeared to be in a similar range (**Figure 17 B**).

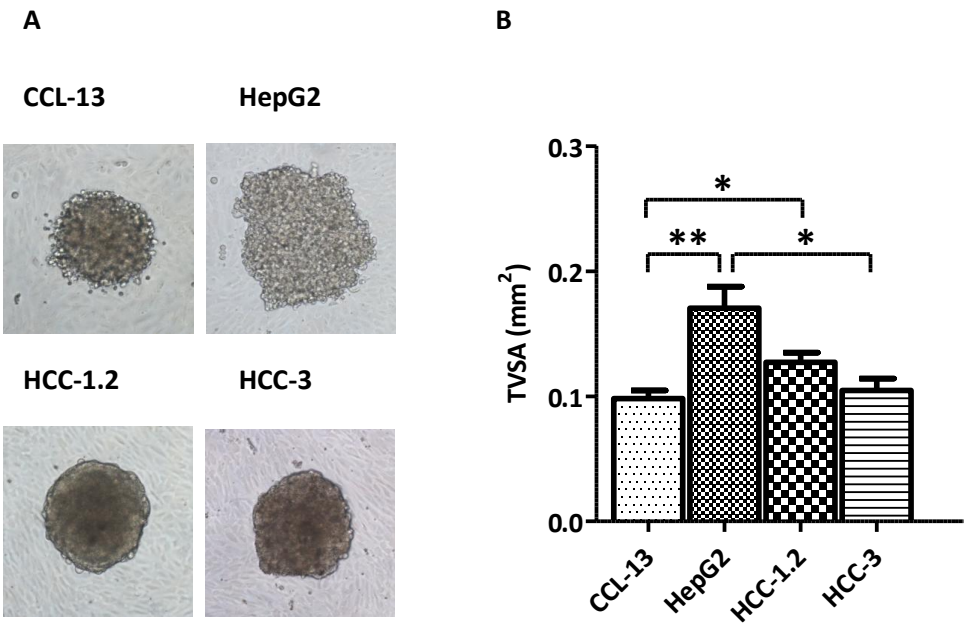


Figure 17: Mean spheroid area of HCC model cell lines. *3x10^3 cells were incubated in 96-well plates with round bottom and medium containing 20% methylcellulose for 48 hours. In (A) representative pictures of spheroids are shown. In (B) the mean top view spheroid area (TVSA) was measured by ImageJ software. Data are given as mean ± SD of four independent experiments. Significant differences between cell lines are indicated as follows: unpaired t-test, *p<0.05, **p<0.01.*

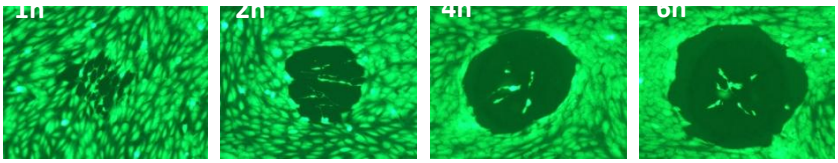
As depicted in **Figure 18 A**, CCL-13 spheroids caused time-dependent formation of gaps in the BEC or LEC monolayer growing underneath. Thereby, BECs were more sensitive to spheroid-induced gap formation than LECs. A comparison of all cell lines revealed large defects in blood endothelial monolayers being greatest in size for CCL-13 cells (**Figure 18 B**). Significantly smaller gaps were detected for HCC-3 cells followed by HepG2 and HCC-1.2 cells respectively. Similarly, CCL-13 induced gap formation in LECs was significantly more pronounced when compared to the other cells lines. HepG2 cells generated larger gaps than HCC-1.2 and HCC-3 spheroids. During spheroid transfer to monolayers, HepG2 spheroids were often disrupted. Consequently, only CCL-13 and HCC-1.2 cells were used for further assays.

Recent studies showed that inhibition of NF-κB by application of BAY 11-7082 reduced MCF-7 induced CCID formation in LEC monolayers (Vonach, 2011). BAY 11-7082 is known to be an irreversible inhibitor of I-κBα phosphorylation thus preventing the translocation of NF-κB (Pierce, 1997). Here, we investigated whether NF-κB is involved in BEC or LEC retraction induced by HCC spheroids. Treatment of CCL-13 or HCC-1.2 spheroids with 5µM of BAY 11-7082 revealed no impact on gap formation in BEC or LEC monolayers. Inhibitor treatment at concentration of 10 µM blocked gap formation of BECs by ~5 % and of LECs by ~10 % when incubated with CCL-13 spheroids.

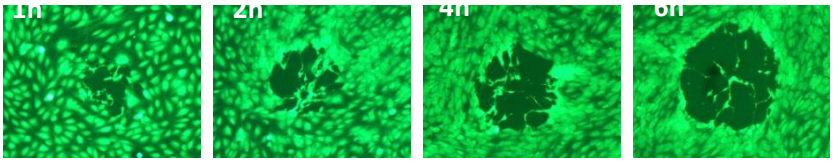
Treatment of HCC-1.2 spheroids with 10 µM BAY 11-7082 reduced gap formation of BECs by ~20% and of LECs by ~30% (**Figure 19**).

A

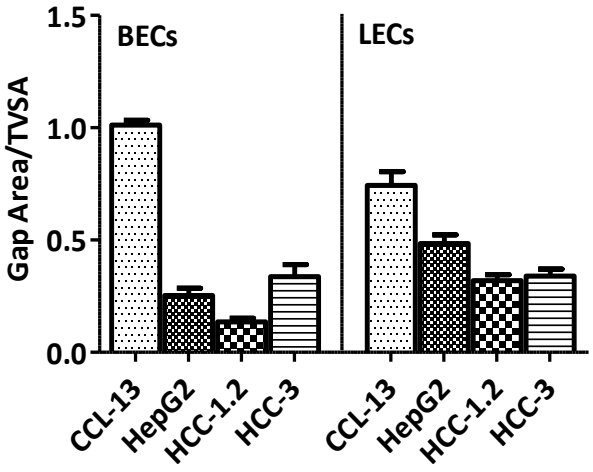
BECs



LECs



B



C

BECs	HepG2	HCC-1.2	HCC-3
CCL-13	<0.0001	<0.0001	<0.0001
HepG2		0.0154	0.2313
HCC-1.2			0.0116
HCC-3			

LECs	HepG2	HCC-1.2	HCC-3
CCL-13	0.0114	0.0007	0.0011
HepG2		0.0124	0.0282
HCC-1.2			0.6457
HCC-3			

Figure 18: Interaction of HCC model cell lines with endothelial cells. *$3x10^3$ cells were incubated in96 well plates with round bottom and medium containing 20% methylcellulose for 48 hours. Spheroids were placed on the top of a blood (BEC) or lymphatic (LEC) endothelial cell monolayer. (A) The gradual increase of gap areas underneath CCL-13 spheroids was depicted after the time points indicated. (B) The ration of gap areas to top view spheroid area (TVSA) of HCC model cell lines is given after 4 hours co-culture. Data are given as mean ± SD of four independent experiments. (C) Significant differences between cell lines, indicated by p-values, were determined by unpaired t-test.*

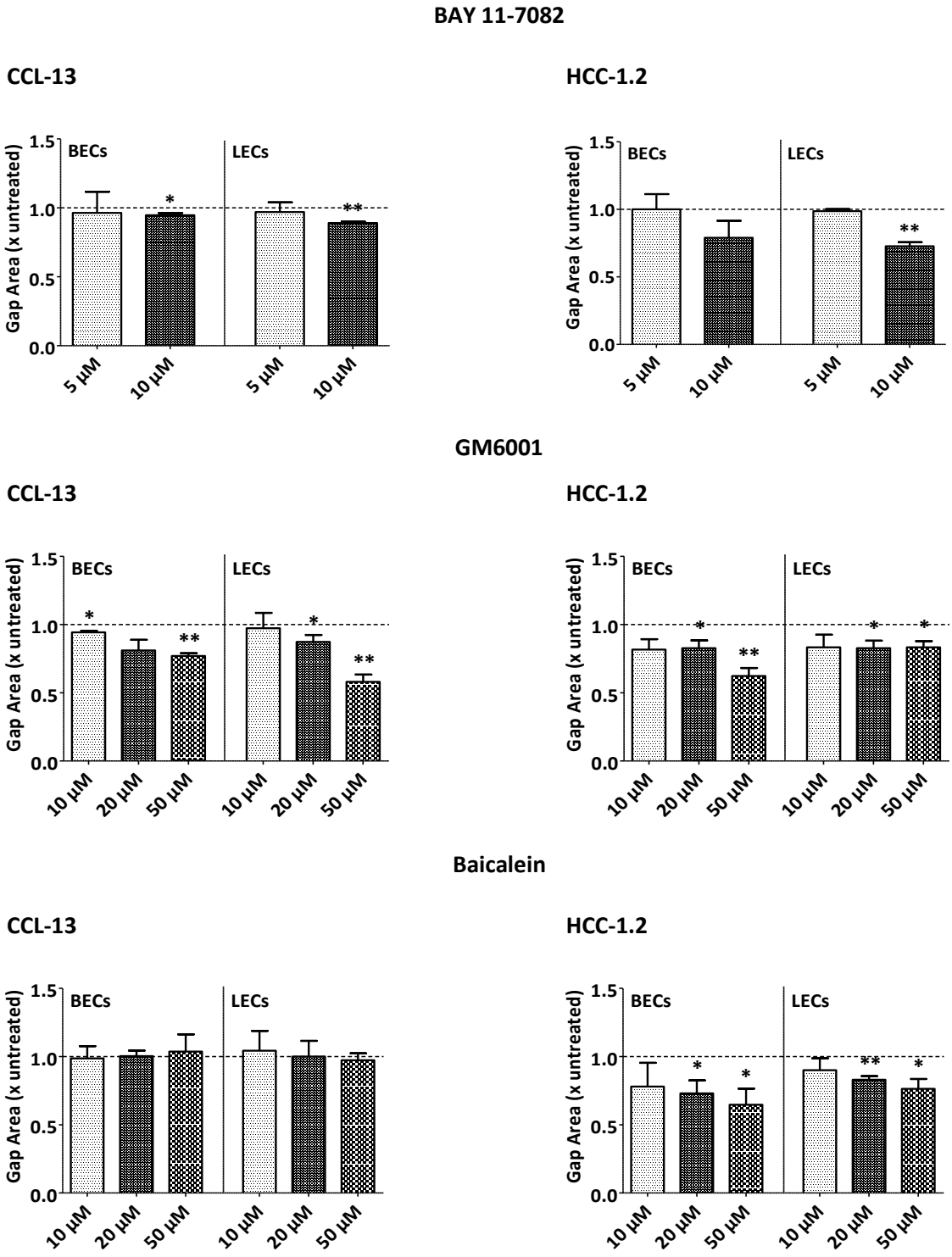


Figure 19: Inhibition spheroid induced gap formation of BEC and LEC monolayers by treatment with BAY 11-7082, GM6001 and Baicalein. *CCL-13 and HCC-1.2 cells were incubated in 96-well plates with round bottom (3x10^3 cells per well) and medium containing 20% methylcellulose for 48 hours. Spheroids were treated with different concentrations of BAY-11-7082, GM6001 and Baicalein for 1 hour. Spheroids were placed on the top of a blood (BEC) or lymphatic (LEC) endothelial cell monolayer and incubated for 2-5 hours. Pictures of gap areas were taken and areas were measured by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences to the untreated control are indicated as follows: unpaired t-test, *p<0.05, **p<0.01.*

Activities of metalloproteases are involved in the complex process of metastasis. Therefore, MMP activity was pharmacologically inhibited by the pan-matrix-metalloprotease inhibitor GM6001. Results shown in **Figure 19** revealed a significant reduction of gap formation with both cell lines. When co-incubating CCL-13 spheroids with BECs, the higher the concentrations of the inhibitor, the more reduced gap areas could be observed. Furthermore, CCL-13 spheroid induced retraction of LECs was seen to be reduced by about 20 % with 10 µM, 20 µM and 50 µM of the inhibitor. A similar effect was seen with HCC-1.2 spheroids, exhibiting a decrease of gap formation of BECS by ~5 % to ~20 % and of LECs by ~5 % to ~40 % when applying different concentrations of GM6001.

Recently, it has been shown that formation of gaps in a confluent LEC monolayer caused by MCF-7 spheroids is mediated by secretion of 12(S)-HETE which is a product of arachidonic acid metabolized by lipoxygenases (LOXs) (Kerjaschki, 2011). To investigate, whether 12-HETE also is involved in HCC spheroid gap formation, CCL-13 and HCC-1.2 spheroids were treated with different concentrations of the pan-LOX inhibitor Baicalein. As a result, a blockade of gap generation in BECs by about 20 % to 35 % and in LECs by about 10 % to 35 % was seen with HCC-1.2 spheroids No effect was detected with CCL-13 spheroids on both endothelial cell lines (**Figure 19**).

In general, all inhibitors acted in a dose-dependent manner, indicating that retraction of endothelial cells is mediated by MMPs, lipoxygenases and the NF-κB pathway. These effects were more pronounced in LECs compared to BECs.

5.1.3. Impact of bile acids on gap forming capacities of HCC model cell lines

Since several studies showed an association of bile acids with increased invasiveness and metastasis (Halvorsen, 2000; Debruyne, 2002), we were interested in studying the impact of bile acids on the ability of HCC cells to interact with endothelial cells.

Treatment of CCL-13 spheroids with different concentrations of CA had no effect on gap formation in BECs when compared to the untreated control (**Figure 20**). On LEC monolayers, results showed an insignificant increase in gap areas with 50µM of CA. HCC-1.2 spheroid induced gap formation in BECs was slightly enhanced by increasing concentrations of CA, while gap areas in LECs appeared to be unaffected.

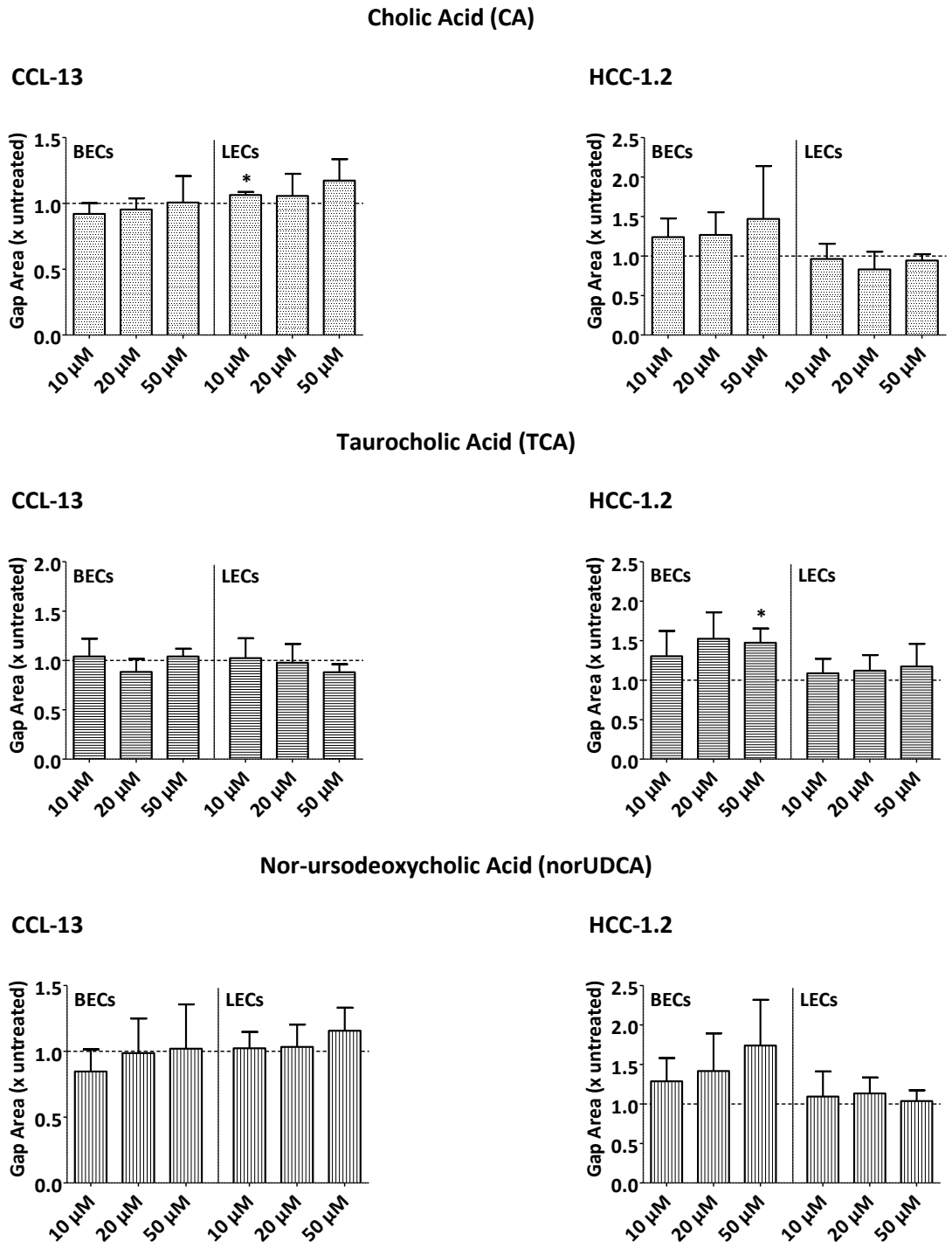


Figure 20: Influence of cholic acid (CA), taurocholic acid (TCA) and nor-ursodeoxycholic acid (norUDCA) on gap formation. *CCL-13 and HCC-1.2 cells were incubated in 96-well plates with round bottom (3x10^3 cells per well) and medium containing 20% methylcellulose for 48 hours. Spheroids were treated with different concentrations of CA, TCA and norUDCA for 1 hour. Spheroids were placed on the top of a blood (BEC) or lymphatic (LEC) endothelial cell monolayer and incubated for 2-5 hours. Pictures of gap areas were taken and areas were measured by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences to the untreated control are indicated as follows: unpaired t-test, *p<0.05.*

CCL-13 cells incubated with TCA at concentrations of 20µM showed a ~10% decrease of gap formation in BECs, whereas treatment of spheroids with lower or higher concentrations of TCA had no effect (**Figure 20**). On LECs, gap areas tended to be diminished with increasing concentrations of TCA. HCC-1.2 spheroids induced an increase of BEC retraction by about 50% with 20µM and 50µM of the bile acid. The increase in gap areas with higher concentrations of TCA was also detected in LECs although less pronounced than in BECs.

Treatment of CCL-13 spheroids with 10 µM of norUDCA decreased marginally gap formation whereas higher concentrations were ineffective (**Figure 20**). On LECs, CCL-13 spheroids slightly increased gap formation at a concentration of 50 µM. HCC-1.2 spheroids treated with norUDCA enhanced BEC retraction to about 130% with 10 µM, ~140% with 20µM and ~170% with 50µM of the bile acid. On LEC monolayers, norUDCA treatment had no effect on gap formation.

5.2. Part II: Impact of FGFR4 on the pro-metastatic behaviour of HCC model cell lines

We investigated whether FGFR4 is relevant for HCC cells to gain a metastatic phenotype. For this purpose, the receptor was either overexpressed by transient or stable transfection or was blocked by infection with adenoviruses expressing dominant negative forms of FGFR4.

5.2.1. Impact of transient FGFR4 overexpression on mRNA and protein levels

For transient overexpression of FGFR4, CCL-13 and HCC-1.2 cells were transfected with an empty vector (LV) serving as negative control and plasmids encoding either the wildtype version of FGFR4, i.e. FGFR4-G388 (Gly), or the mutant version FGFR4-R388 (Arg). To evaluate the efficiency of the transfection, a GFP expressing construct was used. In CCL-13 cells, a GFP signal was detected in nearly every cell, pointing to a high and very sufficient transfection efficiency (**Figure 21**), whereas in HCC-1.2 cells the GFP signal was clearly weaker. Anyway, approximately 60 % of HCC-1.2 cells were transfected efficiently.

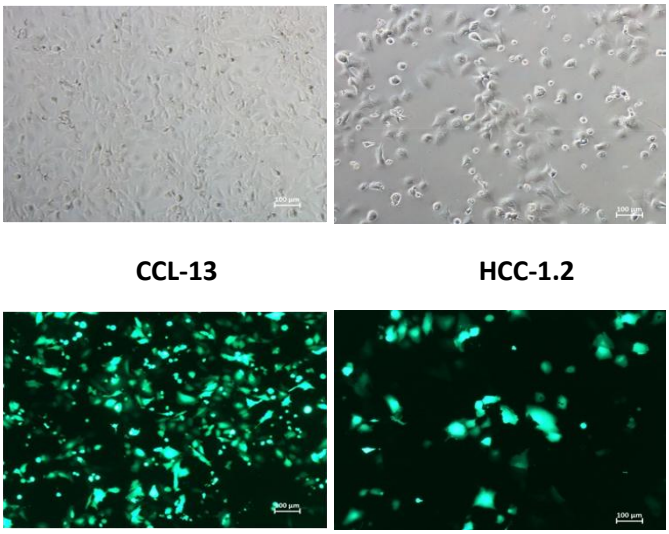
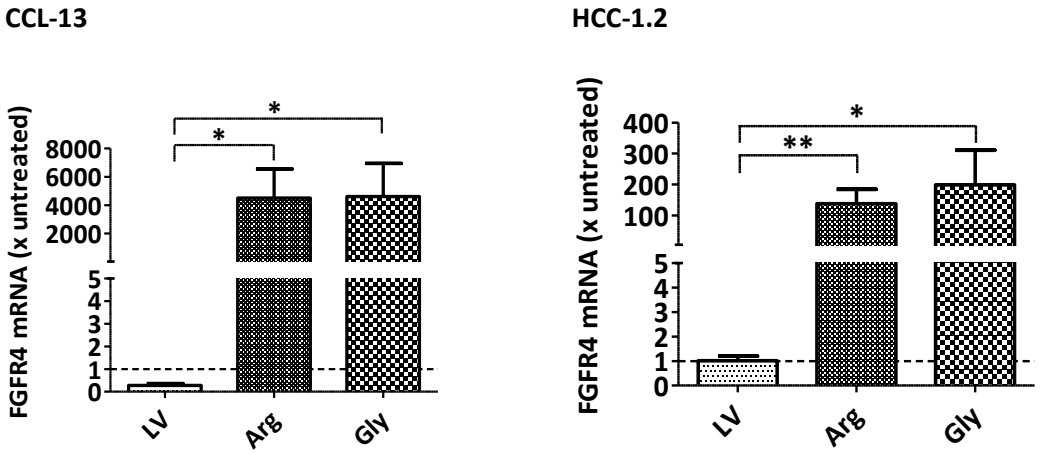


Figure 21: Transfection efficiency in HCC model cell lines. *CCL-13 and HCC-1.2 cells were transiently transfected with a plasmid coding for GFP. For evaluation of transfection efficiency, GFP positive cells were compared to total cells 48 hours after transfection.*

Overexpression of the receptor was confirmed by determining mRNA and protein levels of transfected cells using quantitative real-time PCR and western blot analysis, respectively. Analysis of mRNA levels revealed a ~5000-fold overexpression of both, FGFR4-G388 and FGFR4-R388, in CCL-13 cells (**Figure 22 A**). Comparatively, mRNA expression of FGFR4-G388 and FGFR4-R388 in HCC-1.2 cells was elevated approximately 200-fold.

A



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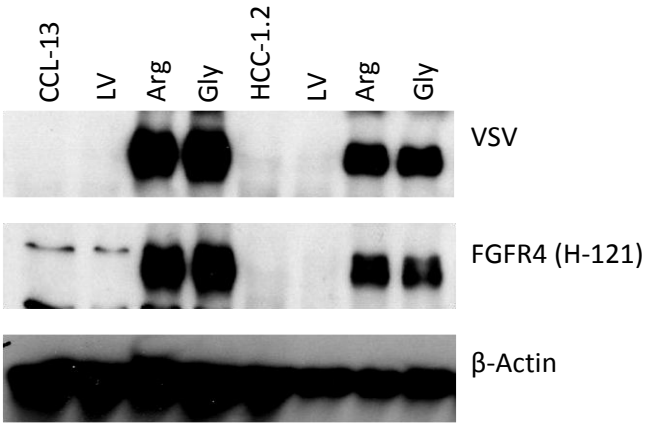


C

VSV	CCL-13	HCC-1.2
Untreated	1.00	1.00
LV	1.43	0.81
Arg	9.42	5.62
Gly	8.46	4.75

FGFR4 (H-121)	CCL-13	HCC-1.2
Untreated	1.00	1.00
LV	0.93	0.75
Arg	3.97	4.32
Gly	3.58	3.52

Figure 22: Impact of FGFR4 overexpression on mRNA and protein levels. *CCL-13 and HCC-1.2 cells were transiently transfected with an empty host vector (LV), plasmids coding for FGFR4-G488 (Gly), or FGFR4-R388 (Arg). (A) RNA was harvested 48 hours after transfection and FGFR4 expression was analyzed via qRT-PCR, using β2 microglobulin as housekeeping gene. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences to the empty vector control (LV) are indicated as follows: unpaired t-test, *p<0.05, **p<0.01. (B) Proteins were harvested 48 hours after transfection and analyzed via western blot. Proteins encoded by the plasmid constructs were detected by anti-VSV-tag antibody, total FGFR4-protein was detected by anti-FGFR4 H-121 antibody. (C) Densitometric quantification of western blots shown in (B) by ImageJ software. β-actin was used as loading control. Results are shown as fold untreated control and data represent results of one experiment.*

Western blot analysis indicated increased protein levels in both cell lines transfected with either FGFR4-G388 or FGFR4-R388 when compared to the negative control and endogenous protein

expression (**Figure 22 B**). As expected, FGFR4 levels appeared to be more elevated in CCL-13 cells as a result of the inherent low FGFR4 expression and the efficacy of transfection in contrast to HCC-1.2 cells. Quantification of the protein levels revealed a ~9.5/8.5-fold overexpression of FGFR4-Arg^{388} and FGFR4-Gly^{388} in CCL-13 cells, respectively, when analyzing the anti-VSV-tag stained blots. HCC-1.2 plasmid transfection yielded a ~5.5/5-fold overexpression. Applying the FGFR4 (H-121) antibody, FGFR4-R388 was found to be ~4 and 3.5-fold increased in both CCL-13 and HCC-1.2 cells, respectively.

5.2.2. Impact of transient FGFR4 overexpression on cell aggregation and cell-matrix adhesion

Alterations in cell-cell interactions by transient FGFR4 overexpression in CCL-13 and HCC-1.2 cells were tested in the hanging drop assay. As presented in **Figure 23**, aggregation of cells transfected with the empty host vector appeared to be impaired, pointing to an effect caused by the transfection itself. Compared to LV transfected cells, overexpression of the receptor had no influence on aggregation capabilities of CCL-13 and HCC-1.2 cells. Additionally, there was no difference between Gly- and Arg-overexpressing cells.

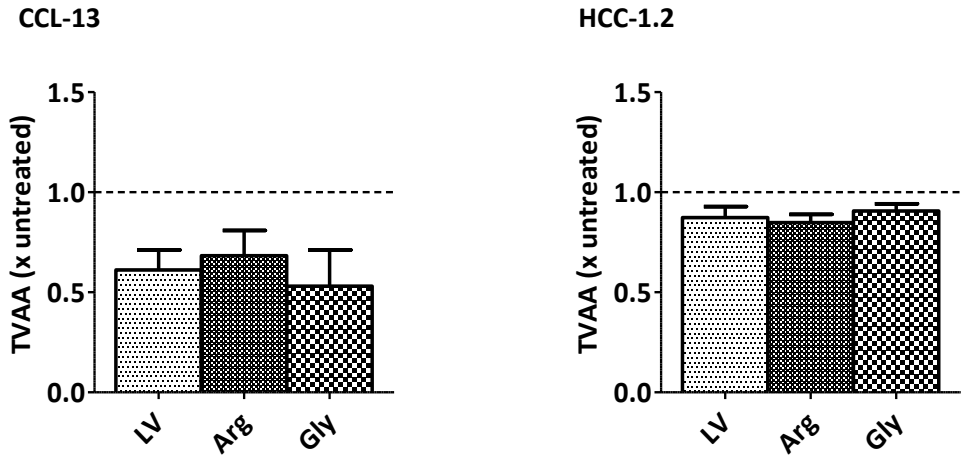


Figure 23: Impact of transient FGFR4 overexpression on cell aggregation. *CCL-13 and HCC-1.2 cells were transiently transfected with an empty host vector (LV), plasmids coding for FGFR4-G488 (Gly), or FGFR4-R388 (Arg). 24 hours after transfection, 1x10^4 cells were suspended in a volume of 20 µl. A drop of 20 µl was placed on the inner site of the lid of a cell culture plate. The lid was then placed on the culture plate, which generated a hanging drop. Hanging drops were incubated for 72 hours and pictures of cell aggregates were taken. The mean top view aggregate area (TVAA) was evaluated by measuring the TVAA of all cell aggregates found in a drop by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of four independent experiments.*

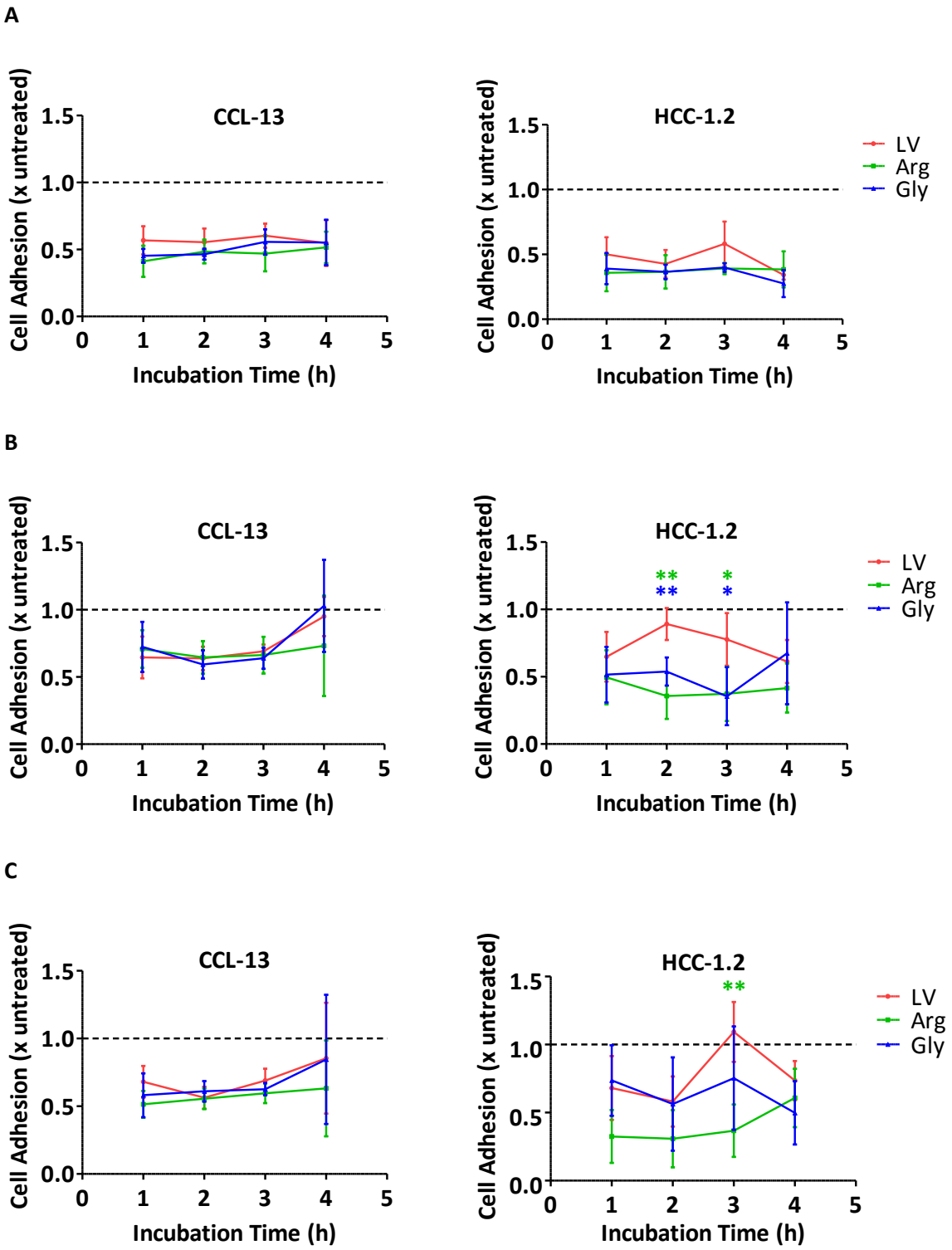


Figure 24: Impact of transient FGFR4 overexpression on cell-matrix adhesion. *CCL-13 and HCC-1.2 cells transiently transfected with an empty host vector (LV), plasmids coding for FGFR4-G488 (Gly), or FGFR4-R388 (Arg). 48 hours after transfection, 1x10^4 cells were seeded in 96-well plates on polystyrene (A), collagen (B) and fibronectin (C). At timepoints indicated, medium was discarded and cells were washed twice with PBS to remove non attached cells. The number of adherent cells was determined by MTT assay. Results are shown as fold untreated control. Data are given as mean ± SD of four independent experiments. Significant differences to the empty vector control (LV) are indicated as follows: unpaired t-test, *p<0.05, **p<0.01.*

Cell-matrix interactions of both cell lines transfected with FGFR4-Gly or FGFR4-Arg were studied on polystyrene, collagen and fibronectin surfaces. Overall, transfection with the empty vector control reduced the number of attached cells in both cell lines on all surfaces when compared to the untreated control (**Figure 24**). CCL-13 cell overexpressing FGFR4/Gly or FGFR4/Arg showed no change in adhesion properties when compared to the empty vector control neither on polystyrene nor on collagen- or fibronectin-coated surfaces. Cell-matrix adhesion of HCC-1.2/FGFR4 cells was slightly decreased on polystyrene, but was significantly decreased after 2-3 hours of incubation on collagen and fibronectin. Adhesion values for Gly- and Arg-overexpressing HCC-1.2 cells were similar in all experiments.

5.2.3. Impact of transient FGFR4 overexpression on interaction with endothelial cells

Next, we studied the interaction of FGFR4 overexpressing HCC cells with blood or lymphatic endothelial cells. For this purpose, spheroids of transiently transfected cells were formed, which were then placed on the top of a BECs or LECs monolayer. After a certain period of interaction, the area of gaps in the endothelial layer underneath was measured.

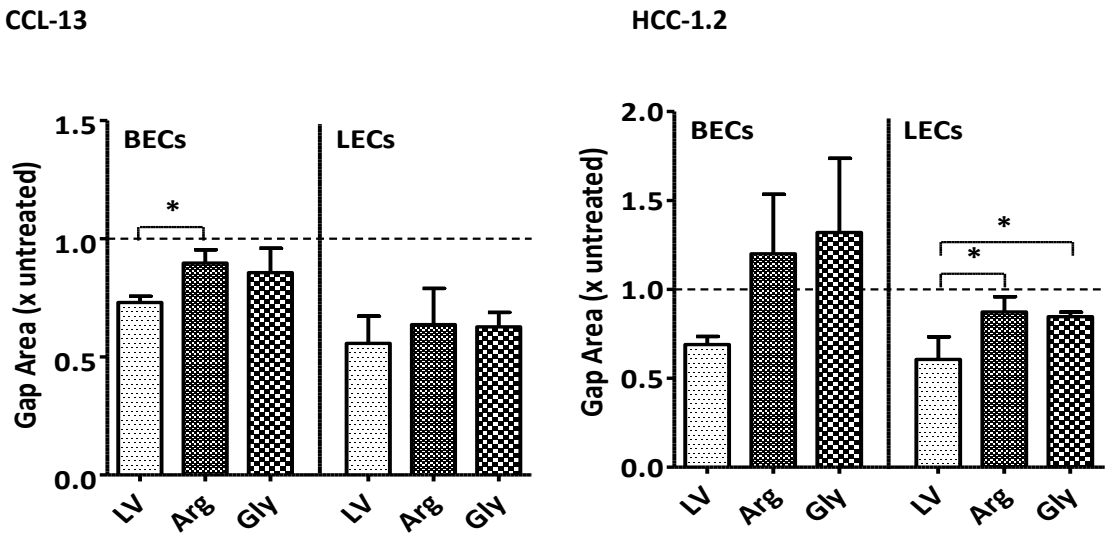


Figure 25: Impact of transient FGFR4 overexpression on gap formation. *CCL-13 and HCC-1.2 cells were transfected with an empty host vector (LV), plasmids coding for FGFR4-G488 (Gly), or FGFR4-R388 (Arg). 24 hours after transfection, 3x10^3 cells were incubated in 96-well plates with round bottom and medium containing 20% methylcellulose for 48 hours. Spheroids were placed on the top of a blood (BEC) or lymphatic (LEC) endothelial monolayer and incubated for 2-5 hours. Pictures of gap areas were taken and areas were measured by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences to the empty vector control (LV) are indicated as follows: unpaired t-test, *p<0.05.*

As illustrated in **Figure 25**, transfection of CCL-13 and HCC-1.2 cells with the empty vector control (LV) decreased spheroid induced gap formation on BECs and LECs when compared to the untreated control. Transfection with both versions of FGFR4 reversed this effect. In detail, CCL-13/Arg spheroids significantly enhanced gap formation when compared to the empty vector control. Transfection of CCL-13 cells with the wild type version of FGFR4 also increased gap formation but results were not significant. No differences in gap formation were detected between CCL-13/LV and CCL-13/Arg or CCL-13/Gly spheroids put on the top of LECs. HCC-1.2 spheroids overexpressing FGFR4/Arg or FGFR4/Gly significantly increased gaps in LEC monolayers when compared to FGFR4/LV, in BECs a tendency to enlarged gaps could be observed. No differences were seen caused by the single nucleotide polymorphisms.

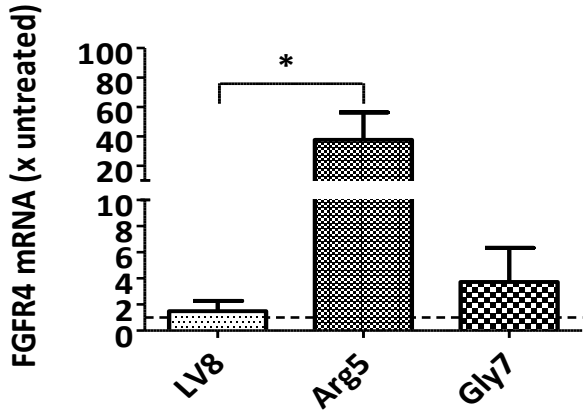
5.2.4. Impact of stable FGFR4 overexpression on mRNA and protein levels

To confirm the results from transient overexpression experiments and determine the long-term effects of FGFR4 overexpression, HCC-1.2 clones (HCC-1.2/Arg5 and –Gly7), stably expressing FGFR4-encoding plasmids or an empty host vector (HCC-1.2/LV8) were used to investigate cell aggregation, cell-matrix interactions and interactions with BECs and LECs.

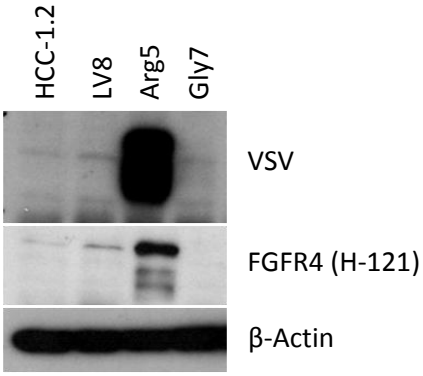
First, FGFR4 overexpression was verified by determination of mRNA levels by qRT-PCR. Results indicated approximately 40-fold and 4-fold elevated mRNA levels for HCC-1.2/Arg5 and HCC-1.2/Gly7 clones, respectively (**Figure 26 A**). On protein level, FGFR4/Arg5 expression appeared to be approximately 16-fold increased when detected with the anti-VSV-tag antibody. Using the anti-FGFR4 antibody, FGFR4-R388 expression was detected to be ~10-fold elevated (**Figure 26 B**). FGFR4/Gly7 expression could not be detected by western blot analysis.

Figure 26: Impact of FGFR4 overexpression on mRNA and protein levels. *(A) FGFR4 mRNA levels of stable overexpressing HCC-1.2 clones were analyzed via qRT-PCR, using β2 microglobulin as housekeeping gene. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences are indicated as follows: unpaired t-test, *p<0.05. (B) FGFR4 overexpressing was analyzed by western blot using anti-VSV-tag and FGFR4 H-121 antibodies for detection of plasmid encoded and total protein respectively. (C) Densitometric quantification of bonds shown in (B) by ImageJ software. β-actin was used as loading control. Results are shown as fold untreated control and data represent results of one experiment.*

A



B



C

HCC-1.2	VSV	FGFR4 (H-121)
Untreated	1.00	1.00
LV8	1.52	1.89
Arg5	15.82	10.10
Gly7	1.39	0.43

5.2.5. Impact of stable FGFR4 overexpression on cell aggregation, cell-matrix interactions and gap formation

Cell aggregation capabilities of HCC-1.2 clones (FGFR4/Arg5 or FGFR4/Gly7), stably overexpressing FGFR4, were investigated by performing hanging drop assays. Thereby, HCC-1.2/Arg5 showed an approximately 2-fold higher mean cell aggregate area than HCC-1.2/Gly7 cells, which appeared to be unaffected in comparison to HCC-1.2/LV8 (**Figure 27 A**).

As illustrated in **Figure 27 B-D**, FGFR4/LV8 enhanced cell-matrix adhesion on polystyrene, collagen and fibronectin when compared to the untreated control. Adhesive capabilities of HCC-1.2/Arg5 and –Gly7 clones were shown to be significantly lower than the empty vector control when testing on polystyrene and collagen surfaces. Adhesion to fibronectin surfaces was also decreased, although not significantly. However, adhesion of FGFR4/Arg5 and –Gly7 clones to all surfaces tested appeared to be in the range of the untreated control. Additionally, differences in cell adhesion of Arg5 and Gly7 clones were detected. Cell-matrix adhesion to collagen and fibronectin was more decreased in HCC-1.2/Arg5 than in HCC-1.2/Gly7.

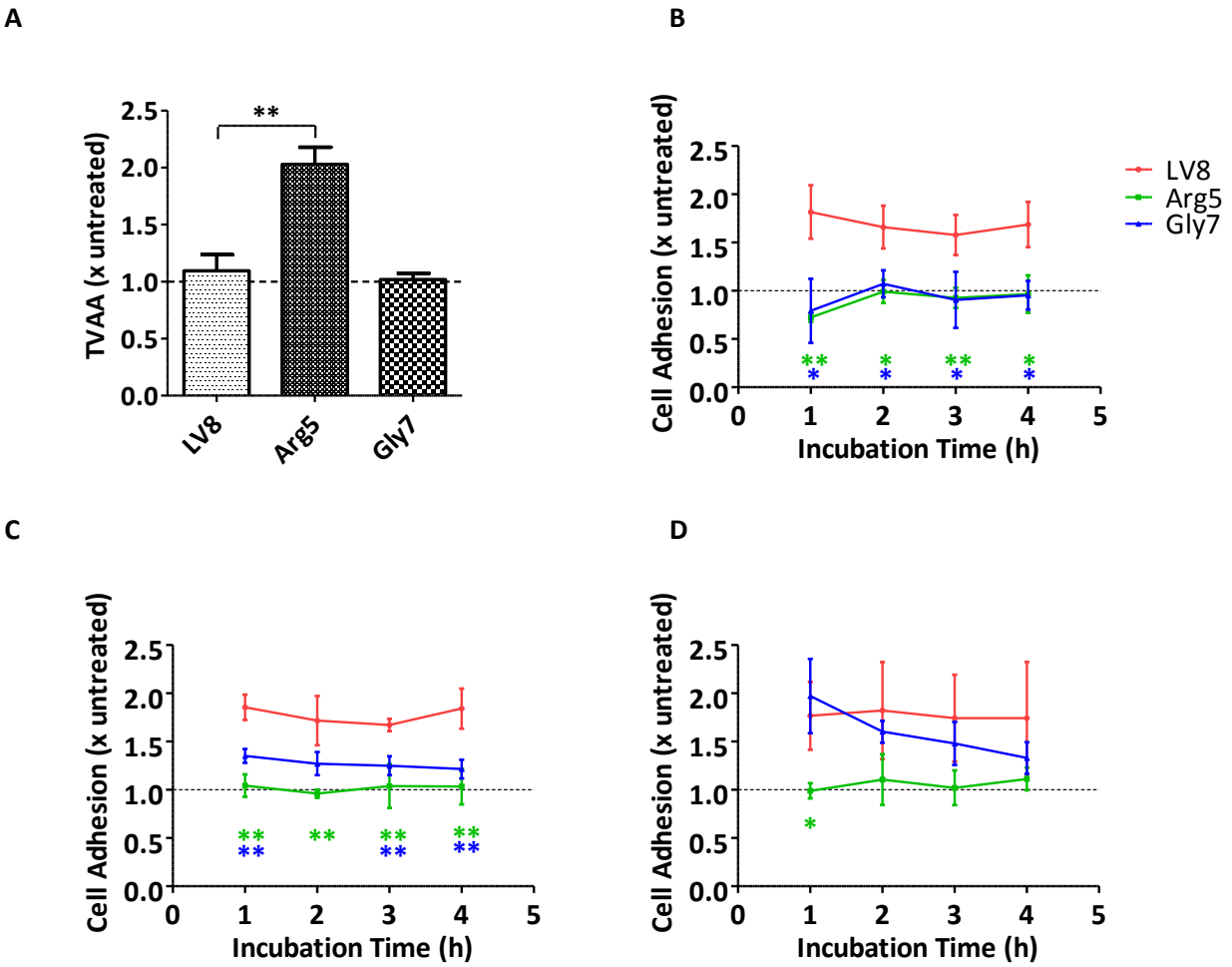


Figure 27: Impact of stable FGFR4 overexpression on cell aggregation and cell-matrix adhesion. *(A) 1x10^4 cells of HCC-1.2 clones (FGFR4/Arg5 or FGFR4/Gly7), stably overexpressing FGFR4, were suspended in a volume of 20µl. A drop of 20µl was placed on the inner site of the lid of a cell culture plate. The lid was then placed on the culture plate, which generated a hanging drop. Hanging drops were incubated for 72 hours and pictures of cell aggregates were taken. The mean top view aggregate area (TVAA) was evaluated by measuring the TVAA of all cell aggregates found in a drop by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences are indicated as follows: unpaired t-test, **p<0.01. (B-D) 1x10^4 FGFR4/Arg5 or FGFR4/Gly7 cells, stably overexpressing FGFR4, were seeded in 96-well plates on polystyrene (B), collagen (C) and fibronectin (D). At timepoints indicated, medium was discarded and cells were washed twice with PBS to remove non attached cells. The number of adherent cells was determined by MTT assay. Results are shown as fold untreated control. Data are given as mean ± SD of four independent experiments. Significant differences to the empty vector control are indicated as follows: unpaired t-test, *p<0.05, **p<0.01.*

Spheroid dependent gap formation revealed a significant increase in gap areas with HCC-1.2/Arg5 cells both on blood (1.5 fold) and lymphatic (1.1 fold) endothelial cells. In contrast, HCC-1.2/Gly7 cells did not show any effect on endothelial cell retraction (**Figure 28**).

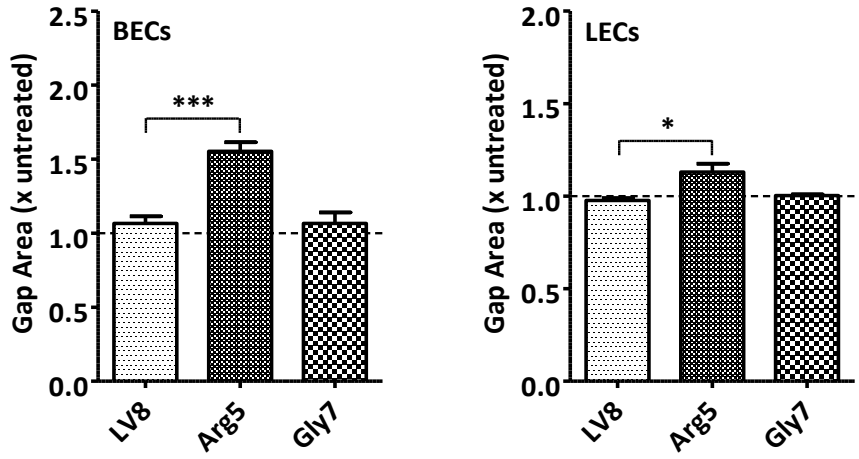


Figure 28: Impact of stable FGFR4 overexpression on gap formation. *$3x10^3$ cells were incubated in 96-well plates with round bottom and medium containing 20% methylcellulose for 48 hours. Spheroids of HCC-1.2 cells stably overexpressing FGFR4 were placed on blood (BEC) and lymphatic (LEC) endothelial cell monolayers and incubated for 2-5 hours. Pictures of gap areas were taken and areas were measured by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences are indicated as follows: unpaired t-test, *p<0.05, ***p<0.001.*

5.2.6. Impact of blocking endogenous FGFR4 signaling on mRNA levels

Blockade of FGFR4 function was achieved by transient infection of CCL-13 and HCC-1.2 with two different adenoviral constructs, referred as solFGFR4 and kdFGFR4. Both forms inhibit FGFR4-mediated signaling in a dominant negative way. SolFGFR4 is a truncated and secreted isoform of FGFR4 interfering with receptor-ligand interactions (Ezzat, 2001), whereas kdFGFR4 harbours a kinase-dead form of FGFR4, in which the kinase domain is substituted by CFP leading to the formation of non-functional dimers. A viral construct expressing the antisense mRNA of COX2 served as negative control. Infection efficiency with adenoviruses was tested by the use of a GFP-encoding constructs.

As illustrated in **Figure 29**, nearly all cells evaluated showed a GFP signal 48h after infection pointing to a very high efficiency of infection. Further evaluation of FGFR4 knock-down was done by investigating the expression of the constructs by qRT-PCR. CCL-13 cells showed an approximately 40 fold overexpression of kdFGFR4 when compared to the negative control (**Figure 30**). Similarly, kdFGFR4 was overexpressed in infected HCC-1.2 cells. SolFGFR4 expression was not detected by qRT-PCR as the primers used for the gene expression assay bind to a region of the receptor, which is deleted in this form.

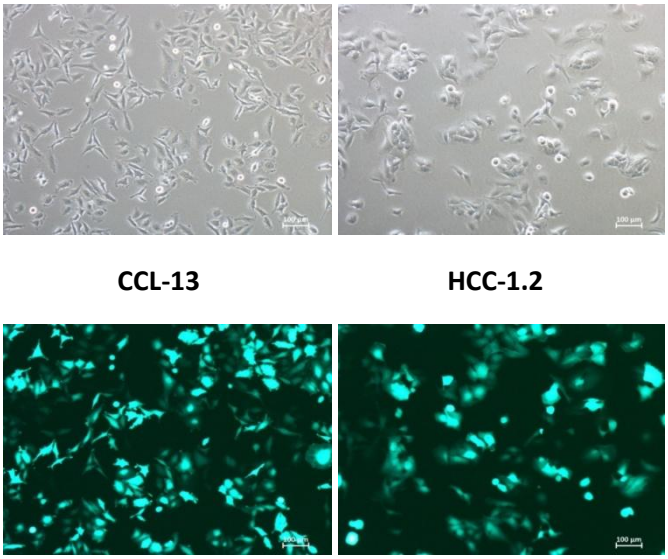


Figure 29: Infection efficiency of HCC model cell lines. *CCL-13 and HCC-1.2 cells were transiently infected with a construct coding for GFP. For evaluation of infection efficiency, GFP positive cells were compared to total cells 48 hours after infection.*

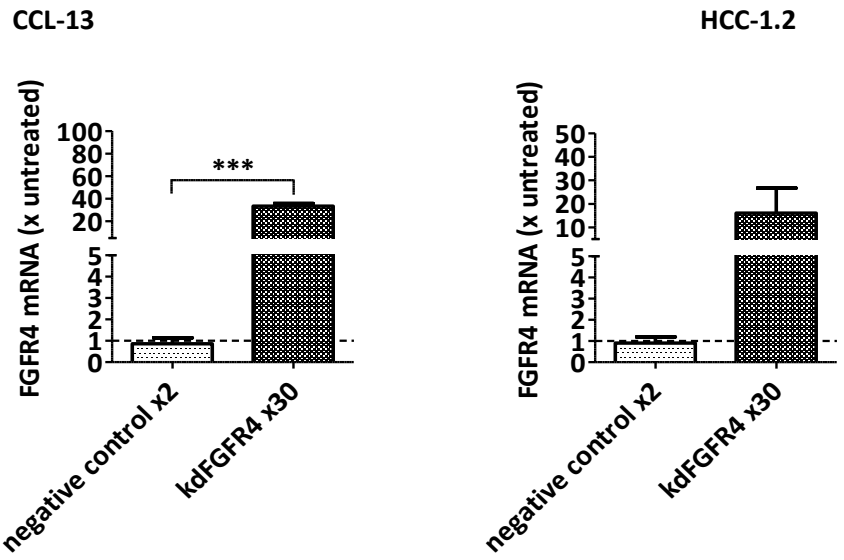


Figure 30: Impact of blocking endogenous FGFR4 signaling on mRNA levels. *CCL-13 and HCC-1.2 cells were transiently infected with a COX2 antisense construct (negative control) or a dominant negative form of FGFR4 (kdFGFR4). RNA was harvested 48 hours after infection and FGFR4 expression was analyzed via qRT-PCR, using β2 microglobulin as housekeeping gene. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences are indicated as follows: unpaired t-test, ***p<0.001.*

5.2.7. Impact of blocking endogenous FGFR4 signaling on cell-cell and cell-matrix adhesion

As experiments with transient and stable overexpression of FGFR4 revealed a tendency towards enhanced cell aggregation and decreased cell-matrix adhesion, results were expected to be reversed by transient knock-down of the receptor.

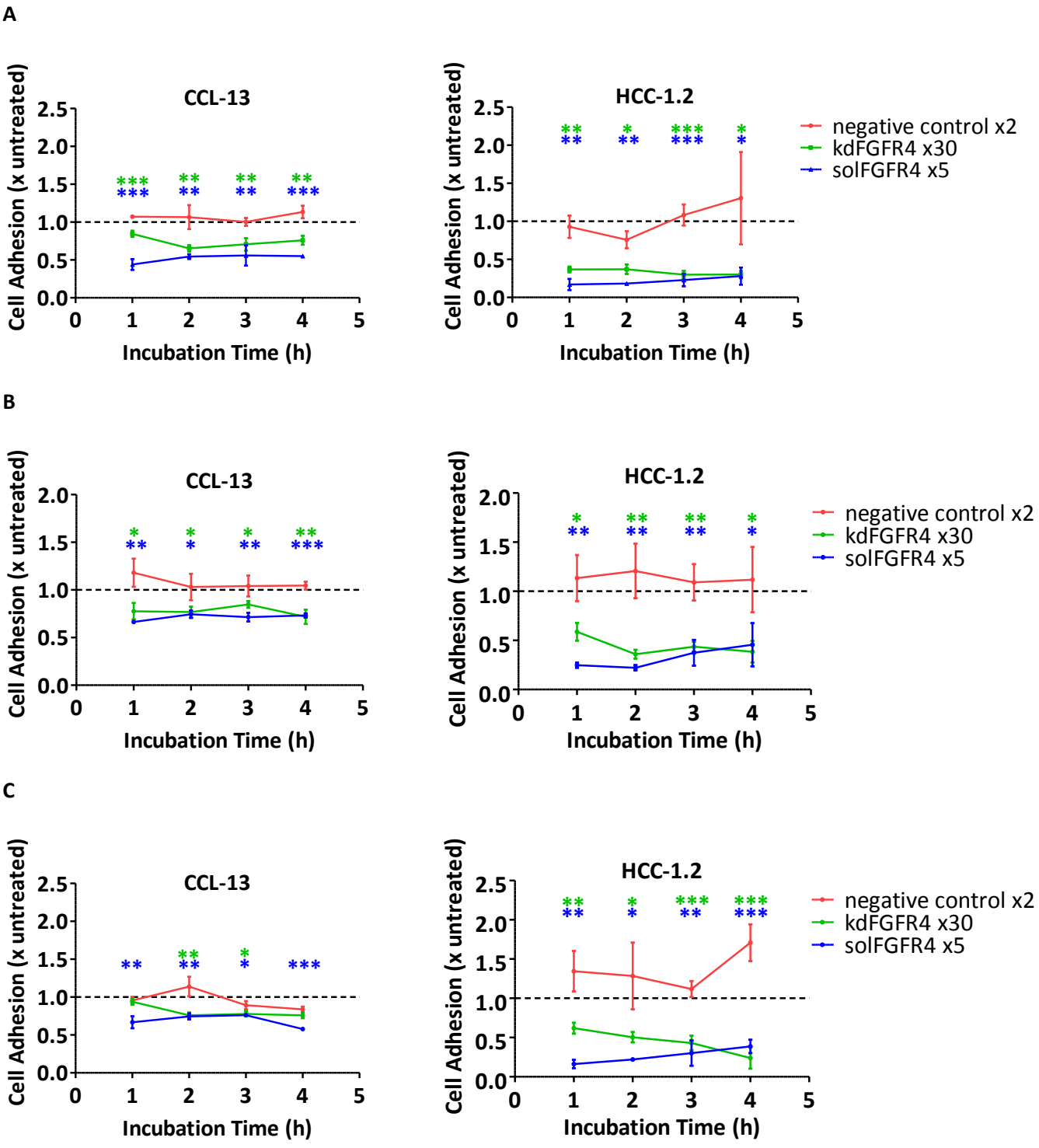


Figure 31: Impact of blocking endogenous FGFR4 signaling on cell-matrix adhesion. *CCL-13 and HCC-1.2 cells were transiently infected with a COX2 antisense construct (negative control) or dominant negative forms of FGFR4 (kdFGFR4, solFGFR4). 48 hours after infection, 1x10^4 cells were seeded in 96-well plates on polystyrene (A), collagen (B) and fibronectin (C). At timepoints indicated, medium was discarded and cells were washed twice with PBS to remove non attached cells. The number of adherent cells was determined by MTT assay. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences to the negative control are indicated as follows: unpaired t-test, *p<0.05, **p<0.01, ***p<0.001.*

Cell-matrix adhesion of CCL-13 cells infected either with kdFGFR4 or solFGFR4 was significantly decreased on polystyrene, collagen and fibronectin surfaces (**Figure 31**). HCC-1.2 cells infected with the adenoviral constructs also exhibited a decreased cell-matrix interaction on all surfaces used in the experiments. Overall, in solFGFR4 infected cells cell adhesion seemed to be more affected than in cells infected with kdFGFR4.

As shown in **Figure 32**, the kinase-dead form of FGFR4 (kdFGFR4) significantly decreased cell aggregation abilities of CCL-13 cells by about 80%. SolFGFR4 infected cells showed a nearly complete inhibition of cell-cell interactions when compared to the negative control. In HCC-1.2 infected with kdFGFR4 adenoviruses, the mean aggregate area was reduced about 30%, whereas infection with solFGFR4 had no effect on cell aggregation.

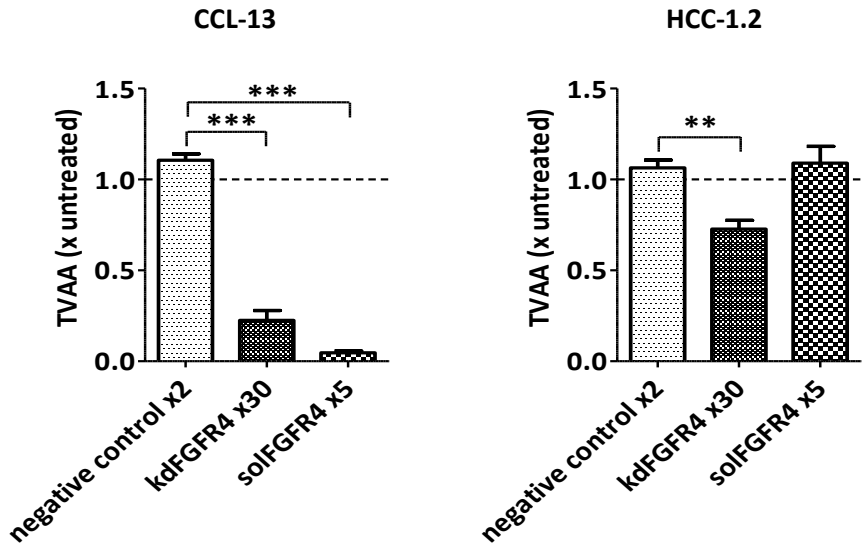


Figure 32: Impact of blocking endogenous FGFR4 signaling on cell aggregation. *CCL-13 and HCC-1.2 cells were transiently infected with a COX2 antisense construct (negative control) or dominant negative forms of FGFR4 (dnFGFR4, solFGFR4). 24 hours after infection, 1x10^4 cells were suspended in a volume of 20 µl. A drop of 20 µl was placed on the inner site of the lid of a cell culture plate. The lid was then placed on the culture plate, which generated a hanging drop. Hanging drops were incubated for 72 hours and pictures of cell aggregates were taken. The mean aggregate area was evaluated by measuring the size of all cell aggregates found in a drop by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of four independent experiments. Significant differences are indicated as follows: unpaired t-test, *p<0.05, ***p<0.001.*

5.2.8. Impact of blocking endogenous FGFR4 signaling on gap formation

The influence of blockade of endogenous FGFR4 signaling on gap formation in endothelial cell monolayers was studied. For that, CCL-13 and HCC-1.2 cells expressing dominant negative forms of the receptor were used. Spheroids expressing the COX2 antisense construct revealed gaps in a similar

range as the untreated control (**Figure 33**). Compared to the negative control, CCL-13 spheroids infected with dnFGFR4 showed a slightly reduced gap area in BEC monolayers. On LECs, kdFGFR4 expressing CCL-13 cells only marginally reduced gap formation. Expression of solFGFR4 had no effect on gap formation in BEC or LEC monolayers. Spheroid-induced gap formation of HCC-1.2 cells expressing kdFGFR4 was significantly decreased to about 50% on BECs and about 75% on LECs when compared to COX2 AS infected cells. HCC-1.2 cells infected with solFGFR4 tended to reduce gap areas only in BECs but not in LECs.

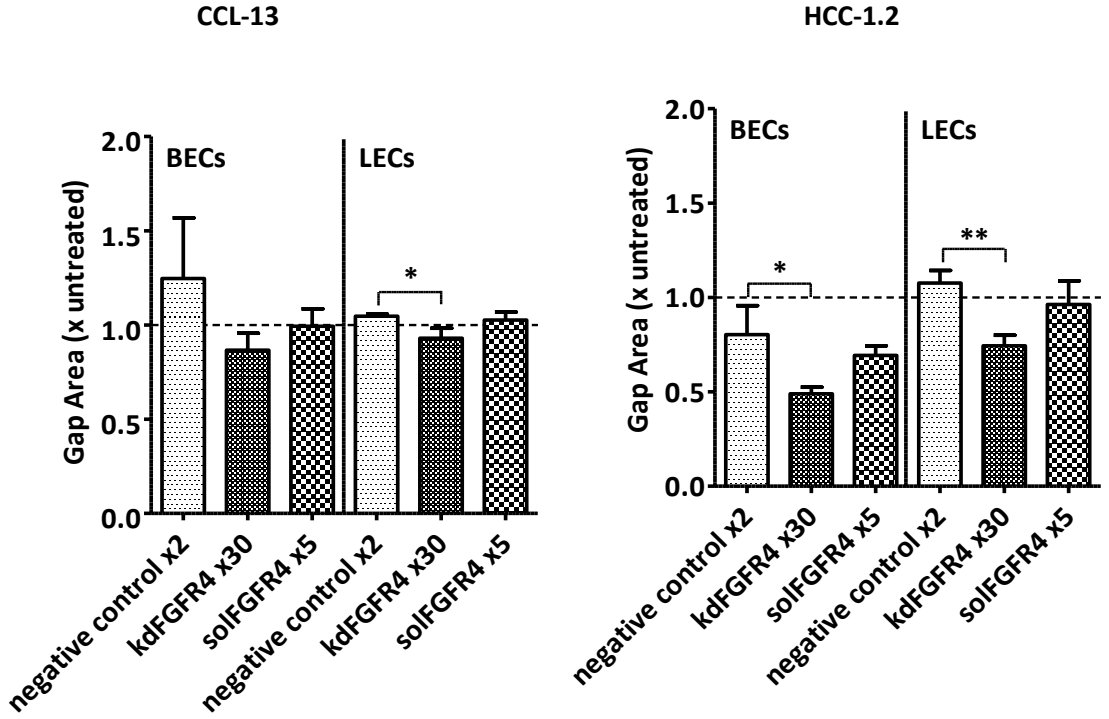


Figure 33: Impact of blocking endogenous FGFR4 signaling on gap formation. *CCL-13 and HCC-1.2 cells were transiently infected with a COX2 antisense construct (negative control) or dominant negative forms of FGFR4 (kdFGFR4, solFGFR4). 24 hours after infection, $3x10^3$ cells were incubated in 96-well plates with round bottom and medium containing 20% methylcellulose for 48 hours. Spheroids were placed on a blood (BECs) or lymphatic (LECs) endothelial cell monolayer and incubated for 2-5 hours. Pictures of gap areas underneath were taken and areas were measured by ImageJ software. Results are shown as fold untreated control. Data are given as mean ± SD of three independent experiments. Significant differences are indicated as follows: unpaired t-test, *p<0.05, **p<0.01.*

6. Discussion

Progression of cancer is a stepwise process from a normal cell to a malignant cell involving the deregulation of various growth factor signalling pathways. Amongst others, the FGF/FGFR signalling system has been implicated in the pathogenesis of many cancer entities as a result of inappropriate expression of genetic alterations (Ahmad, 2012). The motivation for investigating the role of FGFR4 in human diseases relies on several recent findings. First, FGFR4 belongs to the FGFR family which is crucial for tumour invasiveness and angiogenesis (Eswarakumar, 2005; Wang, 2004). Second, FGFR4 transcript expression was detected to be significantly upregulated in human HCC (Ho, 2009; Gauglhofer, 2010), which potentially links the receptor to progression of hepatocytes to a malignant state.

Metastasis is described as a major cause for human mortality given that tumorigenesis is associated with metastasis in 90% of all cancer deaths (Sporn, 1996). During the last decades, the malignant progression of tumour has been intensively investigated, as the prevention of the formation of secondary tumours is a prior goal for cancer therapy. In particular, the acquisition of migratory and invasive capabilities as well as connection of the tumour to the vasculature has been identified as prerequisite for cells to form metastases at distal locations (Gotzmann, 2004).

FGFR4 has been recently associated with deregulation of adhesion and invasion, which are crucial steps in tumour metastasis (Johnston, 1995; Bange, 2002; Sugiyama, 2010/1; Sugiyama, 2010/2). Moreover, elevated FGFR4 expression was shown to promote colony formation, anchorage-independent growth, tumorigenicity of hepatocarcinoma cells and to decrease cell-matrix adhesion (Gauglhofer, 2010; Naegelen, 2010; Schrottmaier, 2012). The identification and characterization of a single nucleotide polymorphism in many cancer patients even more points to a role of FGFR4 in progression and invasion. Accordingly, the FGFR4-Arg^{388} allele increased cell motility and ECM degradation (Bange, 2002; Sugiyama, 2010/1; Sugiyama, 2010/2; Stadler, 2006). In this regard, the present study was aimed to get further insight in the importance of FGFR4 for tumour progression and pro-metastatic events in HCC.

HCC model cell lines show differences in cell-matrix adhesion and cell aggregation

The progression of a tumour to a metastatic stage is fatal in most cancer entities. Metastasis is a complex, multi-step process in which migratory and adhesive capabilities of cancer cells are crucial for invasion into adjacent tissues or vessels, cells have to migrate for which they have to disintegrate their tight cell-matrix and cell-cell adhesion (Spano, 2012).

Cell-cell interactions were analyzed by means of the hanging drop assay, which was developed on the basis of a method used for the production of embryoid bodies. Generally, we were confronted with several problems in the evaluation of the assay. We decided to measure the top view areas of all aggregates formed in a single hanging drop. Further we assumed that the aggregate area is directly proportional to the ability of the cells to establish cell-cell interactions.

Three HCC model cell lines were investigated with regard to their cell aggregation capabilities. Thereby, HepG2 cells showed the highest ability to form cell aggregates, followed by HCC-1.2 and CCL-13 cells. Results agree with the observation that HepG2 cells often from three-dimensional foci due to their strong intercellular adhesion. On the opposite site, CCL-13 and HCC-1.2 cells grow into epithelial monolayers pointing to a less pronounced disposition for the formation of cell aggregates. Cell-matrix adhesion also differed between the HCC cell lines, i.e. CCL-13 cells attached faster to a polystyrene surface than any other cell line investigated. HCC-1.2 and HepG2 cells behaved very similar with regard to their adhesive capabilities.

BAY 11-7082, GM6001 and Baicalein inhibit gap forming capabilities of HCC model cell lines

The process of intravasation into the blood or lymphatic vasculature through direct interaction of the tumour cells with vascular endothelial cells is an early event in the metastatic process (Kramer, 1979). Hence, the ability tumour/endothelial cell interaction were investigated using a 3D co-culture system, which mimics the breakthrough of tumour cells into the blood or lymphatic vasculature. Basically, all cell lines investigated were able to form gaps in endothelial cell monolayers. Spheroid-induced gap formation was lowest with HCC-1.2 cells pointing to a less aggressive phenotype of the cell line concomitant with the findings of Gauglhofer et al. that HCC-1.2 cells are non-tumorigenic in vivo. In contrast, CCL-13 and HepG2 cells showed rapidly growing tumours in SCID mice and also enhanced endothelial cell retraction in gap assay experiments.

NF-κB activation was shown to play direct or indirect roles in cell proliferation, survival, migration, tumorigenesis and metastasis in most of the major forms of human cancers (Brown, 2008). Inhibition of the NF-κB cascade has already been shown to reduce tumour growth and to induce apoptosis in a variety of carcinomas such as head and neck, prostate, pancreatic and gastric carcinomas (Williams, 2003; Adachi, 2004; Lun, 2005; Nawrocki, 2006). In Caov-3 ovarian cancer models, irreversible inhibition of I-κBα phosphorylation with BAY 11-7082 (Pierce, 1997) was demonstrated to block NF-κB activation leading to anti-tumour effects in vivo and in vitro (Mabuchi, 2004). Additionally, inhibition of NF-κB translocation with BAY 11-7082 reduced gap forming capacity of MCF-7 spheroids on lymphatic endothelial cells in a dose-dependent manner (Vonach, 2011). Accordingly, Flister et al.

suggested that activation of NF-κB is responsible for lymphatic endothelial cell activation hence NF-κB activity may be necessary for endothelial cell motility required for cancer cell intravasation (Flister, 2010). Although NF-κB often is involved in tumour promotion, experimental evidence suggests also an inhibitory role of this factor in early carcinoma development (Dajee, 2003; Sakurai, 2006; Luedde, 2007). Here, treatment of HCC spheroids with BAY 11-7082 inhibited gap formation of BECs and LECs in a dose-dependent manner and at low concentrations.

Matrix metalloproteinases (MMPs) have been clearly connected to the proteolytic degradation of ECM components and cancer cell invasion by modulation of cell-cell and cell-ECM interactions, therefore assisting tumour cells during metastasis (Deryugina, 2006). Various studies report a blockade of tumour growth and local invasion through extracellular matrices by natural or synthetic inhibition of MMPs (Conway, 1996; Baker, 2002). Kerjaschki et al. also noted a decrease in gap formation with MCF-7 spheroids by inhibition of metalloproteinases with GM6001 (Kerjaschki, 2011), which was suggested to be a result of degradation of the VE-cadherin meshwork at inter-endothelial junctions and matrix attachment (Baker, 2002; Deryugina, 2006). In this study, inhibition of metalloproteinases by the pan-matrix inhibitor GM6001 revealed a contribution of metalloproteinases to tumour invasion and metastasis in HCC, since CCL-13 and HCC-1.2 spheroid-induced gap formation of BECs and LECs was significantly reduced.

Overexpression of 12-LOX resulted in enhanced expression of integrins in various tumour cell lines (Tang, 1993; Pidgeon, 2003) implicating a general role of 12-LOX in modulation of adhesion and survival by regulating a subset of integrins. In the opposite, Bhattacharya et al. described anti-carcinogenic effects of 15-LOX in colorectal cancer (Bhattacharya, 2009).12(S) hydroxyeicosatetraenoic acid (HETE), which is produced by lipoxygenases (12- and 15-LOX) was identified to increase endothelial cell motility and retraction of human umbilical cord endothelial cells (Honn, 1994). In MCF-7 tumour spheroids, 12(S)-HETE was identified as one of the major factors in the process of endothelial cell retraction as inhibition of the enzymatic activity of 15-LOX with the LOX inhibitor baicalein reduced spheroid-induced gap formation on lymphatic endothelial cell monolayers (Kerjaschki, 2011). Fitting to the observations of Kerjaschki et al., treatment of HCC spheroids with baicalein significantly inhibited gap formation on blood and lymphatic endothelial cells in a dose-dependent manner. Taken together, these findings suggest a role for 15-LOX and its product 12(S)-HETE in the interaction of tumour cells with vascular endothelial cells. Thus, induction of endothelial cell migration and modulation of inter-endothelial adhesion may contribute to focal disruption of the vasculature necessary for tumour invasiveness (Kerjaschki, 2011).

Bile Acids have no significant effect on gap formation

Bile acids are endogenous molecules which have been involved in regulation of cholesterol homeostasis, lipid dissolubility and metabolic signalling (Trauner, 2010; Baptissart, 2012). Since bile acids also have been implicated in tumorigenesis, a number of studies report the different effects of BAs on normal and neoplastic growth of epithelial cells (Cook, 1940; Bernstein, 2005; Nagengast, 1995; Zimber, 2000). Tsuda et al. indicated that elevated bile acid levels might act as promoters of hepatocarcinogenesis in rats (Tsuda, 1984). However, the impact of BAs is still controversial as both beneficial and undesirable effects have been described but most often they were associated with enhanced invasiveness and metastasis (Nagengast, 1995; Zimber, 2000).
It was of great interest to investigate whether bile acids influence the ability of HCC cell to interact with endothelial cell as this might point to stimulating or preventive effects of BAs in the metastatic process of hepatocarcinogenesis.

Recently, it has been found that CA stimulates the invasion of human colorectal cancer cells into collagen type I gels (Debruyne, 2002). Here, some effects of CA treatment on the ability of spheroids to form gaps were only seen with CCL-13 spheroids on LECs and HCC-1.2 spheroids on BECs. Both times, CA treatment slightly increased gap formation with increasing concentrations on the BA.
In 1984, Salmon et al. published that TCA significantly promoted gastric carcinogenesis leading to the development of invasive adenocarcinomas or angiosarcomas (Salmon, 1984). In this study, TCA treatment of HCC-1.2 spheroids increased the gap forming capability on BECs.

UDCA appears to stimulate bile flow, render the bile composition less toxic and reduce the retention of potentially toxic bile acids in hepatocytes and liver injury (Flickert, 2013) Moreover, UDCA has been reported to reduce invasive tumours in rat models of colon carcinogenesis (Earnest, 1994). Oyama et al. reported reduced hepatocarcinogenesis in rats through inhibition of proliferation and induction of apoptosis upon UDCA treatment (Oyama, 2002). Trauner et al. showed that norUDCA, which is a side chain-shortened homologue of UDCA, improves fatty liver in Western diet-fed ApoE(-/-) mice, pointing to an beneficial effect of norUDCA in fatty liver diseases (Trauner, 2010). Treatment of HCC cell spheroids with norUDCA produced variable results. Gap forming capabilities of CCL-13 were slightly enhanced on LECs, while HCC-1.2 spheroids showed increased gap formation on BECs. Still, results were not significant. Nevertheless, norUDCA might enhance the ability of HCC cells to interact with endothelial cells.

Concluding, all results presented were not significant, hence it remains questionable whether BAs influence pro-metastatic processes in hepatocarcinogenesis and the behaviour of HCC cells.

FGFR4 overexpression reduces cell-matrix adhesion

Receptor tyrosine kinases (RTKs) are known to interact with cell adhesion molecules, including cadherins, cell adhesion molecules of the immunoglobulin family (Ig-CAMs), CD44 and integrins. Thus, they are possibly involved in modulating lateral cell-cell adhesions and ECM interactions (Cavallaro, 2004). Deregulation therefore may cause pathogenic effects in a number of diseases, including cancer (Cavallaro, 2004). Studies about the role of FGFR4 in adhesion and migration are controversial. Some researchers related activation of FGFR4 to an increase in cell migration and α-fetoprotein (AFP) production and thus to a worse prognosis (Ho, 2009). Motoda et al. support the suppressive role of FGFR4 in pancreatic ductal adenocarcinoma cells as they found inhibition of cell migration and invasion by stimulation of the receptor *in vitro* (Motoda, 2011). Moreover, the presence of the R^{388}-polymorphism was shown to increase the motility of MDA-MB-231 mammary tumour cells whereas FGFR-G388 suppresses invasion and motility of breast cancer cells pointing to a different impact of the allele variants in the metastatic process (Bange, 2002; Stadler, 2006).

In previous studies, stable overexpression of FGFR4 was found to cause reduced adhesion to polystyrene and collagen (Naegelen, 2010). Otherwise, transient overexpression of FGFR4 and knockdown of the receptor did not seem to affect cell-matrix adhesion (Schrottmaier, 2012). In agreement, data published by Ezzat et al. demonstrated that NIH-3T3 fibroblasts and GH4 pituitary cells overexpressing FGFR4 showed no significant loss of cell adhesion to fibronectin and collagen IV extracellular matrices (Ezzat, 2004). Here, the impact of FGFR4 on cell-matrix adhesion was investigated with a modified version of the assay used before. Transient overexpression of FGFR4 had no effect on cell-matrix adhesion of CCL-13 cells. In contrast, HCC-1.2 cells displayed a decreased adhesion on collagen and fibronectin surfaces, whereby FGFR4-R388 showed an even more pronounced effect than the wildtype. In accordance with previous results, adhesive capacities of HCC-1.2/FGFR4 clones were diminished, but here, adherence of FGFR4/Arg5 clones to collagen and fibronectin was even more decreased than of FGFR4/Gly7 clones. Unexpectedly, also adenoviral blockade of FGFR4 signaling significantly reduced cell-matrix adhesion of both cell lines on polystyrene, collagen and fibronectin compared to the negative control. Regardless, results are not conflicting, since blockade of FGFR4 signalling clearly increases apoptosis rates (Naegelen, 2010).

FGFR4 might increase cell aggregation

In the course of carcinoma progression, epithelial cells become invasive through the process of epithelial-to-mesenchymal transition (EMT) resulting in loss of cell polarization, dissociation of intercellular interactions and gain of an invasive protein expression pattern (Yilmaz, 2009). This phenotypical conversion of cells has been described in various cancer entities including HCC

(Gotzmann, 2004; Thiery, 2006; Polyak, 2009). In vitro, epithelial cells, including hepatocytes, undergo EMT triggered by the treatment with TGF-β, resulting in the switch to a mesenchymal phenotype which is more aggressive when injected into animals (Huber, 2005; Gotzmann, 2006). Otherwise, it has been reported that metastasis in inoculated tumour could be increased in the absence of a demonstrable EMT (Biname, 2008). Moreover, those epithelial tumours are able to migrate collectively and intravasate into blood vessels as sheets or cohorts (Friedl, 2004; Wicki, 2006). Recently, a MT-1-MMP/FGFR4 complex was observed to be expressed in the leading cells of collectively invading tumour fronts leading to the assumption that the function of FGFR4 in tumour cell invasion is dependent on the levels of MT1-MMP induction at the invasive tumour edges (Sugiyama, 2010/1). During collective invasion, FGFR4-R388 expressing cells sustain cell-cell adhesion and promote tumour expansion via MT1-MMP (Rowe, 2009). Otherwise, FGFR4-R388 expression abolishes cell-cell interactions during single cell invasion (Rowe, 2009).

In this study, cell aggregation, representing the ability for intercellular interactions, tended to be increased only in HCC-1.2/Arg5 clones. Transient overexpression of FGFR4 seemed to have no impact on cell aggregation abilities of HCC cell lines. Blockade of FGFR4 signaling appeared to massively decrease intercellular adhesion of CCL-13 cells, whereas cell aggregation of HCC-1.2 cells only seemed to be decreased by infection with the kinase-dead version of FGFR4 but not with the soluble form and the effect was far less profound than in CCL-13 cells. In the context of describing the impact of FGFR4 on intercellular adhesion capabilities, Bange et al. observed that cells expressing FGFR4-R388 appeared to be more individual, whereas cells expressing FGFR4-G388 grew tightly connected to each other (Bange, 2002). Additionally, HCC-1.2/Arg clones were described to be more aggregated on the surface of cell culture dishes compared to vector controls or HCC-1.2/Gly clones (Gauglhofer, 2010) but it has to be considered, that this may be independent of the receptor's overexpression as it could be a result of the altered morphology gained in the course of the establishment of the clones. Taken together, results are not very meaningful, since the effects detected cannot be clearly accredited to an altered FGFR4 expression. However, the receptor may contribute to the maintenance of intercellular interactions, which would support the model of collective invasion during the metastatic process.

FGFR4 affects gap forming capacity of HCC model cell lines

To metastasize, tumour cells have to escape the primary tumour, get access to the blood and/or lymphatic vessels, adhere to the endothelium of capillaries at the target side and escape the vasculature to proliferate at the secondary site (Spano, 2012). In general, it is suggested that cancer cells actively invade into the vasculature, but recent studies indicate an invasion-independent

pathway for HCC where tumour nests are surrounded by blood vessels and enter the circulation as endothelial-coated tumour emboli (Sugino, 2004). Thereby, tumour cells may be the initiating force in tumour metastasis, but surrounding cells, especially endothelial cell, are important in facilitating tumour growth and spread (Sugino, 2008). To gain insight into the mechanisms underlying invasion of HCC cells into the vasculature and information about the influence of FGFR4 in this process, an in vitro assay was performed that mimics features of the in vivo situation. Tumour cell spheroids correlate to invasive tumour aggregates, and to imitate intra-metastatic vessels, endothelial monolayers were used. Although current evidence presents blood vessels as the preferred entrance into the vasculature in HCC metastasis, gap forming capacity of HCC model cell lines with altered FGFR4 expression were tested with both, blood and lymphatic endothelial cells (Mitsunobu, 1996).

Generally, HCC cells tended to show pronounced effects on blood endothelial cell, which is in agreement with the observation that HCC cells metastasize preferentially through blood vessels (Mitsunobu, 1996). Sugiyama et al. showed a decrease of vascular and lymph node invasion after FGFR4-R388 silencing in vivo whereas silencing of the wildtype form of FGFR4 provoked increased vascular invasion (Sugiyama, 2010/2). As a result, FGFR4 appears to be involved in tumour cell intravasation into the vasculature and, second, the FGFR4 G388R single nucleotide polymorphism may have different impact on the aggressiveness of the metastatic process (Sugiyama, 2010/2). Here, transient overexpression of both versions of FGFR4 equally enhanced spheroid induced gap formation on blood and lymphatic endothelial cells. With FGFR4/Arg5 clones gap areas were detected to be increased whereas overexpression of the wildtype form of FGFR4 did not have any effect. Infection of cells with a kinase-dead version but not with the soluble form of FGFR4 significantly reduced gap areas on both endothelial cell lines.

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8. Abbreviations

AFB1	aflatoxin B1
APS	ammonium persulfate
BCLC	Barcelona Clinic Liver Cancer
BSA	bovine serum albumin
CAM	cell adhesion molecule
cDNA	complementary DNA
CFP	cyan fluorescent protein
CHD	CAM homology domain
cRNA	complementary RNA
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFRL1	FGFR like 1

FRS2-α	FGFR substrate 2 alpha
GFP	green fluorescent protein
GRB2	growth factor receptor bound protein 2
GAB1	GRB2 associated binding protein 1
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HRP	horseradish peroxidase
HSPG	heparan sulphate proteoglycans
Ig	immunoglobulin
IGF-1	insulin like growth factor 1
IP_3	inositol-triphosphate
kdFGFR4	kinase-dead form of FGFR4
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MKP1	MAPK phosphatase 1 / 3
MMLV	Moloney Murine Leukemia Virus
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
LOH	loss of heterozygosity
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCAM	neural cell adhesion molecule
P	phosphorylation
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PEG	polyethylene glycol
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLCγ	phospholipase Cγ
Rb	retinoblastoma
RPMI	Roswell Park Memorial Institute

RTKs	receptor tyrosine kinases
SDS	sodium dodecyl sulfate
SEF	similar expression to fibroblast growth factor
SH2	Src homology 2
SH3	Src homology 3
SNP	single nucleotide polymorphism
solFGFR4	soluble form of FGFR4
SOS	son of sevenless
SPRY	sprouty
STAT	signal transducer and activator of transcription
TACE	transaterial chemoembolization
TEMED	tetramethylethylenediamine
TERT	telomerase reverse transcriptase
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VSV	vesicular stomatitis virus

9. Figures and tables

9.1. List of figures

9.2. List of tables

10. Curriculum vitae

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Fibroblast growth factor receptor 4: A putative key determinant for the aggressive phenotype of hepatocellular carcinoma. Christine Gauglhofer, Waltraud Schrottmaier, Bettina Wingelhofer, Daniela Huber, Jakob Paur, Isabelle Naegelen, Christine Pirker, Christine Heinzle, Klaus Holzmann, Brigitte Marian, Rolf Schulte-Hermann, Walter Berger, Georg Krupitza, Michael Grusch, Bettina Grasl-Kraupp. *Manuskript in Vorbereitung.*

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Poster: *Impact of FGFR4 Gly388Arg single nucleotide polymorphism on malignancy in hepatocarcinogenesis.* Schrottmaier W.*, Wingelhofer B.*, Gauglhofer C., Naegelen I. and Grasl-Kraupp B. *contributed equally

vFWF-Universitätsvorlesung 2011 (2. Dez. 2011)

Poster: *FGFR4-G388R polymorphism affects clonogenic growth, anchorage-independency, cell-cell and cell-matrix adhesion in hepatocarcinoma model cell lines*. Wingelhofer B.*, Schrottmaier W.*, Gauglhofer C., Naegelen I., Marian B., Heinzle C., Grusch M., Berger W., Holzmann K. and Grasl-Kraupp B. *contributed equally