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Table of content

1 Abstract

Epithelial ovarian cancer is a rapidly progressive, highly lethal disease with low prevalence. It is responsible for 20% of all cancer-related deaths in the United States, since few patients are diagnosed early and subsequently cured. Over the last decades, there has been poor therapeutic improvement besides cytotoxic chemotherapy and oophorectomy, but recent clinical trials with inhibitors of receptor tyrosine kinases have been very promising. Fibroblast growth factor receptors (FGFR) are involved in malignant transformation, angiogenesis and chemoresistance. They have been identified as valuable targets for cancer therapy and inhibition of FGFR-dependent signaling was able to overcome resistance to standard therapies, but the underlying molecular fundamentals have not been evaluated yet. The aim of this study was to characterize the FGF signaling system and its influence on malignancy-related cell properties in epithelial ovarian cancer cells.

RT-PCR analysis of FGF receptors and ligands revealed, that de novo expression of fibroblast growth factors and their receptors leading to autocrine signaling loops with a strong mitogenic potential is a very common event in epithelial ovarian cancers (70%). As evaluated by immunoblotting, the FGF signaling system in these cells is functional and conditioned growth medium was able to induce phosphorylation of ERK1/2 by different degrees. Proliferation assays showed a significant increase in 50% of evaluated ovarian cancer cells when treated with recombinant FGF-2. FGF-1 induced migration in 66% of the cells in a non-significant manner. The dependency of ovarian cancer cells on FGF receptor signaling was evaluated by growth inhibition assays using two different small molecule inhibitors – PD173074, a specific inhibitor of FGF receptors and Dovitinib (CHIR-258), a more promiscuous inhibitor of receptor tyrosine kinases. A-2780 cells were found to be extremely sensitive to FGF inhibition, while HEY and SKOV-3 cells showed moderate sensitivity.

OVCAR-3 cells were figured out as resistant to inhibition of class III, IV and V receptor tyrosine kinases.

Together, these data suggest that FGF receptor inhibitors have a good potential in treatment of ovarian cancers alone and possibly even more in combination with cytotoxic agents. However, the variance in response to the evaluated inhibitors underlines the necessity for reliable serum markers for the improvement of therapeutic strategies against epithelial ovarian cancer.

2 Zusammenfassung

Das Ovarialkarzinom ist eine sich rasch entwickelnde, tödliche, aber seltene Erkrankung. Trotzdem ist sie für 20% aller mit Krebs im Zusammenhang stehenden Todesfälle in den Vereinigten Staaten verantwortlich, da die Krankheit in den meisten Fällen spät diagnostiziert wird. In den letzten Jahrzehnten gab es nur wenig Verbesserung in den therapeutischen Strategien neben der zytotoxischen Therapie und Ovariektomie, aber neue Ergebnisse aus klinischen Studien mit niedermolekularen Inhibitoren der Familie der Rezeptortyrosinkinasen sind sehr vielversprechend. Die Fibroblastenwachstumsfaktoren (FGFs) und ihre Rezeptoren sind involviert in maligne Transformation, Angiogenese und Chemoresistenz und stellen vielversprechende Zielstrukturen in der Krebstherapie dar, insbesondere für die Überwindung von Chemoresistenz gegen Zytostatika. Die zugrunde liegenden molekularen Mechanismen wurden allerdings noch nicht erforscht. Das Ziel dieser Arbeit war die Charakterisierung des Signalsystems der Fibroblastenwachstumsfaktoren und sein Einfluss auf Zelleigenschaften im Ovarialkarzinom die mit Malignität im Zusammenhang stehen.

Die Analyse des FGF Rezeptors und seiner Liganden mit RT-PCR ergab, dass die de novo-Expression von FGFs sowie von FGF Rezeptoren im Ovarialkarzinom häufig zu finden ist (70%) und zu autokrinen Signalschleifen mit hohem mitogenen Potential führt. Wie durch Immunoblotting gezeigt wurde, ist das Signalsystem in diesen Zellen funktionell, und konditioniertes Wachstumsmedium war unterschiedlich stark in der Lage, die Phosphorylierung von ERK1/2 zu induzieren. Wachstumsassays zeigten eine signifikante Steigerung der Proliferation bei der Behandlung mit FGF-2 in 50% der untersuchten Zelllinien. FGF-1 zeigte eine nicht-signifikante Steigerung der Motilität in 66% dieser Zellen. Die Abhängigkeit von Ovarialkarzinomzellen von Signalen der FGF Rezeptoren wurde durch Wachstumsinhibierungsassays untersucht. Dabei wurden zwei

unterschiedliche, niedermolekulare Inhibitoren verwendet – PD173074, ein spezifischer Inhibitor der FGF Rezeptorfamilie und Dovitinib (CHIR-258), ein Inhibitor mit geringerer Spezifität für einzelne Rezeptoren aber mit höherer Bandbreite. A-2780 Zellen zeigten eine extrem hohe Sensitivität gegen FGFR-Inhibierung, während HEY und SKOV-3 Zellen eine moderate Sensitivität aufwiesen. OVCAR-3 Zellen zeigten eine hohe Resistenz gegenüber der Inhibierung von Rezeptortyrosinkinasen der Klassen III, IV und V.

Zusammen zeigen diese Daten, dass Inhibitoren der FGF Rezeptoren ein gutes Potential in der Behandlung des Ovarialkarzinoms haben, möglicherweise aber noch mehr in Kombination mit Zytostatika. Allerdings unterstreicht die starke Varianz in der Reaktion der unterschiedlichen Zellen auf die FGFR-Inhibierung die Notwendigkeit zuverlässiger Serummarker um die therapeutischen Strategien für die Behandlung des Ovarialkarzinoms zu verbessern.

3 Introduction

3.1 Ovarian cancer

Ovarian cancer is a heterogeneous, rapidly progressive and highly lethal disease of low prevalence (Schorge et al 2010).

Worldwide each year, 200.000 women are diagnosed with ovarian cancer and 125.000 die from this disease (Parkin et al 2005, Sankaranarayanan and Ferlay 2006). In the United States, it is the fifth leading cause of all cancer-related deaths, but only the eighth leading cause of cancer in women (Jemal et al 2010) since few patients are diagnosed early and subsequently cured (Schorge et al 2010).

The etiology of ovarian cancer is poorly understood and the exact tissue origin is not clear. It has been thought that the majority of ovarian cancers derive from inclusion cysts from a single cell-layer of epithelium surrounding the ovary (Auersperg et al 2001). In fact ovarian, fallopian tube and primary peritoneal carcinomas have identical histological and morphological features and it is believed that a high percentage of "ovarian cancers" actually arise in the fimbrinated end of fallopian tube or from components of the secondary Müllerian system with metastasis to the ovary (Dubeau 2008, Kindelberger et al 2007). Primary peritoneal cancers might also derive from these tissues (Muto et al 1995, Schorge et al 1998).

Ovarian cancers can roughly be separated into two categories. Low-grade tumors grow slowly, are less responsive to chemotherapy and have a low malignant potential. High-grade carcinomas exhibit great genetic instability, are rapidly metastatic and more chemosensitive (Landen et al 2008).

Risk factors. Up to 10% of all ovarian cancer patients have inherited germ-line mutations in BRCA1, BRCA2, MLH1 or MSH2 (Smith et al 2001). These

genes encode tumor suppressors involved in DNA strand-break repair. The other 90% of women exhibit increased proliferation-associated DNA-damage during their reproductive years (Purdie et al 2003, Schildkraut et al 1997). Women, who never gave birth, have double risk of developing ovarian cancer (Titus-Ernstoff et al 2001) and the probability decreases with each birth plateauing at about 5 delivers (Hinkula et al 2006). It is generally believed that the risk increases with every menarche during a woman's lifetime and this number decreases with every pregnancy while breast feeding additionally delays resumption of menarche and therefore has a protective effect (Yen et al 2003).

The opposite applies to early menarche, late menopause and nulliparity, which is often associated with infertility (Tworoger et al 2007). Long-term use of contraceptives reduces the risk by 50% and it is believed that 100.000 deaths have already been prevented by those drugs (Beral et al 2008). White women have an elevated risk of 30-40% compared to black or Hispanic women for developing ovarian carcinomas. The striking influence of ethnic background is illustrated by European Jewish women, where 35-40% diagnosed with ovarian cancer possess a mutation in BRCA1 or BRCA2 (Smith et al 2001). Prophylactic oophorectomy can be considered after the age of 40 and is associated with a more than 90% decreased risk for the development of ovarian cancer.

Over the last decades there was minimal progress in detecting ovarian cancer at a more curable early stage. The screening for serum markers, sonograms and pelvic examinations did not decrease mortality (Hogg and Friedlander 2004). CA125 tumor antigen is the most widely studied biomarker as it was initially found to be elevated in 83% of ovarian cancer patients (Bast et al 1983), but further studies demonstrated high false-positive and false-negative rates due to several other factors modulating CA125-serum-levels (Schorge et al 2010). A large prospective study demonstrated that the combination of CA125 serum-levels with

transvaginal ultrasonography would lead to many unnecessary surgical interventions with a surgery-to-cancer-ratio of 19.5 to 1 (Stirling et al 2005). More than 30 serum markers have been identified during the last years and some of them were able to provide evidence of ovarian cancer up to 3 years before clinical diagnosis (Anderson et al 2010) but rigorous validation studies are still pending.

Staging and treatment of ovarian cancer

Stage I	Growth limited to the ovaries

Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on external surface; capsule intact
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact
Ic	Tumor either stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings

Stage II	Growth involving one or both ovaries with pelvic extension

IIa	Extension and/or metastases to the uterus and/or tubes
IIb	Extension to other pelvic tissues
IIc	Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings

Stage III	Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive regional lymph nodes. Superficial liver metastasis equals Stage III. Tumor is limited to the true pelvis, but with malignant extension to small bowel or omentum

IIIa	Tumor grossly limited to the true pelvis, with negative lymph nodes, but with microscopic seeding of abdominal peritoneal surfaces, or extension to small bowel or mesentery
IIIb	Tumor of one or both ovaries with implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; regional lymph nodes are negative
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive regional lymph nodes

Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV

Table 1 | **Carcinoma of the ovary: FIGO nomenclature**. Adapted from FIGO 2009

Borderline and low-grade ovarian cancers (Stage Ia, table 1): Cyto-reductive surgery is generally recommended, but can be restricted to the affected ovary and corresponding fallopian tube to maintain fertility in young women. Early and late stage ovarian cancers are managed by cytoreductive surgery and chemotherapy. Neoadjuvant chemotherapy is used regularly to reduce tumor volumes before surgery in advanced stages. Typical chemotherapy for ovarian cancers is carboplatin alone or carboplatin and paclitaxel and achieves clinical complete remission in the majority of patients. The poor 5-year survival rate of patients with advanced ovarian cancers of 68,1% (Stage III) and 29,1% (Stage IV) (American Cancer Society 2007) is due to high recurrent rates with progressive chemotherapy resistance, underlining the necessity for further research in biological treatment strategies (Bookman 2010).

Current clinical research of targeted therapies

Angiogenesis is crucial for all solid tumors exceeding 1-2mm and vascular endothelial growth factor (VEGF) signaling is the most dominant pathway in new vessel formation promoting migration, proliferation and survival of endothelial cells (Folkman 1971). High levels of VEGF are found in many ovarian tumors, sera and ascites (Boss et al 2001, Kraft et al 1999, Zebrowski et al 1999) and inhibition of VEGF signaling in animal models demonstrated reduction in ascitic fluid accumulation, vessel formation and tumor growth (Byrne et al 2003). Bevacizumab binds human VEGF-A and is approved for treatment of colorectal, renal, breast and non-small cell lung cancer and glioblastoma and showed high response rates in ovarian carcinomas in clinical trials (Burger et al 2007, Cannistra et al 2007). Several small

molecule VEGF receptor inhibitors (Cediranib, Sorafenib, Vargatef, Pazopanib) are currently investigated in ovarian cancers (Matulonis et al 2009, Rauh-Hain and Penson 2008). Some of these inhibitors target related classes of receptors. Vandetanib, a VEGFR/EGFR inhibitor is currently tested in recurrent ovarian cancer besides several other ongoing or planned clinical trials (Ledermann and Raja 2010).

Epidermal growth factor (EGF) receptor overexpression is frequently found in ovarian cancers and nuclear expression is associated with a poor prognosis, but the relationship between overexpression and prognosis remains unclear (de Graeff et al 2009, Xia et al 2009). Many monoclonal antibodies (Trastuzumab, Cetuximab, Pertuzumab, Panitumumab) and small molecule inhibitors (Erlotinib, Gefitinib) have been investigated in ovarian cancers. Overall, they show only modest activity as monotherapy and achieve only non-significant improvement of progression-free-survival in combination with chemotherapy. The response of a particular patient to Gefitinib due to an EGFR mutation underlines the necessity for predictive markers (Ledermann and Raja 2010).

Src kinases are non-receptor tyrosine kinases involved in intracellular signaling. Overexpression is linked to a poor prognosis (Wiener et al 2003), inhibition of Src reverses chemoresistance in ovarian cancer cell lines (Chen et al 2005) and exhibits antiangiogenic properties (Han et al 2006) suggesting an attractive therapeutic target strategy for ovarian cancers.

Poly-ADP-ribose polymerase (PARP) proteins fulfill diverse functions and are involved in DNA repair leading to accumulation of single-strand breaks at PARP inhibition (Hoeijmakers 2001) further resulting in double-strand breaks, which are normally corrected by BRCA-dependent homologous recombination. Cells carrying homozygous mutations in BRCA1 and BRCA2 rely to the more error-prone and PARP-dependent non-homologous end joining pathway leading to chromosomal instability and malignancy (Farmer et al 2005) and are highly sensitive to PARP inhibition (Ledermann

and Raja 2010). The PARP inhibitor Olaparib demonstrated dose-dependent high response rates in BRCA1/BRCA2 mutation carriers (Fong et al 2009), but hypermethylation of BRCA1 is also found in 31% of sporadic, non-BRCA-mutant ovarian cancers indicating the general utility of PARP inhibitors in cancer cells with aberrations in the homologous recombination repair pathway (Ledermann and Raja 2010, McCabe et al 2006). PARP inhibitors MK4827, AGO14699 and ABT888 are currently being studied in early phases of clinical trials (Ledermann and Raja 2010).

a-folate receptor was found to be overexpressed in a range of tumor types and in 70% of primary and 82% of recurrent ovarian carcinomas while levels correlated with the stage of the tumor (Kalli et al 2008). Inhibition with monoclonal antibodies (Farletuzumab) demonstrated reduction in growth rates and cellular and complement-mediated toxicity and is currently investigated in a phase II clinical trial (Ledermann and Raja 2010).

Many novel molecular targets are being evaluated in the treatment of ovarian cancer and most of them demonstrate promising clinical activity. The wide range of newly available drugs underlines the necessity for reliably predictive serum markers for the improvement of ovarian cancer therapy.

3.2 Fibroblast growth factor signaling

3.2.1 Fibroblast growth factor receptors

The fibroblast growth factor receptor family consists of four highly related members, FGFR-1, FGFR-2, FGFR-3 and FGFR-4 (Johnson and Williams 1993). In 2000, a fifth member (FGFR-5, FGFR-L1) was identified, which lacks the tyrosine kinase domain and is believed to be involved in negative regulation (Wiedemann and Trueb 2000). The amino acid sequences are highly conserved, but the receptors differ in ligand specifity and tissue distribution. FGF signaling is crucial for the development of almost all tissues and organs (Powers et al 2000) as well as homeostasis in adult body tissues, leading to pathological conditions, cell transformation and cancer when normal FGFR functions or expression levels are altered. FGF receptor 1-3 gene knockouts are embryo-lethal due to failure in formation of the primitive streak (Jaskoll et al 2002). The phenotype of activating FGF receptor mutations, which result in abnormal activation or ligand independency are therefore the primary source of knowledge for their role in embryonic development.

Fibroblast growth factor receptor 1

In the evolving embryo, FGFR-1 has a central role in the development of the nervous system and is involved in growth regulation of long bones. Loss-of-function mutations in FGFR-1 lead to a dysfunction of nerve cell migration causing the "Kallmann syndrome", which is characterized by anosmia and hypogonadotrophic hypogonadism. It is still uncertain how FGFR-1 mutation leads to other varying symptoms of the "Kallmann syndrome", like cleft palate or abnormal tooth development. Pfeiffer syndrome is associated with gain-of-function mutations in FGFR-1. Dysregulation of bone development leads to premature fusion of skull

bones (craniosynostosis) and cutaneous syndactyly (Chen and Deng 2005, Dode et al 2007, Pitteloud et al 2006).

FGFR-1 expression is widespread over the human adult organs. High levels were found in the skin, cornea, lung, heart and placenta, moderate levels in testis and ovary (Hughes 1997).

Fibroblast growth factor receptor 2

FGFR-2 is involved in the regulation of bone growth and bone cell determination. Loss-of-function mutations in FGFR-2 lead to the lacrimo-auriculo-dento-digital (LADD) syndrome which is characterized by abnormal development of lacrimul ducts and salivary glands, abnormally shaped ears, hearing loss, tooth abnormalities and malformations of hands and feet. Many syndromes for gain-of-function mutations of FGFR-2 have been described, including Apert, Beare-Stevenson, Crouzon, Jackson-Weiss and Pfeiffer (table 2). They are characterized by premature fusion of skull bones (craniosynostosis) and limb abnormalities (Carinci et al 2005, Shams et al 2007, Wilkie 2005).

Syndrome	Craniosynostosis	other characteristics
Apert	+	bony syndactyly
Beare-Stevenson	+	cutis gyrata
Crouzon	+	graded severity of limb abnormalities (nil, broad first digits, elbow fusion)
Jackson-Weiss	+	
Pfeiffer	+	

Table 2 | **Syndromes caused by FGFR-2 gain-of-function mutations**. (Wilkie 2005)

FGFR-2, similar to FGFR-1, is widely expressed in human adult organs. Abundant expression was found in the prostate and stomach but not in pancreas, ovary, cornea and placenta (Hughes 1997).

Fibroblast growth factor receptor 3

FGFR-3 is involved in the regulation of ossification by limiting the transformation of cartilage to bone cells. Abnormal receptor activity is often associated with skin disorders, indicating an important role in keratinocyte development. Gain-of-function mutations show different phenotypes depending on the degree of FGFR-3 activation, but achondroplasia, premature skull bone fusion ("Muenke syndrome") and thickened and/or dark skin are most common (Chen and Deng 2005, Coumoul and Deng 2003, Horton and Lunstrum 2002).

FGFR-3 has a more restricted pattern of tissue-distribution. It was found in the appendix, colon, liver, sublingual gland, placenta and cervix but not in the stomach, duodenum, ileum, kidney and ureter. It is worth noting, that the overall immunoreactivity for FGFR-3 was much lower than for FGFR-1 and FGFR-2 (Hughes 1997).

Fibroblast growth factor receptor 4

FGFR-4 is involved in the maturation of limb muscles and skull bones. Foveal cones show high levels of FGFR-4 indicating an important role of FGFR-4 in development and maintenance of the retina. Knock-out mice are viable but show reduced limb muscles (Marics et al 2002) and elevated cholesterol metabolism (Yu et al 2000).

FGFR-4 has a restricted pattern of expression in the human adult body and is highly expressed in the liver, sublingual gland ducts, kidney, ureter and media of arterioles and veins of different organs including the ovaries (Hughes 1997).

3.2.2 Structure of fibroblast growth factor receptors

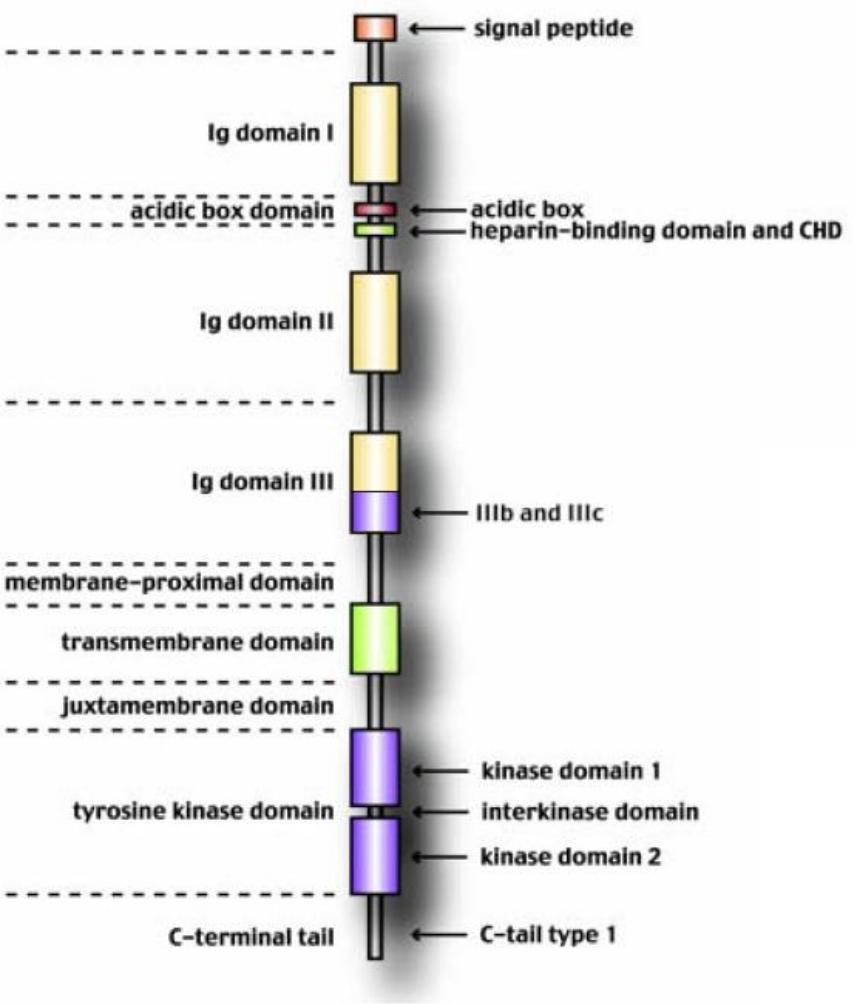


Figure 1 | **Domain structure of FGFR-1**. Adapted from Groth and Lardelli 2002, sizes are not to scale.

The general structure of FGF receptors is highly conserved (Johnson and Williams 1993). Figure 1 (Groth and Lardelli 2002) shows a schematic diagram of the domain structure of FGFR-1 as an example of the general FGF receptor structure. More detailed schematics of exon arrangements of FGF receptors 1-4 can be found in figures 9-12.

Extracellular, there is a signal peptide followed by three immunglobuline-(Ig-) like domains. The acidic box, a heparin-binding and a cell adhesion molecule (CAM) homology domain (CHD) are located between IgI and IgII (Green et al 1996). The transmembrane region is followed by a

juxtamembrane domain, a split tyrosine-kinase domain and a short c-terminal tail (Johnson and Williams 1993).

Alternative splicing generates a great diversity of FGF receptor isoforms. The signal peptide is important for the transport of the receptor to the cell surface, indicating that isoforms lacking the signal peptide will stay in the cytosol or modulate transcription factor activity in the nucleus. The acidic box forms an autoinhibitory loop with the immunglobuline-like domain I and excision of one or the other during the alternative splicing process potentially leads to faster and/or prolonged response to ligand binding (Groth and Lardelli 2002, Olsen et al 2004).

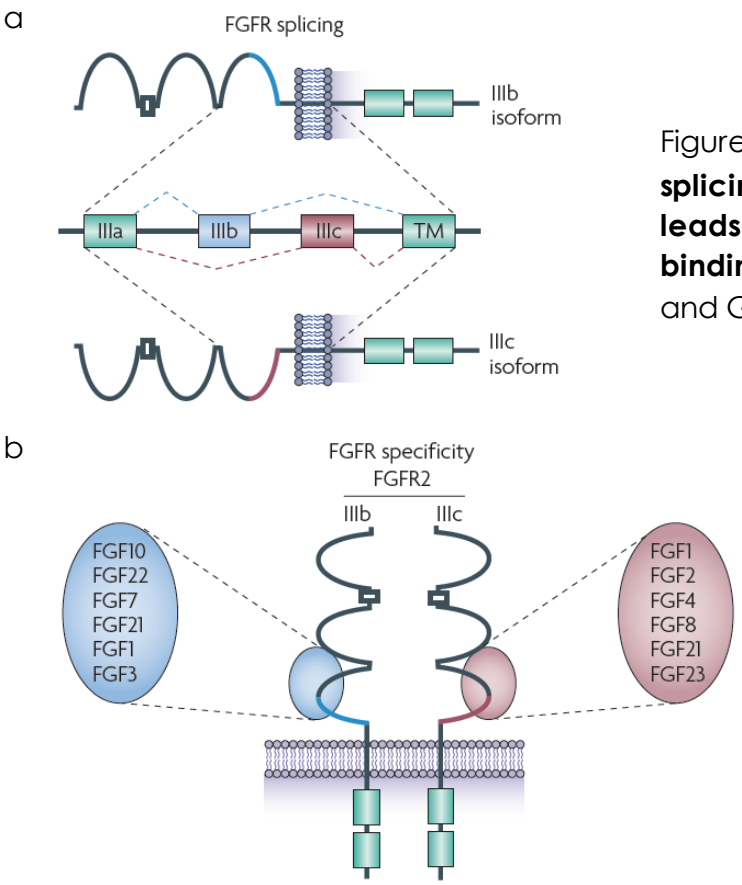


Figure 2 | **Alternative splicing of FGF receptors leads to different ligand-binding specifities**. (Turner and Grose 2010)

Alternative splicing of the immunglobuline-like domain III leads to the extensively studied isoforms "IIIa", "IIIb" and "IIIc" which do only exist for the FGF receptors 1-3. IIIa is composed of Ig-domain II and the first half of Ig-domain III (IIIa, figure 2a). It is therefore soluble and incapable to

activate downstream signaling, but has a high affinity for fibroblast growth factors indicating a regulatory mechanism. Isoforms IIIb and IIIc are generated by exon skipping resulting in different amino acid sequences in the second half of the immunglobuline-like domain III (figure 2a) which leads to different ligand binding specifities (figure 2b). Isoforms lacking the transmembrane domain are soluble and may act in the cytoplasm as well as in the nucleus. FGF receptor variants without the split tyrosine kinase domain are believed to act as negative regulators due to their capability to form dimers with active receptors (Johnson and Williams 1993, Turner and Grose 2010).

Expression of fibroblast growth factor receptor splice variants

Rubin et al 1989 were the first to notice that keratinocytes express receptors for FGF-7, but fibroblasts and endothelial cells do not. On the other hand, endothelial cells and fibroblasts do express receptors for FGF-2, which cannot be found on epithelial cells. Johnson et al 1991 examined the expression of fibroblast growth factor receptor 1 splice variants in more detail and showed that variants IIIa, IIIb and IIIc are expressed exclusively as well as simultaneously by different cell lines, but expression levels of variant IIIc always exceeded those of variant IIIb by far.

Extensive in situ hybridization analysis on tissue-specific expression of FGFR splice variants have been performed in rat (Wanaka et al 1991), chicken (Patstone et al 1993) and mouse embryos (Kornbluth et al 1988) and showed distinct cell-type-specific spatial and temporal patterns of receptor expression during development. Unfortunately, there is very limited data of tissue-specific cell-distribution of particular FGFR splice variants in normal human adult tissues at protein level as it was only evaluated in a small number of tissues without any cellular localization and in most cases for FGF receptors 1 and 2 only (Hughes 1997).

Different splicing variants of FGF receptors 1-4 will be abbreviated as follows (table 3).

Receptor	Abbreviation
FGFR-1 IIIb	1b
FGFR-1 IIIc	1c
FGFR-2 IIIb	2b
FGFR-2 IIIc	2c
FGFR-3 IIIb	3b
FGFR-3 IIIc	3c
FGFR-4	4Δ

Table 3 | **Abbreviations for FGF receptors 1-4**

3.2.3 Fibroblast growth factors

22 fibroblast growth factors have been identified and are clustered into 7 subfamilies due to their sequence similarities and functional properties. Their evolutionary relationship is illustrated as a phylogenetic tree shown in figure 3 (Itoh and Ornitz 2004).

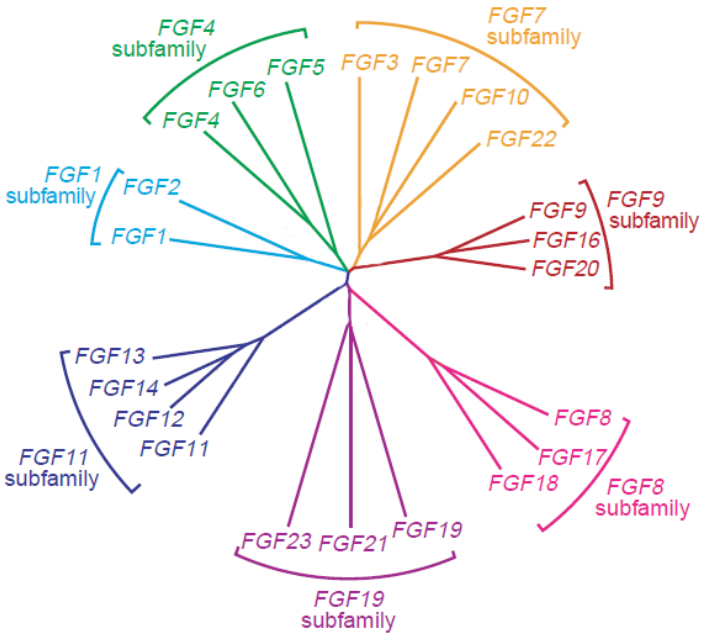


Figure 3 | **Evolutionary relationship of human fibroblast growth factors**. FGFs are divided into 7 subfamilies, each with 2-4 members due to sequence similarities and functional properties. Branch lengths represent evolutionary distances between the genes. Adapted from Itoh and Ornitz 2004.

Although they share a high sequence similarity, the members of the FGF-11 subfamily are not able to activate FGF receptors. They were renamed to fibroblast homologous factors (FHF) and are not generally considered members of the FGF family (Olsen et al 2003). FGF-15 is the murine paralogue of human FGF-19 and therefore not shown in figure 3.

3.2.4 FGF-FGFR signaling system

All FGFs except FGF-1, FGF-2 and the FGF-9 subfamily have a signal peptide determining their secretion. FGF-1 and FGF-2 are secreted via a direct protein export mechanism (Nickel 2005), while the FGF-9 subfamily is sequestered through the endoplasmatic reticulum and the subsequent Golgi pathway (Revest et al 2000).

In order to bind to FGF receptors, FGFs need heparin sulphate proteoglycans (HPSG). A functional FGF-FGFR unit consists of 2:1:2 FGF:HPSG:FGFR subunits as shown in figure 4.

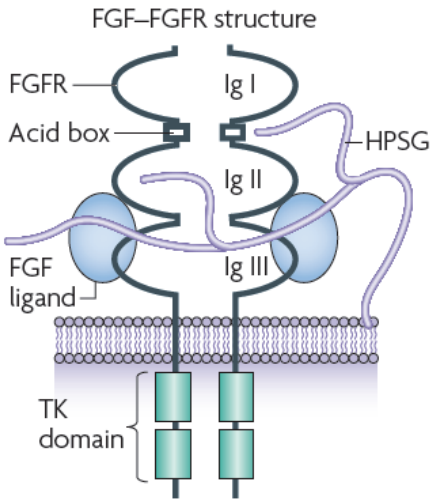


Figure 4 | **Basic structure of the FGF:HPSG:FGFR complex**. A functional FGF-FGFR unit consists of 2:1:2 FGF:HPSG:FGFR subunits. Adapted from Turner and Grose 2010.

Beside HPSG availability, FGF signaling is regulated through various mechanisms. As described before and shown in table 4, FGF receptors have different ligand-binding properties. This is among others due to

alternative splicing in the IgIII-domain. Autocrine signaling is prevented by tissue-specific expression patterns of FGFs and FGF receptors. Generally, epithelial-like cells tend to express IIIb-variants, while mesenchymal-associated cells do express IIIc-variants (Johnson and Williams 1993).

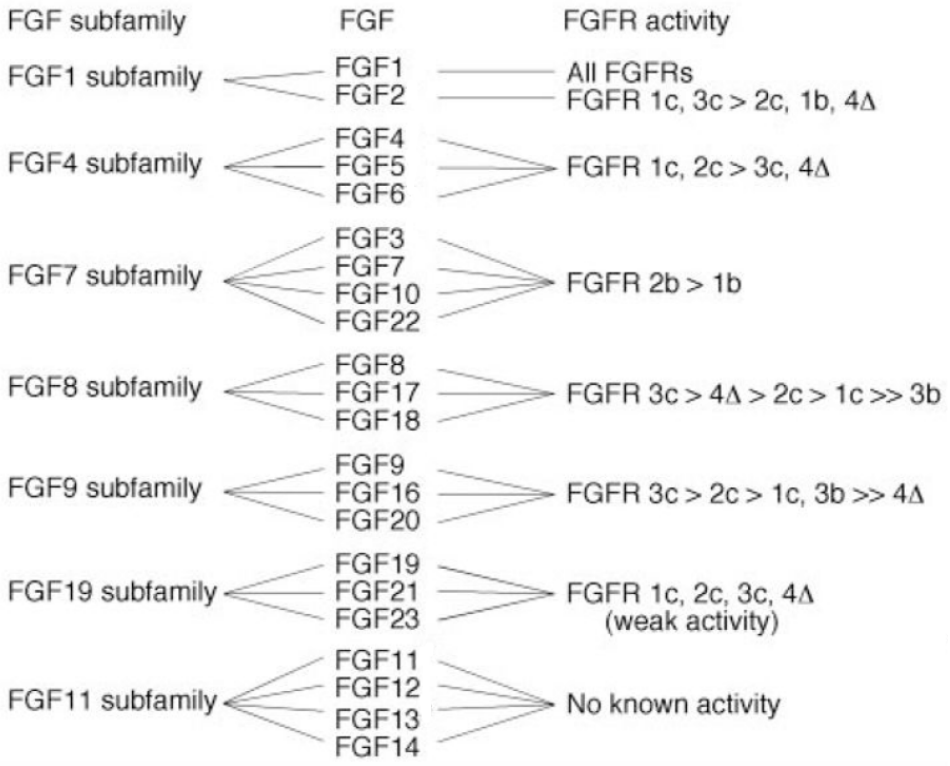


Table 4 | **Relative activity of fibroblast growth factors**. FGFs are arranged by subfamily identity. FGF-11 subfamily corresponds to fibroblast homologous factors. FGF-19 subfamily's activity is weak due to its dependence on Klotho-proteins. Abbreviations are explained in table 3. Adapted from Zhang et al 2006.

Zhang et al 2006 were not able to find any significant activation of FGF receptors by the FGF-19 subfamily. This is due to their dependence on Klotho-proteins which enhance FGF receptor binding and are expressed in a tissue-dependent manner (Kurosu et al 2006). It turned out that FGF-19, FGF-21 and FGF-23 act in an endocrine instead of a paracrine manner (Beenken and Mohammadi 2009).

3.2.5 FGF receptor downstream signaling

Fibroblast growth factor receptors act as dimers in a ligand dependent manner. Activation and subsequent dimerization of the receptors leads to transphosphorylation of tyrosine-kinase domains and the c-terminus, which act as docking sites for adaptor proteins and results in activation of multiple signaling pathways as shown in figure 5 (Turner and Grose 2010).

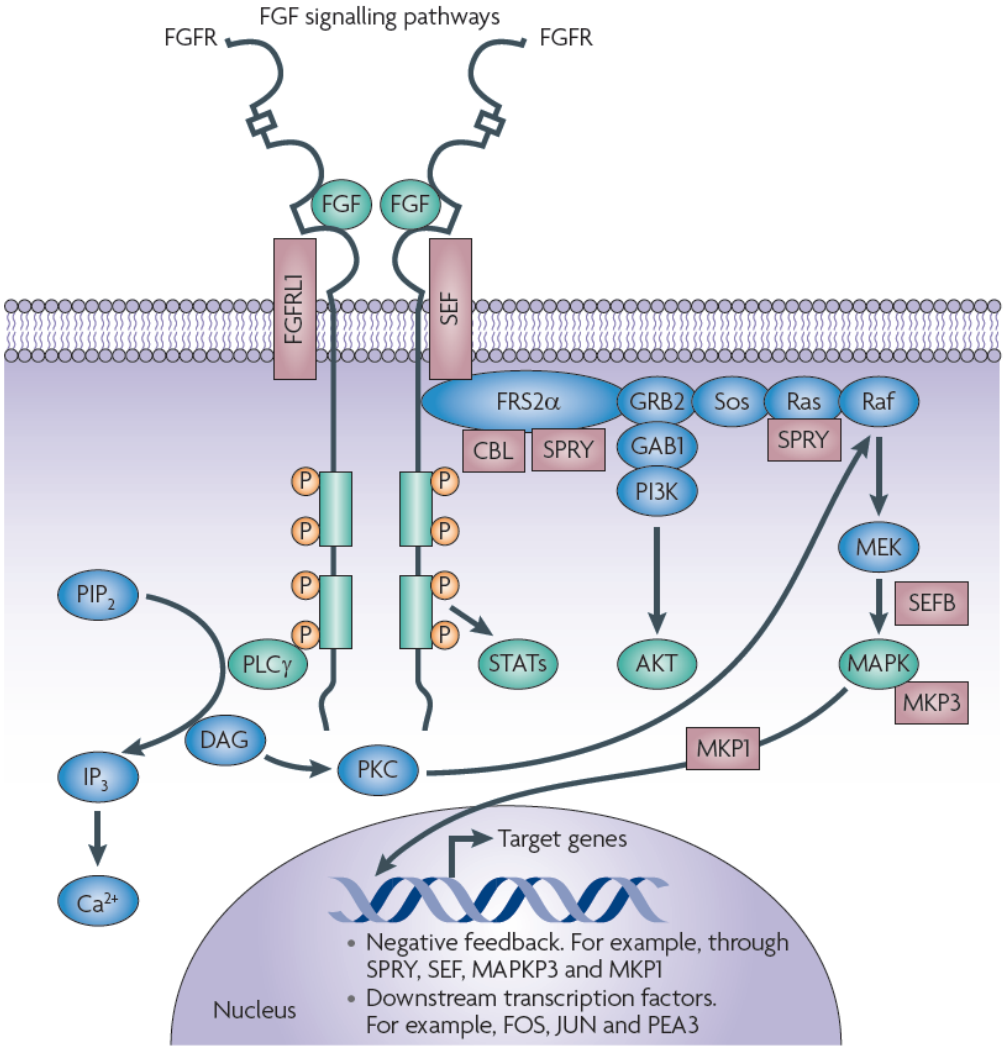


Figure 5 | **FGFR signaling network**. Signaling pathways are described in the text. Abbreviations: FGF, fibroblast growth factor, FGFR, fibroblast growth factor receptor, SEF, similar expression to fgf genes, FGFRL1, FGFR-like 1, FRS2a, FGFR substrate 2a, GRB2, growth factor receptor bound protein 2, SOS, son of sevenless, SPRY, Sprouty, GAB1, GRB-associated-binding protein 1, PI3K, phosphoinositide 3-kinase, MEK, MAP/ERK kinase, MAPK, mitogen activated protein kinase, MKP3, MAPK phosphatase 3, MKP1, MAPK phosphatase 1, AKT,

protein kinase B, STATs, signal transducers and activators of transcription, PIP_2, phosphatidylinositol 4,5-bisphosphate, PLCγ, phosphoinositide phospholipase C, DAG, diacylglycerol, PKC, protein kinase C, IP_3, inositol triphosphate, FOS, FBJ murine osteosarcoma viral oncogene homolog, JUN, jun proto-oncogene, PEA3, polyoma enhancer activator 3. Turner and Grose 2010.

FGFR substrate 2 (FRS2) is a key adaptor protein of FGF signaling and mostly specific to FGF receptors (Gotoh 2008). It binds to the juxtamembrane domain of the FGF receptor and is phosphorylated at multiple sites upon activation. The following recruitment of SOS and GRB2 activates Ras, Raf and the MAPK signaling pathway (Eswarakumar et al 2005), which is activated primarily in response to FGF treatment and leads to cell proliferation in the majority of tissues. PI3K pathway and AKT signaling is activated independently of SOS through GAB1 (Altomare and Testa 2005) and generally promotes anti-apoptotic mechanisms.

PLCγ is triggered independently of FRS2 and activates PKC, which can directly interact with Raf, activating MAPK pathway (Klint and Claesson-Welsh 1999). Other activated pathways are p38 MAPK, Jun kinase, STATs and RSK2 depending on the cellular context (Kang et al 2009).

Negative regulation of FGF signaling is insufficiently studied yet. Activated receptors are internalized and degraded or recycled. FRS2 is phosphorylated at Threonine and Serine residues leading to inhibition of GRB2-binding (Gotoh 2008). SPRY, SEF, MAPKP3 and MKP1 modulate downstream signaling at different points of the signaling cascade.

3.2.6 Aberrant FGF signaling and cancer

Activating mutations. Somatic mutations of FGFR-3 can be found in about half of all bladder cancers (Cappellen et al 1999). A single mutation in the extracellular domain of FGFR-3 (S249C) leads to constitutive homodimerization and subsequent activation of the receptor and corresponds to 50% of all mutations in FGFR-3 (di Martino et al 2009, Naski

et al 1996). Activating mutations identified in cancer cells frequently exhibit the same nucleotide aberrations as seen in skeletal dysplasias (Ornitz and Marie 2002).

Gene amplifications are common in FGFR-1 and FGFR-2 but not in FGFR-3 (Nord et al 2010). 10% of gastric cancers show an amplification of FGFR-2 and are highly sensitive to FGFR inhibition (Kunii et al 2008, Takeda et al 2007). 10% of all breast cancers show amplification of FGFR-1.

Chromosomal translocations are found in 15% of multiple myelomas and these cells are highly sensitive to FGFR inhibition (Qing et al 2009, Trudel et al 2006). Many translocations in FGF receptors have been identified and normally show a fusion of a transcription factor with the tyrosine-kinase domain of the receptor leading to constitutive dimerization and activation of FGF downstream effectors (Roumiantsev et al 2004, Xiao et al 1998, Yagasaki et al 2001).

Autocrine/Paracrine signaling. Melanomas express high levels of FGFR-1 and FGF-2 and inhibition leads to growth regression in human melanoma xenografts, suggesting an autocrine signaling loop (Wang and Becker 1997). FGF-1 is frequently amplified in ovarian cancers which could promote angiogenesis (Birrer et al 2007). Additionally, an autocrine FGF-2/FGFR-1IIIc signaling loop was identified in non-small cell lung cancer (Marek et al 2009). Paracrine signaling is difficult to model, but several FGFs have been found to be overexpressed in various cancer types (Poon et al 2001).

Increased signaling in prostate cancer cells was found due to loss of expression of *negative regulators* (SPRTY (Fritzsche et al 2006), SEF (Darby et al 2006)) and is believed to promote androgen-independency (Kwabi-Addo et al 2004).

3.2.7 Carcinogenic mechanisms of aberrant FGF signaling

Proliferation. Enhanced proliferation is one important characteristic of cancer cells. Mouse models have shown that overexpression of FGF-10 and FGF-8 in the murine prostate promotes epithelial hyperproliferation, but a second mutation (AKT and PTEN, respectively) was necessary to induce tumorigenesis (Abate-Shen and Shen 2007, Memarzadeh et al 2007).

Survival. Depending on the cellular context, FGFs can trigger activation of anti-apoptotic pathways (PI3K, STAT). High FGF-2 serum-levels are associated with poor prognosis in small cell lung cancer (SCLC), which is due to upregulation of anti-apoptotic proteins (Pardo et al 2002, Pardo et al 2006).

Migration, Invasion. A constitutively active FGFR-1 was shown to induce invasive mammary lesions (Welm et al 2002) and prostatic intraepithelial neoplasia (Freeman et al 2003). FGFR-2IIIb – FGF-10 dependent invasion was found in pancreatic cancer cells (Nomura et al 2008).

Angiogenesis. FGF signaling comprises a key function in epithelial repair and FGF-2 possesses an essential role in blood vessel formation at the wound site (Werner and Grose 2003). Various FGFs were shown to be involved in tumor angiogenesis (Kandel et al 1991, Presta et al 2005). They stimulate vessel formation and maturation, facilitate degradation of extracellular matrix (ECM) and alter intercellular adhesion and communication (Presta et al 2005). FGF-1 is regularly upregulated in ovarian cancer cells (Birrer et al 2007) and release of FGF-2 from tumor cells acting on endothelial cells has been described previously (Schweigerer et al 1987).

3.2.8 Inhibition of FGF signaling

Drug name	Company	Range of activity or target
Small molecular tyrosine kinase inhibitors		
SU5402	*In vitro* reagent	Selective FGFR inhibitor (now superseded by availability of PD173074)
PD173074	*In vitro* reagent	Selective FGFR inhibitor
TKI258	Novartis	FGFR, PDGFR and VEGFR inhibitor
BIBF 1120	Boehringer Ingelheim	FGFR, PDGFR and VEGFR inhibitor
BMS-582,664 (Brivanib)	Bristol-Myers Squibb	FGFR and VEGFR inhibitor
E7080	Eisai	FGFR, PDGFR and VEGFR inhibitor
TSU-68	Taiho Pharmaceutical	FGFR, PDGFR and VEGFR inhibitor
FGFR antibodies and FGF ligand traps		
IMC-A1	ImClone	FGFR1-IIIc-specific antibody
PRO-001	ProChon Biotech	FGFR3-specific blocking antibody
R3Mab	Genentech	FGFR3-specific antibody
1A6	Genentech	FGF19-specific antibody
FP-1039	Five Prime Therapeutics	FGF ligand trap (multiple FGFs)
FGF ligand for mucosal chemoprotection		
Palifermin (Kepivance)	Biovitrum AB	Recombinant FGF7 (activates FGFR2-IIIb)

Table 5 | **FGF receptor inhibitors**. Adapted from Turner and Grose 2010

All FGF receptor tyrosine kinase inhibitors are ATP-competitive and also target VEGFR-2 due to their structural similarity. Targeting FGFR as well as VEGFR has striking advantages, inhibiting proliferation and angiogenesis simultaneously. In contrast, multi-kinase inhibitors are not very potent against FGF receptors and achieving sufficient inhibition might be challenging (Turner and Grose 2010). Table 5 gives an overview on FGF receptor inhibitors. One major side-effect of FGF receptor inhibition is hyperphosphatemia by blocking FGF-23 signaling, which is the key regulator of phosphate homeostasis (Shimada et al 2004). Antibodies exhibit antiproliferative effects in bladder cancer cells and t(4;14) myeloma (Qing et al 2009). FGF-7 is licensed for treatment of mucositis induced by myelotoxic therapy (Turner and Grose 2010).

PD173074, a small molecule inhibitor, originally published as an FGFR-1 and VEGFR-2 inhibitor (Mohammadi et al 1998) was later shown to inhibit FGF receptors 1-3 vigorously and FGFR-4 at high doses (Ezzat et al 2005). It reversibly binds to the receptor tyrosine kinase (RTK) ATP cleft and inhibits autophosphorylation of the dimerized receptors. Although it was shown, that PD173074 inhibits survival and growth in FGFR-dependent cancer cells (Byron et al 2008, Kunii et al 2008, Trudel et al 2004), it was dropped from clinical trials due to toxicity issues and is now being used as a research tool.

CHIR-258 (TKI258, Dovitinib) is a multi-kinase inhibitor targeting RTK-classes III (platelet derived growth factor receptor, PDGFR), IV (FGFR) and V (vascular endothelial growth factor receptor, VEGFR). It is currently in phase II of clinical development. Recent analyses with Kinomescan, a competition binding assay, indicate that CHIR-258 is much more promiscuous and that FGFR-2 is inhibited too. Nonetheless, FGFR-4 is poorly affected by CHIR-258 (Karaman et al 2008).

Receptor	IC_{50}, µM
FLT3	0,001
c-KIT	0,002
CSF-1R/c-fms	0,036
FGFR-1	0,008
FGFR-3	0,009
VEGFR-1	0,01
VEGFR-2	0,013
VEGFR-3	0,008

Receptor	IC_{50}, µM
PDGFR-ß	0,027
PDGFR-a	0,21
InsR	2
EGFR-1	2
c-Met	>3
EphA2	4
Tie2	4

Table 6 | **CHIR-258 in-vitro kinase assay** (Trudel et al 2005)

4 Materials and Methods

Human cell lines

Human ovarian cancer cell lines, SV-40 immortalized ovarian surface epithelium and primary ovarian surface epithelium. Citations refer to the first announcement in literature if the isolation of the cells was not published independently.

Cell line	Description	isolation site	Reference
A-2774	adenocarcinoma	unknown	(Vigani et al 1990)
A-2780		ascites	(Eva et al 1982)
CAOV-3		unknown	(DiSaia et al 1975)
H-134		ascites	(Broxterman et al 1987)
HEY		primary	(Buick et al 1985)
HOC-7		ascites	(Buick et al 1985)
OVCAR-3		ascites	(Hamilton et al 1983)
SKOV-3		ascites	(DiSaia et al 1975)
TR-170		ascites	(Hill et al 1987)
PA-1	teratocarcinoma	primary	(Giovanella et al 1974)
IOSE-80	SV-40 immortalized ovarian surface epithelium	primary	(Maines-Bandiera et al 1992)
IOSE-364		primary	(Maines-Bandiera et al 1992)
IOSE-386		primary	(Maines-Bandiera et al 1992)
OSE	primary ovarian surface epithelium	primary	Innoprot, Derio, Spain

Growth media of cell lines

Cell line	growth medium (GM)	freezing medium
A-2774	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
A-2780	RPMI +10% FCS +1% PSG	GM +5% FCS +10% DMSO
CAOV-3	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
H-134	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
HEY	DMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
HOC-7	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
OVCAR-3	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
SKOV-3	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
TR-170	DMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
PA-1	aMEM +10% FCS +1% PSG	GM +5% FCS +10% DMSO
IOSE-80	Medium 199/MCDB 105 (1:1) +5% FCS +1%PSG	GM +35% FCS +6% DMSO

IOSE-364	Medium 199/MCDB 105 (1:1) +5% FCS +1%PSG	GM +35% FCS +6% DMSO
IOSE-386	Medium 199/MCDB 105 (1:1) +5% FCS +1%PSG	GM +35% FCS +6% DMSO
OSE	Basal medium +5ml OEpiCGS +5ml PS-solution	GM +15% FCS + 10% DMSO

Thawing of cells

The cryotube containing cells of interest was thawed in aqua bidest at room temperature and transferred to 20ml precooled growth medium. After centrifugation for 5 min at 4°C, 1000rpm, the supernatant was decanted and the cell pellet reconstituted in 14ml growth medium at room temperature. The obtained cell suspension was split up into two T25 tissue culture flasks and incubated as described below.

Maintenance of cell culture

All cell lines were cultured in T25 tissue culture flasks in 7ml of the respective growth medium (see table above), incubated at 37°C, 5% CO_2, 95% humidity and passaged when confluence reached 80-90% (preferably once a week). Cells were carefully washed with Dulbecco's PBS (D-PBS), 800µl Trypsin/EDTA was added and the flask was incubated at 37°C until cells detached. After tapping the flask gently, the cells were diluted in accordance to their growth rate in two T25 tissue culture flasks containing growth medium and were incubated as described.

Cryopreservation of cells

Cells were grown in growth medium containing 10% FCS until they reached 70-80% confluence and were harvested by trypsinization and centrifugation (5min at 1000rpm). After resuspending in freezing medium, cells were counted (Neubauer chamber, cells diluted 1:10 in trypan blue) and a suspension containing twice as much the final cell concentration was prepared. Cryogenic tubes were placed on ice, 500µl of the

prepared cell suspension was added to every vial and left on ice for 15min. After 500µl of precooled freeze medium containing 12% or 20% DMSO, respectively, were added and mixed gently, the suspension was left on ice for 30min. Vials were put into "Mr. Frosty" freezing container (Fisher Scientific, Rochester NY) overnight at -80°C and transferred to liquid nitrogen the next day.

Cell proliferation and cytotoxicity assay

Solutions

Paraformaldehyde solution. 0,4g Paraformaldehyde was added to 10ml DPBS. Solution was heated in a fume hood while taking care that it did not exceed 60°C. After all solid particles were dissolved, 5N NaOH was added until the solution cleared (~2µl). Finally, the solution was filter sterilized, aliquoted and stored at -20°C.

Destaining solution. 10% acetic acid, 25% methanol in aqua bidest

Crystal violet solution. 0,5% crystal violet, 25% methanol in aqua bidest

Cells were grown in growth medium containing 10% FCS until they reached 70-80% confluence and harvested by trypsinization and centrifugation (5min at 1000rpm). After resuspending in growth medium containing 5% FCS, cells were counted (Neubauer chamber, cells diluted 1:10 in tryphan blue), the desired concentration was prepared and the cells seeded in a 96-well plate using a Dispenser Multipette. After incubation overnight, growth medium was removed and 100µl growth medium containing twice as much the desired final FCS concentration was added. A dilution series was prepared in growth medium containing 0% FCS with twice as much the desired final concentration of the substance of interest and added to the wells in triplicates. Note: Care was taken to ensure consistent amounts of solvent (e.g. DMSO) when the

substance of interest was not solubilized in water.
Cell number was determined after 72 hours using paraformaldehyde-fixation with crystal violet staining as described below.

Fixation / Crystal violet staining

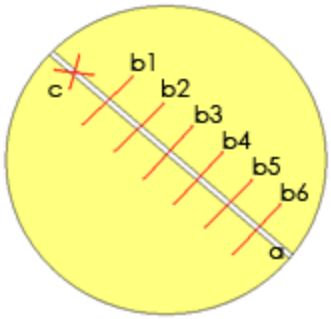
Medium was removed and cells were washed with cold D-PBS. 50µl of paraformaldehyde solution were added and cells were incubated for 30min at room temperature. After washing with cold DPBS, 50µl of crystal violet solution were added and incubated for 10min at room temperature. Subsequently, cells were washed three times with aqua bidest and plates were allowed to dry for at least two hours or overnight.

Detection

100µl of destaining solution were added and incubated for 20min at room temperature. Optical density was determined using a micro-plate reader (absorbance 570nm/reference 405nm).

Scratch-assay (adapted from Liang et al 2007)

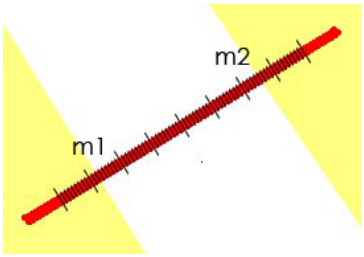
Cells were seeded in 35mm dishes with different cell numbers to create a confluent monolayer (A-2780: $4{,}7 \cdot 10^6$, HEY: $1{,}5 \cdot 10^6$, SKOV-3: $7{,}5 \cdot 10^5$. OVCAR-3 cells do not adhere when seeded at high densities) in 3ml medium containing 5% FCS and allowed to adhere overnight.



Next day, the cell monolayer was evenly scratched using a yellow pipette tip (a). Growth medium was removed and cells were washed with 1ml D-PBS and 1ml growth medium. Treatment was performed in 2ml medium.

Dishes were marked with 6 scratches in the plastic

bottom (b1-b6) perpendicular to the scratch in the cell monolayer (a) with a sharp scalpel. The starting point for the measurement was marked too (c).



Measurement was done using an eyepiece micrometer at 10x optical magnification: For each measuring point b1-b6, the scratch in the bottom of the plate was focused first and positioned coincidentally to the scale of the micrometer. Then, without moving the dish, the cell layer was focused and the distance between the margins of the monolayer (m1, m2), which is equal to the width of the scratch (a) was noted at 0-2-4-6-8-24 hours after scratching the cell monolayer.

Analysis of data: For normalization of cell migration data, the width of each scratch at the "0 hour" time-point was subtracted from the corresponding "2, 4, 6, 8, 24 hours" time-points. For each time-point, the mean value and the standard deviation was calculated from the 6 individual measurements per time point.

Western Blot Analysis

Solutions

	Reagent	Stock conc.	Quantity	Final conc.
RIPA	NaCl	5M	3ml	150mM
	Tris pH7,4	1M	5ml	50mM
	DOC (Sodium deoxycholate)	10%	5ml	0,5%
	EGTA	50mM	4ml	2mM
	EDTA, pH7,4	50mM	10ml	5mM
	NaF	500mM	6ml	30mM
	ß-Glycerophosphate pH7,2	400mM	10ml	40mM
	Tetrasodium pyrophosphate	100mM	10ml	10mM
	Benzamidine	30mM	10ml	3mM

Nonidet P-40	pure	1ml	1%

adjust pH to 7,4 and fill up with aqua bidest to 95ml. Store at 4°C.

RIPA+

1,9ml RIPA
20µl 200mM Na-Orthovanadate (heat-activated)
80µl 25x Complete stock solution (Roche, Boston MA)

4x Sample buffer

Reagent	Stock conc.	Quantity	Final conc.
Glycerol	pure	5ml	50%
Tris-HCl pH6,8	1M	1,25ml	125mM
SDS	20%	2ml	4%
Bromophenol blue	1%	1,25ml	0,125%
ß-Mercaptoethanol*	pure	0,5ml	5%
total		10ml	

*to be added just before use

10x running buffer

Tris base	pure	30,3g	25mM
Glycine	pure	144,2g	192mM
SDS	pure	10g	0,1%

fill up with aqua bidest to 1000ml, store at 4°C

1x TB

Glycine	pure	16,8g	150mM
Tris pH8,3	1M	75ml	50mM
SDS	10%	7,5ml	0,05%
Methanol	pure	300ml	20%
Aqua bidest		1117,5ml	
total		1500ml	

10x TBS

Tris pH7,5	1M	500ml	50mM
NaCl	5M	300ml	150mM
Aqua bidest		200ml	

1x TBS: add 900ml aqua bidest to 100ml 10x TBS (final concentration for 1xTBS)

1x TBS-T

TBS	10x	100ml	1x
Tween20	pure	1ml	0,1%
Aqua bidest		900ml	

10x BS

TBST	1x	100ml	1x
BSA	pure	4g	4%

	Reagent	Quantity	Volume	Final conc.
Antibody solution (first antibody)	TBS-T	1,053x	14,25ml	1x
	Blocking Solution	4,21%	4,75ml	4%
	Sodium azide solution	1%	1ml	0,05%
	total		20ml	
Antibody solution (second antibody)	TBS-T	1x	30ml	1x
	BS	4%	10ml	1%
	total		40ml	
A/B solution	Acrylamide	40%	7,4ml	30%
	Bis Solution	2%	2ml	0,4%
	Aqua bidest		0,6ml	
4% stacking gel	A/B solution	30%/0,4%	1,673ml	
	Tris pH6,8	0,5M	3,15	
	SDS	10%	125µl	
	APS	10%	125µl	
	TEMED	pure	12,5µl	
	Aqua bidest		7,46ml	
10% running gel	A/B solution	30%/0,4%	6,7ml	
	Tris pH8,8	1,5M	5ml	
	SDS	10%	200µl	
	APS	10%	100µl	
	TEMED	pure	10µl	
10% APS	Ammonium persulfate	pure	0,1g	
	Aqua bidest		1ml	

Antibodies	Target protein	Provider	Catalogue no.
	Actin	Santa Cruz	sc-1616
	pFRS2Y196	Cell Signaling	3864S
	pFRS2Y436	Cell Signaling	3861S
	FRS2	Abnova	H00010818-B01
	p-AKTS473	Cell Signaling	9271S
	AKT	Cell Signaling	9272
	p-ERK1/2	Cell Signaling	9101

ERK1/2	Cell Signaling	9102
p-S6	Cell Signaling	2215
S6	Cell Signaling	2317
a-Rabbit (Donkey), HRP	Promega	V7951
a-Mouse (Chicken), HRP	Santa Cruz	sc-2954

Medium was removed and cells were washed twice with cold D-PBS. After removing D-PBS completely, 100µl RIPA+ was added, the dish was left on ice for at least 5min and the cells were scraped using a cell scraper. The cell lysate was transferred to a microtube and left on ice for at least 10min while being vortexed regularly. Samples were centrifuged for 30min at 4°C, 12500rpm and the supernatant was transferred to a fresh microtube and stored at -80°C.

Protein concentration was determined using Biorad Protein Assay Standard II, BSA (10-0,156mg/ml) was used as a reference. 5µl of RIPA+/BSA dilution/sample was mixed with 20µl Reagent A' (1ml Reagent A +20µl Reagent S) and 200µl Reagent B and incubated at room temperature (RT) for 20min, optical density was determined using a micro-plate reader (absorbance 655nm/reference 450nm). Samples were adjusted to 1µg/µl in 1x sample buffer and stored at -20°C.

SDS-PAGE: Protein samples in 1x sample buffer were boiled for 10min at 95°C and vortexed. Thirty µl of those lysates were then applied to a 4% stacking/10% separation SDS-polyacrylamide gel. 2µl of the molecular weight marker "Magic Mark XP" (Invitrogen, Carlsbad CA) were loaded in parallel for estimation of molecular mass separation within the gel. Separating conditions were 100V constant for about 2 hours in a Biorad Minigel Electrophoresis Chamber.

Protein transfer: Separated proteins were transferred to a methanol-activated PVDF membrane using a Biorad Mini Transblot Module. Transfer conditions were 290mA constant at 4°C overnight.

Immunostaining: Membranes were blocked in blocking solution +4% FCS for 1h at RT on a shaker, washed 3 times, 5min each in TBS-T and incubated overnight with 10ml of the first antibody at 4°C on a shaker. First antibody was saved, membranes washed 3 times, 5min each in TBS-T, incubated with the second antibody for 1h at RT on a shaker and finally washed 4 times (2x5min, 2x10min) with TBS-T and 4 times (2x5min, 2x10min) with TBS.

Detection: Protein bands were visualized by chemiluminescent detection through horseradish peroxidase conjugated to the secondary antibody. 2,5ml Detection Solution 1 and 2,5ml Detection Solution 2 (ECL-Substrate) were added to the membrane and incubated for 5min on a shaker. Autoradiography films were exposed to the membrane for different times and developed with a Canon table developer.

RNA-Isolation

Cells were grown in growth medium containing 10% FCS in a T25 tissue culture flask.

Homogenization: Medium was completely removed and cells were incubated with 3ml of TRI Reagent (Sigma-Aldrich, St. Louis MO) for at least 5min at RT while shaking the flask vigorously. Cell lysate was homogenized, transferred into a microtube and stored at -80°C.

RNA-Extraction/Precipitation: 150µl of cold bromo chloropropane (BCP) were added per sample, vortexed for 15sec, left at RT for 15min and centrifuged 15min at 4°C, 12500rpm. The aqueous phase was transferred into a new microtube; 1ml of isopropanol was added per sample,

vortexed for 15sec and centrifuged 10min at 4°C, 12500rpm. The supernatant was aspirated, 1,5ml 75% EtOH were added, vortexed for 15sec and centrifuged 10min at 4°C, 12500rpm. The supernatant was aspirated again and the remaining RNA pellet was air-dried. Finally, RNA was dissolved in 20µl DEPC-treated water and stored at -80°C.

Quantification and quality control of RNA: RNA was quantified spectrophotometrically measuring a 1:500 diluted sample against aqua bidest at 260/280nm twice in a quartz cuvette. Calculation of RNA-concentration: OD260 x 40 = µg/µl. Estimation of purity: 260/280 ratio ≥ 1,5. Quality/integrity of RNA was determined by agarose gel electrophoresis. 0,3g agarose in 30ml 1xTAE (40mM Tris-acetate, 1mM EDTA in aqua bidest) were heated for 40 seconds in a microwave oven. After adding 3µl of Gel Red (Biotium, Hayward CA), the fluid was poured into the assembled electrophoresis chamber. When the gel had polymerized, 250ml 1xTAE were added. Subsequently, prepared samples (0,5µl RNA in 12µl aqua bidest and 3µl 5x gel loading solution) were applied and separated using 70V/210mA for 30 to 45min. RNA was photographed using a Herolab gel documentation system and checked for a clear appearance of the 18S/28S ribosomal RNA bands.

cDNA-Synthesis

Quantitated RNA was diluted to 0,2µg/µl.

Mastermix

	Vol./sample (µl)	Final conc.
5x reaction buffer (250mM Tris-HCl pH8,3, 375mM KCL, 15mM Mg_2Cl_2)	4	1x
DTT (100mM)	2	10mM
BSA (RNAse/DNAse free), 2,9mg/ml	1	0,28mg/ml
Protector RNAse Inhibitor (40U/µl)	0,315	0,62U
DEPC-treated water	0,185	
dNTP Mix 100mM	0,8	7,8mM
Random hexamer primers (100µM)	1	5µM
200 UM-MLV reverse transcriptase (200U/µl)	1	10U
total	10,3	

10µl RNA (0,2µg/µl) were added to 10,3µl Mastermix and incubated for 60min at 37°C. cDNA and diluted RNA were stored at -80°C.

Polymerase Chain Reaction

GoTaq Green Master Mix (Promega, Madison WI) in a 25µl reaction volume was used for all PCRs.

Mastermix

	Vol./sample (µl)	Final conc.
GoTaq Green Master Mix	12,5	1x
Forward primer	1	0,8µM
Reverse primer	1	0,8µM
PCR water	9,5	0,62U
cDNA	1	
total	25	

PCR programs

Hot start 95°C, algorithm measurement

1x	1'	95°C
cycle	30''	95°C
	30''	annealing temp.
	1'	72°C
1x	7'	72°C
1x	∞	4°C

Program name	annealing temp. (°C)	cycles
50anneal	50	30
51anneal	51	30
52anneal	52	30
53anneal	53	30
56anneal	56	30
60anneal	60	30
56a40c	56	40
57a45c	57	45
45a45c	45	45
56a45c	56	45
61a40c	61	40
60a40c	60	40
60a45c	60	45
54a40c	54	40
53a35c	53	35
63a40c	63	40
65a40c	65	40

Primer sequences

Name	Sequence	PCR program	Reference	Target
FGF1s	GAAGCCCAAACTCCTCTACTG	57a45c	Fischer et al 2008	FGF1
FGF1as	TGTTGTAATGGTTCTCCTCCA			
FGF2s	CTGTACTGCAAAAACGGG	45a45c		FGF2
FGF2as	AAAGTATAGCTTTCTGCC			
FGF3s	CCTAATCTGGCTGCTACTG	53anneal	Self-made	FGF3
FGF3as	CCTCCACTGCCGTTATCTC			
FGF4s	ACTACCTGCTGGGCATCAAGCG	56a45c	Fischer et al 2008	FGF4
FGF4as	TCTTGCTCAGGGCGATGAACATG			
FGF5s	CCCGGATGGCMAGTCAATGG	61a40c		FGF5
FGF5as	TTCAGGGCAACATACCACTCCC			
FGF6s	AACGTGGGCATCGGCTTTCACCT	56a40c		FGF6
FGF6as	CCCGCTTTACCCGTCATTTGC			
FGF7s	CTTTGCTCTACAGATCATGCTTTC	60a40c		FGF7
FGF7as	TTGCCATAGGAAGMAGTGGGCT			
FGF8s	TGAGCTGCCTGCTGTTGCACTTG	60a40c		FGF8
FGF8as	CTTGGCGATCAGCTTCCCCTTCTT			
FGF9s	AATGTGCCCGTGTTGCCGGTG	56a40c		FGF9
FGF9as	AATTTTCTGGTGCCGTTTAGTCCTA			
FGF10s	CCAAGAAGGAGAACTGCC	54a40c		FGF10
FGF10as	CCATTCAATGCCACATAC			

FGF16s	ATCTACACGGCTTCTCCTC	50anneal	Self-made	FGF16
FGF16as	TCTCCTCGCTCATTCATTC			
FGF17s	TGCTGCCCAACCTCACTC	53a35c	Fischer et al 2008	FGF17
FGF17as	TCTTTGCTCTTCCCGCTG			
FGF18s	ACTTGCCTGTGTTTACACTTCC	53a35c		FGF18
FGF18as	CCAGAACCTTCTCGATGAAC			
FGF19s	CGGATCTCCTCCTCGAAAG	60a45c		FGF19
FGF19as	GTGTGGTGGTCCACGTATG			
FGF20s	ACAGCCTCTTCGGTATCT	56a40c		FGF20
FGF20as	GGATCCACTGGTCTAGGTAA			
FGF21s	TTCTGTGCTGGCTGGTCTT	56a40c		FGF21
FGF21as	CACAGGAACCTGGATGTCTTG			
FGF22s	CCTCTTCTCCTCCACTCACTTC	50anneal	Self-made	FGF22
FGF22as	TGCTTTGATGACCACGAC			
FGF23s	CTGGCTTTGTGGTGATTA	50anneal		FGF23
FGF23as	GGAAGTGATACTGAGGAGAG			

GAPDHs	GAGAACGGGAAGCTTGTCAT	51anneal	Grunt et al 2005	GAPDH
GAPDHas	TTCAGCTCAGGGATGACCTT			

FR5	TCCAGTGGCTAAAGCACATC		Fischer et al 2008	
FR7	CCGCATCCGAGCTATTAATC	FR5-FR7 52anneal		FGFR-1b
FR8	CGCCAAGCACGTATACTC	FR5-FR8 50anneal		FGFR-1c
FR11	AACGGGAAGGAGTTTAAGCAG			
FR12	TGGCAGAACTGTCAACCATGC	FR11-FR12 52anneal		FGFR-2c
FR13	CTCGGTCACATTGAACAGAG	FR11-FR13 51anneal		FGFR-2b
FR17	AACGGCAGGGAGTTCCGCGGC			
FR19	CCCGTCCCGCTCCGACACATTG	FR17a-FR19 60anneal		FGFR-3b
FR20	CCCGGCGTCCTCAAAGGTG	FR17a-FR20 56anneal		FGFR-3c
FR25	GATGGACAGGCCTTTCATGG			
FR26	TGCTGCGGTCCATGTGGGGTCCTC	FR25-FR26 52anneal		FGFR-4

Self-designed primers were checked for secondary structures using NetPrimer (Premier Biosoft Int., Palo Alto CA). T_M was calculated by the nearest neighbour analysis formula provided by OligoCalc (Kibbe 2007).

Detection (Agarose gel electrophoresis)

1,5g agarose in 100ml 1xTAE (40mM Tris-acetate, 1mM EDTA in aqua bidest) were heated for 80 seconds in the microwave oven. After adding 10µl of Gel Red, the fluid was poured into the assembled electrophoresis chamber (15- or 20-slot comb). After the gel had polymerized, 750ml 1xTAE were added. Subsequently, 25µl of the DNA-Marker and the complete PCR products were applied and separated using 70V/210mA. The run was stopped after the yellow dye ran out of the gel and PCR products were photographed using a Herolab gel documentation system.

Identification of signal peptides and transmembrane domains

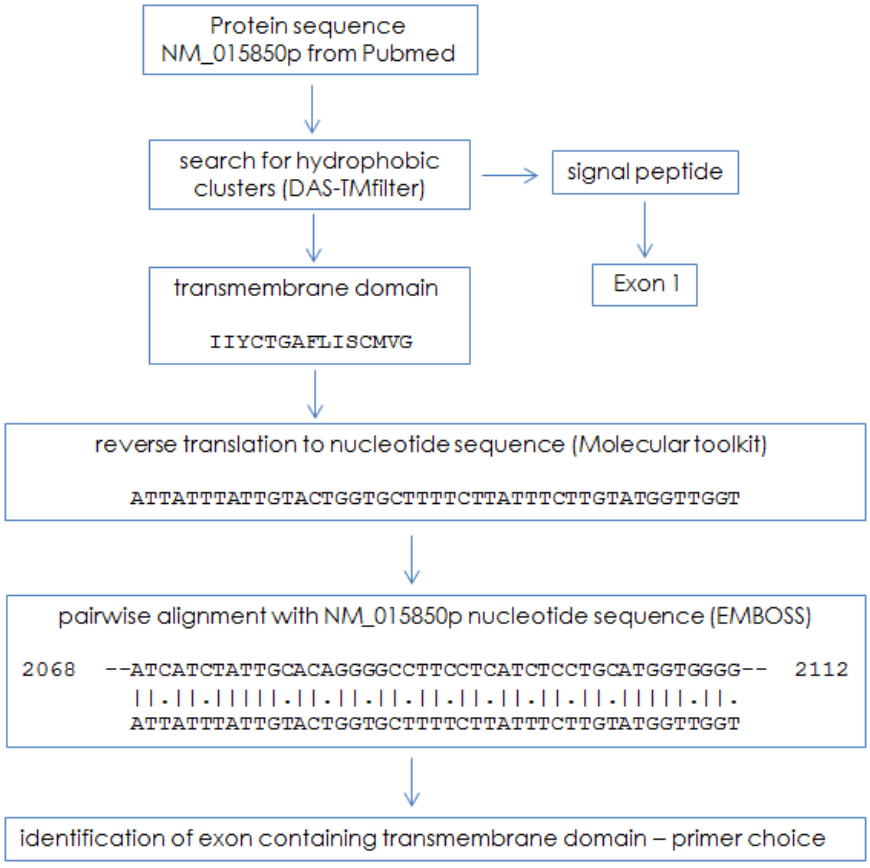


Figure 6 | **Overview of working steps for identification of transmembrane domains and signal peptides, exon identification and primer choice** by the example of FGFR-1 transcript variant 2 (accession number NM_015850). Details are described in the text.

Figure 6 gives a short overview of the workflow for identification of the transmembrane domain, exon identification and resulting primer choice. Every single step is described below in more detail. The whole procedure was done for all existing transcript variants of FGF receptors 1-4 (data not shown).

Hydrophobic cluster prediction

MWSWKCLLFWAVLVTATLCTARPSPTLPEQAQPWGAPVEVESFLVHPGDLLQLRCRLRDDVQSINWLRDGVQLAESNRTRITGEE
VEVQDSVPADSGLYACVTSSPSGSDTTYFSVNVSDALPSSEDDDDDDDSSSEEKETDNTKPNPVAPYWTSPEKMEKKLHAVPAAKTV
KFKCPSSGTPNPTLRWLKNGKEFKPDHRIGGYKVRYATWSIIMDSVVPSDKGNYTCIVENEYGSINHTYQLDVVERSPHRPILQAGLP
ANKTVALGSNVEFMCKVYSDPQPHIQWLKHIEVNGSKIGPDNLPYVQILKTAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNSIG
LSHHSAWLTVLEALEERPAVMTSPLYLEIIIYCTGAFLISCMVGSVIVYKMKSGTKKSDFHSQMAVHKLAKSIPLRRQVTVSADSSASMN
SGVLLVRPSRLSSSGTPMLAGVSEYELPEDPRWELPRDRLVLGKPLGEGCFGQVVLAEAIGLDKDKPNRVTKVAVKMLKSDATEKD
LSDLISEMEMMKMIGKHKNIINLLGACTQDGPLYVIVEYASKGNLREYLQARRPPGLEYCYNPSHNPEEQLSSKDLVSCAYQVARG
MEYLASKKCIHRDLAARNVLVTEDNVMKIADFGLARDIHHIDYYKKTTNGRLPVKWMAPEALFDRIYTHQSDVWSFGVLLWEIFTLG
GSPYPGVPVEELFKLLKEGHRMDKPSNCTNELYMMMRDCWHAVPSQRPTFKQLVEDLDRIVALTSNQEYLDLSMPLDQYSPSFPDT
RSSTCSSGEDSVFSHEPLPEEPCLPRHPAQLANGGLKRR

Table 7 | **Protein sequence of FGFR-1 transcript variant 2** (accession number NM_015850)

DAS-TMfilter (dense alignment surface) is able to identify hydrophobic clusters at high currency and with lower false positive rates than classic programs because it compares the results to experimentally documented proteins (Cserzo et al 2002, Cserzo et al 2004). Table 7 shows the complete protein sequence of FGFR-1 transcript variant 2 (NM_015850p).

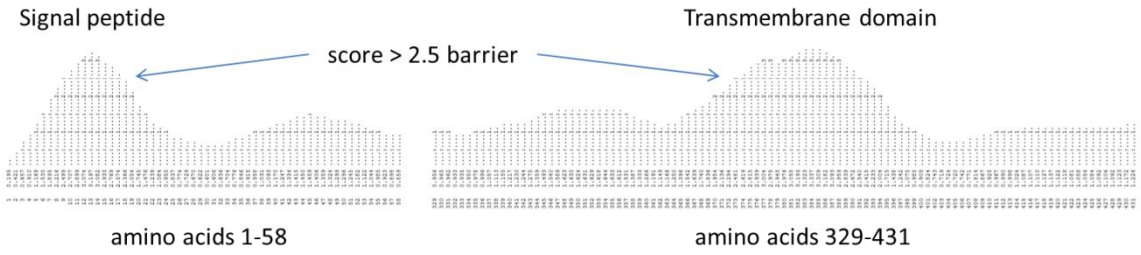


Figure 7 | **Summarized output of DAS-TMfilter for FGFR-1 transcript variant 2**. Hydrophobic clusters reaching a score of 2.5 are tagged as transmembrane regions.

DAS-TMfilter identified two hydrophobic clusters in this protein sequence. The first one has its maximum at 13 amino acids indicating a signal peptide and the second maximum is found at amino acid 384 (figure 7).

Reverse translation of proteins

The predicted transmembrane domain of FGFR-1 has the amino acid sequence "IIYCTGAFLISCMVG" and was translated back into the nucleotide sequence for comparison purposes. Molecular toolkit is a small package of useful programs for translation of nucleotide sequences into amino acid sequences and vice versa (Bowen 2008). The reverse translated nucleotide sequence of the predicted transmembrane domain is "ATTATTTATTGTACTGGTGCTTTTCTTATTTCTTGTATGGTTGGT".

Figure 8 | **Cutout of FGFR-1 transcript variant 2 with binding prediction for primers FR3, FR5, FR6 and FR8 established with Sequence extractor** (Stothard et al 2006). Numbers show the nucleotide position, green-colored primers are "forward" while red-colored primers are "reverse". Grey-colored text reflects the antisense-strand of the nucleotide sequence. Fragment lengths can be determined by clicking the respective primers.

Exon identification

"EMBOSS pairwise alignment" (Rice et al 2000) was used for sequence alignments of the predicted transmembrane domain with the complete nucleotide sequence of FGFR-1 transcript variant 2. Exon 10 was identified to contain the transmembrane domain of FGFR-1 (figure 6).

Primer binding and fragment length prediction

Sequence extractor (Stothard 2006) was used for primer binding and fragment length prediction (figure 8 shows a cutout of FGFR-1 transcript variant 2 with primers FR3, FR5, FR6 and FR8). The results were validated with UCSC in-silico PCR (Kent et al 2002).

5 Results

5.1 Analysis of fibroblast growth factor receptor splice variants

As shown in figures 9-12, fibroblast growth factor receptors exhibit a great variety in expression of splice variants. Although every single variant likely has its features, receptors with a transmembrane domain and a signal peptide for transport to the cell membrane were of major interest in our studies. In order to span cell membranes, transmembrane domains as well as signal peptides contain hydrophobic clusters which can be identified by bioinformatic methods as described before.

5.1.1 Fibroblast growth factor receptor 1

Exon 1 corresponds to the signal peptide and is present in all known splice variants of FGFR-1. Exons 2-9 represent immunglobuline (Ig)-like ligand binding domains with exons 8 and 9 determining IIIb- and IIIc-variants and therefore specificity in binding FGFs. Exon 10 contains the hydrophobic transmembrane domain. Exons 11-19 are situated in the cytoplasm and include the split tyrosine kinase domain and cofactor binding sites as described before. Variants 'b' of exons 3, 4, 7, 17 and 18 consist of corresponding variant 'a' plus a few nucleotides. Primers FR5 (forward) and FR7/FR8 (reverse) were chosen for expression analysis resulting in detection of all splice variants with a transmembrane domain while distinguishing between splice variant IIIb and IIIc.

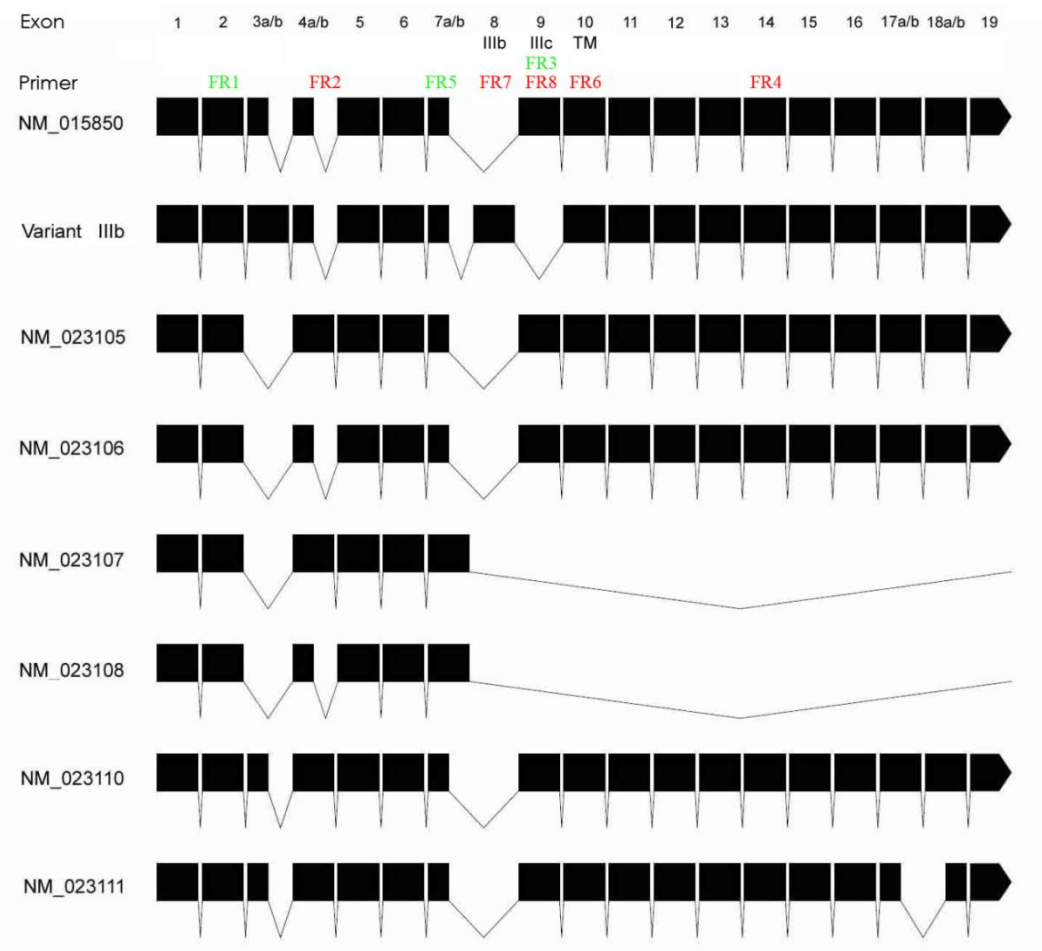


Figure 9 | **FGFR-1 splice variants and primer binding**. Green-colored primer names indicate forward, red-colored primer names reverse primers. Variant IIIb was identified by BLAT (Kent 2002) search with FGFR-1 transcript variant 2 (NM_015850) as input and does not have an accession number yet.

5.1.2 Fibroblast growth factor receptor 2

The signal peptide is only present in 4 out of 8 splice variants with a transmembrane domain. Exons 2-10 represent ligand binding domains, where exons 9 and 10 determine splice variants IIIb and IIIc. Both forms of exon 11, 'a' and 'b', include the functional transmembrane domain. 'B'-forms of exons 3 and 21 contain the corresponding 'a'-form plus a few nucleotides. Exons 12-21 encode the split tyrosine kinase domain and cofactor binding sites as described before. Primer pairs FR11 (forward) and FR12/FR13 (reverse) enabled detection of all splice variants containing the transmembrane domain and a IIIb/IIIc-determining exon.

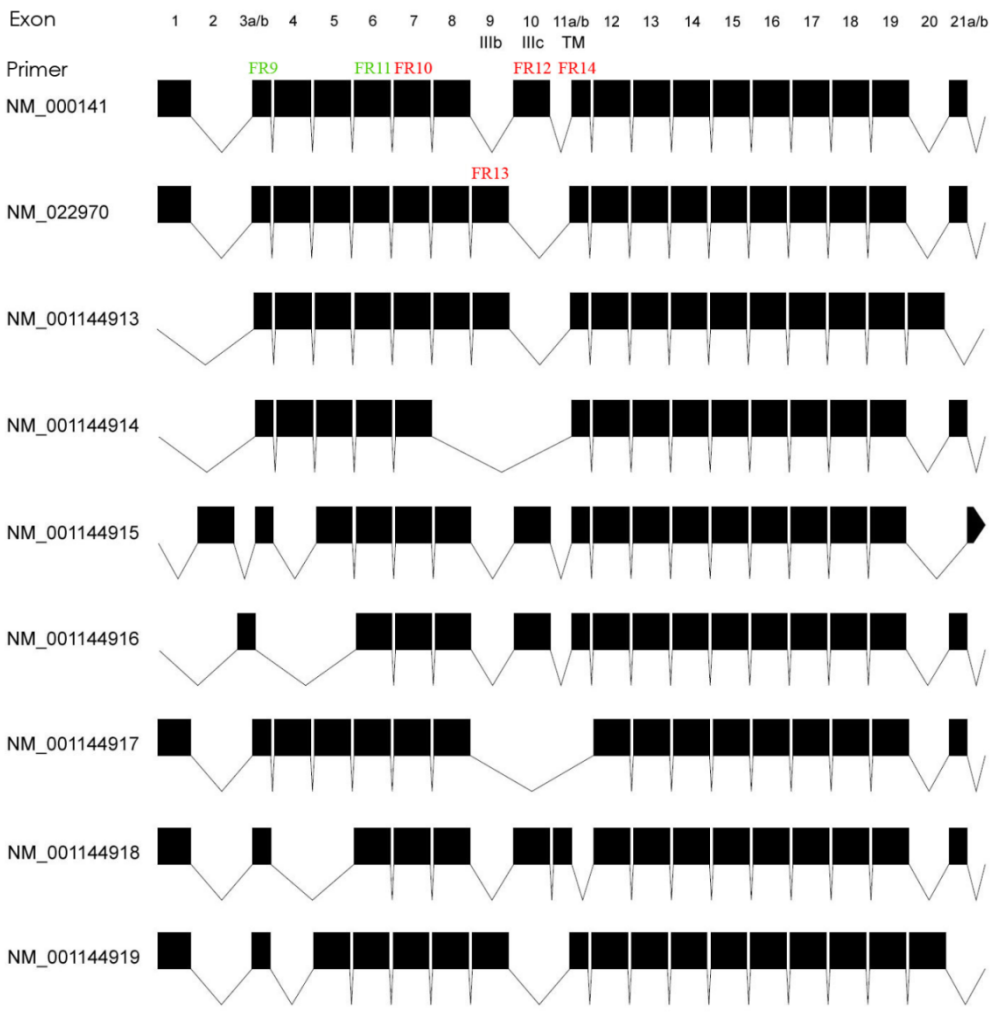


Figure 10 | **FGFR-2 splice variants and primer binding**. Green-colored primer names indicate forward, red-colored primer names reverse primers.

5.1.3 Fibroblast growth factor receptor 3

There exist only three different splice variants of FGFR-3. NM_000142 and NM_022965 are membrane-bound and represent isoforms IIIb and IIIc (exons 8 and 9). NM_001163213 does not exhibit a transmembrane domain as well as no exon for determining the IIIb/IIIc splice variant. Therefore, primers FR17a (forward) and FR19/FR20 (reverse) were chosen for expression analysis.

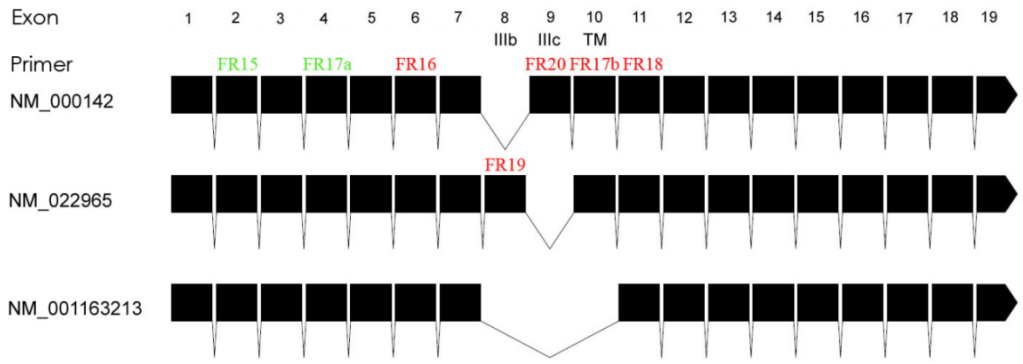


Figure 11 | **FGFR-3 splice variants and primer binding**. Green-colored primer names indicate forward, red-colored primer names reverse primers.

5.1.4 Fibroblast growth factor receptor 4

As described above, no IIIb/IIIc splice variants of FGFR-4 do exist. The 'b'-form of exons 1 and 10 contain the complete 'a'-form plus a few nucleotides. As shown in figure 12, splice variant NM_022963 does not contain a signal peptide or a transmembrane domain. To exclude this isoform, primers FR25 (forward) and FR26 (reverse) were chosen for expression analysis.

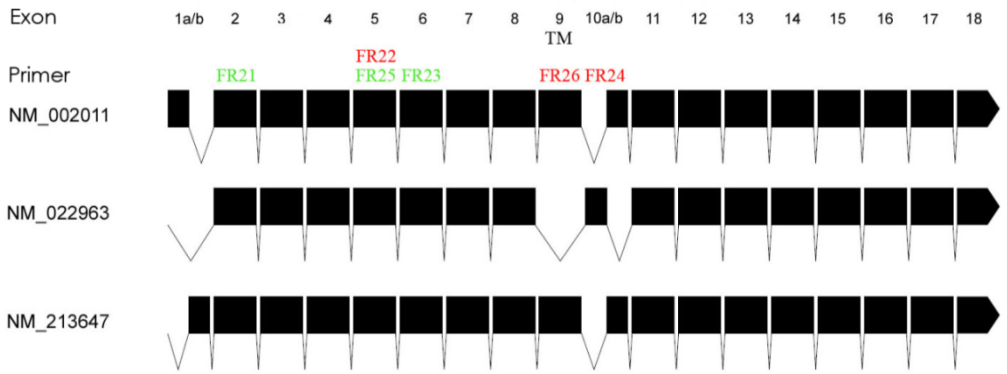


Figure 12 | **FGFR-4 splice variants and primer binding**. Green-colored primer names indicate forward, red-colored primer names reverse primers.

5.2 Expression profile of FGFR isoforms in ovarian cancer cell lines

Figure 13 shows the expression pattern of FGFR isoforms in ovarian cancer cell lines. The two images represent independent experiments with different exposure times to account for the lower mRNA expression of primary ovarian surface epithelium (OSE) cells.

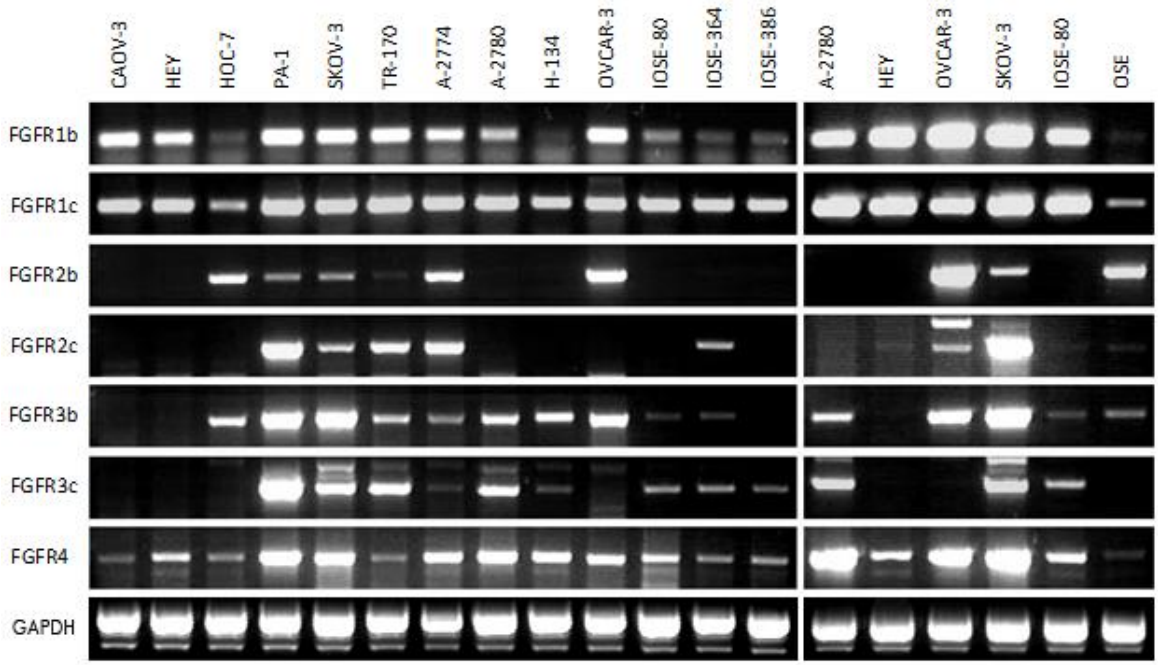


Figure 13 | **RT-PCR expression profile of fibroblast growth factor receptor (FGFR) isoforms in human ovarian cancer cell lines.** CAOV-3, HEY, HOC-7, SKOV-3, TR-170, A2774, A-2780, H-134 and OVCAR-3 represent cancer cell lines derived from ovarian adenocarcinomas. PA-1 is derived from a teratocarcinoma. IOSE-80, IOSE-364 and IOSE-386 cell lines were obtained by immortalization of human ovarian surface epithelium. OSE cells were isolated from primary ovarian surface epithelium. The two images represent independent experiments with different exposure times to account for the lower FGF receptor mRNA expression of primary ovarian surface epithelium (OSE) cells.

FGFR-1b is expressed by all cell lines, but cancer cell lines except H-134 and HOC-7 show much stronger expression than OSE cells. FGFR-1c is evenly expressed among cancer and immortalized cell lines and shows a stronger expression than in OSE cells. FGFR-4 gives a similar picture of expression among cancer cell lines and immortalized cells, which do

express considerably more FGFR-4 than OSE cells. When taking a closer look to the expression of FGFR-2 and FGFR-3 isoforms, cancer cell lines can be divided into 6 groups from expressing none to all FGFR-2 and FGFR-3 transcript variants (Table 8).

	FGFR-2b	FGFR-2c	FGFR-3b	FGFR-3c
CAOV-3	-	-	-	-
HEY	-	(+)	-	-
HOC-7	+	-	+	-
OVCAR-3	+	+	+	-
A-2780	-	-	+	+
H-134	-	-	+	(+)
PA-1	+	+	+	+
SKOV-3	+	+	+	+
TR-170	(+)	+	+	+
A-2774	+	+	+	(+)

Table 8 | **Different expression patterns of FGFR-2 and FGFR-3 isoforms in ovarian cancer cell lines.** "+" normal expression, "(+)" borderline expression, "-" no expression.

Due to the distribution in expressing different FGFR isoforms and favorable growth properties, A-2780, HEY, OVCAR-3 and SKOV-3 were chosen for further investigations (Table 9)

	1b	1c	2b	2c	3b	3c	4
A-2780	+	+	-	-	+	+	+
HEY	+	+	-	(+)	-	-	+
OVCAR-3	+	+	+	+	+	-	+
SKOV-3	+	+	+	+	+	+	+

Table 9 | **Expression of FGFR transcript variants in A-2780, HEY, OVCAR-3 and SKOV-3 cancer cell lines**. "+" normal expression, "(+)" borderline expression, "-" no expression.

5.3 Expression profile of FGFs in ovarian cancer cell lines

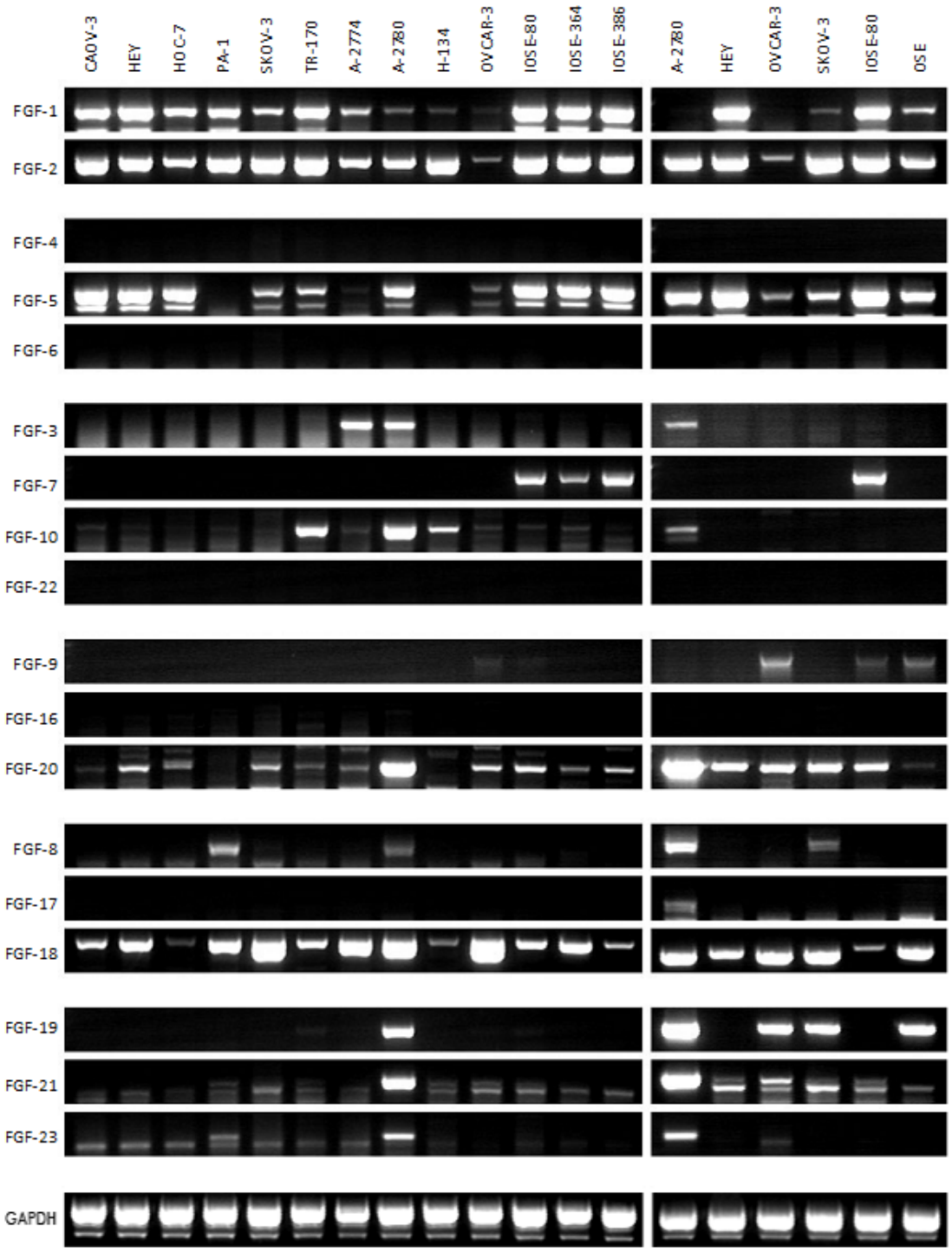


Figure 14 | **RT-PCR expression profile of fibroblast growth factors (FGFs) in human ovarian cancer cell lines.** CAOV-3, HEY, HOC-7, SKOV-3, TR-170, A2774, A-2780, H-134 and OVCAR-3 represent cancer cell lines derived from ovarian adenocarcinomas. PA-1 is derived from a teratocarcinoma. IOSE-80, IOSE-364 and IOSE-386 cell lines were obtained by immortalization of human ovarian

surface epithelium. OSE cells were isolated from primary ovarian surface epithelium. FGFs are arranged by intrinsic relationship as described in figure 3. The two images represent independent experiments with different exposure times to account for the lower FGF mRNA expression of primary ovarian surface epithelium (OSE) cells.

As previously described, fibroblast growth factors can be arranged in subfamilies by homology and binding properties. The expression of FGF mRNAs was therefore arranged by subfamily identity as mentioned before. FGF-1, FGF-2, FGF-5, FGF-20 and FGF-18 are expressed by the majority of ovarian cancer cell lines (figure 14). FGF-3, FGF-8, FGF-10, FGF-19, FGF-21 and FGF-23 are only expressed by some cell lines. A-2780 is expressing the greatest number of FGFs by far (figure 14). It has to be noted that binding of FGF-19, FGF-21 and FGF-23 to its receptors is dependent on the presence of a co-receptor (α-/β-Klotho). The relevance of these latter growth factors regarding survival, proliferation and migration of ovarian cancer cells can therefore be questioned (Sinha et al 2008, Urakawa et al 2006).

5.4 Potential autocrine signaling loops in ovarian cancer cells

Fibroblast growth factors and their receptors show a distinct pattern of tissue-specific expression, enabling cell communication while preventing undesired autocrine stimulation. As was shown for many different tissues, cancer cells corrupt this system.

All ovarian cancer cell lines show de novo expression of FGF-10, a strong activator of FGFR-2 IIIb, which can be found in normal ovarian surface epithelial cells. CAOV-3, HEY and H-134 are the only ovarian cancer cells that do not express a combination of FGFs/FGFRs with a high mitogenic potential. HOC-7, A-2774, TR-170 and OVCAR-3 cells do express the combination FGF-10/FGFR-2 IIIb, but FGF-10 or FGFR-2 IIIb expression is very low. SKOV-3 cells do express low levels of FGF-10 and normal levels of

CAOV-3

	5 +	10 (+)	20 (+)	18 +
1b +		39,4		
1c +	59		28,1	
4 (+)			26,6	52,8

HEY

	5 +	10 (+)	20 +	18 +
1b +		39,4		
1c +	59		28,1	
2c (+)	25		68,4	28,9
4 +			26,6	52,8

HOC-7

	5 +	10 (+)	20 +	18 +
1b (+)		39,4		
1c +	59		28,1	
2b +		217		
3b +			44,3	
4 (+)			26,6	52,8

SKOV-3

	5 +	10 (+)	20 +	8 +	18 +
1b +		39,4			
1c +	59		28,1	57,5	
2b +		217			
2c +	25		68,4	91,6	28,9
3b +			44,3	18,6	
3c +			89,5	209	77,7
4 +			26,6	102	52,8

Table 10 | **Potential autocrine signaling loops of different ovarian carcinoma cells**. All cell lines show de novo expression of FGF-10, a strong activator of FGFR-2 IIIb, which is expressed in healthy ovarian surface epithelial cells. Several other potential autocrine signaling loops can be identified and are examined in detail in the text. + normal expression, (+) borderline or very low expression, green caption: approximately-equal expression level of ovarian surface epithelium (OSE) cells, green numbers: combination of FGF/FGFR found in OSE cells too; blue: overexpression in comparison to OSE cells, red: not expressed in OSE cells. Numbers are adapted from Zhang 2006 and describe relative mitogenic activities of FGFs at the respective FGF receptor proportional to the mitogenic activity of FGF-1 at this FGF receptor. White boxes: relative mitogenic activity <15% . 1b: FGFR-1 IIIb, 1c: FGFR-1 IIIc, 2b: FGFR-2 IIIb, 2c: FGFR-2 IIIc, 3b: FGFR-3 IIIb, 3c: FGFR-3 IIIc; 4: FGFR-4; 3: FGF-3, 5: FGF-5, 8: FGF-8, 9: FGF-9, 10: FGF-10, 17: FGF-17, 18: FGF-18, 20: FGF-20.

A-2774

	5 (+)	3 +	10 (+)	20 +	18 +
1b +		34,4	39,4		
1c +	59			28,1	
2b +		44,6	217		
2c +	25			68,4	28,9
3b +				44,3	
3c (+)				89,5	77,7
4 +				26,6	52,8

TR-170

	5 +	10 +	20 +	18 +
1b +		39,4		
1c +	59		28,1	
2b (+)		217		
2c +	25		68,4	28,9
3b +			44,3	
3c +			89,5	77,7
4 (+)			26,6	52,8

A-2780

	5 +	3 +	10 +	20 +	8 +	17 +	18 +
1b +		34,4	39,4				
1c +	59			28,1	57,5	22,7	
3b +				44,3	18,6		
3c +				89,5	209	111	77,7
4 +				26,6	102	85,5	52,8

OVCAR-3

	5 (+)	10 (+)	9 +	20 +	18 +
1b +		39,4			
1c +	59			28,1	
2b +		217			
2c +	25		57,2	68,4	28,9
3b +			42,7	44,3	
4 +				26,6	52,8

PA-1

	10 (+)	8 +	18 +
1b +	39,4		
1c +		57,5	
2b +	217		
2c +		91,6	28,9
3b +		18,6	
3c +		209	77,7
4 +		102	52,8

OSE

	5 +	9 +	20 (+)	18 +
1b (+)				
1c +	59		28,1	
2b +				
2c (+)	25	57,2	68,4	28,9
3b (+)		42,7	44,3	
4 (+)			26,6	52,8

H-134

	10 +	18 +
1b (+)	39,4	
1c +		
3b +		
3c (+)		77,7
4 +		52,8

FGFR-2 IIIb but show high de novo expression of FGFR-3 IIIc and overexpression of FGFR-4 together with their highly activating ligand FGF-8. A-2780 is not expressing FGFR-2 IIIb but shows de novo expression of FGFR-3 IIIc together with its highly activating ligands FGF-8 and FGF-17 and overexpression of FGFR-4 which is activated by FGF-8 too. Numerous other combinations of ligands and receptors can be found where one or both parts are overexpressed in different ovarian cancer cells. Since their mitogenic potential is below or approximately on par with autocrine signaling loops found in normal ovarian surface epithelial cells they are not considered as high potential candidates for the identification of autocrine signaling loops in ovarian cancer cells.

5.5 Modulation of Ras/MAPK and PI3K downstream effectors by FGF-1, FGF-2 and conditioned growth medium in ovarian cancer cells

One of the first questions when studying a signaling pathway is to verify its functionality in the investigated cell lines. As described previously, FGF-1 is the most promiscuous FGFR ligand and capable of activating all FGFRs. FGF-2 is only binding to IIIc-variants of FGF receptors 1-3 and to FGFR-4 but provides strong proliferation signals in different cell lines. As shown in figure 14, ovarian cancer cells express various FGF ligands suggesting autocrine mechanisms providing survival or proliferation signals. Therefore, serum-depleted cells were either treated with 5nM FGF-1 or FGF-2 or 1ml of conditioned growth medium (CM) for 5 or 10 minutes.

5.5.1 FGF-1 and FGF-2 trigger activation of the Ras/MAPK pathway, but not of the PI3K-pathway in A-2780, OVCAR-3 and SKOV-3 ovarian cancer cells

As shown in figure 15, FGF-1 and FGF-2 trigger activation of the Ras/MAPK (ERK1/2), but not the PI3K (AKT, S6) pathway. Interestingly, FGF-2 causes a much stronger phosphorylation of ERK1/2 than FGF-1 in A-2780 and OVCAR-3, but not in SKOV-3 cells. Although the situation with conditioned growth medium (CM) is not as clear, ERK1/2 is phosphorylated in all three cell lines when treating cells with conditioned growth medium.

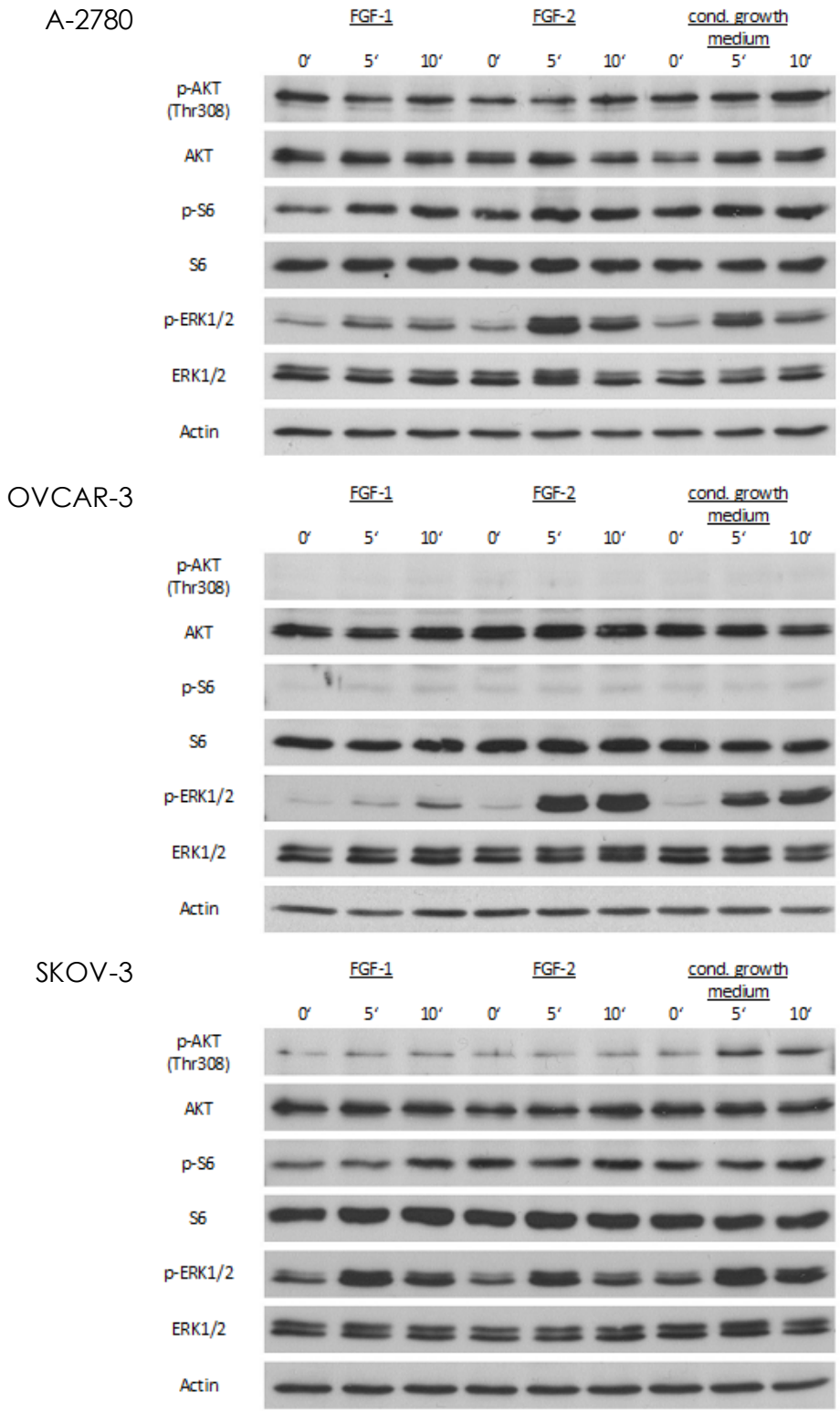


Figure 15 | **Ligand-dependent phosphorylation of fibroblast growth factor receptor (FGFR) downstream effectors in ovarian cancer cells in vitro**.
FGF-1, FGF-2 and conditioned growth medium induce phosphorylation of ERK1/2 by different degrees, but do not trigger activation of the PI3-kinase pathway (AKT, S6). Serum depleted cells were stimulated with 5nM of FGF-1 or FGF-2 or 1ml of conditioned growth medium for 5 or 10 minutes.

5.5.2 FGF-1 and FGF-2 are not capable of activating Ras/MAPK in HEY ovarian cancer cells

FGF-1 and FGF-2 are not able to trigger activation of Ras/MAPK pathway and only show minor induction of PI3K pathway in HEY cells in vitro. Failure of Ras/MAPK activation is due to the fact that phosphorylation levels of ERK1/2 are already very high (exposure time of pERK in figure 16 is as short as feasible). Conditioned growth medium is able to activate the PI3K pathway to a higher degree than FGF-1 or FGF-2 alone, suggesting that HEY cells release growth factors besides FGFs which are capable of signaling in an autocrine manner.

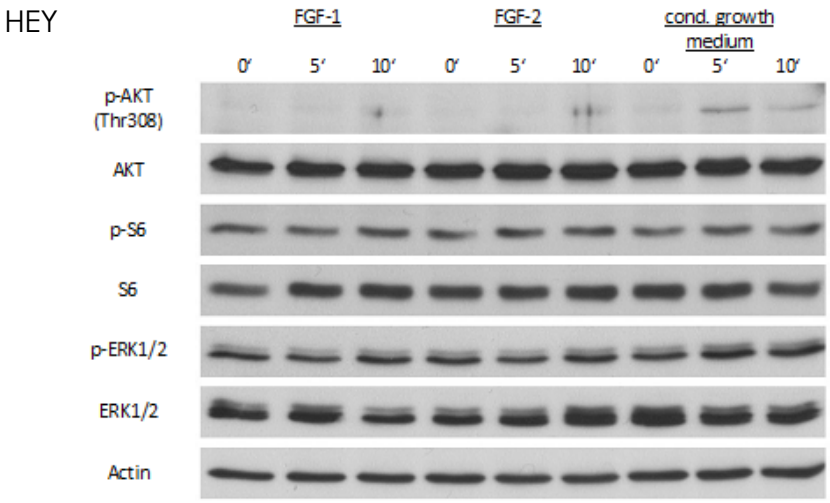


Figure 16 | **Ligand-dependent phosphorylation of fibroblast growth factor receptor (FGFR) downstream effectors in HEY ovarian cancer cells**. FGF-1, FGF-2 and conditioned growth medium are not able to induce phosphorylation of ERK1/2 because it is already highly activated in HEY cells. Conditioned growth medium triggers phosphorylation of PI3K pathway (p-AKT). Serum depleted cells were stimulated with 5nM of FGF-1 or FGF-2 or 1ml of conditioned growth medium for 5 or 10 minutes.

5.6 Influence of FGF-1 and FGF-2 on proliferation of ovarian cancer cells in vitro

To work out the effects of FGF ligands on cell growth, a cell proliferation assay was performed. Cells were seeded and allowed to adhere overnight before treating them with different concentrations of FGFs (0-0,1-0,5-1-5-10 nM) for 72 hours.

5.6.1 FGF-2 but not FGF-1 induces cell proliferation in A-2780 and HEY ovarian cancer cells

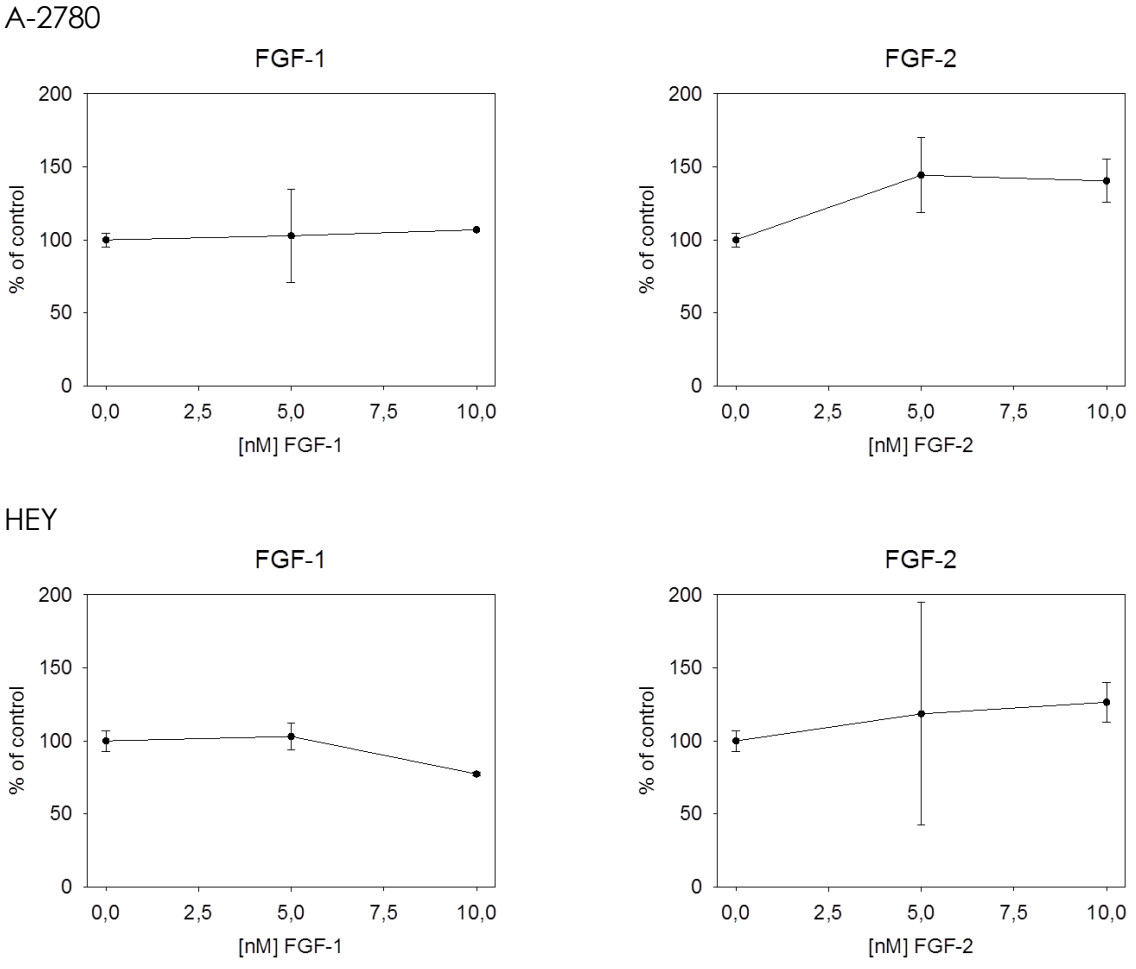


Figure 17 | **Effects of FGF-1 and FGF-2 on growth of A-2780 and HEY ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth stimulation after 72 hours of treatment with FGF-1 or FGF-2.

As shown in figure 17, FGF-2 is able to stimulate proliferation of A-2780 cells by 40% and of HEY cells by 26,5% in proportion to untreated cells while FGF-1 is not capable of doing so.

5.6.2 Neither FGF-1 nor FGF-2 induce cell proliferation in OVCAR-3 and SKOV-3 ovarian cancer cells

As shown in figure 18, neither FGF-1 nor FGF-2 is able to induce proliferation in OVCAR-3 and SKOV-3 ovarian cancer cells in vitro. This is particularly surprising for OVCAR-3 due to the strong phosphorylation of ERK1/2 when stimulated with FGF-2 (figure 15).

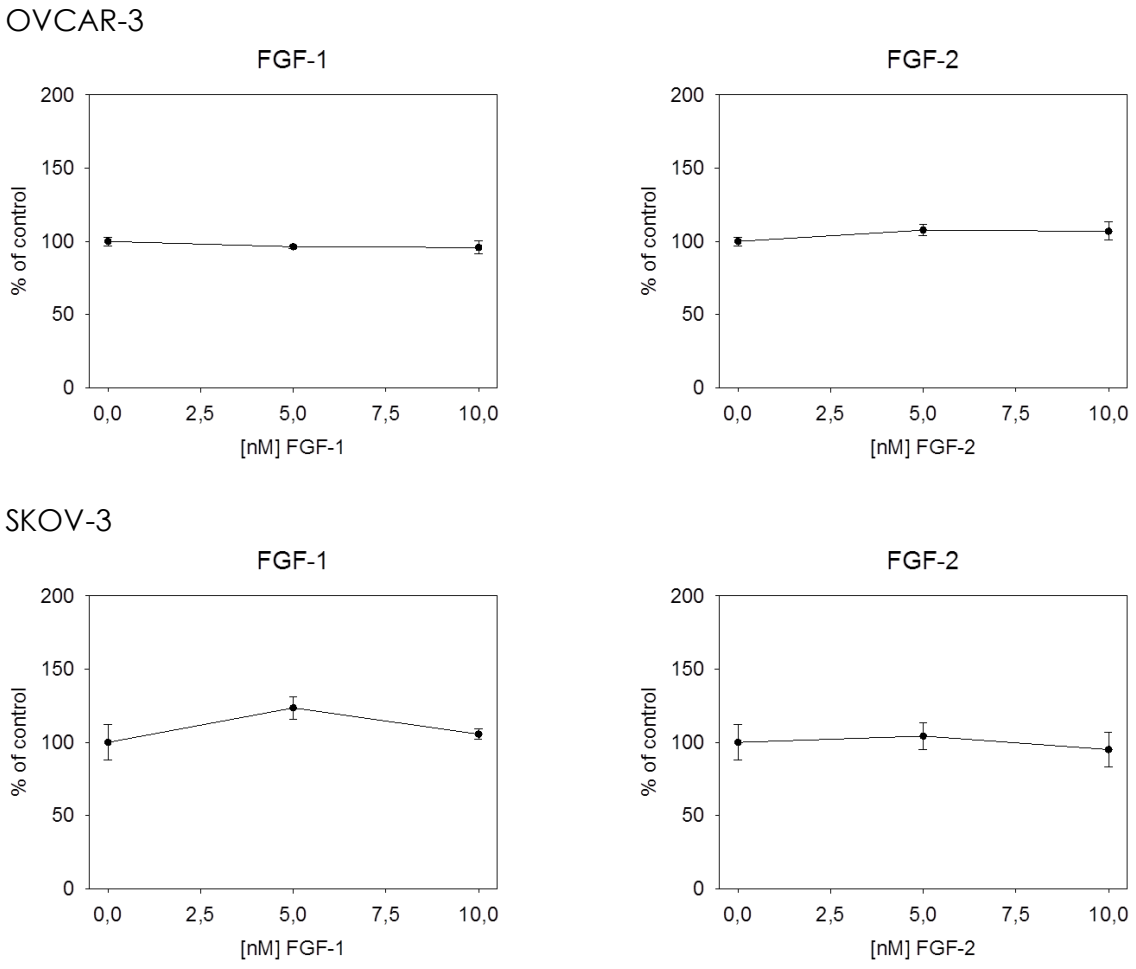
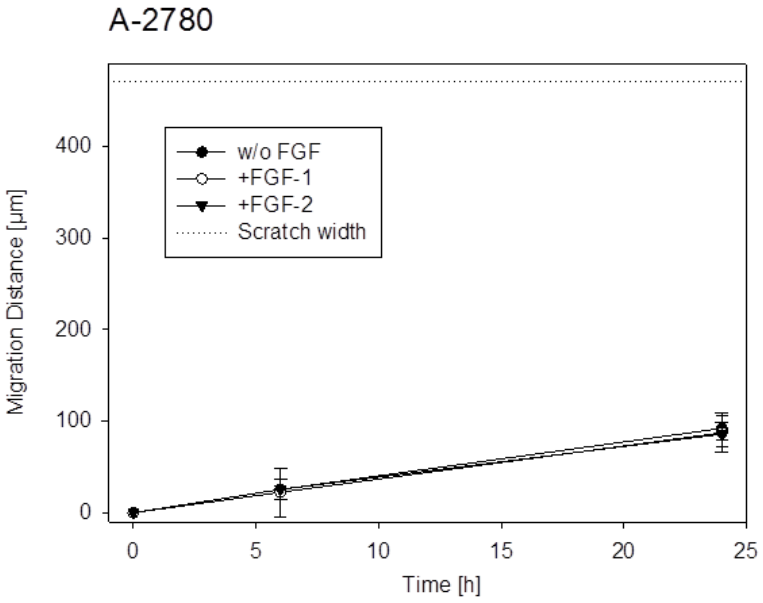


Figure 18 | **Effects of FGF-1 and FGF-2 on growth of OVCAR-3 and SKOV-3 ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth stimulation after 72 hours of treatment with FGF-1 or FGF-2.

5.7 Effects of fibroblast growth factors on migration of A-2780, HEY and SKOV-3 ovarian cancer cells

Tissue invasion and metastasis are the major sources of mortality in cancer patients and this eminently applies to ovarian cancers. To determine the influence of fibroblast growth factors on the migration of ovarian cancer cells, the scratch-assay protocol of Liang et al 2007 was adapted to enable statistical analysis. Unfortunately, it is necessary to seed the cells at very high densities and OVACR-3 cells failed to adhere when doing so (it is not possible to let the cells grow until reaching confluence since they generate an extracellular matrix-like structure which prevents migration, Liang et al 2007). Data can therefore only be shown for A-2780, HEY and SKOV-3 cells. Treatment with FGF-2 was only done in A-2780 cells as the most promising cell line due to the strong phosphorylation of ERK1/2 (figure 15).



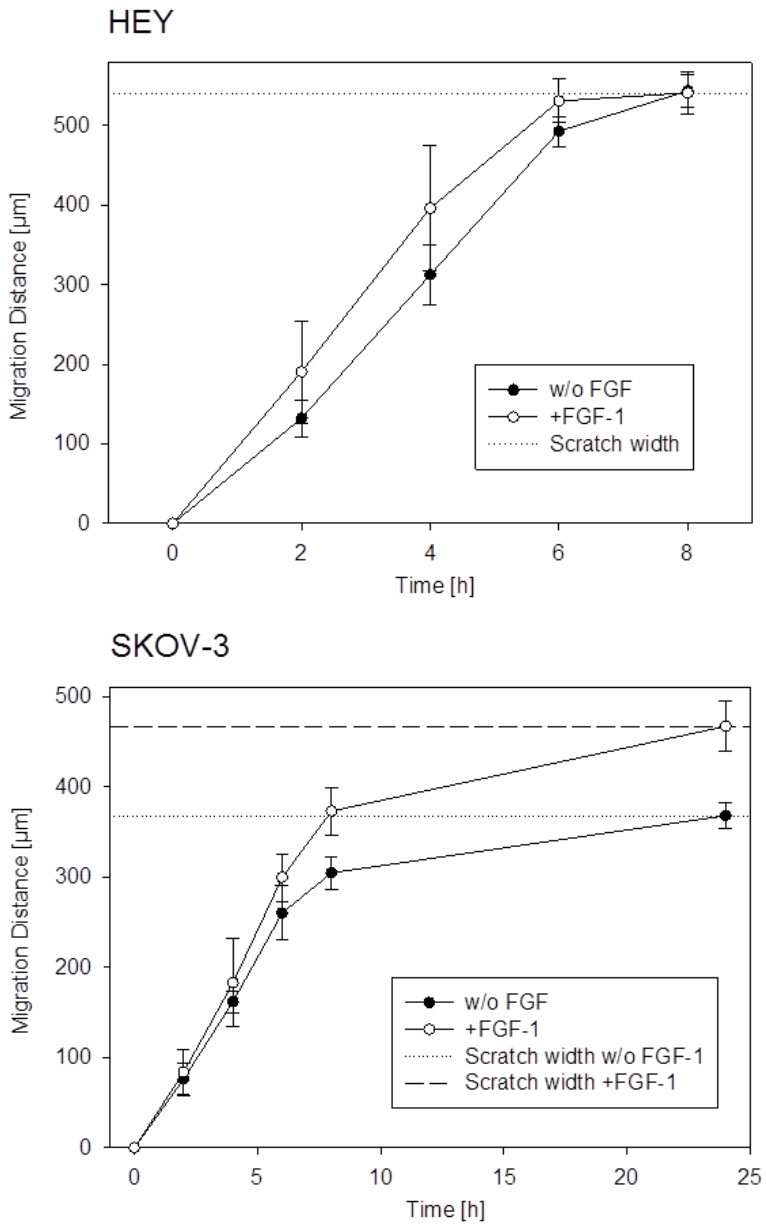


Figure 19 | **Effects of FGF-1 and FGF-2 on migration of A-2780, HEY and SKOV-3 ovarian cancer cells.** Migration in response to 5nM FGF-1 or FGF-2 versus untreated. Calculation of migration distance: mean g_t – mean g_0 (g… gap width, t… time points, n ≥ 5).

A-2780 cells migrate very slowly when compared to HEY and SKOV-3 cells and they are not able to close the scratch in a time-frame of 48 hours (data not shown) and neither FGF-1 nor FGF-2 is able to induce migration in those cells. HEY and SKOV-3 cells are much more versatile and close the

scratch after around 6 hours. Treatment with FGF-1 activates migration in SKOV-3 and to a higher degree in HEY cells, but the increase is non-significant in both cases and further experiments are necessary to confirm these results.

5.8 Impact of FGFR inhibition on survival and proliferation of ovarian cancer cells in vitro

To study the dependence of ovarian cancer cells on FGF signaling, two different tyrosine-kinase inhibitors were used. PD173074 is inhibiting FGFR 1-3 and FGFR-4 at higher doses, while Dovitinib (CHIR-258) is a more promiscuous inhibitor (see table 6 for in vitro kinase assay data). However, the major targets of Dovitinib are FGFR-1, FGFR-3 and VEGFR-2. Cell lines were treated with different concentrations of inhibitors (0,1 – 0,5 – 1 – 5 – 10µM) and cell numbers were determined after 72 hours of treatment.

5.8.1 Fibroblast growth factor receptor signaling is crucial for proliferation of A-2780 ovarian cancer cells

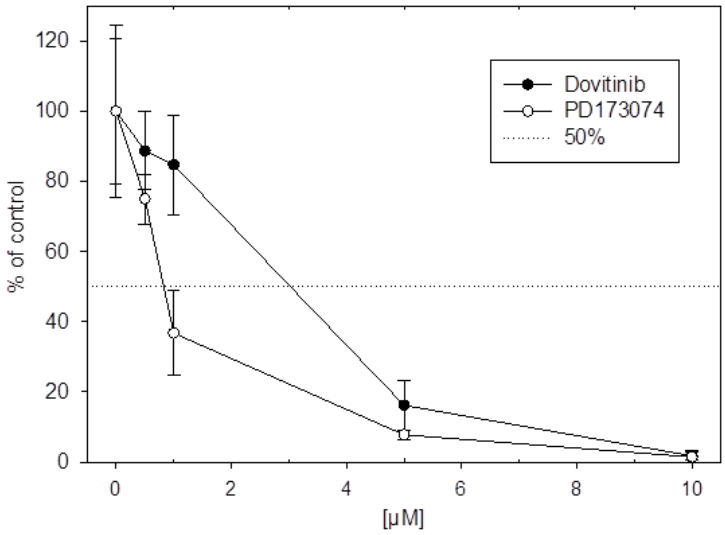


Figure 20 | **Effects of tyrosine-kinase inhibitors Dovitinib (CHIR-258) and PD173074 on growth of A-2780 ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth inhibition after 72 hours of treatment with Dovitinib or PD173074.

FGFs are able to induce proliferation of A-2780 cells in vitro (figure 17) and trigger strong phosphorylation of ERK1/2 (figure 15). As shown in figure 20, A-2780 cells are highly sensitive to inhibition of FGFR in vitro. Corresponding IC_{50}-values are 0,83µM for PD173074 and 3,03µM for Dovitinib.

5.8.2 PD173074 but not Dovitinib (CHIR-258) inhibits HEY ovarian cancer cell proliferation

Although it is not possible to stimulate proliferation of HEY cancer cells with FGFs, FGF receptor signaling might provide ligand-independent benefits for cell proliferation and survival. As shown in figure 21, HEY cells are sensitive to PD173074 (IC_{50} = 3,29µM) but resistant to treatment with Dovitinib.

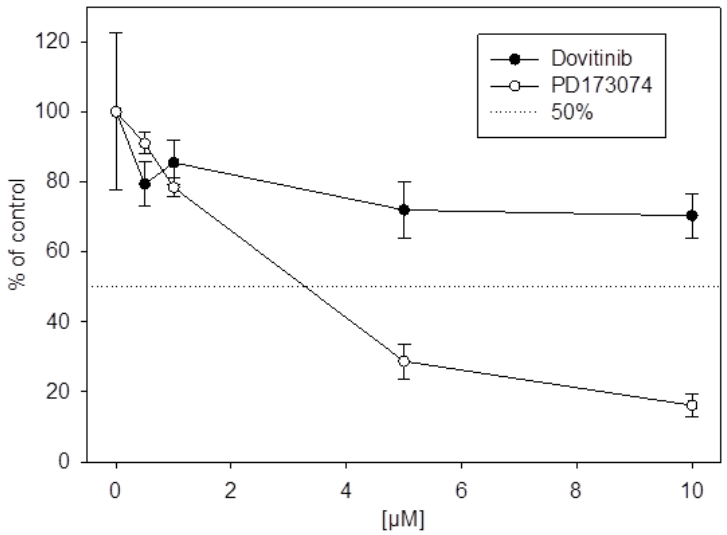


Figure 21 | **Effects of tyrosine-kinase inhibitors Dovitinib (CHIR-258) and PD173074 on growth of HEY ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth inhibition after 72 hours of treatment with Dovitinib or PD173074.

5.8.3 Neither PD173074 nor Dovitinib (CHIR-258) inhibits OVCAR-3 ovarian cancer cell proliferation at clinical relevant concentrations

As shown in figure 22, OVCAR-3 cells are resistant to PD173074 treatment and show only minor sensitivity to treatment with Dovitinib (IC_{50} = 8,44µM).

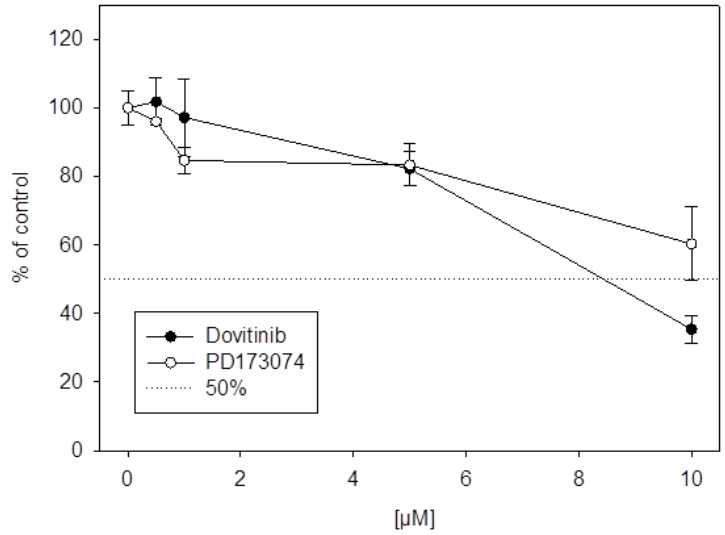


Figure 22 | **Effects of tyrosine-kinase inhibitors Dovitinib (CHIR-258) and PD173074 on growth of OVCAR-3 ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth inhibition after 72 hours of treatment with Dovitinib or PD173074.

5.8.4 PD173074 and Dovitinib (CHIR-258) exhibit similar dose response curves on inhibition of SKOV-3 ovarian cancer cell proliferation

As shown in figure 23, PD173074 and Dovitinib show dose-responsive inhibition of SKOV-3 cell proliferation at doses that can be reached clinically. Corresponding IC_{50}-values are 4,62µM for PD173074 and 4,81µM for Dovitinib.

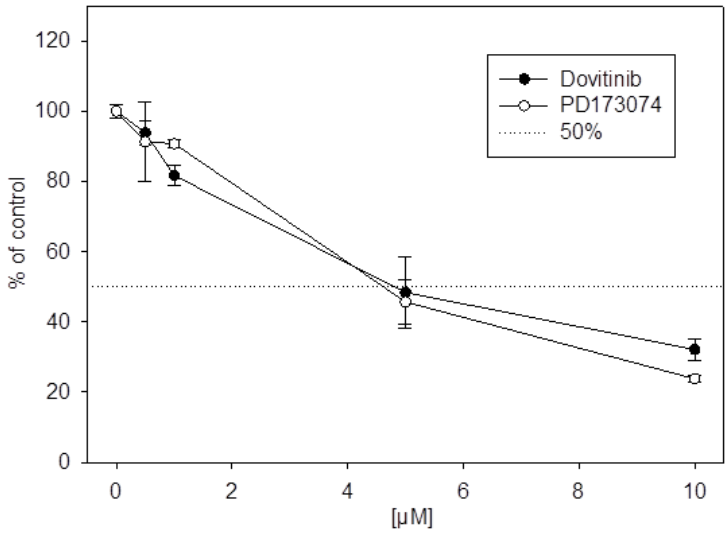


Figure 23 | **Effects of tyrosine-kinase inhibitors Dovitinib (CHIR-258) and PD173074 on growth of SKOV-3 ovarian cancer cells.** Crystal violet staining. Means ± SD, n=3. Dose-dependent growth inhibition after 72 hours of treatment with Dovitinib or PD173074.

6 Discussion

The majority of ovarian cancer cell lines show overexpression of FGFR-1c (9/9, 100%), FGFR-1b (7/9, 78%) and FGFR-3b (7/9, 78%) in comparison to OSE cells and at least half of them (>5/9, >56%) overexpress FGFR-4 and show de novo expression of FGFR-3c. Additionally, 44% of the investigated cell lines lost expression of FGFR-2b. When taking a closer look at potential signaling loops, some restrictions have to be considered. Although FGF-1 and FGF-2 are translocated through the cell membrane, they normally stay bound to the cell surface and are only released if the cell is damaged. This makes it unlikely that they trigger autocrine downstream signaling (Malecki et al 2004). FGF-5 was originally published as a proto-oncogene with transforming potential (Zhan et al 1988) but was later shown to be induced by serum growth factors like EGF, PDGF and TGF-a (Werner et al 1991). The expression must therefore be considered a cell culture derived artifact, but as shown in table 10, FGF-5 is not a strong activator of the expressed FGF receptors anyway. The de novo expression of FGF-7 in immortalized ovarian surface epithelium cell lines (IOSE-80, IOSE-364, IOSE-386) might be a result of the genomic transformation during the immortalization process eliminating their use for comparison when evaluating fibroblast growth factor receptor and ligand expression in ovarian cancer cells. As mentioned before, the FGF-19 subfamily (FGF-19, FGF-21 and FGF-23) is dependent on the expression of Klotho proteins in the target cells in order to activate downstream signaling which have not been found in other tissues than kidney and liver. De novo expression of FGF-10, although very low in most cells, was found in all evaluated cell lines and the majority of them expressed its high affinity receptor FGFR-2b, leading to an autocrine signaling loop with a strong mitogenic potential. Several other potential autocrine loops could be identified too. In fact, CAOV-3, HEY and H-134 cells are the only evaluated cell lines without any

potential autocrine signaling loop with a strong mitogenic potential (compare table 10 and belonging text).

A-2780 ovarian cancer cells are expressing FGFR-4 and both splice variants of FGFR-1 and FGFR-3 (figure 13). They also express a wide variety of FGF family members (figure 14). Nevertheless, the MAP kinase signaling system is yet not highly active in these cells, but can be induced by FGF-1, to a much higher degree by FGF-2 and by conditioned growth medium (figure 15). This correlates with the induction of cell growth by FGF-2 (figure 17). Correspondingly, cells are highly sensitive to inhibition of FGF receptor and to multi-kinase inhibition (figure 20) with IC_{50}-values of 0,83µM for PD173074 and 3,03µM for Dovitinib. PD173074 is inhibiting FGF receptors 1-3 with high affinity, indicating that FGFR-1, FGFR-3 or both provide crucial signals for the proliferation of A-2780 cancer cells. Dovitinib is, amongst other targets, inhibiting FGFR-1 and FGFR-3 suggesting that the growth suppression is due to inhibition of the same target. As previously mentioned, more promiscuous inhibitors like Dovitinib are less potent against FGF receptors than specific FGF receptor inhibitors. This could explain the discrepancy in growth inhibition of Dovitinib and of PD173074. A-2780 cells are expressing the broadest range of FGFs of all tested cell lines. Surprisingly, they do not exhibit the former described FGFR-2b / FGF-10 autocrine signaling loop but instead show a unique de novo expression of FGF-8 and FGF-17 in combination with de novo expression of FGFR-3c and overexpression of FGFR-4 resulting in an autocrine signaling loop with a high mitogenic potential (table 10). As shown in figure 19, A-2780 cells are not very motile compared to HEY and SKOV-3 cells. Neither treatment with FGF-1 nor treatment with FGF-2 induced migration in these cells.

HEY ovarian cancer cells express both variants of FGFR-1, small amounts of FGFR-2c and moderate levels of FGFR-4 (figure 13) and no autocrine signaling loop with a high mitogenic potential was found (table 10). Phosphorylation of Ras/MAP kinase pathway could not be elevated by

treatment with FGF-1 or FGF-2 due to the high basal levels of ERK1/2 phosphorylation (figure 16, exposure times for pERK are as short as feasible), suggesting a constitutive activation within the Ras/MAP kinase pathway. Besides, a slight activation of the PI3 kinase pathway could be observed. As shown in figure 19, HEY cells are very motile and are able to close the scratch in the cell monolayer in a time-frame of 5 hours. Treatment with FGF-1 triggers migration in a non-significant manner while FGF-2 induces proliferation significantly in these cells ($p<0,05$, figure 17). HEY cells are resistant to treatment with Dovitinib, but exhibit sensitivity to PD173074. As mentioned before, PD173074 inhibits FGF receptors 1-3 with high affinity and FGF receptor 4 at high doses but Dovitinib only affects FGF receptors 1-3. The sensitivity to treatment with PD173074 could therefore be due to an activating mutation of FGFR-4 similar to the one found in MDA-MB-453 breast cancer cells (Roidl et al 2010).

OVCAR-3 ovarian cancer cells are expressing all FGF receptor splice variants except FGFR-3 IIIc (figure 13). De novo expression of FGF-10 leads to an autocrine signaling loop with a very high mitogenic potential (table 10). The FGF signaling system is highly inducible by FGF-2 and by conditioned growth medium and to a lesser extent by FGF-1 triggering phosphorylation of ERK1/2. Surprisingly, neither treatment with FGF-1 nor with FGF-2 is able to induce proliferation in these cells (figure 18) and they are resistant to FGF receptor inhibition by PD173074 and exhibit only minor growth suppression when treated with Dovitinib, suggesting signaling mechanisms that are independent from platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGF) and vascular endothelial growth factor receptor (VEGFR).

SKOV-3 ovarian cancer cells are expressing all seven splice variants of the FGF receptor. The simultaneous expression of FGF-8 and FGF-10 leads to multiple potential autocrine signaling loops (FGF-10 / FGFR-2b, FGF-8 / FGFR-3c and FGF-8 / FGFR-4). The FGF receptor signaling system in these

cells can be slightly induced by FGF-1, FGF-2 and conditioned growth medium as evidenced by elevated phosphorylation of ERK1/2. Neither treatment with FGF-1 nor with FGF-2 was able to induce proliferation in these cells (figure 18), but a non-significant increase in motility could be observed when treating with FGF-1 (figure 19). Treatment with tyrosine kinase inhibitors PD173074 and Dovitinib showed equal growth reduction curves. Expecting, that PD173074 inhibits FGF receptors at lower doses than Dovitinib due to its higher affinity, this indicates that SKOV-3 cells receive proliferation signals not only from FGFR-1, FGFR-2 and FGFR-3 but from other receptors inhibited by Dovitinib as well.

Future directions

Recombinant, dominant negative FGF receptors are lacking the intracellular tyrosine kinase domain, but are capable to dimerize with functional receptors preventing transphosphorylation and subsequent downstream signaling and are available as a research tool for all four FGF receptors. The introduction of dominant negative receptors in future experiments would be very useful for a more precise characterization of the single receptors. However, it is not possible to knock down a single transcript variant with this method and short interference RNA (siRNA) could be used to characterize the function of promising transcript variants in more detail.

As mentioned before, mutations in the extracellular domain of FGF receptors are quite common and lead to constitutive activation of the affected receptor. HEY ovarian cancer cells expose high basal phosphorylation-levels of ERK1/2 but are sensitive to inhibition by FGF receptor inhibitors, indicating that this strong activating signal originates from one of the expressed FGF receptors. Sequencing of the FGF receptors could therefore be used to evaluate an activating mutation in these cells.

FGF receptors have been identified as targets for cancer therapy and inhibition of FGFR-dependent signaling was able to overcome resistance to standard therapies and showed synergistic growth inhibition effects when combined with EGF receptor inhibitors (Fischer et al 2008, Pardo et al 2002). Ovarian cancer patients usually respond well to the standard therapy Cisplatinum, Paclitaxel and their derivatives, but recurrence accompanied by resistances to those chemotherapeutics is quite common. Multi-kinase inhibitors like Dovitinib and BIBF 1120 are currently in evaluation for the treatment of relapsed ovarian cancer patients and the combination of standard therapies with different FGF receptor and multi-kinase inhibitors should therefore be considered in future studies.

As was discussed before, de novo expression of fibroblast growth factors in combination with FGFR-2b, which is expressed in normal ovarian surface epithelium as well as de novo expression of FGF receptors is a common event in ovarian cancer cells and conditioned growth medium was able to trigger activation of the Ras/MAP kinase pathway by different degrees in the evaluated cells. Matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry enables analysis of complex composites of proteins relatively cheap and fast and would be a convenient method for the evaluation of fibroblast growth factors in conditioned growth medium of ovarian cancer cells.

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8 Curriculum vitae

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Education

2003 – 2011	Studies of molecular biology at the University of Vienna
1997 – 2002	College for structural and civil engineering (HTBLuVA Leberstraße)
1993 – 1997	Lower level secondary academic school (Wiener Sängerknaben)
1991 – 1993	Third and fourth form of elementary school (Wiener Sängerknaben)
1989 – 1991	First and second form of elementary school (Pädagogische Akademie Wien)

Work experience

2011	Tutor (University of Vienna)
2009 – 2010	Diploma student (Medical university of Vienna)