Investigating the contribution of FGF receptors in breast tumorigenesis using models of mammary cancer

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2. Summary

Breast cancer, the main cancer in women, occurs in approximately 27% of all yearly diagnosed cancer cases. It is estimated that one out of eight women will develop breast cancer in her lifetime. Therefore it is not surprising that this disease ranks second as a cause of cancer death in women, after lung cancer (American Cancer Society, Cancer Facts and Figures 2009). Based on these numbers, many studies have been undertaken in the area of breast cancer and since 1990, death rates from breast cancer have been decreasing, mainly due to earlier detection and improved treatments. Since the 1970s, hormonal therapies targeting the estrogen receptor have been very successful for treatment of estrogen receptor positive (ER+) breast cancers that consist of about 60% of the cases. More recently, therapies targeting the ErbB2 receptor tyrosine kinase that is overexpressed in about 20% of breast cancer have shown to be of benefit for this subset of breast cancer patients. However, not every patient responds to these treatments or patients become resistant, thus for both therapies there is a high risk of relapse. Consequently, novel therapies are required and will likely arise from an improved understanding of the disease biology.

Recent studies have shown that members of the fibroblast growth factor receptor (FGFR) family of tyrosine kinase receptors are deregulated in breast cancer. Indeed *FGFR1* gene is amplified in 9% of breast cancers and single nucleotide polymorphisms in *FGFR2* gene are strongly associated with an increased probability to develop breast cancer. Using models of breast carcinoma (4T1, 4TO7, 168FARN and 67NR cell lines), our study aimed at a better understanding of how FGFRs contribute to breast tumorigenesis. Furthermore we analysed the effects of blocking the activity of these receptors in cell lines and primary tumors.

In vitro, we showed that 4T1, 4TO7, 168FARN and 67NR cell lines co-express FGF ligands and FGFRs, suggesting an autocrine loop activating FGFRs which leads to the constitutive basal activation of downstream signaling pathways, mainly MAPK and PI3K/AKT. Using TKI258, a tyrosine kinase inhibitor blocking FGFRs, we inhibited the basal activity of FGFRs. This inhibition resulted in a decrease of ERK1/2 and AKT activity, confirming that FGFR signaling maintains the activity of MAPK and PI3K/AKT pathways. Furthermore, interfering with autocrine FGFR signaling dramatically impaired proliferation of the four cell lines and apoptotic cell death was observed in 4T1 and 4TO7. Using constitutively active mutants, we demonstrated that Ras and AKT contribute to cell survival downstream of FGFRs and that 4T1 cells expressing these constructs are partially rescued from the effects of TKI258. Interestingly, when we

combined inhibitors of MEK (UO126) or PI3K (LY294002) with TKI258, we increased the sensitivity of 4T1 cells to TKI258 induced cell death.

In vivo, we describe that TKI258 treatment inhibits FGFR signaling in mice bearing 4T1-induced tumors. In addition, daily oral treatment of mice bearing 4T1- or 67NR- induced tumors with TKI258 over 14 days, significantly reduces tumor outgrowth and decreases 4T1 lung metastasis, showing that blockade of FGFR has strong anti-tumor and anti-metastatic activities.

Two microarray analyses performed on treated 4T1 cells or 4T1 tumors, led to the identification of genes that were regulated after TKI258 treatment. Detailed analyses showed that some of these genes were known to contribute to the metastatic process (matrix metalloproteinases and extracellular matrix proteins) or to cell cycle progression (cyclins and E2F transcription factors). Comparison of these TKI258-regulated genes with publicly available databases of breast cancer patients identified a cohort of patients showing overexpression of genes down-regulated upon TKI258 treatment, and these patients have a higher probability of metastatic disease compared to the other patients. These highly expressed genes might therefore reflect activation of receptor tyrosine kinase signaling pathways like FGFR in 4T1 tumors.

In summary our results show that targeting FGFRs using a TKI has an impact on various biological characteristics of FGFR driven models of breast cancer, including proliferation and survival. In addition, our observations show that blockade of FGFR signaling can be achieved in vivo and this leads to reduction of tumor outgrowth as well as decreased metastases formation. Finally, our meta-analysis on genes that are changed in 4T1 treated cells and tumors provides evidences that results obtained using animal models of a diseases are meaningful in terms of prognostic and can be translated to breast cancer patients.

3. Introduction

3.1. The Fibroblast Growth Factor (FGF) tyrosine kinase receptor family

In complex organisms, cells have to behave in an appropriate and well controlled manner and they should only respond to specific stimuli. These stimuli are varied, ranging from soluble secreted factors, to molecules bound in the plasma membrane of a neighboring cell. These extracellular signals have to be sensed by the cells, integrated across the plasma membrane, amplified and interpreted in a way that induces a correct response. During evolution, different strategies have been developed in order to accomplish this challenge. One of these is the presence of transmembrane receptors that interact with extracellular stimuli and integrate them in the internal cell compartment. One class of receptors is the family of receptor tyrosine kinases (RTK). This family consists of 58 members that can be divided into 20 different subfamilies based on sequence homologies and conserved structural features.

The subfamily IV is comprised of the Fibroblast Growth Factor (FGF) Receptors and includes four structurally related type I growth factor receptors: FGFR1, FGFR2, FGFR3 and FGFR4. Common to all members is an extracellular domain composed of two or three immunoglobulin-like (Ig-like) loops, a stretch of eight consecutive acidic residues (the acidic box) situated between the first and the second Ig-like fold, a single hydrophobic transmembrane region and a cytoplasmic tail containing a split tyrosine kinase domain (Johnson et al., 1990; Johnson & Williams, 1993). Ligand binding to the extracellular domain induces formation of receptor homo or hetero-dimers (Bellot et al., 1991), leading to activation of the tyrosine kinase domain, phosphorylation of specific residues within the cytoplasmic domain of the activated receptors and phosphorylation of adaptor proteins. These phosphorylated residues serve as docking sites for specific signaling complexes that activate different signal transduction cascades, which in turn regulate key cellular processes including cell growth, proliferation, migration and differentiation (Klint & Claesson-Welsh, 1999).

3.2. FGFs and their receptors (FGFRs) in evolution

The components of the FGFR signaling pathway, including the ligands (FGFs) and receptors (FGFRs), are conserved through evolution and genes encoding FGFs and FGFRs have been

identified in multicellular, but not in unicellular organisms (Itoh & Ornitz, 2004; Ornitz & Itoh, 2001) .

The nematode *Caenorhabditis elegans* is the first species that possess components of the FGF signaling pathway. Two *fgf*s (*egl-17 and let-756*) and one *fgfr* (*egl-15*) have been identified in this organism. Mutations in the *egl-15* gene affect the migration of sex myoblasts (SM) and also result in larval arrest and scrawny body morphology (DeVore et al., 1995). The *egl-17* null mutants partially recapitulate the phenotype of *egl-15* mutants with respect to the defect in SM migration, suggesting that this gene acts as a ligand for *egl-15* during SM migration and that at least one other ligand is responsible for the other phenotypes (Burdine et al., 1997). Indeed, mutants for the *let-756* gene do not display a SM migration defect, however they show similarity to other aspects of the *egl-15* mutant phenotype, in particular with the stage of lethality and the physiology of the worms, suggesting that *let-756* is another ligand for *egl-15* (Roubin et al., 1999). More recent studies confirmed that *let-756* is probably acting as ligand for *egl-15* (Huang & Stern, 2004).

In the fruitfly *Drosophila Melanogaster*, three *fgf*s (*branchless, pyramus* and *thisbe*) and two *fgfr*s (*breathless* and *heartless*) have been identified. The *breathless* receptor has been shown to play a role in anterior-posterior migration of the midline glial cells (Klambt et al., 1992) and, together with its ligand *branchless*, plays essential roles in the migration of tracheal cells out of the tracheal pits and in branch patterning (Sutherland et al., 1996). There is evidence to suggest that *pyramus* and *thisbe* are ligands for the *heartless* receptor and that together they regulate the migration of early mesodermal cells and patterning of the early mesoderm in the embryo (Beiman et al., 1996; Gisselbrecht et al., 1996; Stathopoulos et al., 2004).

In the widely used vertebrate model organism zebrafish (*Danio Rerio*), the *fgf* family is comprised of 27 members and the *fgfr* family of 4 receptors (Itoh & Konishi, 2007; Scholpp et al., 2004; Sleptsova-Friedrich et al., 2001; Thisse et al., 1995; Tonou-Fujimori et al., 2002). Several *fgf* mutants have been generated and described. The phenotypes are multiple and highlight the roles of the *fgf* genes in brain development, pectoral fin bud formation, haematopoiesis, erythrocyte differentiation and formation of the otic placode and vesicle. Approaches with

membrane-bound dominant negative and constitutively active receptors have been used to study the functions of the different receptors. The use of constitutive active mutants revealed an increase in dorsalisation of the embryo, increased formation of the posterior brain and inhibition of forebrain formation for all four Fgfrs. Dominant negative mutants for Fgfr1, 2 and 3 showed impaired development of posterior structures, brain anomalies and small heads, whereas dominant negative FGFR4 showed only defect in the posterior structure (Ota et al., 2009).

3.3. FGF/FGFR network in mammals

In mammals, four FGF receptors have been characterized and can be activated by 22 FGFs. Moreover, in comparison to invertebrates, further levels of complexity and regulation of the FGF/FGFR interactions have been achieved. On one hand, alternative splicing has increased the functional diversity of FGFRs by regulating the total number of Ig-like domains present on the receptors (two or three) and by allowing different isoforms of the C-terminal part of the third Ig-like loop. On the other hand, ligand-receptor interactions have also been refined in space by the added requirement of specific cofactors like glycosaminoglycan, Klotho or βKlotho in order to initiate a response. Both the increase in complexity of the receptors, and the need for spatially restricted cofactors, diversifies FGF signaling, permitting it to contribute to development, angiogenesis, metabolism and regulation of cell proliferation, cell differentiation and migration. Importantly, deregulation of FGF signaling has been associated with several human diseases like dwarfism, rickets and cancer.

3.3.1. FGFR ligands

There are 22 mammalian FGFs, each encoded by a single gene. These 22 FGFs share 13 to 71% amino acid identity and are divided into 7 subfamilies (Figure 3-1) (Itoh & Ornitz, 2004), ranging in size from ~160 to 260 amino acid residues and have molecular weights from 17 to 34 kDa. Structural studies have shown that the conserved FGF core region consists of 12 antiparallel β-strands, of which the β1-β2 loop and β10-β12 region are involved in binding to the major co-receptors heparin and heparan sulfate proteoglycan (HSPG) (Ornitz & Itoh, 2001). Expression of the different FGFs has been widely studied, mainly in development. A summary of the FGF classification is presented below.

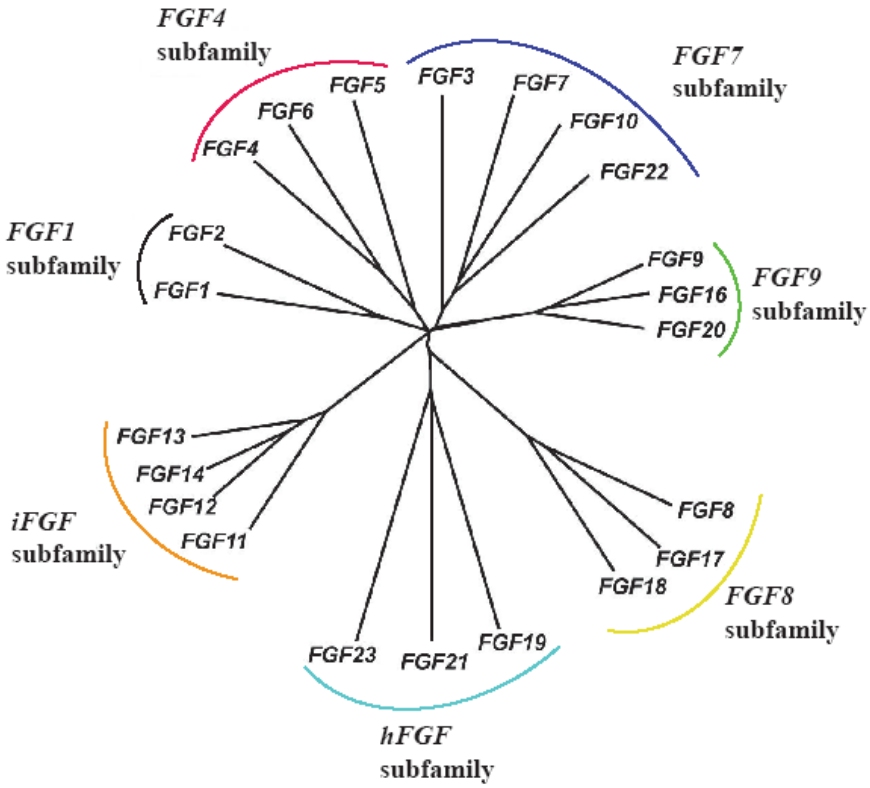

Figure 3-1 Phylogenetic analysis of the human FGF gene family

The family of the human FGF genes is divided in 7 subfamilies. The canonical FGFs are clustered in the subfamilies 1, 4, 7, 8 and 9. They bind to heparin and most of them are secreted into the extracellular space using the classical endoplasmic reticulum-Golgi secretion pathway. FGF1, 2, 9, 16 and 20 use another, not yet characterized, secretion mechanism. Members of the hFGF subfamily act as hormones in an endocrine manner and do not interact with heparin, but require co-receptors like kotho and βklotho. The iFGF subfamily members are not secreted and remain in the intracellular compartment. Interestingly, their activity appears to be independent of FGFRs. Adapted from (Itoh, 2007).

The FGF1 subfamily consists of two members, FGF1 and FGF2, also known as acidic FGF (aFGF) and basic FGF (bFGF), respectively. FGF1 has been described as the "universal" ligand, capable of activating all of the FGFR subtypes. FGF2's spectrum of activity is slightly more restricted than that of FGF1, however it is the second broadest activator of the FGFR subtypes (Zhang et al., 2006). Four different FGF2 isoforms can be synthesized by the use of three in-frame CUG codons located upstream of the standard AUG start codon, resulting in longer polypeptides. Neither member of this FGF subfamily has the classical amino-terminal signal peptide required for secretion out of cells, however they are both found on the cell surface and associated with the extracellular matrix, suggesting that they can be released from damaged cells

or that they use a different mechanism than the classical secretory pathway in order to be released from the intracellular compartment (McNeil et al., 1989; Mignatti et al., 1992). Both FGF1 and FGF2 have an amino-terminal nuclear localization motif and are detected in the nucleus, however the biological function of nuclear-localized FGFs is still unclear (Hu et al., 2000; Powers et al., 2000).

Members of the FGF4 subfamily are secreted from the cells and include FGF4, FGF5 and FGF6. Secretion occurs via the classical endoplasmic-reticulum-Golgi pathway and during this process a signal peptide consisting of hydrophobic amino acids, present on the amino-terminal part of newly translated FGFs, is cleaved. Interestingly, all three members of this family were found to be glycosylated. This modification seems to negatively regulate FGF4 activity (Bellosta et al., 1993), however it does not appear to be involved in modulation of FGF5 and FGF6 activity (Clements et al., 1993; Pizette et al., 1991).

Four ligands comprise the FGF7 subfamily, namely FGF3, FGF7, FGF10 and FGF22. Members of this family possess an amino-terminal signal peptide that allows them to be secreted and glycosylation sites. In addition, different isoforms of FGF3 are synthesized, all of which contain a C-terminal nuclear localization sequence. FGF7, also known as keratinocyte growth factor (KGF), and FGF10 are mainly secreted by mesenchymal cells and act on epithelial cells expressing the appropriate receptor subtype. Finally, although FGF22 possesses a signal peptide, overexpression studies showed, that it is mainly localized within the cell and at the cell surface however without being released (Beyer et al., 2003).

The FGF8 subfamily consists of three members (FGF8, FGF17 and FGF18) all of which possess an amino-terminal signal peptide and are secreted. FGF8, also called androgen-induced growth factor (AIGF), is found in at least seven isoforms in the mouse. These isoforms differ in their amino-termini, but retain the signal peptide required for secretion.

All three members of the FGF9 subfamily (FGF9, FGF16 and FGF20) lack a classical cleavable amino-terminal signal peptide nevertheless, these FGFs are still secreted into the extracellular

space, suggesting that the members of the FGF9 subfamily utilize an alternate ER-Golgi-independent pathway for secretion.

The members of the hormone-like (hFGF) subfamily (FGF19, the human ortholog of FGF15, FGF 21 and FGF23) all act in an endocrine manner. Crystallographic studies have shown that the heparin binding site of the hFGFs diverges from that of the canonical FGFs, resulting in a decreased affinity for heparin and thus enhanced diffusion of these factors (Goetz et al., 2007; Harmer et al., 2004). However, specificity of signal transduction to key organs is achieved by hFGFs via their interaction with the alternative co-receptors Klotho or βKlotho, which is necessary to fully activate FGFRs on their target tissues.

Finally, the intracellular FGF (iFGF) subfamily, also named FGF homology factors (FHFs), consists of four members (FGF11, FGF12, FGF13 and FGF 14) that lack the classical signal peptide required for secretion and therefore, as their name suggests, remain localized within the cell. These iFGFs all appear to function in an FGFR independent way and furthermore contain a nuclear localization signal. In addition, FGF13 has been found to be expressed in several isoforms but their role is still not understood.

3.3.2. Architecture of the FGF receptors

All FGFs, with the exception of the iFGFs, mediate their cellular responses by binding to and activating a family of four type I transmembrane receptors (FGFR1-4) (Figure 3-2). These receptors are encoded by four distinct genes located on four different chromosomes: 8p11.2 (*FGFR1*), 10q26 (*FGFR2*), 4p16.3 (*FGFR3*), and 5q35.1 (*FGFR4*). Sequence homology at the amino-acid level is high between the different receptors and *FGFR2*, *3* and *4* share 70%, 61% and 53% amino acid sequence identity respectively with *FGFR1* (Zhang et al., 1999).

The extracellular portion of FGFRs consists of about 360 amino acids and is composed of three Ig-like domains designated D1-D3. A stretch of seven to eight consecutive acidic amino acids (the acid box) is localized between D1 and D2 and serves as a binding site for heparin. A single transmembrane-spanning domain of around 20 amino acids links the extracellular part to the 80 amino acids long intracellular juxtamembrane domain. This juxtamembrane domain is required

for the constitutive, ligand- and phospho-tyrosine-independent, association of FGFRs with two major adaptor proteins, namely fibroblast receptor substrate 2 α and β (FRS2 and FRS2β) (Ong et al., 2000). The tyrosine kinase domain immediately follows the juxtamembrane domain and is composed of approximately 280 amino acids. The particularity of the FGFR kinase domain is the presence of a 15 amino acids long non-catalytic insert that splits the tyrosine kinase into two parts (Klint & Claesson-Welsh, 1999). The 60 amino acid long C-terminal tail of the FGFR serves as binding site for signaling molecules.

Various isoforms of the FGFRs are produced via differential splicing or exon skipping (Figure 3-2). Soluble receptors were first characterized for FGFR1 (Johnson et al., 1990). These receptors are secreted and contain two or three Ig-like domains, however they lack the transmembrane and intracellular domains. They are thought to act as modulators of FGFR signaling by binding to free FGFs and sequestering them from activatable full length receptors (Hanneken, 2001). To date, soluble receptors were described for all four members of the FGFR family (Jang, 2002; Kishi et al., 1994).

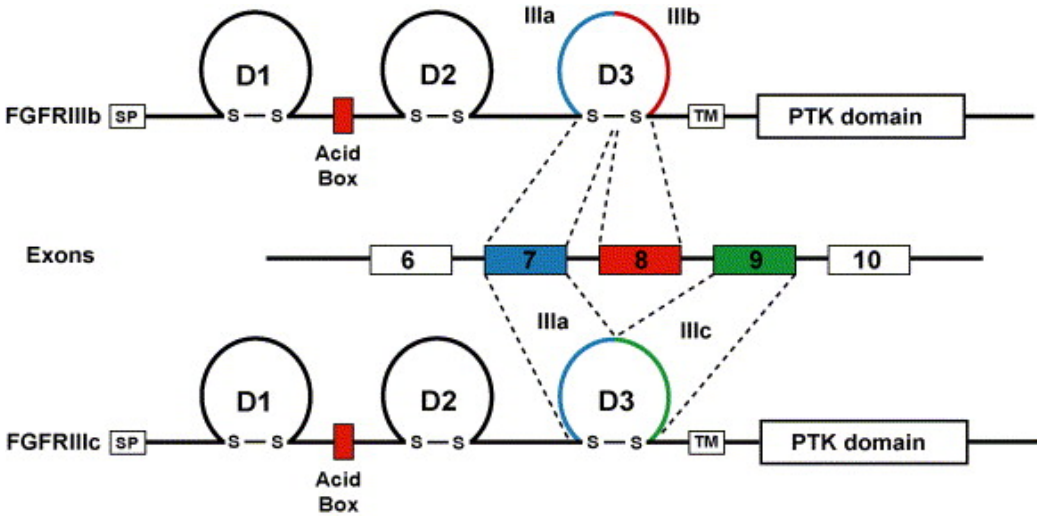

Figure 3-2 Structure of FGFRs.

FGFRs contain an N-terminal signal peptide which is required for their membrane localization. The extracellular part consists of 3 Ig-like loops (D1-D3) and an acid box between D1 and D2. The extracellular domain of FGFRs is subject to differential splicing. The main splicing event occurs in the D3 loop and affects ligand binding properties. The intracellular tyrosine kinase domain is linked to the ligand binding domain through a unique transmembrane part. (Eswarakumar et al., 2005)

Isoforms lacking the D1 domain have also been described (Johnson et al., 1990) and are more sensitive to ligand induced signaling. Indeed, D1 plays an autoinhibitory role by acting as a competitor of FGF for receptor binding, thereby lowering the affinity of FGFRs for their ligands (Kiselyov et al., 2006; Wang et al., 1995). Interestingly, alternative splicing of exons 8 and 9 of FGFR1, 2, 3 but not 4, leads to changes in the C-terminal part of their D3 domains and gives rise to the so-called IIIb and IIIc receptor isoforms (Figure 3-2) (Johnson et al., 1991; Miki et al., 1992; Werner et al., 1992a). These alternative forms display different ligand binding properties and their expression pattern is well defined and hardly overlap. Indeed, it has been shown that the IIIb isoforms are generally expressed in epithelial cells, whereas the IIIc isoforms are found on mesenchymal cells. This split pattern of expression allows interesting cross-talk to occur between epithelial and mesenchymal layers during development.

Figure 3-3 shows a summary of the ligand-receptor interactions between the FGFs and the different splice variants of the FGFRs.

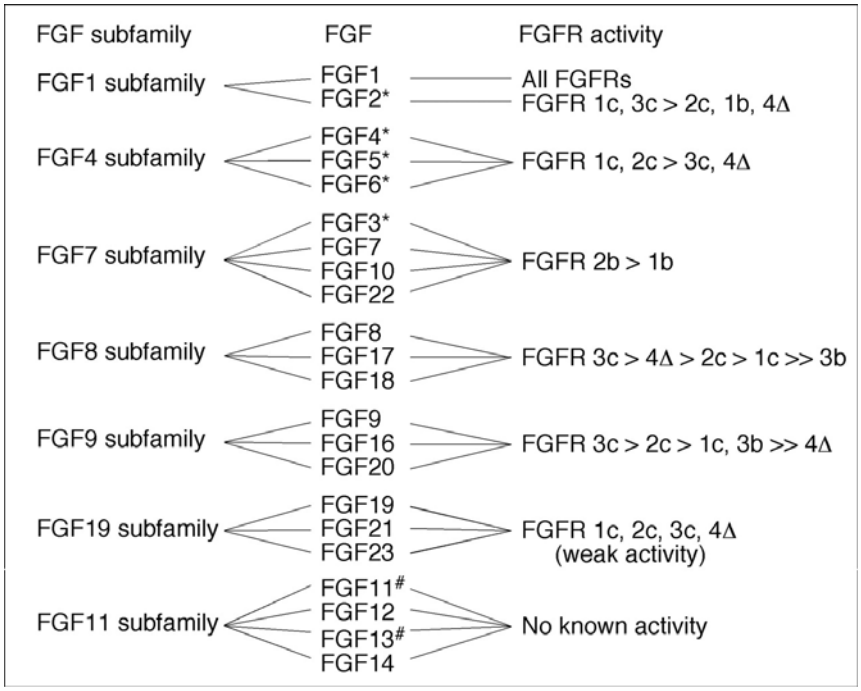

Figure 3-3 Relative activity of FGFs on FGFRs

Mitogenesis-based comparison of receptor specificity of the FGF family. (Zhang et al., 2006)
*Data from (Ornitz et al., 1996), # were not tested

Intracellular C-terminal splice variants have been described for FGFR2 (Itoh et al., 1994; Tannheimer et al., 2000). These three isoforms have different signaling properties due to the presence or absence of a tyrosine residue required for the binding of PLCγ. Moreover, they confer different invasive phenotypes to breast cancer cells when ectopically expressed (Moffa et al., 2004).

3.3.3. Heparan sulfate proteoglycans, Klotho and βKlotho as coreceptors

Proteoglycans (PG) are membrane bound extracellular matrix proteins containing carbohydrate side chains named glycosaminoglycans (GAG). GAGs are repeated units of disaccharides that possess a negative charge due to the presence of sulfate and carboxyl groups. Heparin and the complex heparan sulfate proteoglycan (HSPG), have been shown to play a key role in the FGF signaling pathway. The repeated disaccharide units of heparin sulfate consist of a 2-*O*-sulfated or unmodified hexuronic acid (β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA)) and either an *N*-sulfated or *N*-acetylated D-glucosamine (GlcN). The *N*-sulfated-glucosamines ($GlcNSO_3$) may in addition be *O*-sulfated at C3, C6 or both and the *N*-acetylated-glucosamine (GlcNAc) may be *O*-sulfated at C6. The combination of these different structural units into disaccharides and their subsequent arrangement into chains creates an extraordinarily large potential for structural diversity (Coombe & Kett, 2005). The interaction between FGFs and HSPG has been shown to be relevant for FGF-dependent binding to and activation of FGFRs. In addition, FGFs fail to activate FGFRs in cells lacking endogenous HSPG or in cells treated with heparanase. This suggests that the interaction between FGFs and HSPG is required for FGFs to signal via FGFRs. Moreover, by binding with high affinity to FGFs, HSPG creates local reservoirs of ligands that can be released in the presence of specific proteases into the extracellular space, therefore, spatially and temporally regulating the availability of these ligands. Alternately, this interaction may stabilize FGFs against proteolytic degradation by keeping them in a constrained environment not accessible to proteases.

The three members of the hFGF subfamily have only weak affinity for HSPG (Goetz et al., 2007; Harmer et al., 2004) and therefore require different co-receptors to activate FGFRs. The Klotho and βKlotho genes encode for homologous, single transmembrane domain proteins that bind to FGFRs. The expression of Klotho is restricted to a few tissues, primarily the kidney and the

chorroid plexus in the brain. Complexes between Klotho and FGFR1-IIIc, FGFR2-IIIc and FGFR4 have been described, and results in a significant increase in affinity of the complex for the FGF23 ligand (Kurosu et al., 2006). The tissue expression of βKlotho differs from that of Klotho as it is found predominantly in the liver and white adipose tissue. Here as well, interaction between FGFR1-IIIc, FGFR4 and βKlotho has been demonstrated. Interaction of βKlotho with these different FGFRs regulates the activities of FGF15/19 and FGF21 on target tissues (Kuro-o, 2008).

3.4. Activation of receptor tyrosine kinases (RTKs)

Activation of receptor tyrosine kinases (RTKs) has been intensively studied and all evidence suggests that dimerization of RTKs is a prerequisite for activation of the tyrosine kinase domain (Schlessinger, 2000). Within the FGFR family, dimerization can occur between two identical receptors (homodimerization) and there are reports suggesting that FGFRs can form heterodimers that are able to transphosphorylate themselves (Bellot et al., 1991). The classical view of RTK activation begins with the binding of ligand to the extracellular domain of a receptor. This step either induces conformational changes within the extracellular parts of the receptors themselves, unmasking domains involved in dimerization (EGFR family), or acts as a crosslink between two receptors, thus bringing them into close proximity of one another (VEGFR family). Ligand mediated interactions between two receptors induce further conformational changes within their extracellular domains, which are transduced to the intracellular part via the transmembrane domain. These intracellular conformational changes result in activation of the tyrosine kinase domains via phosphorylation of tyrosine residues within the intracellular part. These modified residues act then as docking sites for adaptor proteins that activate downstream signaling cascades.

3.4.1. The symmetric Two-End model for FGFR dimerization

The structures of several FGFs were solved (Blaber et al., 1996; Osslund et al., 1998) and the first crystal structure of FGF2 bound to the domains D2 and D3 of FGFR1 was determined in 1999 (Plotnikov et al., 1999). Large amount of data on FGF/FGFR complexes is now available since these initial works were rapidly followed by the structures of other complexes, namely FGF1/FGFR1, FGF2/FGFR2 (Plotnikov et al., 2000) and more recently, FGF10/FGFR2-IIIb

(Yeh et al., 2003). Interestingly, these structures possessed several common features that allowed a better understanding of the molecular mechanisms governing FGF-FGFR binding specificity. In the resulting two-end model, two FGFs, two FGFRs and two heparin oligosaccharides cooperate to interact and form a symmetric functional dimeric unit (Figure 3-4) (Ibrahimi et al., 2005; Mohammadi et al., 2005b).

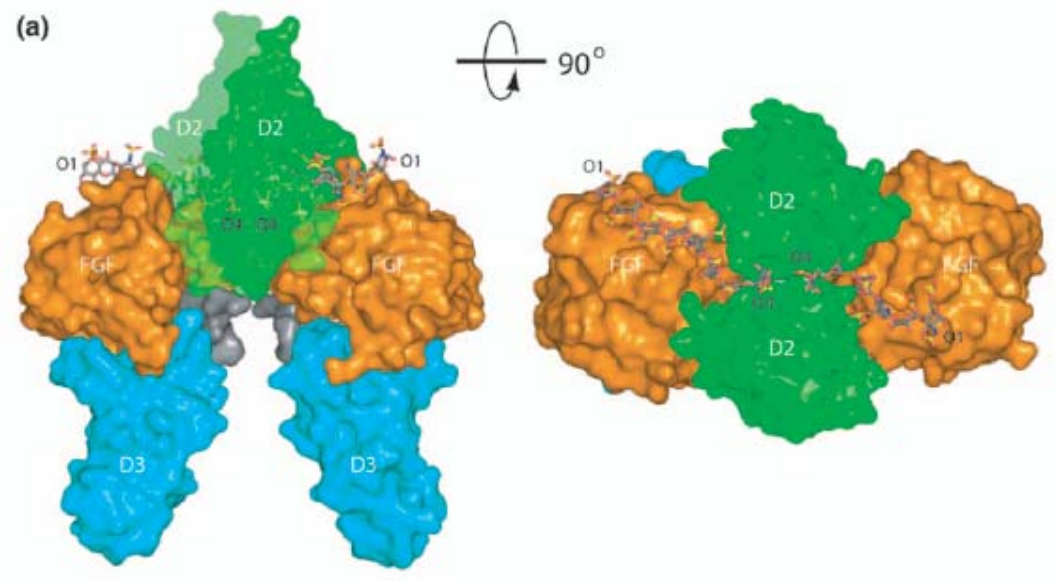

Figure 3-4 The two-end model of FGF-FGFR-heparin complex

One FGF2 (yellow) binds to the D2 (green) and D3 (blue) domains of one FGFR1-IIIc, therefore forming a monomeric ligand-receptor complex. Two monomers interact and form a dimeric complex by direct interaction between the receptors and by interaction between the ligand of one monomer with the D2 domain on the receptor from the other monomer. Heparin stabilizes the complex by interacting with ligand and receptor. (Mohammadi et al., 2005a)

This model proposes that a functional signaling complex forms as follows: a single FGF first interacts with a single FGFR through surfaces of the receptor's D2 and D3 Ig-like domains. Two of these monomers are then brought together via interaction between the ligand from one monomeric unit and the D2 domain of the receptor of the second FGF-FGFR complex. This produces a dimer consisting of two FGFs and two FGFRs. Stabilization of the dimer is further enhanced by direct receptor-receptor contacts. The close proximity of the two FGF-FGFR monomers results in formation of a positively charged canyon between the D2 domains that extends across the adjoining ligands. Two heparin molecules can bind in this canyon, each of

them promoting monomer stability by increasing FGF-FGFR primary interaction, as well as stabilizing the dimeric complex by enhancing secondary receptor-receptor interactions (Ibrahimi et al., 2005; Mohammadi et al., 2005a; Schlessinger et al., 2000).

3.4.2. Activation of the tyrosine kinase domain of FGF Receptors

Following ligand induced dimerization of the receptors, the intracellular tyrosine kinase domains get activated and phosphorylate several tyrosine residues on the kinase domain themselves, on the receptors and on other adaptor proteins. Resolution of the structure of FGFR1 kinase aided in the elucidation of the mechanisms of FGFR activation (Mohammadi et al., 1996). In brief, the FGFR1 kinase domain is divided into two lobes termed the N- and C-terminal domains. ATP is coordinated by residues in the N-terminal lobe, whereas substrate binding and catalysis are achieved by residues in the C-terminal lobe. When the kinase is inactive, the activation loop is positioned in such a manner that the binding site for substrate peptides is blocked, while the ATP binding site is still accessible. In addition to this physical function, autophosphorylation of tyrosine residues within the activation loop is critical for maintaining the kinase in an active state. There are at least seven tyrosine residues within the intracellular part of FGFR1 that have been shown to be phosphotylated; Y463, Y583, Y585, Y653, Y654, Y730 and Y766. The role of the individual phospho-tyrosine residues remains unknown, with the notable exceptions of Y653/Y654 on the activation loop and Y766 on the C-terminal tail, where phosphorylation is needed for activation of the kinase and binding of PLCγ, respectively. However, recent work has shown that the sequential phosphorylation of tyrosine residues in the intracellular part of FGFR1 occurs in three stages. The first step consists of autophosphorylation of Y653, resulting in a 50- to 100-fold stimulation in kinase activity. The second step is the temporal phosphorylation of Y583 in the non catalytic insert of the kinase, Y463 in the juxtamembrane region, Y766 in the C-terminal tail and Y585 in the non catalytic insert of the kinase, potentially creating docking sites for adaptor and signaling proteins. Finally Y654 in the activation loop gets phosphorylated, resulting in a total 500- to 1000-fold increase in the receptor's kinase activity (Furdui et al., 2006; Lew et al., 2009).

3.5. Activation of intracellular signaling pathways

Phosphorylation of the intracellular tyrosine residues provides specific docking sites for Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domain-containing adaptor proteins and signaling molecules (Schlessinger, 2000). Interestingly, of the seven FGFR phosphorylated tyrosine residues, only Y766 has been confirmed to act as docking site. Indeed, this conserved residue is required for the binding of PLCγ, through its SH2 domain, to FGFRs and its subsequent tyrosine phosphorylation and thus activation by the active receptor (Figure 3-5) (Mohammadi et al., 1991). Active PLCγ cleaves phosphatidyl-inositol-4,5-bisphosphate to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 promotes the release of calcium from the endoplasmic reticulum while DAG and calcium activate protein kinase C (PKC), leading to cytoskeletal rearrangement. Indeed it has been shown that mutation of tyrosine 766 of FGFR1 into a phenylalanine inhibits FGF2 induced shape change and stress fiber formation in porcine aortic endothelial cells, without affecting FGF2 induced proliferation (Cross et al., 2000).

With the exception of PLCγ, FGFRs do not directly recruit adaptor or signaling proteins to phosphorylated tyrosine residues. Indeed FGFRs constitutively interact in a phosphorylation independent manner with the family of the FRS2 adaptor proteins (Figure 3-5) (Kouhara et al., 1997). The FRS2 family consists of two highly homologous proteins (FRS2α and FRS2β) that are targeted to the plasma membrane via a myristylated N-terminal domain and contain a PTB domain which mediates its direct and constitutive binding to FGFRs in a ligand and phospho-tyrosine independent manner. Upon activation of the FGFR kinase, phosphorylation of the six and five tyrosine residues of FRS2α and FRS2β, respectively, occurs. These residues then act as docking sites for the adaptor Grb2 and the phosphatase Shp2, both of which mediate activation of the mitogen-activated protein kinase (MAPK) pathway, therefore playing a key role in FGF dependent mitogenesis (Kouhara et al., 1997); (Lundin et al., 2003). Moreover, in response to FGF stimulation, Gab1 is recruited to Grb2, resulting in its phosphorylation on tyrosine residues and activation of the PI3-kinase/AKT survival pathway downstream of active FGFRs (Ong et al., 2000).

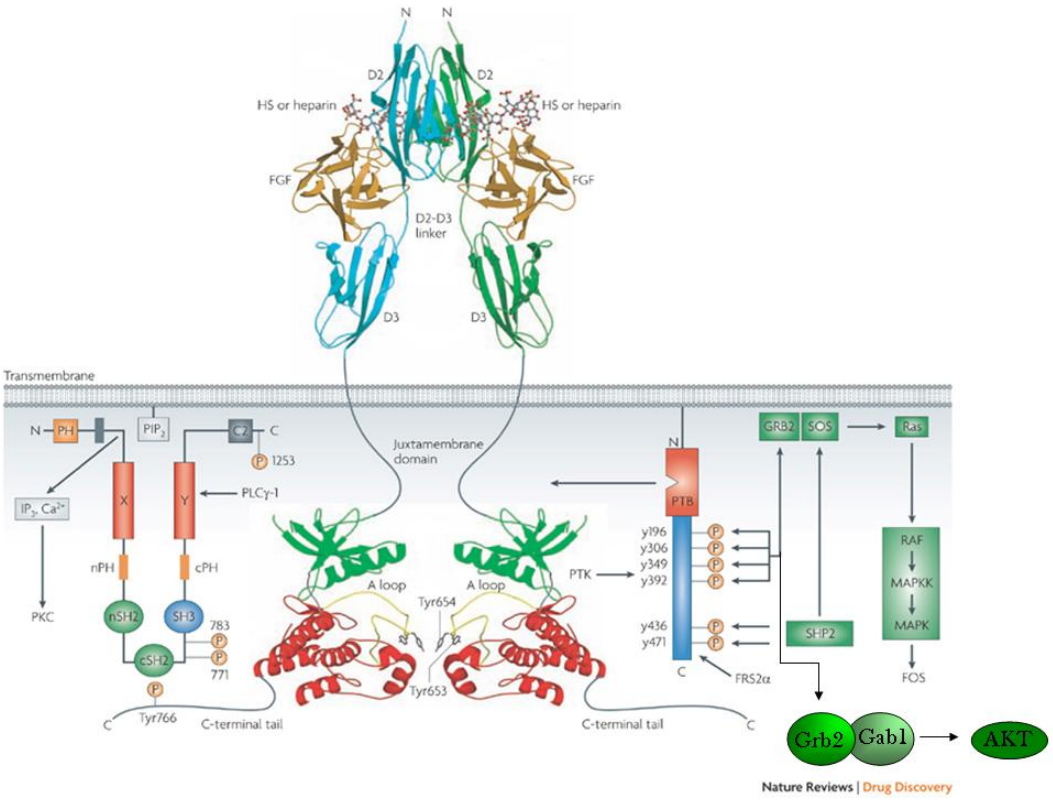

Figure 3-5 Major proteins binding to FGFRs and downstream signaling pathways

Two major proteins directly interact with FGFRs. On one hand, PLCγ binds through its SH2 domain to the phosphorylated tyrosine 766 in the C-terminal tail and gets activated. Active PLCγ cleaves PIP2 into DAG and IP3, leading to regulation of calcium levels. On the other hand, FRS2 constitutively bind FGFRs through its PTB domain. Upon FGF stimulation, FRS2 gets phosphorylated on tyrosine that recruit adaptor required for the activation of MAPK and PI3K-AKT pathways. Adapted from (Beenken & Mohammadi, 2009).

Several other signaling molecules have been described as being activated downstream of FGFRs however, it is still unclear if they directly interact with the receptors:

The non-receptor tyrosine kinase Src is activated in vitro upon FGF stimulation (Landgren et al., 1995; Zhan et al., 1994) and contributes to cell migration (LaVallee et al., 1998). Recent data has shown that Src is recruited to activated FGFR1 through FRS2 and modulates the signaling dynamics of FGFR1 as well as transport of FGFR1 to the plasma membrane (Sandilands et al., 2007).

The adaptor proteins Crk and Shb have been shown to directly interact with the phosphorylated tyrosine 463 and 766, respectively of FGFR1 and become tyrosine phosphorylated. Both these

proteins have been implicated in regulating the activation of the MAPK pathway downstream of FGFRs (Cross et al., 2002; Larsson et al., 1999).

The adaptor protein Grb14 also gets recruited to phosphorylated Y766 of FGFR1 where it acts as a negative regulator of FGF signaling and inhibits MAPK, AKT and PLCγ activation (Cailliau et al., 2005; Reilly et al., 2000).

Finally, activation of Stat1 and Stat3 can be achieved downstream of FGFRs and they are believed to play a role in cell proliferation and survival (Hart et al., 2000).

Once activated, these intracellular signaling pathways converge in the nucleus, where they activate a number of transcription factors, including Ets domain containing factors, c-jun and c-fos. Changes in the level of target genes determine the biological response to receptor activation that, in the case of FGFRs, may vary from mitogenesis, migration, survival and differentiation.

3.6.Regulation of FGFR signaling

FGFR signaling induces key cellular processes which when misregulated can lead to several diseases. Therefore FGFR signaling needs to be tightly controlled in space and time. As previously mentioned, spatial regulation is achieved by the co-expression of specific FGFRs and co-receptors at the surface of FGF responsive tissues and organs. Temporal regulation of FGFR signaling can be achieved in several ways: On the one hand, internalization and subsequent degradation of the catalytically active receptors located at the plasma membrane rapidly decreases the number of molecules present at the cell surface, therefore diminishing the amplitude of the signal. Upon FGF stimulation and activation of the kinase domain, the E3 ubiquitin ligase Cbl is recruited to FRS2 indirectly via Grb2, subsequently leading to ubiquitination of FRS2 and FGFR and thus degradation of these two proteins (Wong et al., 2002; Xian et al., 2007).

On the other hand, active FGF signaling positively influences the expression of several feedback regulators:

The sprouty proteins were the first described feedback modulators of the FGF pathway and their expression is regulated by FGFs. They act as general inhibitors of the Ras-MAPK pathway at different levels and through various mechanisms, of which sequestration and inhibition of Grb2, as well as direct binding and prevention of activation of Raf, are the best characterized (Mason et al., 2006; Thisse & Thisse, 2005).

The Sef protein is a receptor-like glycoprotein that contains a single transmembrane domain and has no known catalytic activity. The prototypic Sef is located at the plasma membrane, but some isoforms are cytoplasmic (Ron et al., 2008). The expression of Sef is induced by FGF signaling and Sef proteins function as feedback-induced antagonists of FGF signaling, however, the mechanisms underlying this activity are still unclear (Furthauer et al., 2002; Lin et al., 2002). It has been proposed that transmembrane Sef could act at the level of the FGFRs themselves, whereas cytosolic Sef specifically regulates the MAPK pathway at the level of MEK (Thisse & Thisse, 2005; Tsang & Dawid, 2004).

The MKP proteins contain an N-terminal, high-affinity ERK binding domain and a C-terminal phosphatase domain. Within this family, expression of MKP1 and MKP3, also called Dusp1 and Dusp6, is positively regulated by FGF signaling. Both members provide feedback mechanisms to attenuate the FGFR signaling pathway, via dephosphorylation and inhibition of ERK1/2 activity (Thisse & Thisse, 2005; Tsang & Dawid, 2004).

3.7. Non canonical FGFR signaling

FGF signaling is also influenced and regulated by other FGF binding proteins without tyrosine kinase activity and by transmembrane proteins that directly interact with and modulate FGFR activity (Figure 3-6) (Murakami et al., 2008).

The syndecans is a family of heparan sulfate proteoglycans that bind to FGFs with low affinity (~100-fold less than the FGF-FGFR interaction). The syndecans were first identified as co-receptors modulating and facilitating the formation of FGF-FGFR signaling complexes (Bernfield & Hooper, 1991). The intracellular tail of these proteins is important for binding and activation of cytoplasmic proteins like Rac1 and PKCα (Zimmermann & David, 1999). Deletion

of the syndecans' tail abrogates FGF induced cell proliferation, suggesting that the intracellular tail of syndecans contributes to FGF signaling (Volk et al., 1999).

Classical integrin ligands are components of the extracellular matrix. However, association of FGF1 with integrins can activate FGFR1 and the classical downstream signaling pathways (Mori et al., 2008).

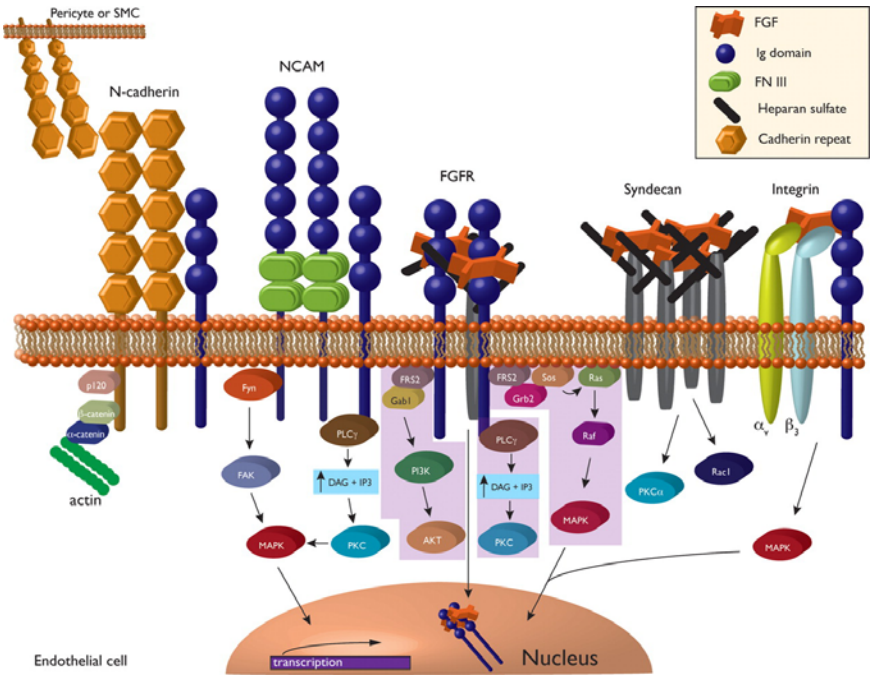

Figure 3-6 Non-canonical FGF signaling

Non-canonical pathways are divided into two groups: On the left of the canonical pathway (purple box) are the ligand independent non-canonical FGFR pathway (N-cadherin and NCAM) and on the right are the ligand (FGF)-dependent pathways (Syndecan and Integrin). From (Murakami et al., 2008).

Neural cell adhesion molecule (NCAM) contains five Ig-like loops and two fibronectin type III domains. NCAMs are mainly involved in homophilic interactions with other NCAM, thereby mediating cell-cell adhesion, but can as well interact with proteins from the extracellular matrix. Recent data reviewed by Hinsby (Hinsby et al., 2004) showed that NCAMs function as signaling receptors for GDNF. NCAM may also serve as a ligand for FGFR1 (Hinsby et al., 2004), and direct interaction between FGFR4 and NCAM, as well as activation of FGFRs upon NCAM binding have been reported (Cavallaro et al., 2001; Kiselyov et al., 2003). Interestingly, on one

hand NCAM's interaction with FGFR stimulates cell-matrix adhesion (Cavallaro et al., 2001) while on the other hand it reduces FGF stimulated signaling as well as proliferation (Francavilla et al., 2007).

Finally, N-cadherins, involved in calcium dependent cell-cell adhesions can bind to FGFRs. The interaction between N-cadherin and FGFR modifies FGFR signaling and inhibits ligand-induced internalization of the receptor and its subsequent degradation, therefore resulting in an increase in FGFR signaling (Cavallaro & Christofori, 2004; Murakami et al., 2008).

3.8.FGF signaling in physiological processes

FGF receptors are widely expressed and almost all tissues express one or several FGFs. FGF signaling has been implicated in various processes: Early patterning and dorso-ventral axis formation (Thisse & Thisse, 2005), embryonic development where FGF signaling controls cell migration and gastrulation through regulation of E-cadherin via the transcription factor snail (Ciruna & Rossant, 2001), limb development (Xu et al., 1999), neuronal induction and patterning of the brain (Ford-Perriss et al., 2001), kidney and lens development (Bates, 2007; Robinson, 2006) as well as regulation of metabolism. To examine the contribution of this signaling pathway to development and its physiological role in vivo, whole body knockouts (KO), conditional knockouts (cKO), as well as knockin and transgenic mouse models expressing dominant negative FGFRs have been generated.

3.8.1. Role of FGF receptors and their ligands in development

In the absence of *Fgfr1*, embryos die prior to, or during gastrulation at day E9.5-12.5. These mutant embryos fail to develop properly due to problems in embryonic cell proliferation and migration, as well as defective pattern formation (Deng et al., 1994). Interestingly, specific knock-out of the *Fgfr1*-IIIc isoform displayed a similar phenotype as the total *Fgfr1* KO, whereas mice with *Fgfr1*-IIIb KO were viable and without obvious phenotype. These results display the important role that the FGFR1-IIIc isoform plays in embryonic development and emphasize that each isoform of FGFR1 has a specific and non overlapping function during development (Partanen et al., 1998; Yamaguchi et al., 1994).

Fgfr2 KO embryos do not survive later than E4.5-5.5 (Arman et al., 1998). Targeted deletion of the third Ig-like loop (D3) of *Fgfr2* results in embryonic lethality no later than E10.5, due to defects in the placenta and limb bud formation (Xu et al., 1998). An isoform specific KO approach has allowed closer examination of the phenotypes related to deletion of the IIIb or IIIc splice variant of *Fgfr2*. The IIIb isoform KO mice are viable until birth but do not survive after birth due to a failure in lung formation. Other defects associated with this model include the lack of anterior pituitary, forelimbs and hindlimbs. In addition, skeletal and skin abnormalities were observed (De Moerlooze et al., 2000). The IIIc isoform KO mice are viable but exhibited delayed ossification, dwarfism and a reduced length of the limb bones proportional to the reduced size of the whole skeleton. Interestingly, and in contrast to *Fgfr2*-IIIb KO, no developmental limb defects were observed in *Fgfr2*-IIIc KO animals (Eswarakumar et al., 2002). Here again isoform specific KO approaches emphasize the different roles played by the individual splice variants of *Fgfr2* in development.

Fgfr3 loss of function is not lethal and the phenotype includes skeletal overgrowth of long bones and vertebrae, kyphosis and inner ear defects (Colvin et al., 1996; Deng et al., 1996). Further analysis of the bone defects showed that it is caused by high proliferation, expansion and hypertrophy of chondrocytes.

Fgfr4 KO animals are viable and do not exhibit obvious abnormalities, suggesting that either *Fgfr4* is not essential during development or that other members of the family can compensate for its ablation (Weinstein et al., 1998).

Many studies have been devoted to the knock-out of *Fgf*s. For clarity, the KO studies described herein are grouped according to the different FGF subfamilies.

FGF1 subfamily: There is no obvious phenotype in the *Fgf1* KO animals (Miller et al., 2000), however the *Fgf2* mutants display neuronal, skeletal and skin phenotypes (Dono et al., 1998). Double KO animals for *Fgf1* and *Fgf2* are not doing worse than the phenotype observed in *Fgf2* KO animals (Miller et al., 2000).

FGF4 subfamily: *Fgf4* deletion is embryonic lethal at day E5.5, due to defects in trophoblast proliferation (Feldman et al., 1995). *Fgf5* is involved in regulation of hair growth and KO animals exhibit abnormally long hair (Hebert et al., 1994). *Fgf6* null animals have impaired muscle regeneration (Floss et al., 1997).

FGF7 subfamily: *Fgf7* ablation results in mice with a hair follicle defect (Guo et al., 1996) and decreases the number of nephrons in the kidney (Qiao et al., 1999). *Fgf3* is required for correct tail and inner ear development (Mansour et al., 1993). *Fgf10* KO animals showed postnatal death due to severely impaired development of the limbs, lungs and kidney (Min et al., 1998; Sekine et al., 1999). No KO of *Fgf22* has been described to date.

FGF8 subfamily: *Fgf8* KO embryos die at day E8.5 as a result of gastrulation failure (Sun et al., 1999). Moreover, mutant animals that possess both a hypomorphic and a null allele for *Fgf8* exhibit deletion of brain region and limb, as well as aberrations in heart, eye and craniofacial development (Meyers et al., 1998). Ablation of *Fgf17* leads to abnormalities in the midline cerebral development (Xu et al., 2000). *Fgf18* KO mice survive embryonic development but die at an early neonatal period. The phenotype of *Fgf18* KO animals shares features with the *Fgfr3* KO animals, with respect to skeletal development (Liu et al., 2002; Ohbayashi et al., 2002). In addition, *Fgf18* KO affects in lung development (Usui et al., 2004).

FGF9 subfamily: *Fgf9* KO led to postnatal lethality and the pups have a striking phenotype that consists in male-to-female sex reversal. The lungs of these animals show extreme hypoplasia, which is the most probable cause of death (Colvin et al., 2001a; Colvin et al., 2001b). *Fgf16* deletion results in embryonic lethality at day E11.5, due to a failure in heart development; facial defects were also observed (Lu et al., 2008b). No KO model of *Fgf20* have been described, however, it is expressed in the developing limbs (Hajihosseini & Heath, 2002) and cochlea. In addition, treatment of cochlear explants cultures with an antibody blocking FGF20 abolished normal hair cell development (Hayashi et al., 2008).

hFGF subfamily: FGF19 expression was detected in human fetal cartilage, skin, retina as well as adult gall bladder (Xie et al., 1999). The phenotype of the *Fgf15* KO (the mouse ortholog of

FGF19) animals consists of many phenotypes: small and depleted gall bladder, reduced neurogenesis and increased proliferation in the cortex (Borello et al., 2008; Wright et al., 2004). Targeted disruption of *Fgf21* has been reported and the mice are viable with no obvious developmental problems (Hotta et al., 2009). The *Fgf23* KO mice are viable but have severe growth retardation, abnormal bone phenotype, metabolism disorder, infertility and a short lifespan (Shimada et al., 2004).

3.8.2. FGF signaling in mammary gland development

Mammary gland development in mice occurs in two distinct phases: the embryonic and the postnatal phases. The embryonic phase initiates with the appearance of the milk line, consisting of a localized thickening of the ectoderm. At day E11.5, five pairs of mammary gland primordia (also called placodes) develop along the milk line. During the following days, these epithelial structures invade the surrounding mesenchyme and by E16 there is formation of a rudimentary ductal tree. From day E18.5 until puberty, the mammary gland does not undergo any striking changes. The postnatal phase starts at puberty when the ducts elongate and invade the mammary fat pad in response to circulating ovarian hormones. Structures called terminal end buds (TEBs) form at the tips of the mammary ducts and are responsible for growth of the ductal trees, ramification and invasion into the adipose tissue. The TEBs are multi-layered highly proliferative structures that contain a cap cell layer at the leading edge and multiple layers of body cells (Figure 3-7). Upon invasion into the fat pad and elongation of the ducts, part of the body cells will undergo apoptotic cell death to allow formation of the hollow lumen of the ducts. The body cells that do not die will generate the luminal epithelial cells of the mature ducts and the cap cells will develop into the myoepithelial cells required for milk secretion during lactation. This process takes place until postnatal weeks 10-12, after which the TEBs regress, leaving a fully functional mammary gland. Further remodeling of the mammary gland occurs during pregnancy, when the epithelial cells become highly proliferative and subsequently differentiate to form the lobuloalveolar structures responsible for milk production. Upon parturition, these structures regress by means of an apoptotic process called involution and remodeling of the stroma occurs. Once involution is complete, the remaining ductal tree structures resembles the one present after puberty. During its lifespan the mouse mammary gland can undergo repeated cycles of pregnancy,

lactation and involution (Dillon et al., 2004; Jackson et al., 1997a; Schwertfeger, 2009; Spencer-Dene et al., 2001).

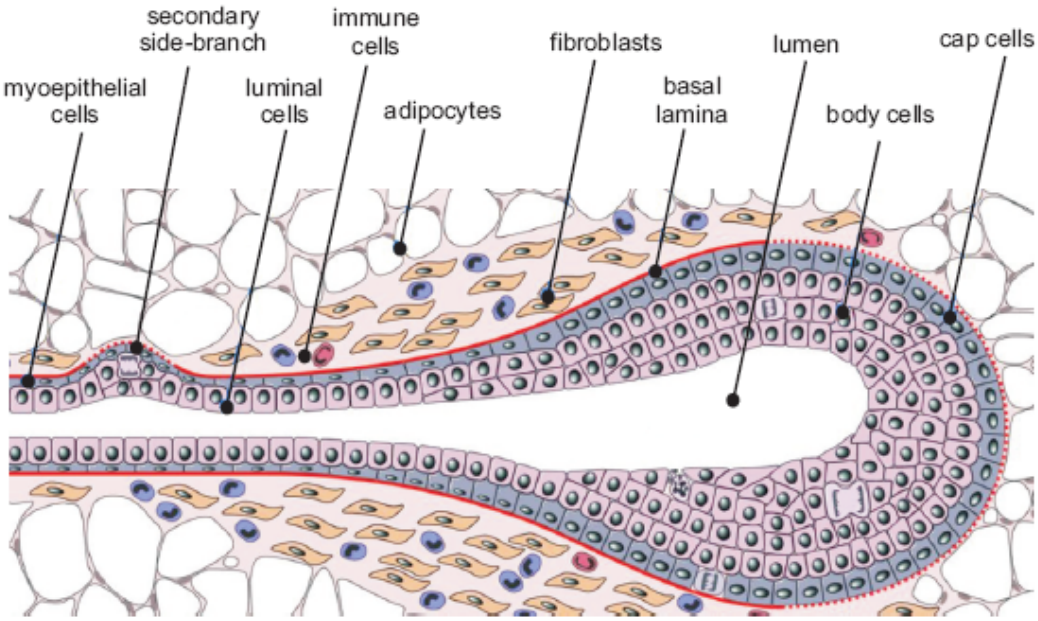

Figure 3-7 Architecture of terminal end bud (TEB)

Terminal end buds (TEBs) consist of highly proliferative multilayered structures. The cells of the cap layer are involved in motility of the end buds and give rise to the myoepithelial cells, whereas the cells from the body layer will form the luminal cells of the ducts. Both layers have a high rate of mitosis, allowing the end bud to be a highly dynamic structure but in addition, the body cells undergo apoptosis, to allow formation of a hollow lumen in the duct. Degradation of the extracellular matrix at the front of the end bud allows elongation of the duct and motility of the end bud. (Sternlicht et al., 2006)

Several FGFs (FGF4, FGF7, FGF8, FGF9, FGF10 and FGF17) and FGFRs (FGFR1-IIIb and FGFR2-IIIb) are expressed during the various embryonic and postnatal steps of mammary gland development and contribute to development of this organ (Coleman-Krnacik & Rosen, 1994; Hens & Wysolmerski, 2005; Pedchenko & Imagawa, 2000).

Transgenic mice with targeted expression of dominant negative FGFR1-IIIc and FGFR2-IIIb in the mammary gland revealed that only FGFR2-IIIb has a role in postnatal lobuloavleolar development of the mammary gland (Jackson et al., 1997a; Jackson et al., 1997b). Further studies showed that FGFR2-IIIb is also required for embryonic development of at least four out of the five placodes and KO animals for *Fgf10* or *Fgfr2*-IIIb fail to initiate and maintain mammary buds

during embryogenesis (Mailleux et al., 2002). In addition, attenuation of endogenous FGFR2-IIIb signaling in the mammary gland by inducible reversible expression of a soluble FGFR2-IIIb that acts as a ligand trap, showed that FGFR2-IIIb signaling plays a critical role during developmental stages of the mammary gland, where it controls the induction, the survival and proliferation of the placodes. Expression of this soluble FGFR2-IIIb at postnatal developmental stages show a decrease in formation and maintenance of the TEBs together with a decrease in proliferation and survival of the luminal epithelial cells with no effects on the regenerative potential of epithelial progenitor cells (Parsa et al., 2008). Another approach using cKO of *Fgfr2*-IIIb in the mammary gland showed duct elongation and branching defect, accompanied by a decrease in proliferation and invasion of the TEBs upon deletion of *Fgfr2*-IIIb (Lu et al., 2008a). These results suggest that FGFR2-IIIb is involved in embryonic as well as postnatal development of the mammary gland.

3.8.3. FGF signaling in skin homeostasis and repair

FGF7 also known as keratinocyte growth factor (KGF) was originally discovered as a mitogenic factor for a mouse keratinocyte cell line, suggesting that it may play an important role in the skin. Targeted expression of a truncated dominant-negative FGFR2-IIIb to suprabasal keratinocytes under the keratin 14 promoter led to epidermal atrophy and disorganization, as well as hair follicles morphology defects. Moreover, the rate of keratinocyte proliferation was strongly decreased (Werner et al., 1994). *Fgfr2*-IIIb KO animals do not survive beyond birth. However, at term, they show severe skin abnormalities like decreased thickness and decreased proliferation of the basal layer keratinocytes and they exhibit defects in number and distribution of hair follicles. Interestingly, *Fgf10* KO animals showed similar but less severe phenotypes (Petiot et al., 2003). These data suggest that FGF10 indeed signals via FGFR2-IIIb and they together contribute to skin development. As *Fgfr2*-IIIb KO animals die short after birth, conditional KO models using the Cre-lox system were developed (Sauer, 1998). Crossing of mice bearing floxed *Fgfr2*-IIIb with mice expressing the *Cre* recombinase under the keratin 5 promoter allowed specific deletion of *Fgfr2*-IIIb in the epidermis. Detailed analysis of the phenotypes demonstrates expression of FGFR2-IIIb is required for correct hair and sebaceous gland development. In addition, cutaneous inflammation was observed and hyperthickening of the epiderm as well as papilloma formation appeared in aging mice (Grose et al., 2007).

The role of skin as a barrier against invading microorganisms is well understood. However, damages or injuries to skin decrease its efficiency to function as a proper barrier. Wound healing is the repair process that is initiated immediately after injury and it has been described that KGF expression is strongly induced during wound healing (Komi-Kuramochi et al., 2005; Werner et al., 1992b). Interestingly, transgenic animals expressing a dominant negative FGFR2-IIIb in the epidermis show a severe delay in wound re-epithelialization, suggesting a role for this receptor in wound repair. Surprisingly, *Kgf* KO animals did not show any phenotype with respect to skin and do not show a defect in wound healing, suggesting that compensation mechanisms probably occur in these animals (Werner & Grose, 2003).

3.8.4. Hormone-like FGFs as metabolism regulators

The metabolic FGFs (FGF19, FGF21 and FGF23) were shown to be involved in glucose, lipid, bile acid, phosphate and vitamin D metabolism in an endocrine manner. The lack of a heparin-binding domain allows these FGFs to be efficiently secreted and released in the circulation (Goetz et al., 2007). Nevertheless, these factors still require a co-factor, Klotho or βKlotho, in order to bind to and activate FGFRs.

The best characterized role of FGF19/FGF15 is in bile acid homeostasis. In the intestine, FGF19/FGF15 expression is upregulated by the farnesoid X receptor (FXR), a nuclear hormone receptor that is a key regulator of bile acid synthesis. FGF19/FGF15 is released in the circulation and binds to FGFR4 through interaction with βKlotho in the liver, where it causes downregulation of CYP7A1, leading to a reduction of bile acid synthesis. In addition to hormonal control of bile acid biosynthesis, FGF19/FGF15 is a regulator of gallbladder filling and both *Fgfr4* and *FGF19*/*Fgf15* KO animals have small, depleted gallbladders (Jones, 2008). Another metabolic contribution of this growth factor was found in transgenic mice expressing FGF19 in the muscle under the myosin light chain promoter (Tomlinson et al., 2002). Indeed, these animals have a decrease in white adipose tissue and retain a lean phenotype under high fat diet (Figure 3-8 a).

FGF21 is released mainly from the liver but is as well found in pancreas, adipose tissue and muscle. Its expression is regulated upon fasting by the peroxisome proliferator-activated

receptors (PPARα and PPARγ) (Kharitonenkov, 2009), and it requires βKlotho as a coreceptor to activate FGFR1-IIIc and FGFR2-IIIc (Kurosu & Kuro-o, 2008; Suzuki et al., 2008). The target tissues of FGF21 are multiple (adipose tissue, pancreas and liver) (Figure 3-8 b) and the effects of FGF21 stimulation were first described in adipocyte, where it induces an increase in glucose uptake by upregulation of the glucose transporter GLUT1 (Kharitonenkov et al., 2005). Similar to the FGF19 transgenic mice, animals with over-expression of FGF21 in the liver were resistant to diet induced obesity, and have improved metabolic profiles. In contrast, FGF21 deficiency led to increased body weight, development of fatty liver and reduced oxygen consumption.

FGF23 primarily originates from bone and requires Klotho as a co-receptor. Interestingly, *Fgf23* KO animals and Klotho mutant mice have similar phenotypes showing premature aging, increased renal expression of *Cyp27b1* (1α-hydroxylase), high vitamin D in the blood, hyperphosphataemia and impaired bone mineralization, as well as ectopic calcification in soft tissues (Shimada et al., 2004; Tsujikawa et al., 2003). As co-expression of Klotho and FGFRs is found in the kidney and in the parathyroid gland (Ben-Dov et al., 2007; Liu et al., 2008), the effects of FGF23 signaling were studied in details in these tissues. In the kidney, FGF23 binds to FGFR1-IIIc and has two major effects: it decreases the levels of *Cyp27b1*/*Cyp24* (24-hydroxylase) that in turn reduces production of active vitamin D (Saito et al., 2003). In the parathyroid gland, FGF23 by activating FGFR1 or FGFR3 inhibits parathyroid hormone (PTH) production and secretion, leading to a drop of PTH concentration in the serum. This decrease in PTH leads to a decrease of *Cyp27b1* in target tissues and a decrease of vitamin D (Urakawa et al., 2006). The effects of FGF23 on parathyroid gland and kidney have in common a lowering of vitamin D, that in turn affects reabsorption of phosphate in the kidney through regulation of the expression of Na/Pi cotransporters (*Npt2a* and *Npt2c*) and absorption of phosphate in the intestine (Figure 3-8 c) (Beenken & Mohammadi, 2009; Kurosu & Kuro-o, 2008; Kurosu & Kuro, 2009).

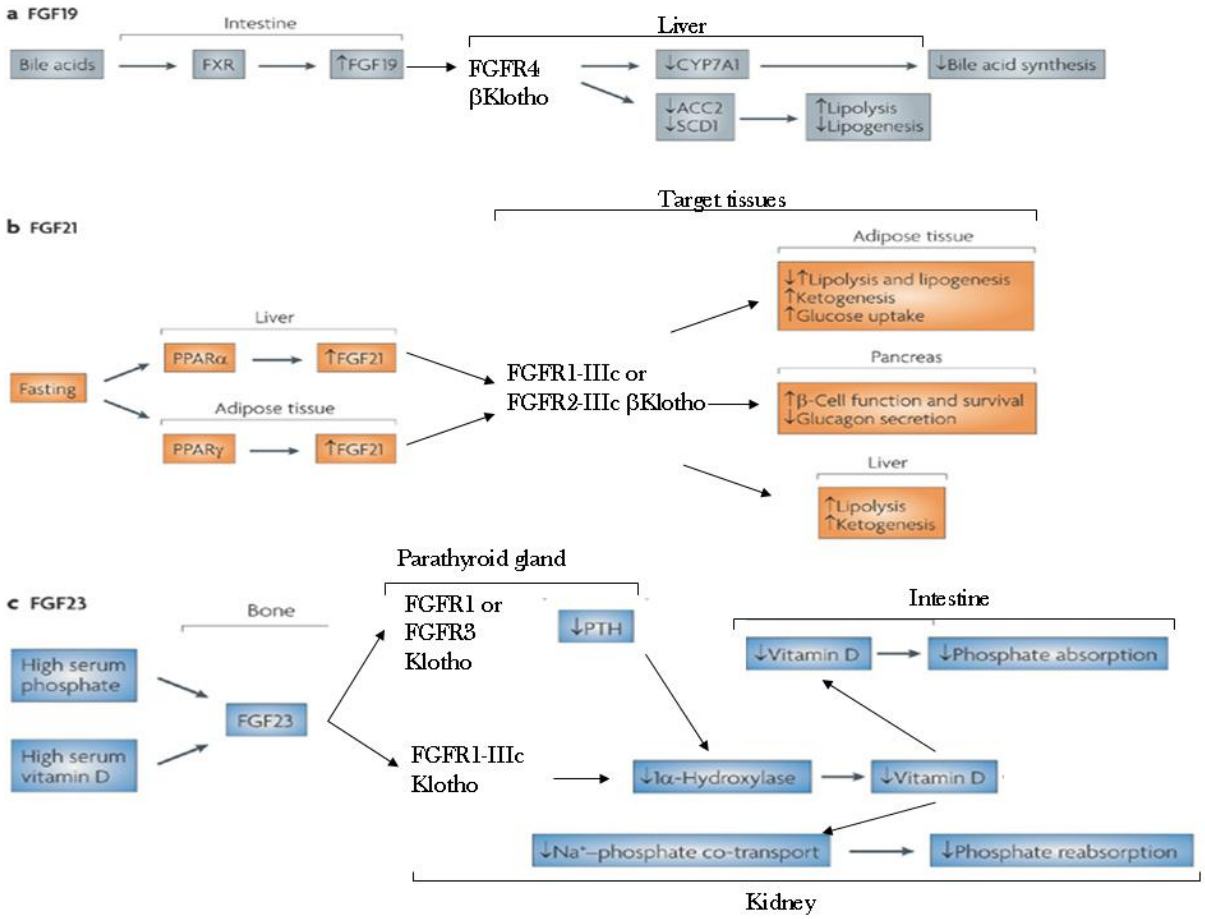

Figure 3-8 hFGFs and their role in metabolism

a) Regulation of FGF19 by FXR receptor in the intestine and its role in the regulation of bile acid synthesis through βKlotho mediated activation of FGFR4 in the liver.

b) FGF21 mediates PPAR induced fasting response through tissue specific βklotho dependent activation of FGFR1-IIIc or FGFR2-IIIc.

c) FGF23 production in the bone is upregulated in response to high serum phosphate and vitamin D. FGF23 activates FGFR1 or FGFR3 with the Klotho coreceptor in the parathyroid gland and decreases PTH release, which downregulate. FGF23 also directly acts on FGFR1-IIIc and Klotho on the kidney where it modulates 1α-hydroxylase levels. This leads to changes in Vitamin D expression and decrease phosphate absorption in the intestine as well as decrease phosphate reabsorption from the Kidney. Adapted from (Beenken & Mohammadi, 2009)

3.9.Deregulation of FGF signaling

FGFs and their receptors have been associated with multiple biological activities and several developmental processes. Knockout and knockin approaches highlighted the fact that tightly regulated FGF signaling is required for growth and development of healthy organisms. However, various mechanisms can lead to uncontrolled and deregulated FGF signaling reviewed in (Eswarakumar et al., 2005; Grose & Dickson, 2005). Deregulation of FGF signaling as well as

other signaling pathway can be achieved by several mean (the biology of cancer, Weinberg, Garland Science): Co-expression of a ligand and its receptor by the same cell leads to a constitutive autocrine activation of the downstream signaling pathways. Overexpression of ligands may increase the signals received by a cell, thus leading to an inappropriate response to the stimuli. Protease mediated shedding of ligands from their co-receptor and their release into the blood stream, allows a broader and uncontrolled activity of the ligands on tissues where they normally do not induce a response. Isoform switching can cause active signaling in inappropriate tissues. Point mutations as well as gain or loss of function mutations can deregulate a signaling pathway. Studies of the kinetics of phosphorylation and activation of the kinase domain of FGFR1, showed that activation of this receptor occurs in a two-step mechanism mediated by ordered and regulated autophosphorylation (Furdui et al., 2006). Interestingly, follow up studies demonstrated that oncogenic point mutation in the kinase domain disrupt this order of autophosphorylation, thereby leading to aberrant activation of downstream signaling molecules (Lew et al., 2009). Ligand independent activation of a receptor can be achieved either by structural alterations leading to constitutive activity of the kinase domain or by overexpression of the receptor. Genetic rearrangements like gene amplification, translocation and fusion can deregulate the expression of a gene or lead to expression of constitutively active mutant lacking regulatory domains. Finally, polymorphisms have been shown to modulate the expression of certain genes as well as the activity of proteins. All these processes of deregulating a signaling pathway have been reported for FGF signaling and contribute to development of several diseases.

3.9.1. Deregulation of FGF signaling in human diseases.

Craniosynostosis occurs with a frequency of approximately 1 in 2500 individuals and consists in the premature fusion of the skull sutures. Nine craniosynostosis syndromes have been described, showing different severities in abnormal skull sutures as well as limb abnormalities. All these syndromes were linked with germ line point mutations in FGFR1 or FGFR2. The majority of mutations are located in the ligand binding area, in the Ig-like domain III (Wilkie, 2005). Most of these mutations consist of a loss or gain of a cysteine residue that induce increase of affinity for the ligand or ligand-independent dimerization and activation of the receptors (Eswarakumar et al., 2005; Marie et al., 2005; McIntosh et al., 2000).

Fgfr3 KO animals showed elongated bones therefore, it is not surprising that activating mutations in *FGFR3* have been associated with human dwarfism (Deng et al., 1996). In the most common form of this disease (achondroplasia), FGFR3 mutations occur in the transmembrane domain and lead to increase of both kinase activity and stability of the mutant FGFR3, thereby activating FGFR signaling in a ligand independent manner. The activating mutations in FGFR3 are thought to act in part through the activation of STAT and lead to a decrease in chondrocyte proliferation and therefore a decrease of the hypertrophic zone in the growth plates (Coumoul & Deng, 2003; L'Hote & Knowles, 2005).

FGF3 mutations are associated with a rare form of sensorineural hearing loss, called Michel aplasia. This disease is characterized by a complete loss of the inner ear structures, however the effect of the FGF3 mutations on FGFR signaling have not yet been studied (Krejci et al., 2009).

Lacrimo-auriculo-dento-digital (LADD) syndrome is an autosomal dominant disease characterized by hearing loss, dental and digital anomalies. Missense mutations in FGF10, FGFR2 and FGFR3 have been found in patients suffering from this syndrome (Milunsky et al., 2006; Rohmann et al., 2006). The contribution of these mutations to the disease is probably through a loss of function mechanism (Lew et al., 2007; Shams et al., 2007).

Loss of function mutations in FGF8 interfering with its binding on FGFR1 as well as mutations in FGFR1 lead to Kallmann's syndrome. This disease is a developmental disorder characterized by absent or delayed puberty, hypogonadism, low serum levels of gonadotropins and defective sense of smell (anosmia) (Dode & Hardelin, 2009; Dode et al., 2003; Hardelin & Dode, 2008).

Parkinson disease which affects approximately 1% of the population has symptoms including tremor, bradykinesia and rigidity. The main cause of the disease is due to a loss of dopaminergic neurons through an apoptotic death. Three single nucleotide polymorphisms (SNPs) were found on *FGF20* and showed significant association with the disease (van der Walt et al., 2004). These SNPs have been shown to increase *FGF20* translation, leading to upregulation of α-synuclein, that is one of the agents causing the disease (Beenken & Mohammadi, 2009; Krejci et al., 2009).

Several disorders are caused by mutations in FGF23. Autosomal dominant hypophosphataemic rickets (ADHR) is a disorder of renal phosphate wasting, defective cartilage mineralization and defective bone mineralization. The mutation responsible for this disease disrupts a cleavage site for subtilisin-like proprotein convertase, rendering FGF23 less susceptible to degradation, therefore increasing the biological activity of FGF23 and leading to the disease (Beenken & Mohammadi, 2009; Krejci et al., 2009). Inactivating mutations of the PHEX endopeptidase in X-linked hypophosphataemic rickets leads to increased level of circulating FGF23. On the other hand, decreased FGF23 signaling due to destabilizing mutations causes pathologies like familial tumoral calcinosis that is characterized by hyperphosphataemia and deposition of calcium crystals in periarticular spaces. Mutations in Klotho have been implicated in the same type of disease, showing the importance of FGF23, Klotho axis in this disease.

3.9.2. Aberrant FGF signaling in human cancers

The effects of inappropriate FGF signaling have been intensively examined in tumorigenesis and it appears that FGFRs and their ligand contribute to progression of various cancers.

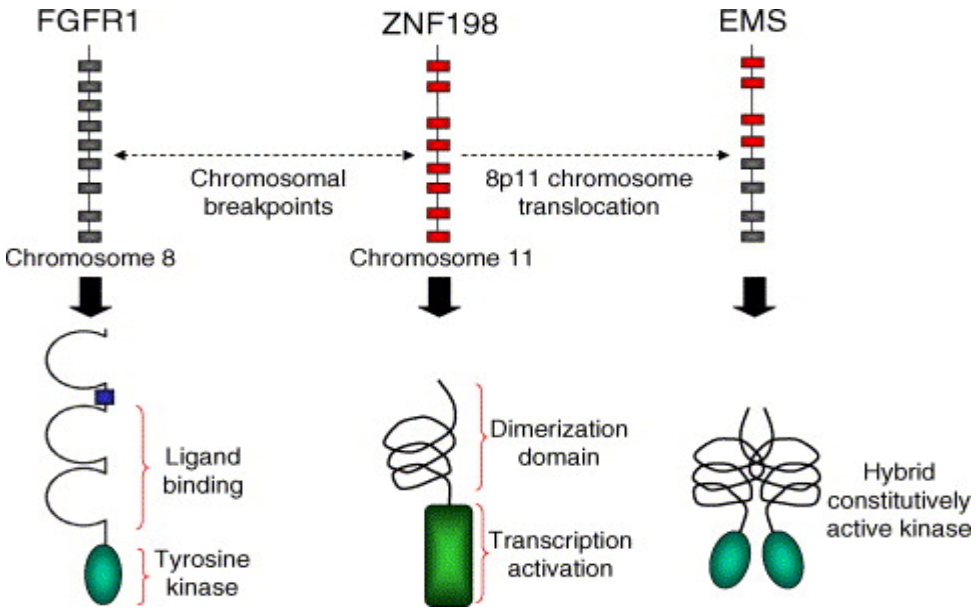

Figure 3-9 Mechanisms of translocation and fusion

Translocation of part the FGFR1 gene (the kinase domain) and fusion with ZNF198 (the dimerization domain) leads to formation of a fusion protein that consist of the dimerisation domain of ZNF198 and the kinase domain of FGFR1. Interaction of two fusion proteins mediated by their dimerization domain allows the kinases to get constitutively activated and therefore signal in an uncontrolled manner (Grose & Dickson, 2005).

In hematological malignancies, chromosomal translocation and fusion of *FGFR1* with different partners has been described and plays an important role in myeloproliferative syndromes (MPS). Translocation between *FGFR1* and a partner gene causes a disease characterized by myeloid hyperplasia, eosinophilia and lymphoblastic lymphoma that is called 8p11 myeloproliferative syndrome (EMS). The cause of this disease results in the fusion between the kinase domain of FGFR1 and a dimerization domain, leading to constitutive tyrosine kinase activity of the fusion protein (Figure 3-9). This in turn protects the cells expressing this fusion protein from apoptosis (Eswarakumar et al., 2005; Grose & Dickson, 2005). In addition to *FGFR1*, translocation and fusion with a dimerization domain, as well as activating mutations, were described for *FGFR3* in multiple myeloma (L'Hote & Knowles, 2005).

In the prostate, FGFs are required for development, growth and maintenance of prostatic tissue. Therefore it is not surprising that deregulated FGF signaling in the prostate leads to malignancy. Indeed, expression of various FGFs (FGF1, FGF2, FGF6, FGF8, FGF9 and FGF17) has been described in prostate cancer. In addition, FGF8 expression correlates with advanced tumor stage and is associated with decreased survival (Cronauer et al., 2003; Dorkin et al., 1999; Mattila & Harkonen, 2007). Expression of FGFR1 was found in 20% of moderately differentiated and 40% of poorly differentiated cancers, arguing that with transformation and progressive loss of differentiation, there is an increase of FGFR1 expression in prostate cancer. In addition, chemical activation of FGFR1 in the prostate epithelium of transgenic animals leads to development of hyperplasia (Acevedo et al., 2007; Kwabi-Addo et al., 2004; Winter et al., 2007). Another interesting observation was made in prostate tumors upon disease progression; namely an isoform switch from the normally epithelia-expressed FGFR2-IIIb, to the mesenchymally-expressed FGFR2-IIIc (Grose & Dickson, 2005). The switch in FGFR2 isoforms to the mesenchymal form may induce a change from paracrine to autocrine activation of the signaling downstream of FGFRs, since the epithelial-cell derived FGF ligands may activate the FGFR2-IIIc isoform in the tumor cells.

FGF1 and FGF2 are increased in the urine of patients with bladder cancers (Cronauer et al., 2003). Expression of other FGFs (FGF5 and FGF8) was also reported in bladder cancer. As for prostate carcinoma, it seems that low expression or loss of the isoform IIIb of FGFR2 is

associated with poor prognosis, suggesting that in bladder cancer, this receptor could act as a tumor suppressor. Activating mutations of FGFR3 located either on the ligand binding domain or in the kinase domain were described in 40% of the cases and FGFR3 has been shown to be required for bladder cancer cell proliferation (Cronauer et al., 2003; Grose & Dickson, 2005; Knowles, 2008).

Several other human cancers show deregulation of FGF signaling. In pancreatic cancer high expression of several FGFs (1, 2, 5 and 7) and most of the FGFRs was observed, leading to an autocrine activation of the receptors and downstream signaling pathway (Kornmann et al., 1998). Activating mutations in FGFR2 have been found in 10% of endometrial cancers (Byron et al., 2008; Byron & Pollock, 2009). Other mutations in FGFRs were found in lung cancer (FGFR2) (Davies et al., 2005), ovarian cancer as well as colorectal and gastric cancer (FGFR2 and FGFR3) (Jang et al., 2001; Katoh, 2008).

3.9.3. FGF signaling in mammary tumors and in human breast cancers

Breast cancer is the main diagnosed cancer in women (27% of the cases) and is the major cause of death in women between 35 and 59 years old (American Cancer Society, Cancer Facts and Figures 2009). However, what we call breast cancer is not a homogenous disease and gene expression studies performed on breast tumors showed that this pathology can be divided in at least 5 subtypes; namely Luminal A, Luminal B, ERBB2+, Basal-like and Normal Breast-like (Figure 3-10) (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003).

Luminal A and B subtypes are estrogen receptor positive (ER+), with the highest expression level of ER in the luminal A, while the other subtypes are all characterized by low or absent expression of ER. The ERBB2+ subtype has an amplification of the *ERBB2* gene, leading to high expression of the receptor tyrosine kinase ErbB2. The basal-like subtype has high expression of keratins 5 and 17 and laminin. Normal breast-like group shows high expression of genes normally expressed by adipose tissues or non-epithelial cell types. Interestingly, these different subtypes of breast cancer show distinctive prognoses, with the best prognosis for luminal A and the worst for the basal-like subtype (Figure 3-10) (Perou et al., 2000; Sorlie et al., 2001).

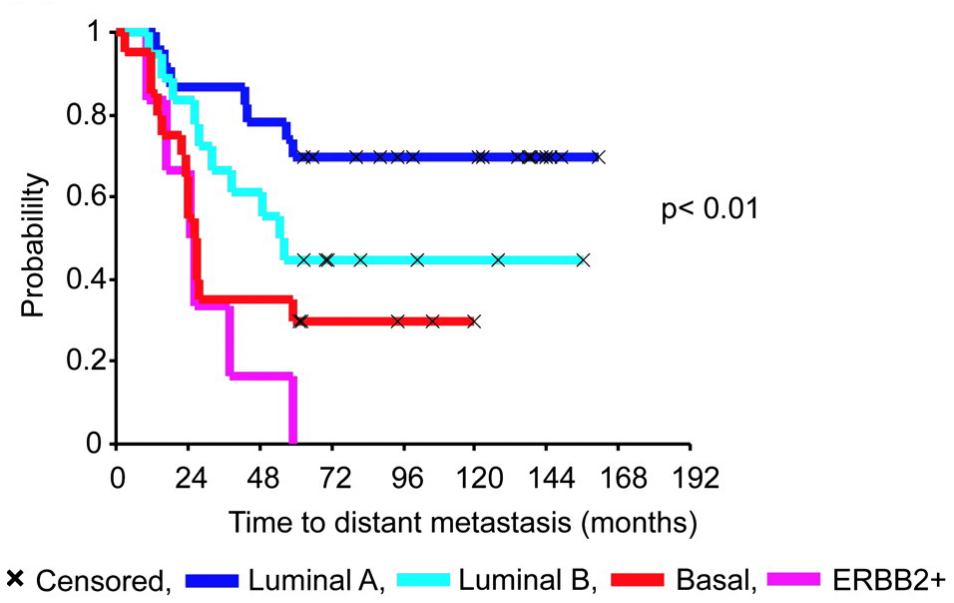

Figure 3-10 Prognoses of different subtypes of breast cancer

Kaplan-Meier analysis of breast cancer outcome. The study was recording time to development of distant metastasis in 97 cases of breast cancer (Sorlie et al., 2003).

The assumption that FGFs could contribute to mammary tumorigenesis started with studies investigating the integration sites of the mouse mammary tumor virus (MMTV) (Nusse & Varmus, 1982). Indeed additional studies showed that the MMTV integrates commonly near genes coding for members of the FGF signaling pathway (*Fgf3, Fgf4, Fgf6, Fgf8, Fgf10* and *Fgfr2*) (Mattila & Harkonen, 2007; Peters et al., 1989; Theodorou et al., 2004; Theodorou et al., 2007). To validate these observations, transgenic animal models were developed that express FGF3 or FGF8 in the mammary gland under the control of the MMTV promoter and all the animals developed mammary tumors (Daphna-Iken et al., 1998; Muller et al., 1990). Another approach expressed a chemically activable FGFR1 kinase domain under the MMTV promoter (Welm et al., 2002). Prolonged treatment of the animals with a chemical inducing dimerization and activation of the kinase domain led to invasive lesions in the mammary gland. Interestingly, an in vitro study comparing the activity and signaling properties of inducible FGFR1 kinase versus inducible FGFR2 kinase showed that both receptors can induce proliferation, however only FGFR1 signaling was sustained and promoted an invasive phenotype. One mechanism proposed for these differences in activity is the increased internalization and down-regulation of

FGFR2 after its activation, possibly via interaction with the ubiquitin ligase Cbl (Xian et al., 2007).

In contrast to the clear contribution of FGFs to mouse mammary tumors, the role of FGFs in human breast cancer is still unclear. Considering the genes involved in MMTV induced mouse mammary tumors, FGF4 and FGF6 are the growth factors about which the least data are available. *FGF4* is located within the 11q13 genetic locus that is commonly amplified in about 18% of breast cancers (Karlseder et al., 1994), however no data have been published concerning the expression of FGF4 in breast tumors. The expression of FGF6 has been described as being more restricted than FGF1 and FGF2 in a panel of breast cancer tumors and cell lines (Penault-Llorca et al., 1995), however no recent study aimed at better analyzing the pattern of FGF4 and FGF6 expression in breast tumors. *FGF3* (*int-2*) is also located in the 11q13 amplicon (Karlseder et al., 1994), and amplification of *FGF3* correlates with increased aggressiveness in node-negative breast carcinoma (Fioravanti et al., 1997). FGF8 was found elevated in malignant compared to non-malignant breast tissues (Marsh et al., 1999) and FGF10 is overexpressed in breast carcinoma (Theodorou et al., 2004). Data concerning FGF2 are ambiguous: it has been shown to be expressed at lower levels in human breast cancer (Colomer et al., 1997; Luqmani et al., 1992), but at the same time, elevated FGF2 expression in breast tumors is associated with more aggressive form of the disease and is elevated in the serum of patient with breast cancer (Sliutz et al., 1995; Visscher et al., 1995). In addition, high level of FGF2 correlates with resistance to paclitaxel (Gan et al., 2006). FGF1 expression data are as well controversial, as FGF1 was found to a lower extent in breast tumor when compared to normal tissue (Bansal et al., 1995) but other studies showed that FGF1 is higher in cancer than in benign tumors (Yoshimura et al., 1998). These controversial results could reflect different types of breast tumors examined and warrant further analysis of the level of FGF1 and FGF2 in breast cancer patients.

With respect to FGFRs, high expression of *FGFR1*, *FGFR2* and *FGFR4* mRNA was detected in 22%, 4% and 32% of breast cancer respectively and gene amplification of FGFR1 was found in 9% of tumors (Jacquemier et al., 1994; Penault-Llorca et al., 1995). Recent studies analyzed in detail the 8p11-12 amplicon, containing *FGFR1*. Because it is not always overexpressed when amplified, it is not clear if *FGFR1* is always the driving oncogene on the 8p11-12 amplicon (Ray

et al., 2004). An interesting observations is that when amplified and overexpressed, *FGFR1* associates with poor prognosis and it is by itself an independent prognostic factor for overall survival (Elbauomy Elsheikh et al., 2007; Gelsi-Boyer et al., 2005). In addition, genetic alterations involving *FGFR1* amplification and overexpression were found in classic lobular carcinomas (Reis-Filho et al., 2006). Despite some conflicting data on its expression in the 8p11-12 amplicon, FGFR1 is an interesting drugable target that needs to be considered in FGFR1 overexpressing tumors.

Recently, two genome-wide association studies identified single nucleotide polymorphisms (SNPs) that located on *FGFR2*. One study looked at alleles associated with breast cancer susceptibility in patients with a strong family history of breast cancer, and found that the most significantly associated SNP was located within the intron 2 of *FGFR2* (Easton et al., 2007). The second study was performed on postmenopausal patients with invasive breast cancer (Hunter et al., 2007). Here, four SNPs were found, all located in the intron 2 of *FGFR2*. A follow up of the study from Easton et al., showed that the SNP on *FGFR2* had a stronger association with: estrogen positive (ER+) than with ER- tumors, lower grade than higher grade and with node positive than negative tumors (Garcia-Closas et al., 2008). Interestingly, it has been reported that the major SNP in *FGFR2* described in the Easton study increases the binding of the transcription factors Oct-1/Runx2, that could leading to an increase in the expression of FGFR2 (Meyer et al., 2008). Finally, another study showed that FGFR2 is up-regulated in luminal A and basal subtypes (Nordgard et al., 2007).

One SNP from Guanine to Adenine was described in codon 388 of *FGFR4*, which generates receptors with either a glycine (Gly) or an arginine (Arg) at the residue 388 in the transmembrane domain of FGFR4 (Bange et al., 2002). In vitro data shows that the FGFR4 Arg^{388} gives a more motile phenotype to breast cancer cells. In patients, the contribution of the Arg^{388} SNP to breast cancer progression is controversial. Bange and colleagues report that this allele is significantly overrepresented in the group of breast cancer patients with positive axillary lymph node and early relapse (Bange et al., 2002), suggesting this allele as a poor prognostic factor in breast cancer. Thussbas et al., showed that the Arg^{388} genotype is a marker for breast cancer progression in patients with adjuvant systemic therapy, particularly chemotherapy, and this could be linked with

therapy resistance (Thussbas et al., 2006). However Jezequel and colleagues found that this SNP is not relevant for breast cancer prognosis (Jezequel et al., 2004). Another interesting study shows that in patients, levels of FGFR4 predict failure on tamoxifen therapy, suggesting that FGFR4 causes tamoxifen resistance in estrogen receptor positive (ER+) tumors (Meijer et al., 2008). It would be interesting to know the status of the, the Arg^{388} SNP in the patients used in this study, to see if it correlates as well with a poorer prognosis than the wild type allele.

3.10. Metastatic spread of cancer cells

The majority of mortality in cancer patients is not due to the primary tumor but is caused by spread of cancer cells that grow in distant organs. The whole process consisting of escape, spread and growth at distant organs is called the metastatic process. In order to establish distant colonies and grow away from the primary tumor, cancer cells have to go through a complex multistep process (Figure 3-11) (Bacac & Stamenkovic, 2008; Gupta & Massague, 2006).

Detachment

Altered cellular adhesion is the first step of the metastatic process. In comparison to normal epithelia, transformed and malignant cells show diminished tight and adherent junctions as well as desmosomes. Cadherins are important mediators of adherent junctions in epithelia. Indeed, it is well documented that the loss of E-cadherin alone or together with an increased expression of N-cadherin drastically changes the adhesive properties of tumor cells (Cavallaro & Christofori, 2004). This switch in cadherin expression is part of a broader program called epithelial-to-mesenchymal transition (EMT). EMT is the conversion of epithelial cells to highly motile cells expressing mesenchymal rather than epithelial markers. Several transcription factors (such as Snail, Slug and Twist) have been implicated in this process, in development but as well in tumor progression (Ciruna & Rossant, 2001; Yang et al., 2004; Yang et al., 2006). Cell-cell interactions are important in regulation of adhesion and integrins that mediate cell-extracellular matrix interactions also emerge as important mediators of the malignant phenotype (Guo & Giancotti, 2004).

Degradation of the basement membrane and extracellular matrix

The basement membrane is the first barrier that protects from invasion. It is composed of well-organized glycoproteins and proteoglycans (collagen, laminin, perlecan). In order to invade into the stroma, tumor cells need to proteolytically disrupt the basement membrane. The major enzymes involved in this process are matrix metalloproteinases (MMPs) (Benaud et al., 1998). MMPs can be membrane bound or secreted proteins and in addition to degradation of the basement membrane, they can release growth factors from the reservoir that in turn can promote tumor growth. Beyond basement membrane, the cells contact the extracellular matrix that they need to disrupt as well. Here again the MMPs as well as other proteases play an important role (Duffy et al., 2000). Degradation and remodeling of the extracellular matrix allows cancer cells to move and invade the environment close to the tumor.

Intravasation

In order to colonize distant organs, cancer cells must enter the vascular network. An active way of intravasation has been reported involving MMPs for the degradation of the vascular basement membrane, thus allowing the cells to enter into the circulation (Kim et al., 1998). Other ways of intravasation, are possible, in which tumor cells take advantage of the leakiness of tumor vessels and enter the blood stream in a more passive way. For both ways of intravasation, it is not yet clear if the tumor cells need to directly interact with the endothelial cells prior to entering the blood stream (Dua et al., 2005). Interestingly, detection of early metastasis usually occurs in the first draining lymph node close to the tumor and only later in other organs. These observations suggest that some tumor cells leave the primary tumor by invading the lymphatic vessels. After invasion of the lymph node, in a passive or active way, the cells are transported to the vena cava and enter the blood stream, thus allowing them to reach distant organs (Sleeman et al., 2009).

Survival in circulation

Once tumor cells have invaded the blood stream, they can on the one hand have access to all organs, but on the other hand they have to survive in a hostile environment. Circulating tumor cells are exposed to several stresses, including physical damage due to shear forces and interaction with killer immune cells. As tumor cells in the circulation lose all adhesive support,

which in a normal cell would induce death by anoikis, they also have to find a way to become resistant to this type of cell death (Bacac & Stamenkovic, 2008; Gupta & Massague, 2006).

Extravasation

The process of extravasation consists of escaping out of the blood stream into a target tissue. The precise mechanisms by which a cell can leave the vasculature are still not fully understood, however, at least two hypotheses have been described. The first is that a tumor cell, due to size constraint (especially if it aggregates with components of the blood, like platelets) gets trapped in the capillaries and can physically not move. Once immobilized, the cancer cell again starts proliferating and the forces applied by the growing metastasis might disrupt the capillary wall and allow the cells to invade the new environment.

The second way cells could escape the vasculature is by using a mechanism similar to those used by immune cells. Indeed, tumor cells do express several chemokine receptors at their plasma membrane, which act as receptors for secreted factors. The different concentration of ligand is sensed by the tumor cells and acts as a guiding factor to determine where the cells will extravasate.

Interestingly several studies on breast cancer have determined sets of key genes that need to be expressed by the tumor cells in order to give organ specific metastasis (lung or bone) (Minn et al., 2005a; Minn et al., 2005b; Sloan & Anderson, 2002). Moreover recent publications have suggested that by the early secretion of certain factors, tumors have the ability to prepare the microenvironment of distant organs to receive metastatic cells and allow or even promote their proliferation (McAllister et al., 2008).

Growth in secondary sites

After extravasation, the tumor cells may proliferate or stay dormant for many years. For some cancer, in particular breast cancer, it has been reported that metastases occur as long as 20 years after removal of the primary tumor. The reasons for this are likely to be multiple: Micrometastases are not able to promote angiogenesis and therefore the balance between cell proliferation and cell death is almost at equilibrium; inability of the metastatic cell to restart the

cell cycle machinery; non permissive environment due to the lack certain factors required by the cells for growth and proliferation (Weinberg, 2008).

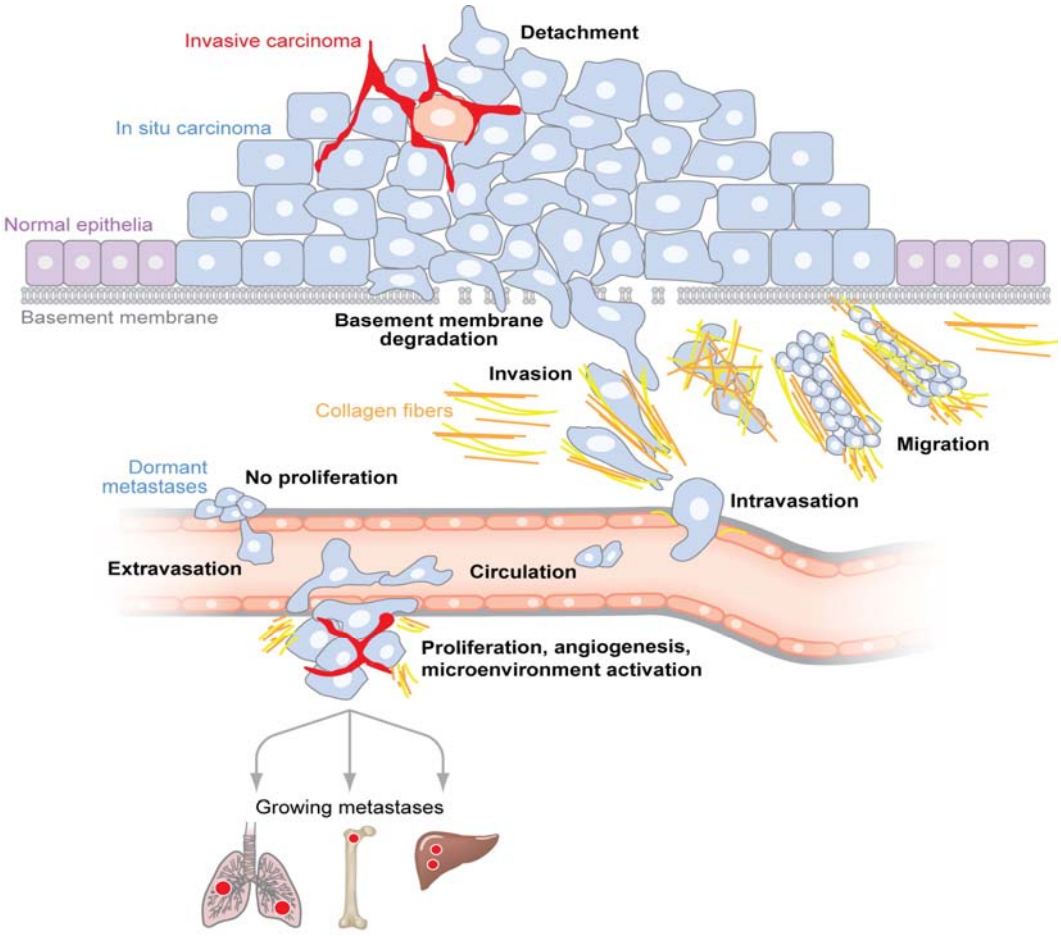

Figure 3-11 Summary of the metastatic process

The progression from a non-invasive in situ carcinoma to growing metastases in distant organs requires several steps. First the cells in the tumor should no longer adhere to each other. Then they need to degrade the basement membrane and the extracellular matrix in order to be able to move away from the tumor. This step requires expression of proteases. Once the motile cells meet a lymph or blood vessel, they need to intravasate, survive into these vessels and extravasate in a distant place. After extravasation, the cells should restart to proliferate in order to grow as metastatses. (Bacac & Stamenkovic, 2008).

3.11. Targeting FGF signaling

As already mentioned, FGF signaling contributes to cell proliferation, cell survival, migration and invasion. When deregulated, these processes are involved in the development of diseases, thus it

is not surprising that both academic and industrial researchers are focusing on targeting FGF signaling. The approaches are multiple, but the most promising ones can be classified into two main categories: The antibody based strategies and the small molecular weight kinase inhibitors.

3.11.1. Antibody based therapies

Several monoclonal antibodies were developed that specifically bind to FGF2 or FGF8. These neutralizing antibodies bind to the ligands and sterically inhibit activation of the FGFRs by these FGFs.

Early studies showed that subcutaneous tumor growth of K1000 cells is suppressed in vivo upon injection of a neutralizing antibody targeting FGF2 (Hori et al., 1991). Interestingly, a strong decrease in tumor angiogenesis was reported in the treated groups. These observations opened the door for investigations of the role that FGFs, in particular FGF2, play in angiogenesis. Other publications report the role of FGFRs and their ligand in proliferation, migration and tubular morphogenesis of endothelial cells. The actual view is that in cancer, FGFs secreted by the tumor act on the one hand in an autocrine manner promoting tumor growth, and on the other hand act in a paracrine manner on endothelial cells, therefore promoting tumor angiogenesis (Compagni et al., 2000; Rusnati & Presta, 2007). It is now well accepted both FGF2 and VEGF play roles in healthy as well pathological angiogenesis and that they can be targeted by neutralizing antibodies (Auguste et al., 2003; Cross & Claesson-Welsh, 2001; Presta et al., 2005).

FGF8 is highly expressed in breast cancers, therefore approaches to neutralize FGF8 using antibodies are obvious (Tanaka et al., 1998). Indeed several studies showed that preventing a specific isoform of FGF8, to bind to its receptors (FGFR2-IIIc, FGFR3-IIIc and FGFR4) efficiently reduces tumor growth in mouse models of breast and prostate cancers (Maruyama-Takahashi et al., 2008; Shimada et al., 2005).

Mice expressing FGF19 in the skeletal muscle develop hepatocellular carcinomas (Nicholes et al., 2002). In addition FGF19 was found to be highly expressed in lung squamous cell and colon carcinomas (Desnoyers et al., 2008). A monoclonal antibody that selectively blocks the interaction of FGF19 with FGFR4 abolished FGF19-mediated activity in vitro, inhibited growth

of colon tumor xenografts in vivo and effectively prevented hepatocellular carcinomas in FGF19 transgenic mice (Desnoyers et al., 2008). Detailed analysis of the molecular mechanisms showed that deregulation of the β-catenin pathway plays an important role in FGF19 mediated growth of colon tumors and colon cancer cell lines. Indeed, treatment with the anti-FGF19 rescued cells from deregulated β-catenin signaling. These findings suggest that the inactivation of FGF19 could be beneficial for the treatment of colon and liver cancer as well as other malignancies involving interaction of FGF19 and FGFR4 (Desnoyers et al., 2008; Pai et al., 2008).

Recently a function-blocking monoclonal antibody specific to FGFR3 (R3Mab) was developed. The epitope recognized by R3Mab prevents FGF binding to the receptor and subsequent dimerization and activation of the FGFR. Remarkably, not only ligand induced activity of wild-type receptors and downstream signaling was blocked, but the disulfide-linked, ligand-independent dimerization of a mutant FGFR3 ($FGFR3^{S249C}$) and subsequent activation of downstream signaling pathways were as well inhibited. In addition, this study showed that in animal models of FGFR3 driven bladder carcinoma and multiple myeloma, R3Mab exert a strong antitumor activity by disrupting FGFR signaling and by inducing antibody-dependent cell-mediated cytotoxicity (Hadari & Schlessinger, 2009; Qing et al., 2009).

Another interesting approach consists in conjugating a cytotoxic drug to antibodies that will specifically interact with mutated or overexpressed targets on cancer cells. This technique allows targeted delivery of the drug and should decrease the side effects.

3.11.2. Small molecule tyrosine kinase inhibitors based therapies

Small molecular weight tyrosine kinase inhibitors (TKIs), that block the activity of the tyrosine kinase and activation of their downstream pathways are nowadays used in the clinics to treat some cancers (Levitzki & Gazit, 1995). Several TKIs showed activity towards FGFRs, among them TKI258 (Renhowe et al., 2009), PD173074 (Pardo et al., 2009; Skaper et al., 2000), SU5402 (Mohammadi et al., 1997), SU4984 (Mohammadi et al., 1997) and BIBF1120 (Hilberg et al., 2008). The structure of the kinase domain of FGFR1 was solved in complex with several inhibitors (Mohammadi et al., 1998) and this led to a better understanding of the mechanism of action of these molecules. Indeed TKIs bind the catalytic pocket of the kinase domain, compete

with ATP, thus rendering the kinase domain catalytically inactive and therefore inhibiting downstream signaling pathways. A tremendous amount of data has been collected about the effects of FGFR inhibitors using several in vitro and in vivo models of diseases. Interestingly, it has been shown that FGFR inhibition in cancer models affects tumor cell growth, survival, migration and invasion, as well as angiogenesis (Hilberg et al., 2008; Koziczak et al., 2004; Koziczak & Hynes, 2004; Reis-Filho et al., 2006).

TKI258 is a promising compound that blocks FGFRs. The effects of this inhibitor have been tested in several model among them multiple myeloma, colon cancer and myeloproliferative disease, where it efficiently block proliferation and tumor growth (Chase et al., 2007; Grand et al., 2007; Lee et al., 2005; Lopes de Menezes et al., 2005; Trudel et al., 2005; Xin et al., 2006). Interestingly, this compound has been given to human in a phase I clinical study on patient with advanced solid tumors (Sarker et al., 2008). TKI258 was well tolerated with an acceptable safety profile. In addition, antitumor activity was seen in melanoma and gastrointestinal tumors. These positive results warrant further trials with this compound.

3.11.3. Alternative therapies

Other approaches have been designed and used to target FGF signaling: Engineering of soluble receptors that bind circulating FGFs and sequester them away from the receptors. Liposome mediated delivery of antisense cDNA targeting FGFs or FGFRs (Wang & Becker, 1997). Inhibition of FGF-FGFR or FGF-HSPG interactions by the use of peptides.

Interestingly, it has been shown that FGFs can as well be used as therapeutics in the treatment of several pathologies. FGF1 is used as recombinant molecule in cardiovascular disorders (Schumacher et al., 1998). FGF4 has been administered by gene therapy techniques but the studies were discontinued (Flynn & O'Brien, 2008). A truncated FGF7 has been approved for the treatment of chemoradiation-induced oral mucositis in patients undergoing bone marrow transplantation (Spielberger et al., 2004). FGF18 is in phase I clinical trial for its effects on osteoarthritis (Ellsworth et al., 2002).

4. Rationale of the work

Targeted therapies are available for breast cancer patients and consist of endocrine treatment for ER positive tumors and trastuzumab treatment for ErbB2 overexpressing tumors. However, not all breast tumors express these targets and in addition, the recurrence of disease upon endocrine therapies is elevated and not all ErbB2 overexpressing cancers are sensitive to trastuzumab. Therefore, new targets are required.

High expression of ligand, amplification and overexpression of FGFR1, SNPs in *FGFR2* are as many possibilities to deregulate FGF signaling that have been described in breast cancer. Several tyrosine kinase inhibitors have been developed, that block the kinase activity of FGFRs. These compounds have been intensively tested in several models of cancer. However, despite the evidence that FGF signaling contributes to breast cancer, only few studies have been performed to validate FGFRs as targets in breast cancer and they were mainly performed in vitro.

Based on this evidence that FGF signaling is important for the development of breast cancer, this work aims at understanding if FGFRs are valid targets in breast cancer and how FGFRs contribute to tumor growth and metastasis in vivo.

To investigate the contribution of FGFRs to the formation of tumors, we used a model consisting of four mouse mammary tumor cell lines named 4T1, 4TO7, 168FARN and 67NR (Aslakson & Miller, 1992). These lines are all derived from the same mouse mammary tumor and all form tumors when injected in the mammary gland of BALB/c animals. However, the metastatic properties of these lines are different: The 67NR are non-invasive due to their incapacity to intravasate. The 168FARN line spreads to the draining lymph node but does not enter the blood stream. The line 4TO7 spreads via the blood to the lungs but is inefficient in growing as metastatic nodules. Finally, the 4T1 line is able to disseminate from the primary tumor and are detected in the lymph node and in the blood. In addition this line grows as visible metastatic foci in the lungs (Aslakson & Miller, 1992).

A preliminary screen performed in our lab showed that the 4T1 cells were sensitive to treatment with PD173074, a known inhibitor of FGFRs. We therefore investigated the expression of FGFRs

and their ligands in this line and the three other sisters lines and tested their sensitivity to FGFR inhibitors.

Using the 67NR cells, we studied in vivo the contribution of FGFR1 to the metastatic process. Finally, using the 4T1 line, we tested in vivo the effect of FGFR inhibition on tumor growth and metastasis formation. To do this, we took advantage of TKI258, a well characterized and promising FGFR inhibitor that is currently in clinical development.

5. Results

5.1. Research article submitted to Cancer research

Fibroblast Growth Factor Receptor maintains survival of breast cancer models: targeting FGFRs blocks PI3K/AKT signaling, induces apoptosis and impairs mammary tumor outgrowth and metastasis

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Conflicts of interest: The authors have no conflicts to declare

Running title: FGFR and mammary cancer
Key words: FRS2, TKI258, 4T1 and 67NR mammary cancer cells, metastasis

Abstract

Members of the fibroblast growth factor receptor (FGFR) family have essential roles in normal physiology and in cancer where they control diverse processes. FGFRs have been associated with breast cancer development and models to study their role in cancer and their targeting potential are desirable. Here, we present an in vitro and in vivo analysis of FGFRs in the mammary cancer cell lines 67NR and 4T1. We show that both cell lines co-express FGFRs and ligands and display autocrine FGFR signaling activity. Fibroblast growth factor receptor substrate 2 (FRS2), a downstream mediator of FGFR is constitutively tyrosine-phosphorylated and multiple signaling pathways are active. Treatment of 67NR and 4T1 cultures with TKI258, an FGFR tyrosine kinase inhibitor caused a rapid decrease in FRS2 phosphorylation, decreased the activity of ERK1/2, AKT and PLCγ, blocked proliferation and induced 4T1 apoptotic cell death via blockade of the PI3K/AKT pathway. In vivo, one dose of TKI258 rapidly lowered FRS2 phosphorylation, ERK1/2 as well as AKT activity in mammary tumors. Long-term treatment of 4T1 and 67NR tumor-bearing mice had a significant impact on primary tumor outgrowth and 4T1 tumor induced lung metastases. A meta-analysis was carried out to identify prognostic markers in human breast tumors and targets with roles in TKI258-anti-tumor activity. Of interest are the down-regulated matrix metalloproteases (MMP), in particular MMP-9, which is essential for metastatic spread of 4T1 tumors.

Introduction

Deregulated activity of receptor tyrosine kinases (RTK) has been implicated in breast cancer development. ErbB2 overexpression has been intensely studied in breast cancer and has been successfully targeted with antibodies and small molecular tyrosine kinase inhibitors (TKI). Considering that only 20% of patients are eligible for ErbB2 directed treatments, it is essential to uncover other therapeutic targets. The association between fibroblast growth factors (FGF) and mammary cancer was first established in MMTV-induced tumors (Peters et al., 1989) and elevated levels of FGF8 have been found in human breast tumors (Mattila & Harkonen, 2007). FGF receptor (FGFR)1 amplification has been reported in sub-types of breast cancer (Reis-Filho et al., 2006) and FGFR1 levels have been linked to poor survival rates (Chin et al., 2006). Intriguingly, genome-wide screens aimed at uncovering breast cancer associated genes identified single nucleotide polymorphisms in *FGFR2* (Easton et al., 2007; Hunter et al., 2007). Based on the increasing evidence supporting the relevance of FGFRs in breast cancer, we have explored the role of this family of receptors in breast cancer models.

There are four FGFR receptors and 22 FGF-related ligands (Beenken & Mohammadi, 2009). Heparin or heparin sulfate proteoglycans mediate ligand-receptor interactions, forming a ternary complex that induces receptor dimerization, autophosphorylation and activation (Eswarakumar et al., 2005; Schlessinger et al., 2000). We show here that 4T1 and 67NR mammary tumor cells (Aslakson & Miller, 1992) co-express multiple FGFRs and ligands and display autocrine FGFR activity. Accordingly, many targets of FGFR signaling: the docking protein fibroblast growth factor receptor substrate 2 (FRS2), Src, PLCγ, Shp-2, STAT3 and PI3K/AKT (Klint & Claesson-Welsh, 1999; Kouhara et al., 1997; Ong et al., 2001) are active in both mammary tumor cell lines.

We investigated the effects of FGFR inhibition using TKI258, an FGFR TKI (Lee et al., 2005). In vitro treatment of 4T1 and 67NR cultures with TKI258 decreased the activity of numerous signaling proteins and blocked cell proliferation. In vivo treatment of tumor-bearing mice with TKI258 led to a strong reduction of mammary tumor growth and for the aggressive 4T1 model, a decrease in lung metastasis. Moreover, we provide evidence that FGFR blockade downregulates key players involved in the metastatic process, in particular MMP9 and the transcription factor

Twist, which have both been shown to be major regulators of lung metastasis in this model (Fayard et al., 2009; Yang et al., 2004).

Materials and Methods

Kinase inhibitors

TKI258 (Lee et al., 2005) was provided by Drs. D. Graus-Porta and C. Garcia-Echeverria, and NVP-BEZ235 was provided by Dr. M. Maira (NIBR, Basel); STI571 and PTK787 were obtained from NIBR, Basel. All inhibitors were prepared as 10mM stocks in DMSO.

Reagents, antibodies, plasmids, cell culture and viral infections

FGF2 was from Sigma-Aldrich (St. Louis, MO, USA); UO126 was from Promega (Madison, WI, USA). The following antibodies were used: FGFR2 (3116), FGFR3 (3163), Pser^{473}-AKT (9271), AKT (9272), Ptyr^{196}-FRS2 (3864), Pthr^{202}/tyr^{204}-ERK1/2 (9101), ERK1/2 (9102), Ptyr^{705}-STAT3 (9131), Asp^{175}-cleaved caspase 3 (9661) and PARP (9542) from Cell Signaling (Danvers, MA, USA); PLCγ (sc-81), FRS2 (sc-8318) and CyclinD1 (sc-20044) from Santa-cruz Biotechnology Inc. (Santa Cruz, CA, USA); alpha-tubulin from NeoMarkers (Fremont, CA, USA); actin from Chemicon (Billerica, MA, USA); MMP9 from Abcam (Cambridge, UK); E-cadherin, p27^{Kip1} and STAT3 from BD transduction lab (San Jose, CA, USA); Ptyr^{418}-Src from Biosource (Carlsbad, CA, USA), Src and Ptyr^{783}-PLCγ from Upstate (Billerica, MA, USA); phospho-tyrosine (Ptyr) was detected with mAb 4G10 (gift from Dr. J. Mestan, NIBR, Basel). cDNA encoding myristylated-AKT was a gift from Dr. B. Hemmings (FMI, Basel). The 4T1 and 67NR mouse mammary carcinoma cell lines, obtained from Dr. J. Yang (University of California, San Diego) and EcoPack-293 (Clontech, Mountain View, CA, USA) were cultured in DMEM, 10% heat inactivated FBS (Sigma), supplemented with penicillin and streptomycin (Sigma). Retroviruses were produced by transient transfection of EcoPack-293 cells 8μg of the following vectors: pBabe-neo or Myr-AKT-pBabe-neo using Fugene (Roche, Basel Switzerland) with a ratio Fugene:DNA 1:6. Three days post transfection, supernatants were collected, filtered and used immediately for over-night infection of 4T1 cells (6cm plates with polybrene at a final concentration of 8μg/ml). Selection was performed with 1000μg/ml G418.

Lysates, immunoprecipitations and western blot analyses

Whole cell lysates were extracted in NP-40 buffer (50mM Hepes (pH 7.4), 150mM NaCl, 25mM β-glycerophosphate, 25mM NaF, 5mM EGTA, 1mM EDTA, 15mM PPI and 1%NP40), supplemented with leupeptin (10μg/ml), aprotinin (10μg/ml), vanadate (2mM), DTT (1mM) and PMSF (1mM) and immunoblotted as previously described (Matsuda et al., 2009). Over-night serum-starved cultures were pre-treated for 60 minutes with 1μM TKI258 or DMSO then stimulated for the indicated time with 50ng/ml FGF2. For prolonged FGFR inhibition cells in serum were treated with 1μM TKI258 or DMSO and harvested at the indicated times. Immunoprecipitations (IPs) were performed following standard procedures (Koziczak et al., 2004). Extraction from tissues and tumors was performed with NP-40 buffer (1ml/100mg). Homogenization of the samples was performed using a polytron and debris was removed by centrifugation. Supernatants were boiled in sample buffer.

Purification of tyrosine-phosphorylated peptides

Peptides containing Ptyr residues were purified from trypsinized cell lysates using a two-step procedure. Briefly, after treatment with TKI258 (1μM for 60 minutes), cells were harvested in lysis buffer (20 mM HEPES pH 8.0, 9 M Urea, 1 mM Sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM sodium beta-glycerophosphate) reduced and alkylated and digested with trypsin after dilution to 2M urea. Tryptic peptides were acidified to 1% TFA and desalted on SepPak C18 cartridges. Immunoaffinity purification using anti-phosphotyrosine antibodies was performed. After elution, peptides were desalted on Poros R3 and further purified on TiO2 microcolumns (Thingholm & Larsen, 2009).

Mass Spectrometry

For LC-MS/MS, the peptides were eluted from the TiO2 column with ammonia (Thingholm & Larsen, 2009), lyophilized, resuspended in 5% formic acid and injected onto a 15 cm x 75 μm ProteoPep 2 PicoFrit column (New Objectives), connected to an LTQ-OrbiTrap XL mass spectrometer (Thermo). Buffer A consisted of H_2O with 0.1 % formic acid and Buffer B of 80 % acetonitrile with 0.1 % formic acid. Peptides were separated using a 120 min gradient from 2% B to 50% B. Data acquisition was done using a 'Top 5 method', where every full MS scan was followed by 5 data-dependent scans on the 5 most intense ions from the parent scan. The

minimal threshold for MS/MS selection was set at 1500, dynamic exclusion was used with repeat count 2 and a 60 sec exclusion window. MSMS spectra were acquired in the ion-trap with 10'000 ions using CID, 30 ms activation time, and 35% normalized collision energy.

Database searches were performed with Mascot Server using the mouse IPI database (version 3.55). Mass tolerances were set at 10 ppm for the full MS scans and at 0.8 Da for MSMS. Search results were validated using Scaffold (Proteome Software) and peptide identifications accepted which exceeded the 95% confidence level. In case of ambiguous assignments, spectra were manually interpreted for confirmation of identity and localization of the phosphorylation site.

Flow Cytometry

Cell cycle profiles were analyzed after 24 hours of DMSO or 1μM TKI258 addition to 4T1 and 67NR cultures as follows. Cells were trypsinized and resuspended in 500μl ice cold PBS, 120μg RNase A and 60μl of propidium iodide (50μg/ml in 50mM Na Citrate pH7.6) were added, then 115μl of Lysis buffer (20mM Na-citrate pH4.0, 26.8mM NaCl, 0.6% NP-40, 30mM EDTA and 30mM EGTA). Cell cycle distribution was measured after 30 minutes on ice with a FACScalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Proliferation and apoptosis assays

Antiproliferative effects of TKI258 were evaluated in 96-well plates over 24-48 hours using a bromodeoxyuridine (BrdU) ELISA kit (GE-healthcare, Little Chalfont, UK) as described (Sini et al., 2005). The cytotoxic effects of TKI258, UO126 and NVP-BEZ235 were evaluated in 96-well plates 24-48 hours after treatment by measuring total cell number and cell death using the YO-PRO assay (Invitrogen, Carlsbad, CA, USA) as described (Beuvink et al., 2005).

In-vivo treatments and analysis of tumor and metastasis formation

Animal experiments were done according to the Swiss guideline governing animal experimentation and approved by the Swiss veterinary authorities. 4T1 and 67NR cells ($5x10^5$ or 10^6) were injected in the fourth mammary fat pad of 10 wk old BALB/c mice (RCC, Basel, Switzerland). Once palpable, tumors were measured daily and volume was calculated using: height x $((\text{diameter}/2)^2 \times \pi)$. Mice were randomly distributed into treated or control groups when tumors reached 50-100 mm^3 and treated with vehicle (water), TKI258 (p.o., once daily at 20, 40

or 50 mg/kg) or with vehicle (PEG300) and PTK787 (p.o. once daily at 25 or 50mg/kg) for the indicated times. For experimental metastasis, $2.5x10^5$ 4T1 cells were injected into tail veins, 5 days later mice were randomized and treated with water or TKI258 (p.o., once daily, 9 days at 40 mg/kg). At the endpoint, mice were sacrificed and tumors and lungs were dissected. Lungs were placed in Bouin's solution to visualize and count metastases. Pictures of the left lungs were taken with a Leica MacroFluo Z6 (Leica, Wetzlar, Germany) and the number of nodules as well as the surface occupied by the metastasis was quantified using image access software.

Immunohistochemistry

Tumors were fixed 24 hours in PBS containing 4% paraformaldehyde then incubated 24 hours in PBS containing 15% sucrose. Fixed tissues were embedded in OCT media and frozen at -80°C. Immunohistochemistry was performed on 8µm thick sections using the antibodies: cleaved-caspase 3 and phospho-histone H3 (Cell signaling) and CD31 (BD Bioscience). Stainings were carried out with the Discovery XT Staining Module (Ventana Medica Systems S.A.).

RNA extraction, RT-PCR and real-time PCR

RNA from growing 4T1 cells or from cells treated with DMSO or 500nM TKI258 for 16 hours was extracted using the Qiashredder and RNeasy Mini Kit coupled with RNase-free DNase set (Qiagen, Venlo, The Netherlands Qiagen) following the manufacturer's instructions. RNA from tissues and tumors were obtained from snap frozen tumor pieces, following extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNAs were washed using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase set (Qiagen). cDNA was obtained from 2.5µg of total RNA, using the Ready-to-go You-Prime First-Strand Beads kit (GE-healthcare, Little Chalfont, UK) with oligos-dT primers (Promega). Semi-quantitative PCR was performed as follow: 2µl of 10X Buffer (Roche), 0.2µl of Taq polymerase (5U/µl Roche), 0.4µl of 10mM dNTP mix (Roche), 0.1µl of each primer (100µM), 1µl of cDNA, filled to a final volume of 20µl with sterile H_2O. Thermal cycling reaction using an Icycler device (Bio-Rad, Reinach, Switzerland) was: 94°C for 2 min; followed by 25 to 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec for detection of FGFR1 and FGFR2: 96°C for 2 min; followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec for detection of FGFR3: 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 45

sec for detection of FGF1. The amplified products were further extended by additional incubation at 72°C for 10 min. PCR products were then loaded on a 1% agarose gel containing ethidium bromide. Quantitative RT-PCR was performed with ABI prism 7000 (Applied Biosystems, Austin, TX, USA) using Absolute QPCR SYBR Green ROX Mix (THERMO Scientific, Waltham, MA, USA) following the manufacturer's guidelines. All quantitations were normalized to β-actin. FGFRs primers, GAPDH and β-actin primers for semi-quantitative PCR and real-time PCR were previously described (Kurosu et al., 2007; Ozawa et al., 1997). Other primers were as follow: MMP1 forward CCTTCCTTTGCTGTTGCTTC, reverse CTCCTTGCCATTCACGTTTT. MMP3 forward CAGACTTGTCCCGTTTCCAT, reverse GGTGCTGACTGCATCAAAGA. MMP9 forward GCATACTTGTACCGCTATGGT, reverse TGTGATGTTATGATGGTCCC. MMP10 forward CAGGTGTGGTGTTCCTGATG, reverse GGAGAAAGTGAGTGGGGTCA. Twist forward ACATCGACTTCCTGTACCAGGTC, reverse AACAATGACATCTAGGTCTCCGG. E-Cadherin forward CAAGGACAGCCTTCTTTTCG, reverse TGGACTTCAGCGTCACTTTG.

Gene expression analysis

First, RNA from TKI258- or vehicle- treated 4T1 cells (triplicate experiments) was amplified and labeled using the Ambion MesageAMP III RNA Amplification Kit (Applied Biosystems). The same protocol was applied on RNA from three tumors treated 14 days with TKI258 or with vehicle. Biotinylated, fragmented cRNA was hybridized to Affymetrix Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA). The data analysis and gene filtering was performed using R/Bioconductor (Gentleman et al., 2004). Signal condensation was performed using only the RMA from the Bioconductor Affy package. Differentially expressed genes were identified using the empirical Bayes method (F-test) implemented in the LIMMA package and adjusted with the False Discovery Rate (FDR) method (Wettenhall & Smyth, 2004). Hierarchical clustering and visualization was performed in R. Probe sets with a Log2 average contrast signal of at least 5, an adjusted p-value < 0.05 and an absolute Log2 fold change of > 0.585 (1.5-fold in linear space) were selected leading to the identification of 2064 and 543 genes changed in cells and tumors, respectively upon TKI258 treatment. The complete microarray data are available in the Gene Expression Omnibus (GEO; accession number,GSE19222)

Second, we analyzed the TKI258-regulated genes in available data sets. The GEO database (www.ncbi.nlm.nih.gov/geo) was used for breast cancer patient cohorts: TRANSBIG (GSE7390) and ERASMUS (GSE2034) and for a tumor model (GSE15299). Data were processed and normalized using MAS 5.0 (GSE7390, GSE2034) or RMA (GSE15299) algorithms as described in the GEO database. Affymetrix probesets were mapped to in vitro and in vivo regulated genes using the NetAffx web site (http://www.affymetrix.com/analysis/index.affx). Hierarchical clustering was done with Cluster 3.0 (de Hoon et al., 2004; Eisen et al., 1998) (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/index.html) on $\log_2$ median centered data using uncentered correlation and average linkage clustering algorithms. Trees were displayed by Java Tree View v1.1.1 (http://jtreeview.sourceforge.net). Kaplan-Meier analysis was done using JMP IN 5.1 (SAS Institute Inc., Building S, Cary, C) and relative p-values calculated with Log-rank test. P-values of the overlaps were calculated using Fisher's exact test. Functional classification analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA). Gene-set enrichment analysis was done with GSEA (Subramanian et al., 2005) using default settings by collapsing probesets to unique genes and taking probesets median expression value. Significance of the enrichment was estimated by 1000 random gene sets permutation.

Results

Constitutive FGFR signaling in 4T1 and 67NR tumor cells

BALB/c mice develop mammary tumors following injection of 67NR and 4T1 tumor cell lines; 4T1 tumors are more aggressive, forming distant lung metastases (Aslakson & Miller, 1992). Both cell lines were examined for FGFR and FGF expression by RT-PCR. 4T1 cells express FGFR1, FGFR2 and FGFR3 (Figure 5-1 A), with FGFR2 expressed at the highest level (not shown). 67NR cells express FGFR2 and FGFR3. FGF1, which activates all receptors was found in both lines (Figure 5-1 A). A western analysis confirmed that FGFR2 and FGFR3 are expressed (Supplementary Figure 1A). It was not possible to examine FGFR1 since commercial antibodies are not available for western analysis. As co-expression of FGFRs and FGF1 was observed in 67NR and 4T1 cells, we tested the hypothesis that these tumor cell lines have autocrine FGFR activity.

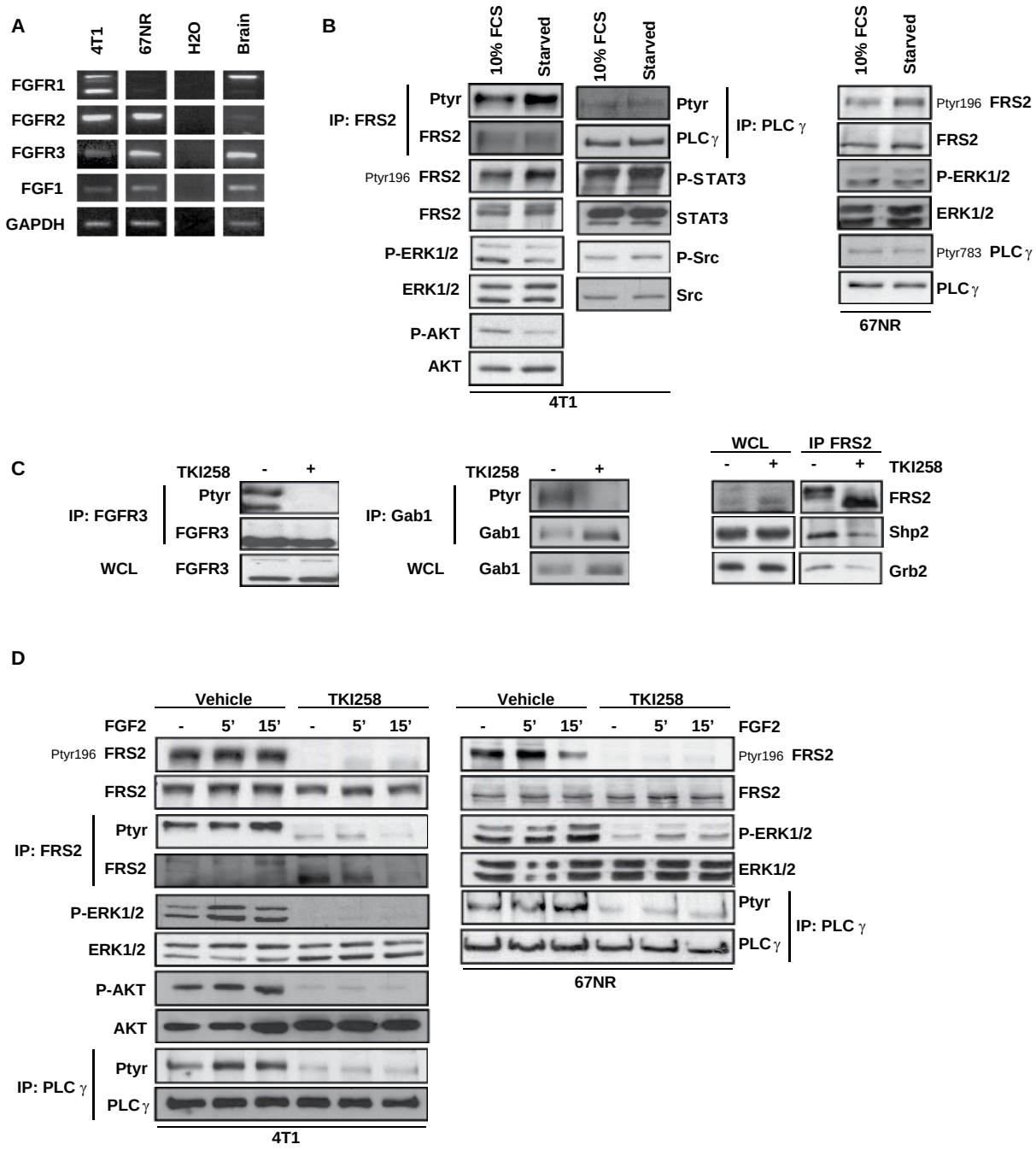

Figure 5-1 FGFR signaling in 4T1 and 67NR cells

A, Semi-quantitative RT-PCR for FGFRs and FGF1, with GAPDH as a loading control. *B*, Lysates were prepared from cells in full serum or serum starved and immunoblot analyses performed with the indicated antibodies. FRS2 and PLCγ IPs were probed for Ptyr content. *C*, Lysates were prepared from cells in full serum, after 1 hour with 1μM TKI258. IPs of FGFR3 and Gab1 were probed for Ptyr content; IPs of FRS2 were probed for associated Shp-2 and Grb2. As controls, whole cell lysates (WCL) and IPs were reprobed with the respective serum. *D*, Serum-starved cultures were pretreated with 1μM TKI258 or DMSO for 1 hour prior to FGF2 stimulation. WCL were prepared and immunoblot analyses performed with the indicated antibodies. FRS2 and PLCγ IPs were probed for Ptyr content.

The activity of signaling proteins downstream of FGFR was measured in lysates of 4T1 or 67NR cells grown in full medium or serum-starved (Figure 5-1 B). FRS2 is a major adaptor protein linking FGFRs to various pathways (Kouhara et al., 1997; Ong et al., 2001). Probing of FRS2 immunoprecipitates (IPs) from 4T1 cells revealed high levels of phospho-tyrosine (Ptyr); the level of Ptyr196-FRS2, a docking site for Grb2 (Kouhara et al., 1997) was also elevated in both cell lines (Figure 5-1 B). Furthermore, total Ptyr and Ptyr783 was detectable in PLCγ, and the levels of P-ERK1/2, P-AKT, P-STAT3 and P-Src were also high (Figure 5-1 B). With the exception of P-AKT, which was slightly lower in serum-starved 4T1 cells, there was little or no effect following serum deprivation on the activity of the other signaling proteins (Figure 5-1 B). Taken together, these results provide strong evidence supporting the hypothesis that 4T1 and 67NR cells possess autocrine FGFR activity.

TKI258 lowers FGFR activity and blocks signaling pathways

To gain more insight into the intracellular pathways controlled by FGFR, we used TKI258, an FGFR inhibitor (Lee et al., 2005; Renhowe et al., 2009). Initially, a global phospho-proteomic screen was undertaken to identify proteins undergoing changes in Ptyr in response to TKI258 treatment of 4T1 cells. Lysates made from controls and cultures treated 1 hour with TKI258 were subjected to tryptic digestion prior to IP with Ptyr specific antibodies. Pulled-down peptides were subjected to LC-MS/MS for detection and quantification of Ptyr changes. Supplementary Table 1 lists peptides that were significantly changed in two independent analyses. One FGFR2-specific Ptyr-peptide from the kinase domain was 1.67-fold decreased, demonstrating that TKI258 blocked FGFR signaling. FRS2, Mapk3 and Gab1-specific Ptyr-peptides were also strongly decreased following FGFR inhibition. FGFR3-specific Ptyr peptides were not detected in this analysis, however, IPs of the receptor from lysates of treated cells revealed a loss of total Ptyr (Figure 5-1 C left panel). FGFR1 levels are low in 4T1 cells, however, manual inspection of the LC-MS/MS data revealed lower levels of one FGFR1-specific Ptyr peptide (data not shown). These results suggest that all the FGFRs in 4T1 cells are active and blocked by TKI258.

Gab1 and FRS2 IPs (Figure 5-1 C middle panel and D) from lysates of inhibitor-treated cells revealed lower Ptyr content compared to controls; Ptyr196-FRS2 levels were also strongly decreased in TKI258-treated 4T1 and 67NR cells (Figure 5-1 D). Moreover, decreased levels of

Shp-2 and Grb2 were complexed with FRS2 in TKI258-treated 4T1 cells (Figure 5-1 C right panel) and FRS2 shifted dramatically on SDS-PAGE (Figure 5-1 C right panel and D), reflecting lower activity of MAPK, which phosphorylates multiple threonine residues on FRS2 (Lax et al., 2002). P-AKT and Ptyr-PLCγ levels were also decreased in inhibitor-treated cells; no effect on constitutive STAT3 or Src activity was seen (Supplementary Fig.1B). Taken together, these results confirm the phospho-proteomic analysis and show that TKI258 has a strong effect on FGFR-mediated signaling.

TKI258 treatment blocks 4T1 and 67NR cell proliferation

Next, effects of FGFR-inhibition on cell proliferation were examined. Using BrdU incorporation, we observed a dose-dependent decrease in S-phase cells, with an 80% decrease at the highest dose (Figure 5-2 A). Furthermore, flow cytometry of propidium iodide stained cells revealed a strong G1 accumulation in TKI258-treated cultures (Figure 5-2 B). Cyclin D1 protein (Figure 5-2 C) and RNA (data not shown) decreased rapidly following TKI258 treatment, and in 4T1 cells there was an increase in p27 levels (Figure 5-2 C). There was also a strong increase in dying cells in TKI258-treated 4T1, but not 67NR cultures, as shown by a YO-PRO assay (Figure 5-2 D). Cell death proceeds via apoptosis since increased levels of cleaved-caspase3 and -PARP were detected (Figure 5-2 C); neither were detected in TKI258-treated 67NR cells (Figure 5-2 C). Together these data show that blockade of autocrine FGFR activity in both mammary cancer cell lines has a strong impact on their proliferation, and decreases survival of 4T1 cells.

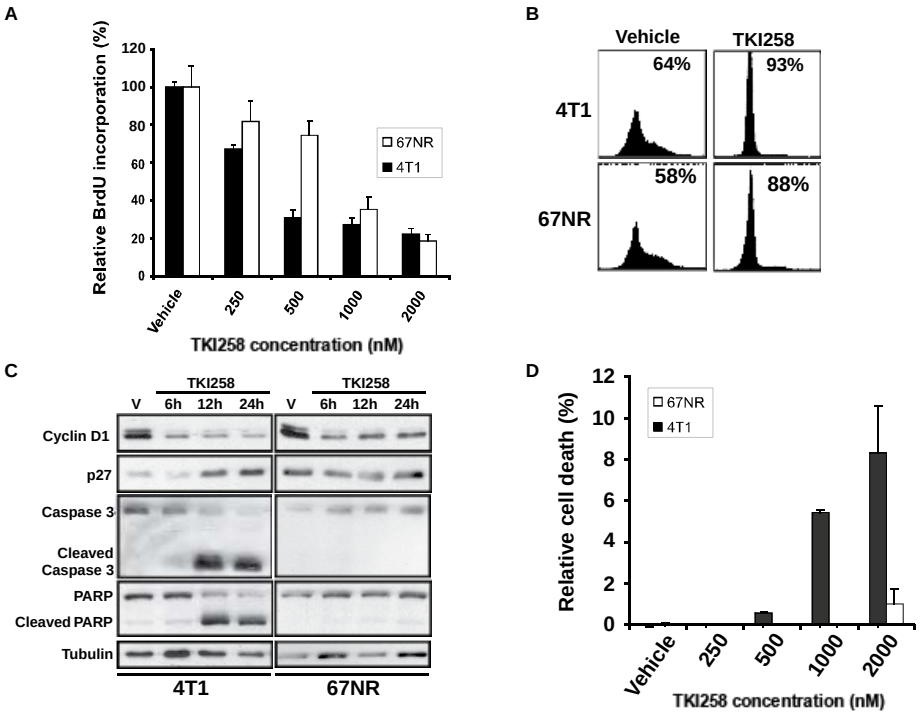

Figure 5-2 Effects of TKI258 on 4T1 and 67NR cells

A, Cells were treated 48 hours with the indicated concentrations of TKI258; BrdU was added 2 hours before experiment-end. The percentage of incorporated BrdU relative to controls is plotted. *B,* Cultures were treated 24 hours with 1μM TKI258 and flow cytometry was performed on propidium iodide stained cells; % of G1 cells is indicated. *C,* Cultures were treated with 1μM TKI258 for the indicated times and WCL were immunoblotted with the indicated antibodies; tubulin served as control. *D,* Cultures were treated 48 hours with different concentrations of TKI258; cell death was detected with the YO-PRO assay and plotted relative to the vehicle-treated control

Rescue of TKI258-mediated 4T1 cell death by expression of Myr-AKT.

TKI258 decreases P-ERK1/2 and P-AKT (Figure 5-1 D). Thus, to analyze the pathways responsible for TKI258's activity, the MEK inhibitor, UO126, and the PI3K inhibitor, NVP-BEZ235 (Maira et al., 2008), were used to target the MAPK and PI3K/AKT pathways, respectively. In UO126-treated 4T1 cultures, proliferation was 50% decreased at the maximal dose (Figure 5-3 A), however, there was no evidence of apoptosis (data not shown). NVP-BEZ235 blocked proliferation by 50% and induced cell death (Figure 5-3 B). These results suggest that active FGFR signaling maintains high PI3K/AKT pathway activity that is essential for 4T1 cell survival. To explore this further, a vector expressing active AKT (Myr-Akt) and a control vector (pBN) were introduced into 4T1 cells and pools were examined for TKI258

sensitivity. Myr-Akt-expressing cells were 2-fold less sensitive than controls to TKI258 treatment (Figure 5-3 C) and maintained high levels of P-Myr-Akt in the presence of TKI258 (Figure 5-3 D). Taken together, these results show the importance of the PI3K/AKT pathway for survival of 4T1 cells.

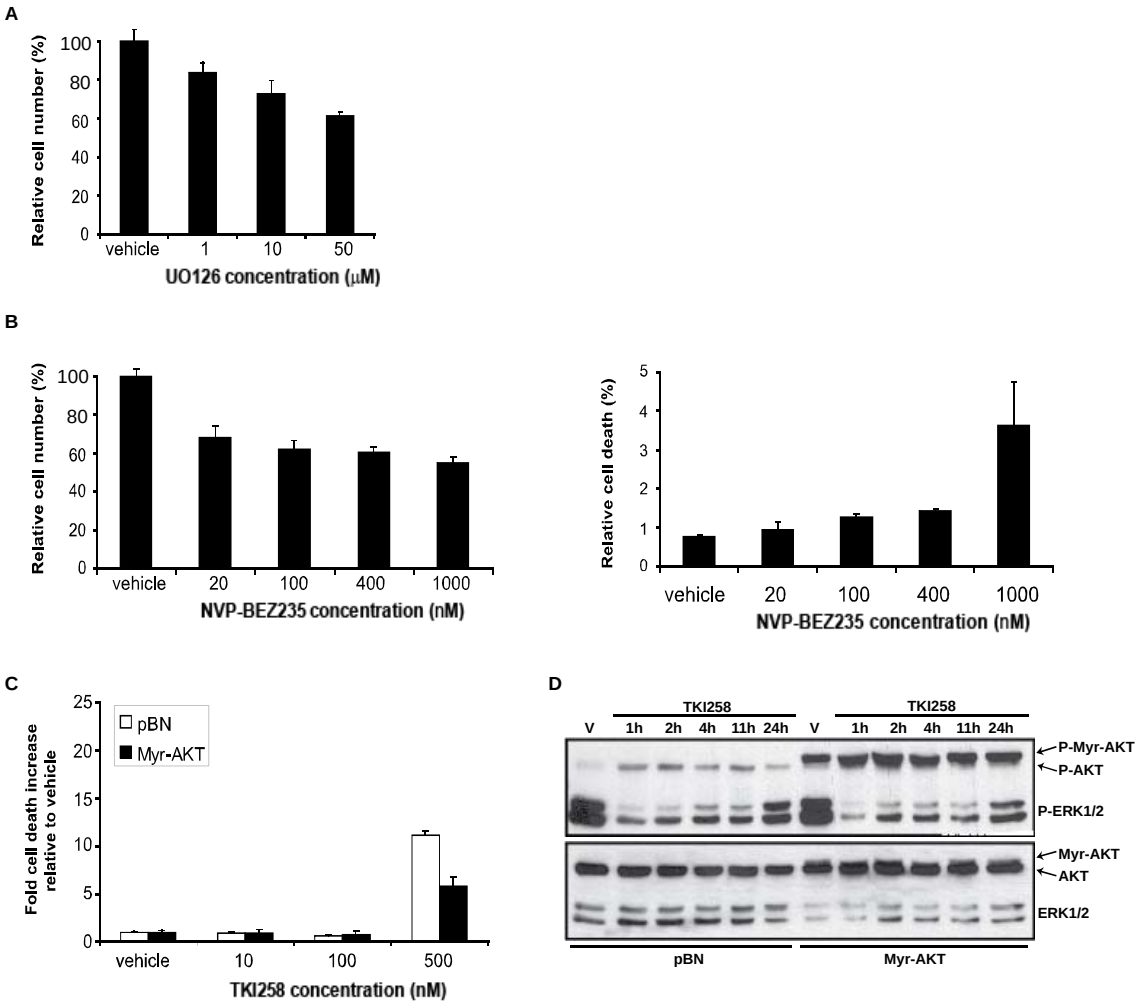

Figure 5-3 PI3K/AKT signaling is required for 4T1 survival

A, Cultures were treated 24 hours with different concentrations of UO126; cell number was determined and plotted relative to control cultures. *B*, Cultures were treated 24 hours with different concentrations of NVP-BEZ235; viable- (left) and dead-cell-number (right) was determined and plotted relative to controls. *C*, Cells stably expressing vector control or Myr-AKT were treated 24 hr with different concentrations of TKI258 and fold-increase in cell death relative to controls was determined using the YO-PRO assay. *D*, WCLs prepared from vector control or Myr-AKT cultures treated with 1μM TKI258 for different times were immunoblotted with the indicated antibodies.

Decreased mammary tumor growth in TKI258-treated mice

Next in vivo effects of TKI258 were examined. First, a single 50mg/kg dose was administered to 4T1 tumor-bearing mice, then tumors were collected 2, 8 and 24 hours after dosing and lysates prepared from 3-tumor bearing mice per time point were analyzed. Vehicle-treated mice had high levels of Ptyr196-FRS2, active ERK1/2 and active AKT (Figure 5-4 A). Importantly, within 2 hours of TKI258 administration there was a significant decrease in Ptyr196-FRS2 and P-ERK1/2 levels in the tumors; P-AKT levels decreased to a lesser extent (Figure 5-4 A). Ptyr196-FRS2 levels remained low 24 hours after treatment; while active ERK1/2 and AKT started to rise by 8 hours (Figure 5-4 A). These results show that TKI258 rapidly blocks the FGFR pathway in vivo in the tumors.

Next we tested long-term effects of TKI258 treatment. Mice bearing 4T1- and 67NR- induced tumors were randomly distributed into treated or control groups. 4T1-tumor-bearing mice were dosed daily at 20 and 40mg/kg for 14 days; 67NR-tumor-bearing mice were dosed at the indicated times with 40mg/kg for 16 days. Non-significant changes in body weight were observed in TKI258-treated animals (Supplementary Figure 2A). Importantly, there was a significant reduction in tumor outgrowth and in tumor weight in the TKI258-treated groups of 4T1- and 67NR-bearing tumors (Figure 5-4 B and C and Supplementary Figure 2B), showing that blockade of FGFR has strong anti-tumor activity.

To determine the mechanisms underlying TKI258 activity, 4T1-induced tumors collected on day 24 were examined for vessel density, proliferation and apoptosis. Quantification of CD31-stained sections and cleaved caspase-3 stained sections revealed a significant decrease in vessel density and a significant increase in cell death in TKI258-treated animals (Figure 5-4 D top and middle). There was no significant change in the mitotic marker P-histone H3 (Figure 5-4 D bottom). Taken together, these results suggest that TKI258 inhibits tumor outgrowth mainly by impairing cell survival, which might result from decreased P-AKT (Figure 5-4 A) and decreased vessel density. TKI258's effects are rapid, showing significant difference in tumor volume in the treated groups within 3 days, however, consistent long-term tumor shrinkage was not observed (Figure 5-4 B and C).

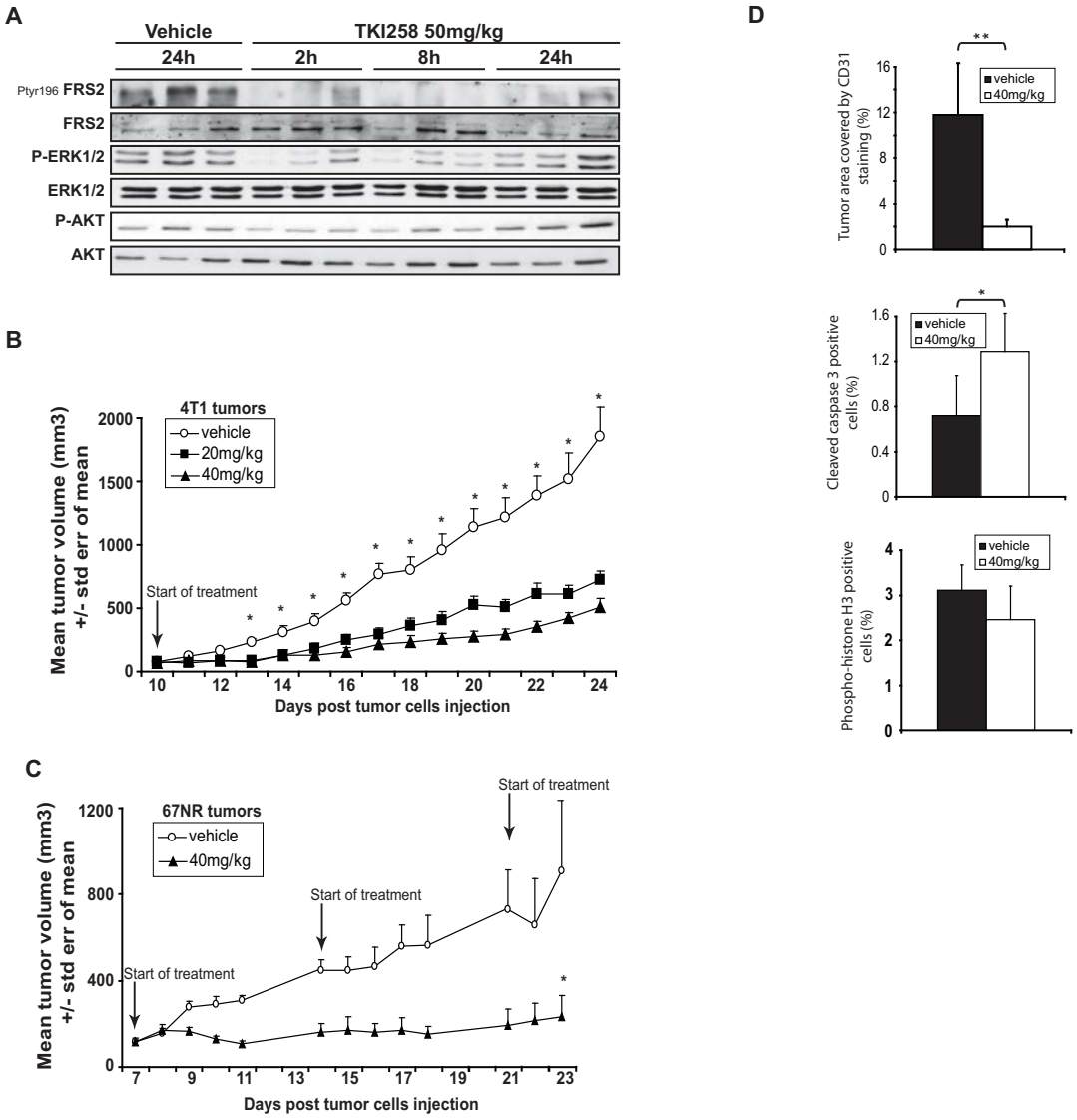

Figure 5-4 In vivo effects of TKI258

A, Mice bearing 4T1 tumors were treated once with TKI258 or water control. At the indicated times, tumors were collected, lysates prepared and immunobloted with the indicated antibodies. Mice bearing *B,* 4T1 and *C*, 67NR tumors were randomized into treatment groups and *B,* 4T1 tumor-bearing mice were treated daily with TKI258 (40mg/kg or 20mg/kg) or water for 14 days. Statistical analysis was performed using one way ANOVA: *, P<0.005. *C*, 67NR tumor-bearing mice were treated with TKI258 (40mg/kg) or water for 5 days, followed by 2 days off-treatment, as indicated. Statistical analysis was performed using Mann-Whitney Test: *, P<0.05. *D,* 4T1 tumors from the 40mg/kg 14-day treated TKI258 or control group were harvested; frozen sections were stained for CD31, cleaved caspase3 or phospho-histone H3. CD31 positive area was measured and plotted as percent of tumor area (top). Cleaved caspase3 positive and total cells were counted and plotted as a %. Phospho-histone H3 positive and total cells were counted and plotted as a %. Statistical analysis was performed using t-test: **, P<0.01; *, P<0.05.

TKI258 treatment decreases lung metastasis

To analyze TKI258's effects on metastasis, lungs of 4T1 tumor-bearing mice sacrificed on day 24 were removed, stained with Bouin's fixative and metastatic nodules were quantified. In vehicle-treated animals multiple large nodules were evident; while the extent of lung metastasis was dramatically reduced in TKI258-treated mice (Figure 5-5 A top). Quantification of foci number and % covered by metastases revealed a significant decrease in both parameters (Figure 5-5 A, lower panels). To assess TKI258's effects directly in the lungs, 4T1 cells were injected through the tail vein and 5 days later TKI258 was administered for 9 days (Figure 5-5 B). TKI258 had a slight, non-significant effect on foci number (Figure 5-5 B top); while the % covered by metastases was significantly decreased (Figure 5-5 B bottom). Thus, FGFR blockade also impairs the ability of 4T1 cells to grow in the lungs following tail vein injection.

Array data on TKI258 treated 4T1 cells and tumor-bearing mice

To find genes changed by TKI258, a genome-wide transcriptome analysis was performed and differentially regulated genes were identified. From triplicates of 16 hours TKI258- or vehicle-treated 4T1 cells, 2064 significantly changed probesets (1648 annotated genes) were identified. The same analysis performed on triplicate tumors from 14-day TKI258-treated versus vehicle-treated mice, led to the identification of 543 genes (254 annotated genes); 65 genes overlapped between the data sets and 61 showed the same trend in vitro and in vivo (Supplementary Table 2). Consistent with a reduction in the extent of lung metastasis, many genes related to cell motility and invasion were identified. In particular, several matrix metalloproteases (MMPs) (MMP1, MMP3, MMP9, MMP10 and MMP13), involved with extracellular matrix degradation (Duffy et al., 1996; Duffy et al., 2000) were down-regulated by TKI258 treatment. A quantitative RT-PCR analysis showed that within 8 hours of TKI258 addition there was >80% decrease in MMP1, 3, 9 and 10 levels in 4T1 cells (Figure 5-5 C); MMP-9 protein was almost undetectable after 24 hours of treatment (Figure 5-5 D). Interestingly, all these MMPs have AP-1 binding sites in their promoter (Yan & Boyd, 2007), connecting TKI258-mediated ERK inhibition to their decreased expression.

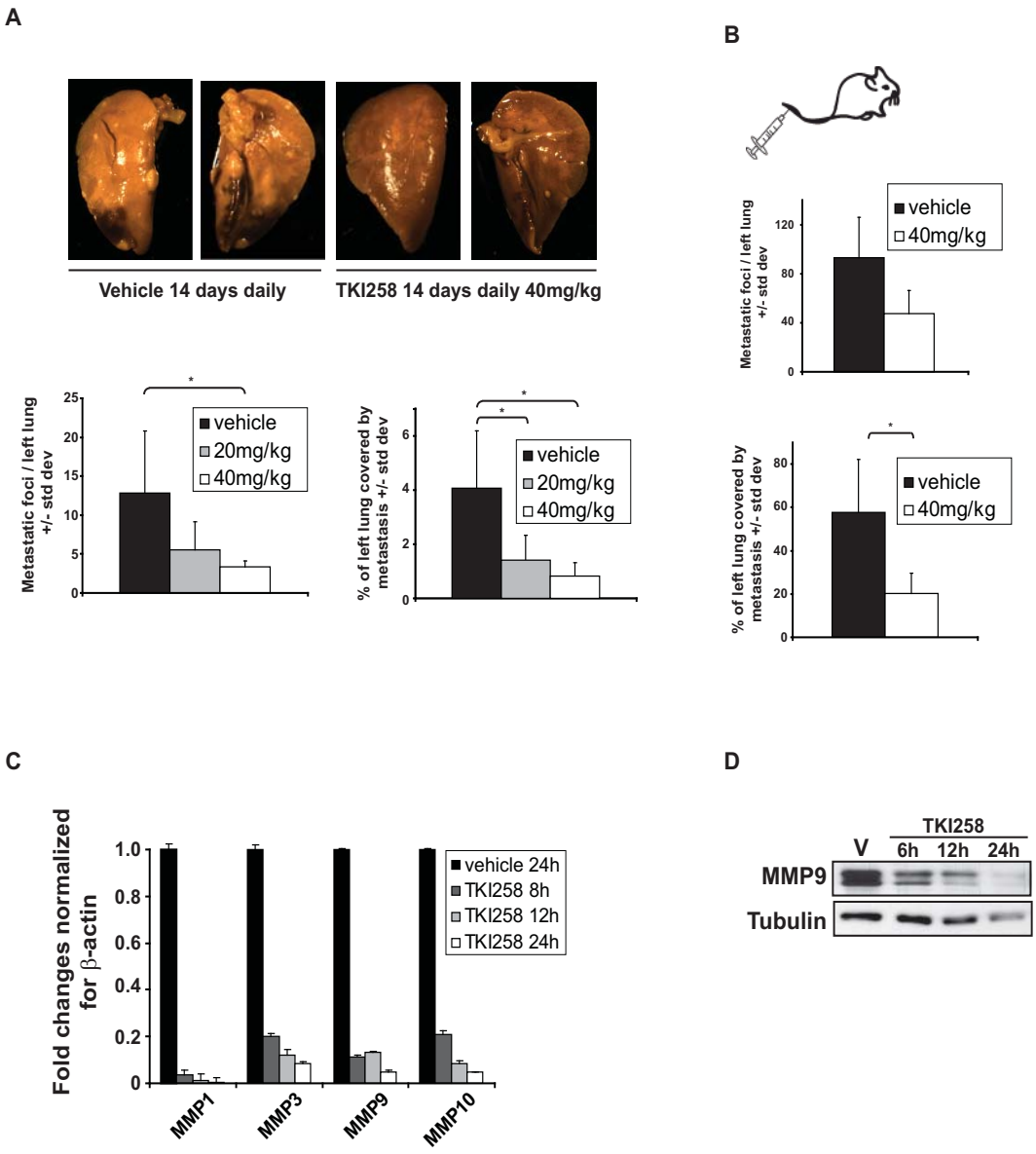

Figure 5-5 Effects of TKI258 on metastasis

A, top, Representative pictures of lungs from 14-day treated TKI258 or control mice; front and back view of left lobes. *Bottom*, Quantification of metastatic foci (*left*) and lung % covered by metastases *(right*) in control and TKI258-treated groups (n=6). Statistical analyses were performed using Kruskal-Wallis test: *, P<0.05 (*right*) or one-way ANOVA test: *, P<0.05 (*left*). *B, top*, 4T1 cells were injected in tail veins and 5 days later mice were treated with 40mg/kg TKI258 or vehicle for 9 days before collecting lungs. Quantification of metastatic foci and % of lung covered by metastases in control and TKI258 treated groups (n=3) performed as in panel *A*. Statistical analyses were performed using student T-test: *, P<0.05. *C*, Quantitative real-time RT-PCR with primers for different MMPs was performed on RNA from TKI258-treated 4T1 cells. *D*, WCLs from TKI258-treated 4T1 cells were immunoblotted for MMP9; tubulin served as loading control.

TKI258-regulated genes identify clusters of human breast tumors with increased metastatic potential.

Based on the ability of TKI258 to reduce metastases and perturb genes involved in cell motility and invasion, we tested whether TKI258-regulated genes might be enriched for prognostic markers in human breast tumors. Two cohorts of breast cancer patients available in the GEO database were used: TRANSBIG (GSE7390) with 198 patients and ERASMUS (GSE2034) with 286 patients. 1648 human orthologous genes affected by in vitro- and 254 genes affected by in vivo-TKI258 treatment were analyzed. Unsupervised hierarchical clustering analysis in both cohorts identified a group of patients characterized by overexpression of the same subset of 64 TKI258- in vitro regulated genes (Cluster 1A); 99.4% of these genes are negatively regulated by TKI258 (Figure 5-6 A and Supplementary Table 3). The clustering analysis was also performed with genes affected by in vivo treatment, which identified two patient groups overexpressing distinct gene-sets (Cluster 1B, 62 merged genes; Cluster 2B, 42 merged genes; Figure 5-6 B) that overlapped in both cohorts (Cluster 1B, 42 overlapping genes, $P = 4.06^{-29}$; Cluster 2B, 15 overlapping genes, $P = 4.14^{-11}$; Supplementary Table 3).

Kaplan-Meier analysis revealed that in both cohorts Cluster 1A and Cluster 2B patients had a worse prognosis compared to other patients (Figure 5-6 C and D upper panels). The difference in metastasis-free survival was even stronger in the sub-group of basal-like patients (Figure 5-6 C and D lower panels). Conversely, patients in Cluster 1B had a better prognosis compared to others (Figure 5-6 D, green line). Of note is the fact that the majority of Cluster 1B genes (49 out of 62 genes, Supplementary Table 3) were up-regulated by the inhibitor, while the majority of genes in Cluster 1A and 2B (99.4% and 65%, respectively; Supplementary Table 3) were negatively regulated by TKI258. Cluster 1A and Cluster 2B cohorts contain almost the same patients (Supplementary Figure 3A and B), while the overlap between the subsets of over-expressed genes characterizing these clusters is very low (3 common genes, Supplementary Figure 3C). This suggested that Cluster 1A and 2B genes might identify distinct pathways and mechanisms that contribute to tumor progression. Of note, Ingenuity Pathway (IPA) functional analysis revealed that cell cycle and DNA replication functions are more enriched in Cluster 1A genes, while cellular movement and inflammatory response functions are more enriched in Cluster 2B genes ($P < 0.05$, Fisher's exact test; Supplementary Figure 4). In summary, our

analysis of genes regulated by TKI258 has led to the identification of breast cancer patients in which a fraction of TKI258-downregulated genes are highly expressed and are prognostic. Indeed, these patients tend to have a poor prognosis, in particular in the basal-like group. This cluster of highly expressed genes might reflect activation of signaling pathways that we identified in our analysis of FGFR in 4T1 tumors.

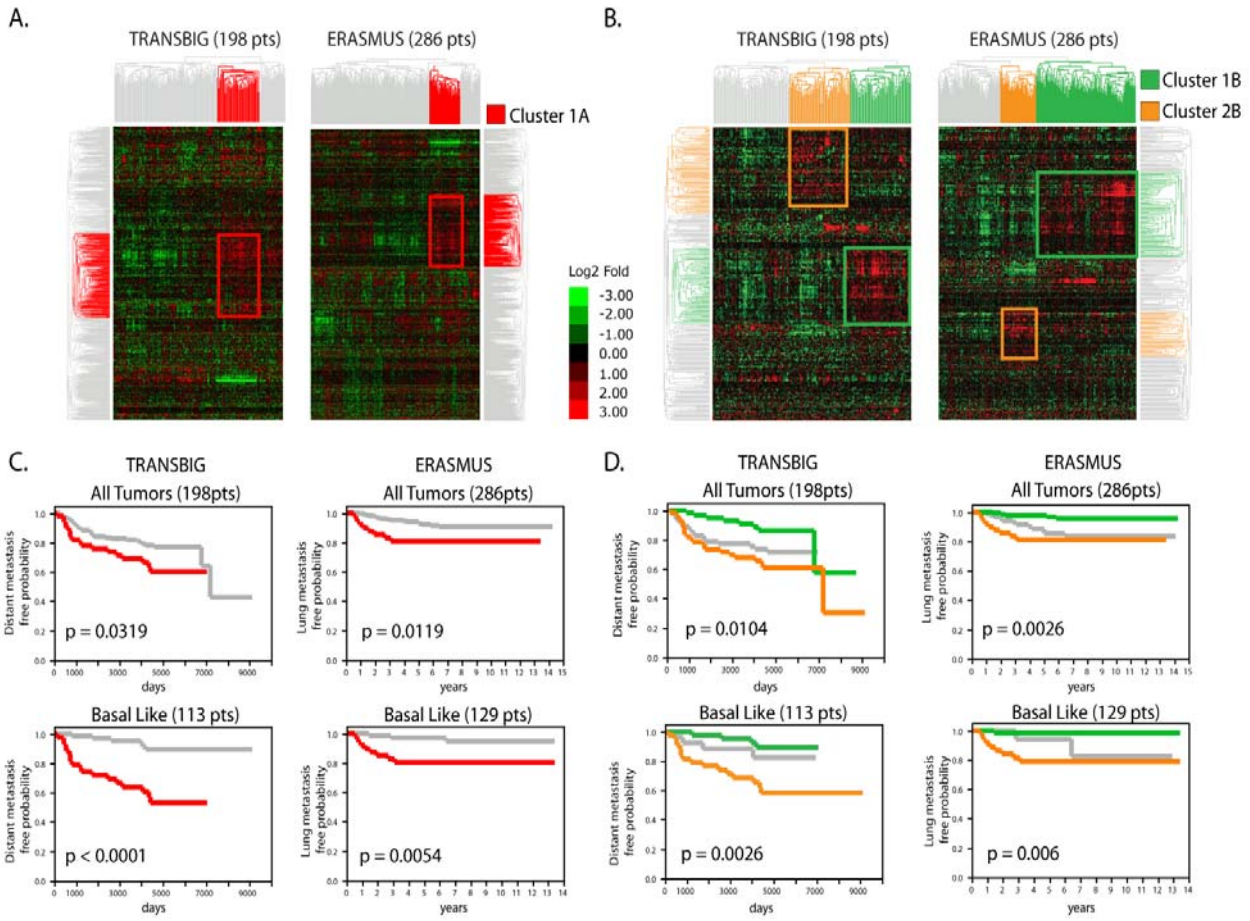

Figure 5-6 Meta-analysis of TKI258-regulated genes in human breast tumors

A, Unsupervised hierarchical clustering of TKI258-regulated genes identified in vitro in 4T1 cells (1648 genes) in the TRANSBIG and ERASMUS cohorts. Red tree and box indicate Cluster 1A patients and relative overexpressed genes in the cluster, respectively. *B*, Unsupervised hierarchical clustering of genes regulated by TKI258 in 4T1 tumors (254 genes) in the TRANSBIG and ERASMUS cohorts. Yellow tree and box and green tree and box indicate Cluster 2B and Cluster 1B patients and relative overexpressed genes in the clusters, respectively. *C*, Kaplan-Meier plots of Cluster 1A patients (red line) compared to the others (grey line) and plots considering only patients with basal-like tumors. P-values were computed using log-rank t-test. *D*, Kaplan-Meier plots of patients in Cluster 1B (green line) and Cluster 2B (orange line) compared to others (grey line) and by considering only patients with basal-like tumors. P-values were computed using log-rank t-test.

Discussion

Despite recent advances in breast cancer treatment there are patients for whom no targeted therapies are available (Rakha & Ellis, 2009). Based on evidence implicating FGFRs as breast cancer risk factors (Easton et al., 2007; Hunter et al., 2007; Meyer et al., 2008) and the identification of *FGFR* amplification and overexpression in specific sub-groups (Nordgard et al., 2007; Reis-Filho et al., 2006), further studies on the potential of targeting this receptor family in breast cancer are warranted. In the work presented here we show that 4T1 and 67NR mammary cancer cells display autocrine FGFR activity due to co-expression of receptors and ligands. Furthermore, we show that multiple signaling pathways activated by FGFRs (Eswarakumar et al., 2005) are inhibited by TKI258 treatment of both tumor cell lines and FGFR inhibition blocks proliferation and causes a strong reduction in mammary tumor outgrowth and lung metastases. We identified the essential role of the PI3K/AKT pathway in 4T1 survival by expressing Myr-AKT in the cells, which rescued them from TKI258-induced apoptosis. Taken together our results suggest that in breast tumors with active FGFR signaling, anti-FGFR therapeutics including TKIs or antibodies might show clinical efficacy.

In addition to FGFRs, TKI258 inhibits VEGFR and PDGFR family members (Lee et al., 2005). Thus, 4T1 cells were tested for sensitivity to the pan-VEGFR inhibitor PTK787 (Wood et al., 2000) and the PDGFR family inhibitor STI571 (Buchdunger et al., 1996). Neither inhibitor had a significant effect on proliferation or survival of 4T1 cultures (Supplementary Figure 5A and B). Moreover, treatment of 4T1 tumor-bearing mice with PTK787 had not effect on tumor outgrowth (Supplementary Figure 5C). Taken together these results suggest that the major effects of TKI258 are related to FGFR blockade. In vivo, TKI258 treatment of both breast tumor models had a significant impact on tumor outgrowth, however, no tumor regression was observed. Despite the significant increase in cleaved caspase-3 observed in vitro in 4T1 cells and in vivo in the tumors, TKI258 only slowed tumor outgrowth. Furthermore, unlike in vitro treatment, TKI258 did not cause a decrease in proliferation markers in the tumors. This suggests that cell death caused by TKI258 was insufficient to overcome growth promoting signaling in the in vivo setting. It is likely that tumor cells use other factors supplied by the tumor environment to survive in vivo. Tumors rarely respond to treatment with a single agent and in the future it will be important to test if FGFR inhibition in combination with other anti-tumor agents causes tumor regression.

We have recently uncovered a novel pathway essential for 4T1 lung metastasis (Fayard et al., 2009). Protease nexin-1 (PN-1), an extracellular serpin, controls MMP-9 transcription via binding its receptor, LRP-1. PN-1 silenced 4T1 cells have decreased MMP-9 levels and impaired metastatic potential, which could be restored by re-expressing MMP-9 in them (Fayard et al., 2009). Interestingly, TKI258 lowers expression of both (Supplementary Tables 4 and 5), showing how FGFR uses multiple pathways to control proliferation, survival and metastatic spread of this breast cancer model. The metastatic process is complex and tumor cells need to overcome many barriers in order to reach and grow in distant organs (Gupta & Massague, 2006). The microarray analysis revealed a number of TKI258-regulated genes that are known to be involved in metastasis (Supplementary Tables 4 and 5), including integrins, extracellular matrix proteins and transcription factors. For example, collagen type IV α5 that has been shown to be down-regulated in invasive cancers (Tanaka et al., 1997) was increased by TKI258 treatment (Supplementary Table 4 and 5). Moreover, two important mediators of epithelial-mesenchymal transition (EMT), E-cadherin and the Twist transcription factor were also altered by TKI258 treatment (Supplementary Table 4 and Supplementary Figure 6A and B). During development FGFR induces EMT (Ciruna & Rossant, 2001), a transition that is characterized by loss of epithelial properties and an increased mesenchymal phenotype (Miettinen et al., 1994; Vincent-Salomon & Thiery, 2003). Interestingly, Twist knock-down 4T1 cells have been shown to form primary mammary tumors, with impaired metastatic potential (Yang et al., 2004).

Gene expression signatures of tumors have become important tools not only to define cancer subtypes and prognosis, but also for defining combined oncogenic pathway activity in tumors (Bild et al., 2006; Potti et al., 2006). It has also been shown that 'biased' approaches (i.e. relying on experimental models) are very powerful in identifying cancer signatures less influenced by tumor genetic heterogeneity (Bianchi et al., 2008). Therefore, we took advantage of the “TKI258 gene signature” to study its expression profile in publicly available breast cancer patient cohorts with long-term follow-up. We identified patient cohorts overexpressing clusters of genes down-regulated by TKI258 (Cluster 1A and 2B), which have a higher probability of metastatic disease compared to the other patients. The two identified genetic signatures are very stable since their prognostic significance was confirmed in both independent cohorts of analyzed patients

Another important aspect of using a 'biased' approach is the possibility to link cancer gene expression signatures to precise molecular alterations, which in the work presented here is TKI258-mediated blockade of FGFR signaling. Interestingly, we found that Cluster 2B, selected from the in vivo experiment, was enriched for genes encoding proteins involved in inflammatory response and cellular movement. This suggests that blocking signaling pathways downstream of FGFR exerts effects not only in the cancer cells but also on the tumor microenvironment. To test this, Cluster 2B genes were compared to genes uncovered in a recent study of an invasive cancer model in which gene expression in the tumor epithelium and the stroma was monitored (Reuter et al., 2009) (GSE15299). Remarkably, when a gene-set enrichment analysis was performed (GSEA), Cluster 2B genes were top scoring in terms of enrichment and significance in the stromal component of the invasive cancer (NES, 2.06; P <0.001; Supplementary Table 6). On the other hand, Cluster 1A genes, which were selected after in vitro TKI258 treatment were top scoring in the epithelial component of the tumor (NES, 2.19; P <0.001; Supplementary Table 6). Taken together, these results suggest that in human tumors inhibition of an RTK like FGFR might result in a concomitant transcriptional re-programming of genes that are important for tumor-stroma interaction and cancer progression.

In summary, FGFRs appear to be a valuable target for treatment of sub-groups of breast cancer patients. We show here that many biological aspects of the tumor, from cell proliferation to invasion and metastasis, are dependent on FGFR signaling and blockade of these receptors has a strong influence on tumor growth and metastasis formation.

Acknowledgements

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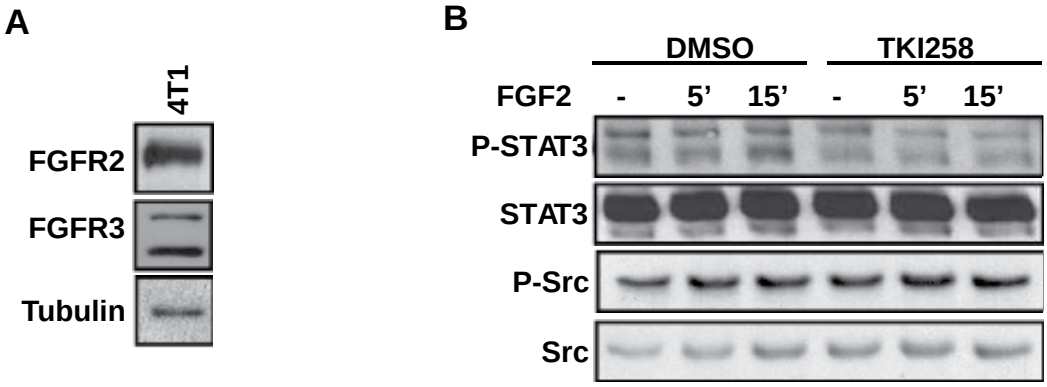
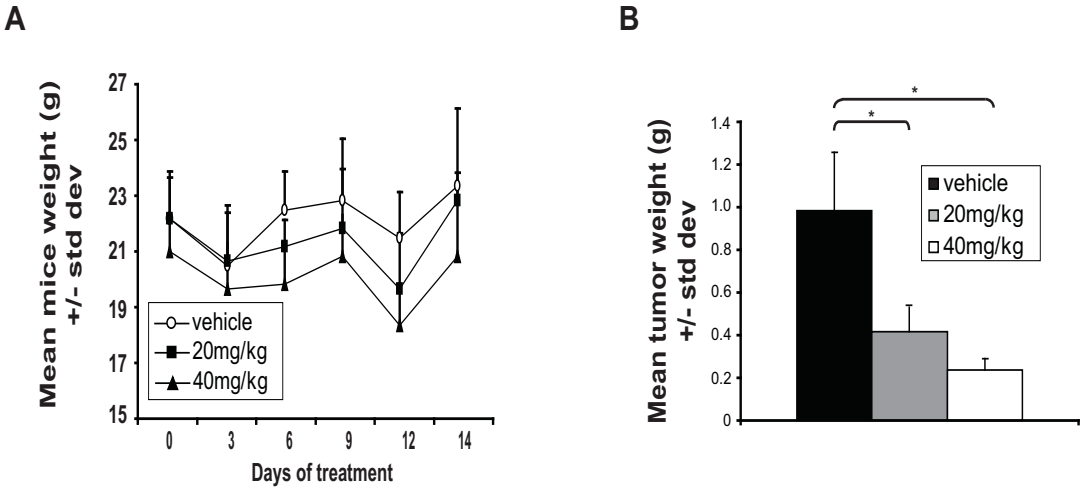
Supplementary figures and tables

Supplementary Figure 1



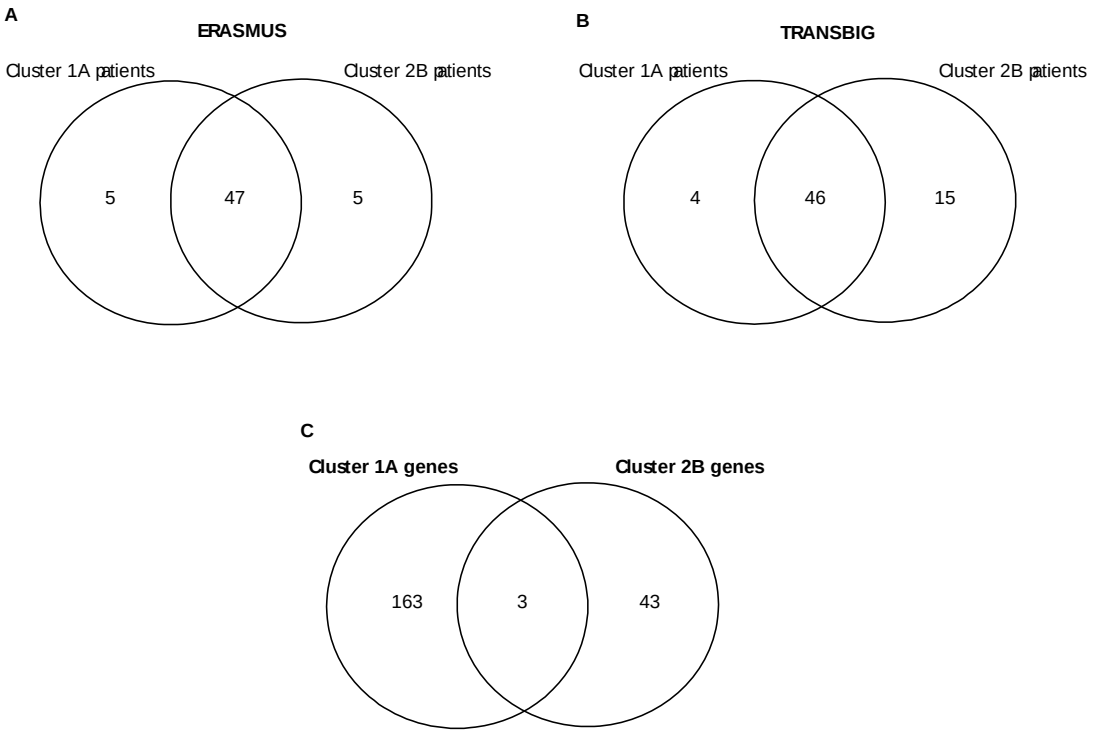
A, Western blot analysis of 4T1 lysates probed with specific antisera for FGFR2, FGFR3 and tubulin as a loading control. *B,* Cells were starved over-night in serum free media and pretreated with 1μM TKI258 or DMSO for 60 minutes prior stimulation with FGF2 for the indicated times. Whole cell lysates were subjected to western blot with antibodies specific for active forms of STAT3 and Src and reprobed with antisera for non-phosphorylated forms.

Supplementary Figure 2



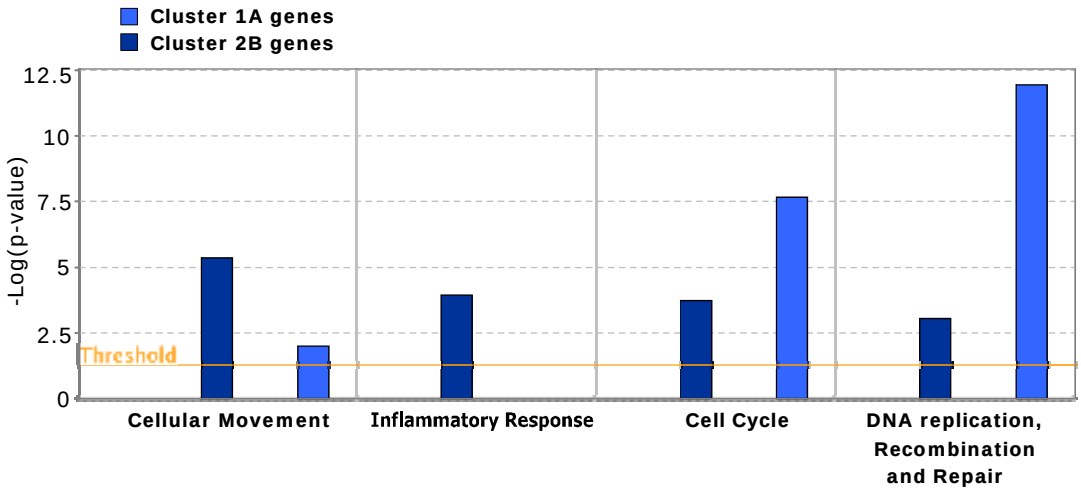
A, Weight of mice bearing 4T1 tumors and upon daily treatment with 20mg/kg, 40mg/kg TKI258 or water for 14 days. *B,* Weight of 4T1 tumors injected into the mammary gland of Balb/C mice. When the tumors reached 100mm^3, the mice were randomized into 3 groups and were orally treated daily with TKI258 (40mg/kg or 20mg/kg) or water for 14 days. Statistical analysis was performed using one way ANOVA: *, P<0.001.

Supplementary Figure 3



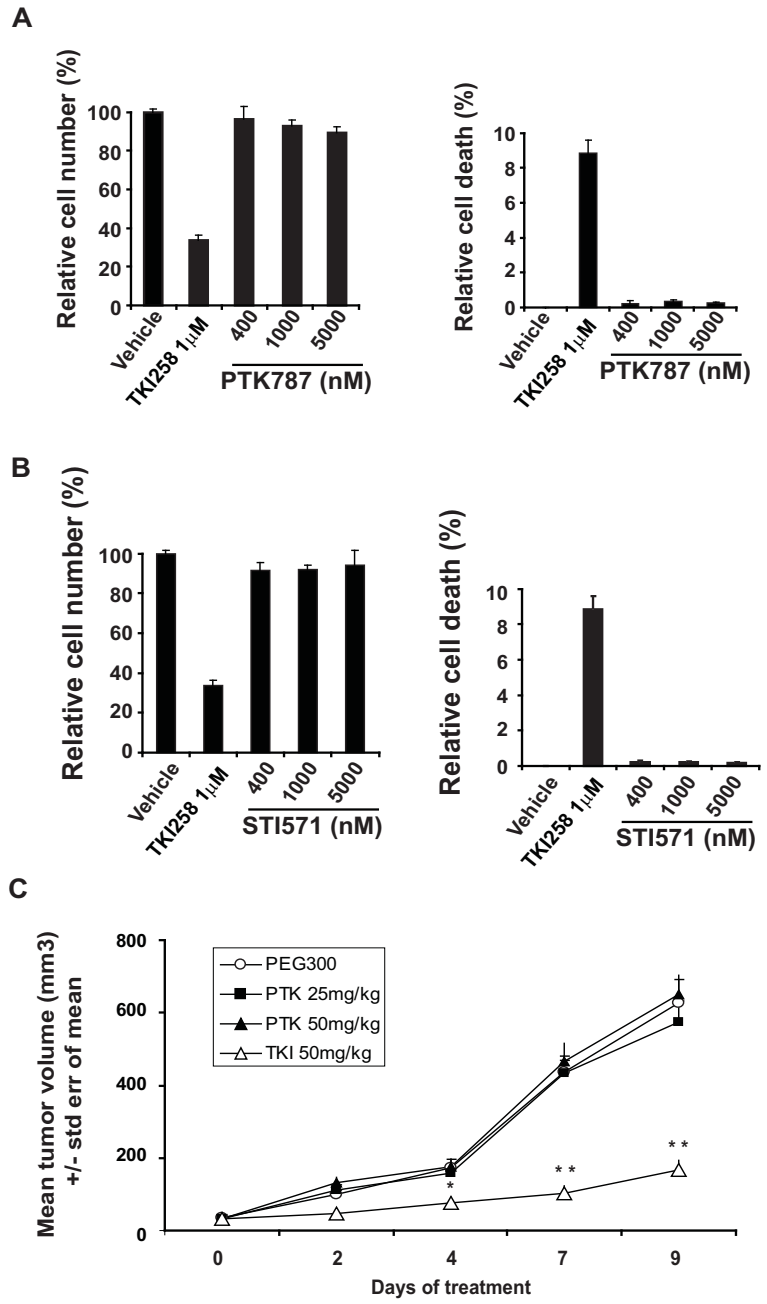
A, Overlap of patients from the ERASMUS cohort, with respect to their belonging to cluster 1A or 2B. *B*, Overlap of patients from the TRANSBIG cohort, with respect to their belonging to cluster 1A or 2B. *C*, Overlap of the genes between cluster 1A and 2B

Supplementary Figure 4



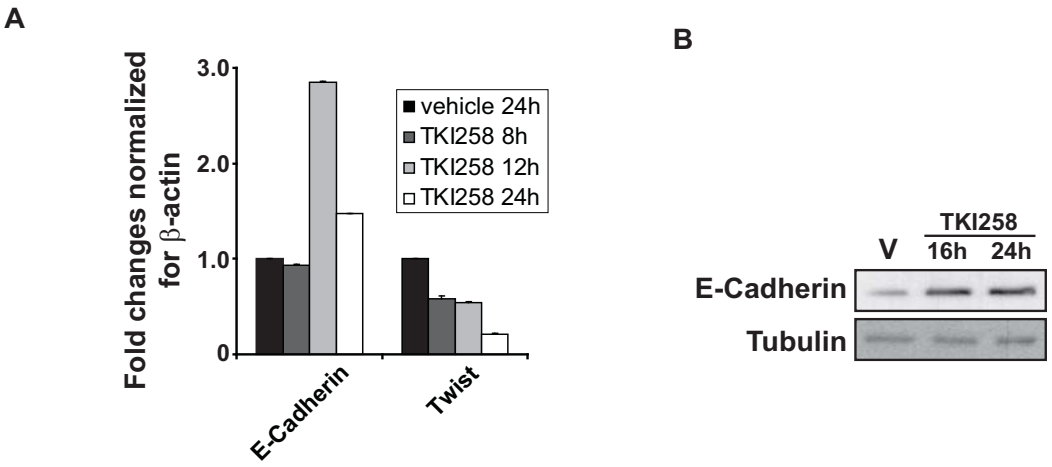
IPA analysis of bio-functions in Cluster 1A and 2B genes. The significance of the enrichment of bio function categories is displayed for both the two gene sets (light blue, Cluster 1A; dark blue, Cluster 2B). Y-axes, the minus logarithm base 10 of p-values (Fisher's exact test). Threshold indicates –Log (P = 0.05) which is used as cutoff.

Supplementary Figure 5



A, Comparison between the effects of 1μM TKI258 and increasing concentration of PTK787 on cell number (left) and cell death (right) using the YO-PRO assay after 24 hours of treatment. The effects were compared to vehicle treatment. *B,* Comparison between the effects of 1μM TKI258 and increasing concentration of STI571 on cell number (left) and cell death (right) using the YO-PRO assay after 24 hours of treatment. The effects were compared to vehicle treatment. *C,* Tumor growth analysis of 4T1 cells injected into the mammary gland of Balb/C mice. When the tumors reached 30mm^3, the mice were randomized into 4 groups (n=6) and were orally treated daily with PTK787 (50mg/kg or 25mg/kg), TKI258 (50mg/kg) or PEG300 for 9 days. Statistical analysis was performed using one way ANOVA: **, P<0.001; *, P=0.002 for TKI258 treated group versus both PTK787 treated groups and PEG300.

Supplementary Figure 6



A, 4T1 cells were treated with 1μM TKI258 for the indicated times. Total RNA was extracted and quantitative real-time RT-PCR was run on cDNA with primers specific for E-cadherin and Twist. *B*, 4T1 cells were treated with 1μM TKI258. At the indicated times, cell lysates were performed and blotted with a specific E-cadherin antibody. Tubulin was used as a loading control.

Supplementary Table 1

Changes in phospho-tyrosine content of peptides in TKI258 versus vehicle treated 4T1 cells

Description	Sequence	log2 ratios Average*
Gab1 GRB2-associated-binding protein 1	657 VDY^{660}VVVDQQK 666	-5.16 ± 0.11
Mapk3 Mitogen-activated protein kinase 3	191 IADPEHDHTGFLTEY^{205}VATR 209	-3.79 ± 0.25
Etl4 Isoform 6 of Sickle tail protein	4 EIVY^{7}AR9	-2.99 ± 0.66
Erh;LOC100042777 Enhancer of rudimentary homolog	89 EKIY^{92}VLLR 96	-2.30 ± 0.22
Frs2 Fibroblast growth factor receptor substrate 2	304 LVY^{306}ENINGLSIPSASGVR 321	-1.84 ± 0.33
Nans N-acetylneuraminic acid synthase	66 ALERPY^{71}TSK 74	-1.73 ± 0.29
Tnk2 Isoform 1 of Activated CDC42 kinase 1	869 VSSTHY^{874}YLLPERPPYLER 886	-1.56 ± 0.77
Fgfr2 fibroblast growth factor receptor 2	650 DINNIDY^{656}YKK 659	-0.74 ± 0.04
Tjp1 Tight junction protein ZO-1	1170 YRPEAQPY^{1177}SSTGPK 1183	1.02 ± 0.48
Mapk14 Isoform 1 of Mitogen-activated protein kinase 14	174 HTDDEMTGY^{182}VATR 186	1.09 ± 0.39
Dok1 Docking protein 1	442 GFSSDTALY^{450}SQVQK 455	1.27 ± 0.47

*Mean ± SD (*n* = 2).

Supplementary Table 2

Genes commonly up- or down-regulated in vivo and in vitro upon TKI258 treatment

Gene symbol	Description	Probeset ID	Ratios in vivo	P-value in vivo	Ratios in vitro	P-value in vitro
Hp	haptoglobin	NM_017370	3.3615	0.0063	3.2595	0.0025
Ogn	osteoglycin	NM_008760	3.2445	0.0079	2.5271	0.0023
Angpt1	angiopoietin 1	NM_009640	2.8679	0.0006	5.3224	0.0001
Adh1	alcohol dehydrogenase 1 (class I)	NM_007409	2.7637	0.0053	2.8519	0.0041
Pdgfc	platelet-derived growth factor, C polypeptide	NM_019971	2.7329	0.0018	6.7471	0.0001
Enpp2	ectonucleotide pyrophosphatase	NM_015744	2.3098	0.0265	3.4469	0.0003
Pdgfrl	platelet-derived growth factor receptor-like	NM_026840	2.2528	0.0123	2.8600	0.0003
C3	complement component 3	NM_009778	2.2293	0.0064	6.5605	0.0001
Vegfc	vascular endothelial growth factor C	NM_009506	2.2040	0.0073	2.1761	0.0020
Slc44a3	solute carrier family 44, member 3	NM_145394	2.1818	0.0003	2.9568	0.0006
Rbp1	retinol binding protein 1, cellular	NM_011254	2.1715	0.0006	2.5176	0.0002
Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	NM_009994	2.1425	0.0085	3.1896	0.0002
Sesn3	sestrin 3	NM_030261	2.0861	0.0015	3.7766	0.0001
Saa3	serum amyloid A 3	NM_011315	2.0620	0.0415	8.6781	0.0009
Tmie	transmembrane inner ear	NM_146260	1.9705	0.0016	1.9888	0.0010
Gdpd2	glycerophosphodiester phosphodiesterase domain containing 2	NM_023608	1.9444	0.0126	12.4670	0.0004
Cldn4	claudin 4	NM_009903	1.8833	0.0195	1.8585	0.0009
Irs1	insulin receptor substrate 1	NM_010570	1.8333	0.0031	1.5176	0.0031
Sned1	sushi, nidogen and EGF-like domains 1	NM_172463	1.7981	0.0180	2.1402	0.0004
Pde5a	phosphodiesterase 5A, cGMP-specific	NM_153422	1.7644	0.0051	2.1471	0.0008
Bdh2	3-hydroxybutyrate dehydrogenase, type 2	NM_027208	1.7382	0.0185	3.7656	0.0003
Dcn	decorin	NM_007833	1.7322	0.0108	4.0357	0.0037
Acox2	acyl-Coenzyme A oxidase 2, branched chain	NM_053115	1.7306	0.0009	2.4702	0.0012
Fmo1	flavin containing monooxygenase 1	NM_010231	1.7129	0.0466	5.3716	0.0007
Adhfe1	alcohol dehydrogenase, iron containing, 1	NM_175236	1.6677	0.0021	3.2613	0.0002
Rasl11b	RAS-like, family 11, member B	NM_026878	1.6669	0.0091	2.2551	0.0022
A330049M08Rik	RIKEN cDNA A330049M08 gene	BC005730	1.6241	0.0087	2.0573	0.0021
Add3	adducin 3 (gamma)	NM_013758	1.5741	0.0363	1.6564	0.0020
Mettl7a1	methyltransferase like 7A1	NM_027334	1.5544	0.0094	1.6628	0.0127
Col4a5	collagen, type IV, alpha 5	NM_007736	1.5441	0.0011	2.2696	0.0004
Tmem176a	transmembrane protein 176A	NM_025326	1.5384	0.0430	2.3997	0.0006
D4Bwg0951e	DNA segment, Chr 4, Brigham & Women's Genetics 0951 expressed	BC030404	1.5150	0.0050	2.6011	0.0005
Inmt	indolethylamine N-methyltransferase	NM_009349	1.5144	0.0042	3.8086	0.0018
Figf	c-fos induced growth factor	NM_010216	1.5091	0.0257	2.2880	0.0203
Tmc4	transmembrane channel-like gene family 4	NM_181820	1.5070	0.0339	2.0344	0.0025
Mgst1	microsomal glutathione S-transferase 1	NM_019946	1.5063	0.0136	3.9337	0.0003
Spry4	sprouty homolog 4 (Drosophila)	NM_011898	0.6569	0.0237	0.3714	0.0012
Angptl2	angiopoietin-like 2	NM_011923	0.6564	0.0015	0.5600	0.0005
Nup35	nucleoporin 35	NM_027091	0.6519	0.0041	0.6072	0.0015
Kif18a	kinesin family member 18A	NM_139303	0.6360	0.0053	0.6240	0.0022
Impdh2	inosine 5'-phosphate dehydrogenase 2	NM_011830	0.6339	0.0228	0.5790	0.0241
Csgalnact1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	NM_172753	0.6269	0.0036	0.3387	0.0001
Mmp9	matrix metallopeptidase 9	NM_013599	0.6257	0.0338	0.3696	0.0005
Il11	interleukin 11	NM_008350	0.6251	0.0013	0.3580	0.0006
Mettl7b	methyltransferase like 7B	NM_027853	0.6151	0.0081	0.4392	0.0028
F630043A04Rik	RIKEN cDNA F630043A04 gene	BC117501	0.6103	0.0010	0.6305	0.0015
3110006E14Rik	RIKEN cDNA 3110006E14 gene	ENSMUST00000065118	0.6057	0.0046	0.2836	0.0004
OTTMUSG00000017540	predicted gene, OTTMUSG00000017540	ENSMUST00000101675	0.5934	0.0217	0.6044	0.0386
Sema7a	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	NM_011352	0.5641	0.0088	0.4689	0.0068
Nptx1	neuronal pentraxin 1	NM_008730	0.5534	0.0018	0.2360	0.0002
Fabp5	fatty acid binding protein 5, epidermal	NM_010634	0.5513	0.0024	0.6020	0.0039
Sema6d	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	NM_172537	0.5500	0.0003	0.6026	0.0015
9030611O19Rik	RIKEN cDNA 9030611O19 gene	BC085288	0.5479	0.0006	0.2358	0.0004
Lipg	lipase, endothelial	NM_010720	0.5405	0.0008	0.2557	0.0004
Mmp10	matrix metallopeptidase 10	NM_019471	0.5304	0.0324	0.4114	0.0012
Mmp1b	matrix metallopeptidase 1b (interstitial collagenase)	NM_032007	0.5203	0.0021	0.1793	0.0001
Mmp3	matrix metallopeptidase 3	NM_010809	0.4561	0.0000	0.1352	0.0003
Mmp1a	matrix metallopeptidase 1a (interstitial collagenase)	NM_032006	0.4115	0.0049	0.2960	0.0003
Mcpt8	mast cell protease 8	NM_008572	0.4083	0.0209	0.4728	0.0010
Calr4	calreticulin 4	NM_001033226	0.3971	0.0020	0.3885	0.0016
Mmp13	matrix metallopeptidase 13	NM_008607	0.3451	0.0036	0.4896	0.0009

Supplementary Table 3

Genes grouping in cluster 1A

Genes in cluster 1A

Gene name	Trend in ERASMUS cohort	Trend in TRANSBIG cohort	Trend in 4T1 cells treated with TKI258
ADD2	UP		DOWN
AMPD2	UP		DOWN
ASF1B	UP	UP	DOWN
BLM	UP	UP	DOWN
C11ORF75	UP		DOWN
C13ORF34	UP	UP	DOWN
C14ORF169	UP		DOWN
C18ORF24	UP	UP	DOWN
C1ORF181	UP		DOWN
C1QBP	UP		DOWN
C21ORF45	UP	UP	DOWN
C6ORF66	UP	UP	DOWN
C6ORF75	UP	UP	DOWN
C9ORF40	UP		DOWN
CCDC86	UP		DOWN
CCDC99	UP	UP	DOWN
CCNB1IP1	UP		DOWN
CCNE1	UP	UP	DOWN
CCNE2	UP	UP	DOWN
CCNF	UP		DOWN
CDC25A	UP	UP	DOWN
CDC45L	UP	UP	DOWN
CDC7	UP	UP	DOWN
CDT1	UP	UP	DOWN
CEP55	UP	UP	DOWN
CGI-115	UP	UP	DOWN
CLSPN	UP	UP	DOWN
CX3CL1	UP		DOWN
CYCS	UP		DOWN
DDX18	UP		DOWN
DDX21	UP	UP	DOWN
DDX39	UP	UP	DOWN
DKC1	UP	UP	DOWN
DNA2L	UP	UP	DOWN
DONSON	UP	UP	DOWN
DPH2	UP		DOWN
DUSP9	UP		DOWN
E2F3	UP	UP	DOWN
E2F8	UP	UP	DOWN
ESPL1	UP	UP	DOWN
ETV5	UP		DOWN
EXO1	UP	UP	DOWN
FABP5	UP	UP	DOWN
FBL	UP		DOWN
FBXO5	UP	UP	DOWN
FEN1	UP	UP	DOWN
FLJ20105	UP	UP	DOWN
FRMD4A	UP		DOWN
GART	UP		DOWN
GEMIN6	UP		DOWN
GFOD1	UP		DOWN
GINS1	UP	UP	DOWN
GINS3	UP		DOWN
GMNN	UP		DOWN
GPATC4	UP	UP	DOWN
HSPD1	UP		DOWN
IFRD1	UP	UP	DOWN
IMPDH1	UP		DOWN
ITGA6	UP		DOWN
JTV1	UP		DOWN
KCNK5	UP		DOWN
KCNN4	UP		DOWN
KIAA0179/Rrp1b	UP		DOWN
KIAA0664	UP		DOWN
KIAA0859	UP		DOWN
KIF18A	UP	UP	DOWN
KLK10	UP		DOWN
LIPG	UP		DOWN
LOC651423	UP		UP
LRP8	UP	UP	DOWN
LSM7	UP		DOWN
MCM10	UP	UP	DOWN
MCM3	UP	UP	DOWN
MDN1	UP		DOWN
ME2	UP	UP	DOWN
METTL1	UP		DOWN
MINA	UP		DOWN
MLF1IP	UP	UP	DOWN
MPP6	UP		DOWN
MTHFD1	UP		DOWN
MYBBP1A	UP		DOWN
MYBL2	UP	UP	DOWN
MYC	UP		DOWN
NASP	UP	UP	DOWN
NAT10	UP		DOWN
NCL	UP		DOWN
NES	UP		DOWN
NKRF	UP		DOWN
NME1	UP		DOWN
NOC4L	UP		DOWN
NSUN5	UP		DOWN
NUP107	UP	UP	DOWN
NUP160	UP	UP	DOWN
NUP43	UP		DOWN
NUP85	UP		DOWN
ODC1	UP		DOWN
ORC1L	UP		DOWN
PA2G4	UP		DOWN
PADI2	UP		DOWN
PBK	UP	UP	DOWN
PCOLCE2	UP		DOWN
PDSS1	UP	UP	DOWN
PHLDA2	UP		DOWN
PLK4	UP	UP	DOWN
POL3G	UP	UP	DOWN
POLQ	UP		DOWN
POLR1E	UP		DOWN
POLR2H	UP		DOWN
POP1	UP		DOWN
PPA1	UP		DOWN
PPAN	UP		DOWN
PRIM1	UP	UP	DOWN
PRKAR2A	UP	UP	DOWN
PTDSR/JMJD6	UP		DOWN
PUS7	UP	UP	DOWN
PVR	UP		DOWN
PWP1	UP		DOWN
PWP2H	UP		DOWN
RAD51AP1	UP	UP	DOWN
RAD54L	UP	UP	DOWN
RAI14	UP		DOWN
RBMX2	UP		DOWN
RCL1	UP		DOWN
RPP30	UP		DOWN
RPP40	UP	UP	DOWN
RRM2	UP	UP	DOWN
RRS1	UP		DOWN
RUVBL2	UP		DOWN
SACS	UP		DOWN
SETD6	UP		DOWN
SFN	UP		DOWN
SHMT2	UP		DOWN
SKP2	UP		DOWN
SLC19A1	UP		DOWN
SLC2A1	UP		DOWN
SLC39A14	UP		DOWN
SNRPA1	UP	UP	DOWN
TBRG4	UP	UP	DOWN
TDP1	UP	UP	DOWN
TFRC	UP		DOWN
TGFA	UP		DOWN
TIMM10	UP		DOWN
TIMM8A	UP		DOWN
TIMM9	UP		DOWN
TIPIN	UP	UP	DOWN
TK1	UP	UP	DOWN
TMEM48	UP	UP	DOWN
TMEM97	UP		DOWN
TNFRSF21	UP		DOWN
TRIM27	UP		DOWN
TRIP13	UP	UP	DOWN
TSR1	UP		DOWN
TUBB6	UP		DOWN
UCHL5	UP	UP	DOWN
UMPS	UP		DOWN
UPP1	UP	UP	DOWN
USP36	UP		DOWN
UTP18	UP		DOWN
WDHD1	UP	UP	DOWN
WDR12	UP		DOWN
WDR43	UP		DOWN
XPO4	UP		DOWN
YARS2	UP		DOWN
YRDC	UP		DOWN
ZNF259	UP		DOWN
ZRF1/DNAJC2	UP		DOWN

Genes grouping in cluster 1B and 2B

Genes in cluster 1B 1st part

Gene name	Trend in ERASMUS cohort	Trend in TRANSBIG cohort	Trend in 4T1tumors treated with TKI258
A2M	UP	UP	UP
ADAMTS5	UP	UP	UP
ADD3	UP	UP	UP
ADH1C		UP	UP
ADIPOQ	UP	UP	UP
AGTRL1	UP	UP	UP
ANGPTL2	UP	UP	DOWN
AOX1	UP	UP	UP
AQP7	UP	UP	UP
BACE1	UP		UP
BDH2		UP	UP
C10ORF10	UP	UP	DOWN
C3	UP	UP	UP
CD36	UP	UP	UP
CD93	UP	UP	DOWN
CFD	UP	UP	UP
CFD		UP	UP
CHGN		UP	DOWN
CHRDL1	UP	UP	UP
CIDEC	UP	UP	UP
COL14A1	UP	UP	UP
COL4A2	UP		DOWN
CXCL12	UP	UP	UP
CYP1B1		UP	UP
DCN	UP	UP	UP
DPT	UP	UP	UP
DUSP1	UP	UP	DOWN
EFEMP1	UP	UP	UP
EMCN	UP	UP	DOWN
ENPP2	UP	UP	UP
FABP4	UP	UP	UP
FIGF	UP	UP	UP
FMO1	UP	UP	UP
FXYD1	UP	UP	UP
GHR		UP	UP
GPD1	UP	UP	UP
GPD1		UP	UP
GPR116	UP	UP	DOWN
GPX3	UP		UP
HP	UP	UP	UP
ITM2A	UP	UP	UP

Genes in cluster 1B 2nd part

Gene name	Trend in ERASMUS cohort	Trend in TRANSBIG cohort	Trend in 4T1tumors treated with TKI258
LEP	UP	UP	UP
LPL	UP	UP	UP
METTL7A	UP		UP
MMP3	UP	UP	DOWN
NID2	UP	UP	DOWN
NNMT	UP	UP	UP
OGN	UP	UP	UP
PCK1	UP	UP	UP
PDGFC		UP	UP
PDGFRL	UP	UP	UP
PGDS	UP		DOWN
RARRES2	UP	UP	UP
ROR1		UP	UP
SERPINE2		UP	DOWN
SFRP4	UP	UP	UP
SFRP4		UP	UP
SNED1	UP		UP
SPRY4	UP		DOWN
STEAP1		UP	UP
TMEM176A		UP	UP
VEGFC	UP	UP	UP

Genes in cluster 2B

Gene name	Trend in ERASMUS cohort	Trend in TRANSBIG cohort	Trend in 4T1 tumors treated with TKI258
ACSL1	UP		UP
ADD3	UP		UP
ANGPT1	UP	UP	UP
APOC1	UP		UP
BMP2	UP		DOWN
BUB1	UP		DOWN
CDC25C	UP		DOWN
CHEK2	UP		DOWN
CHGN	UP		DOWN
COL4A2		UP	DOWN
CXORF15	UP		DOWN
CYP1B1	UP		UP
CYP7B1	UP		UP
DEPDC1	UP		DOWN
DSG1	UP		DOWN
DSG3	UP	UP	DOWN
EGFR	UP	UP	UP
FABP5	UP	UP	DOWN
GPM6B	UP	UP	UP
HMGB3	UP		DOWN
HMMR	UP		DOWN
IGFBP3	UP		DOWN
ITGAX	UP		DOWN
KCNJ2	UP	UP	DOWN
KIF18A	UP		DOWN
LGALS7	UP	UP	DOWN
LIPG	UP	UP	DOWN
MAP2	UP		UP
MMP1	UP	UP	DOWN
MMP13		UP	DOWN
MMP9	UP	UP	DOWN
OIP5	UP		DOWN
PDGFC	UP		UP
PHACTR1	UP		UP
RBP1	UP	UP	UP
ROR1	UP	UP	UP
S100A9	UP		DOWN
SERPINE2	UP		DOWN
SLC1A3	UP		UP
SLC5A3	UP	UP	DOWN
SLC6A2	UP		UP
SRD5A1	UP	UP	DOWN
ST3GAL5	UP		DOWN
TLR7	UP		DOWN
TMEM176A	UP		UP
TRIM29	UP	UP	DOWN

Supplementary Table 4

Genes related to an invasive phenotype modulated upon TKI258 treatment in vitro

Gene symbol	Description	Probeset ID	Ratio (TKI258/control)	P-value
Cdh17	cadherin 17	NM_019753	0.09	0.00005
Mmp3	matrix metallopeptidase 3	NM_010809	0.14	0.00027
Mmp1b	matrix metallopeptidase 1b	NM_032007	0.18	0.00009
Mmp1a	matrix metallopeptidase 1a	NM_032006	0.29	0.00029
Plaur	plasminogen activator, urokinase receptor	NM_011113	0.31	0.00012
Cxcr3	chemokine (C-X-C motif) receptor 3	NM_009910	0.33	0.00048
Mmp9	matrix metallopeptidase 9	NM_013599	0.37	0.00122
Timp1	tissue inhibitor of metalloproteinase 1	NM_001044384	0.38	0.00025
Mmp10	matrix metallopeptidase 10	NM_019471	0.41	0.00115
Itga2	integrin alpha 2	NM_008396	0.42	0.00086
Nes	nestin	NM_016701	0.48	0.00037
Mmp13	matrix metallopeptidase 13	NM_008607	0.49	0.0009
Itgb7	integrin beta 7	NM_013566	0.58	0.0045
Plau	plasminogen activator, urokinase	NM_008873	0.60	0.0037
Cd44	CD44 antigen	NM_009851	0.61	0.002
Bmp7	bone morphogenetic protein 7	NM_007557	0.62	0.0012
Col8a1	collagen, type VIII, alpha 1	NM_007739	0.63	0.0026
Itga6	integrin alpha 6	NM_008397	0.64	0.0022
Jun	Jun oncogene	NM_010591	0.64	0.0018
Twist1	Twist	NM_011658	0.69	0.0001
Timp2	tissue inhibitor of metalloproteinase 2	NM_011594	1.51	0.0013
Cgn	cingulin	NM_001037711	1.51	0.006
Itga1	integrin alpha 1	NM_001033228	1.53	0.0274
Ing4	inhibitor of growth family, member 4	NM_133345	1.58	0.0036
Vcam1	vasculat cell adhesion molecule 1	NM_011693	1.59	0.00076
Itgb3	integrin beta 3	NM_016780	1.72	0.0012
Synpo2	synaptopodin 2	BC158045	1.72	0.0113
Icam1	intercellular adhesion molecule 1	NM_010493	1.73	0.0017
Ptprf	protein tyrosine phosphatase, receptor type, F	NM_011213	1.77	0.0006
Cldn4	claudin 4	NM_009903	1.86	0.0009
Jup	junction plakoglobin	NM_010593	1.88	0.0006
Sdc3	syndecan 3	NM_011520	1.91	0.0009
Krt19	keratin 19	NM_008471	1.91	0.0015
Cdh1	cadherin 1	NM_009864	2.13	0.0003
Col4a5	collagen, type IV, alpha 5	NM_007736	2.27	0.0003
Ephb3	Eph receptor B3	NM_010143	2.35	0.0017
Itgb5	integrin beta 5	NM_010580	2.37	0.0003
Ephb6	Eph receptor B6	NM_007680	2.39	0.0017
Mmp11	matrix metallopeptidase 11	NM_008606	2.54	0.0004
Lamb2	laminin, beta 2	NM_008483	2.73	0.0003
Thbs1	thrombospondin 1	NM_011580	2.75	0.0003
Col6a1	collagen, type VI, alpha 1	NM_009993	2.85	0.0002
Mcam	melanoma cell adhesion molecule	NM_023061	3.16	0.0003
Krt14	keratin 14	NM_016958	3.45	0.0026
Dcn	decorin	NM_007833	4.04	0.0037
Cdon	cell adhesion molecule-related	NM_021339	4.11	0.0002

Supplementary Table 5

Genes related to an invasive phenotype modulated upon TKI258 treatment in vivo				
Gene symbol	Description	Probeset ID	Ratios (TKI258/control)	P-value
Mmp13	matrix metallopeptidase 13	NM_008607	0.34	0.0035
Mmp1a	matrix metallopeptidase 1a	NM_032006	0.41	0.0048
Mmp3	matrix metallopeptidase 3	NM_010809	0.46	0.00003
Angpt2	angiopoietin 2	NM_007426	0.48	0.0167
Mmp1b	matrix metallopeptidase 1b	NM_032007	0.52	0.0021
Mmp10	matrix metallopeptidase 10	NM_019471	0.53	0.0323
Serpine2	serine peptidase inhibitor, clade E, member 2	NM_009255	0.53	0.0463
ItgaX	integrin alpha X	NM_021334	0.55	0.0281
Dsg3	desmoglein 3	NM_030596	0.57	0.0363
Bmp2	bone morphotenetic protein 2	NM_007553	0.58	0.0046
Cldn1	claudin 1	NM_016674	0.59	0.0465
IL11	interleukin 11	NM_008350	0.62	0.0013
Mmp9	matrix metallopeptidase 9	NM_013599	0.62	0.0337
Angptl2	angiopoietin-like 2	NM_011923	0.65	0.0014
Itga9	integrin alpha 9	NM_133721	0.66	0.0030
Col4a2	collagen, type IV, alpha 2	NM_009932	0.66	0.0111
Cdc25c	cell division cycle 25 homolog C	NM_009860	0.66	0.0006
Col4a5	collagen, type IV, alpha 5	NM_007736	1.54	0.0011
Col14a1	collagen, type XIV, alpha 1	NM_181277	1.55	0.0247
Cldn3	claudin 3	NM_009902	1.56	0.0102
Fndc1	fibronectin type III domain containing 1	NM_001081416	1.61	0.0441
Dcn	decorin	NM_007833	1.73	0.0107
Tusc5	tumor suppressor candidate 5	NM_177709	1.78	0.0137
Cldn4	claudin 4	NM_009903	1.88	0.0195
Cxcl12	chemokine (C-X-C motif) ligand 12	NM_001012477	1.96	0.0107
Vegfc	vascular endothelial growth factor C	NM_009506	2.20	0.0072
Angpt1	angiopoietin 1	NM_009640	2.86	0.0005

Supplementary Table 6

	SCC – Stroma			
Gene List	**Size**	**NES**	**NOM p-value**	**FDR q-value**
Cluster 2B	36	**2.06**	<0.001	<0.001
Cluster 1A	97	1.45	0.024	0.028
	SCC – Epithelium			
Gene List	**Size**	**NES**	**NOM p-value**	**FDR q-value**
Cluster 1A	100	**2.19**	<0.001	<0.001
Cluster 2B	45	1.42	0.041	0.027

gene-set enrichment analysis (GSEA) of Cluster 1A and 2B genes in epithelial and stromal component of invasive squamous carcinoma (SCC) (Reuter et al., 2009). Size, number of genes mapped on affymetrix chips and available for the analysis. NES, normalized enrichment GSEA score. Nominal p-values (NOM p-value) and false discovery rate q-values (FDR q-value) were computed based on the probability distribution of enrichment score of 1000 random gene lists.

5.2. Unpublished results

In addition to the results presented in the submitted research article, other experiments and analyses were performed. These additional experiments were aimed at a better understanding the role of FGFR signaling in different cell lines and at studying the individual contribution of the FGFR family members to the tumorigenic and metastatic process. Moreover, we tested if combination of treatments with TKI258 and other inhibitors could be an interesting approach to increase the effects of FGFR blockade.

5.2.1. FGFR inhibition in mouse mammary carcinoma cell lines

Aslakson and Miller described four mouse mammary carcinoma cell lines (4T1, 4TO7, 168FARN and 67NR) that all form mammary tumors when injected into the fat pad of BALB/c mice but display different metastatic properties (Figure 5-7) (Aslakson & Miller, 1992).

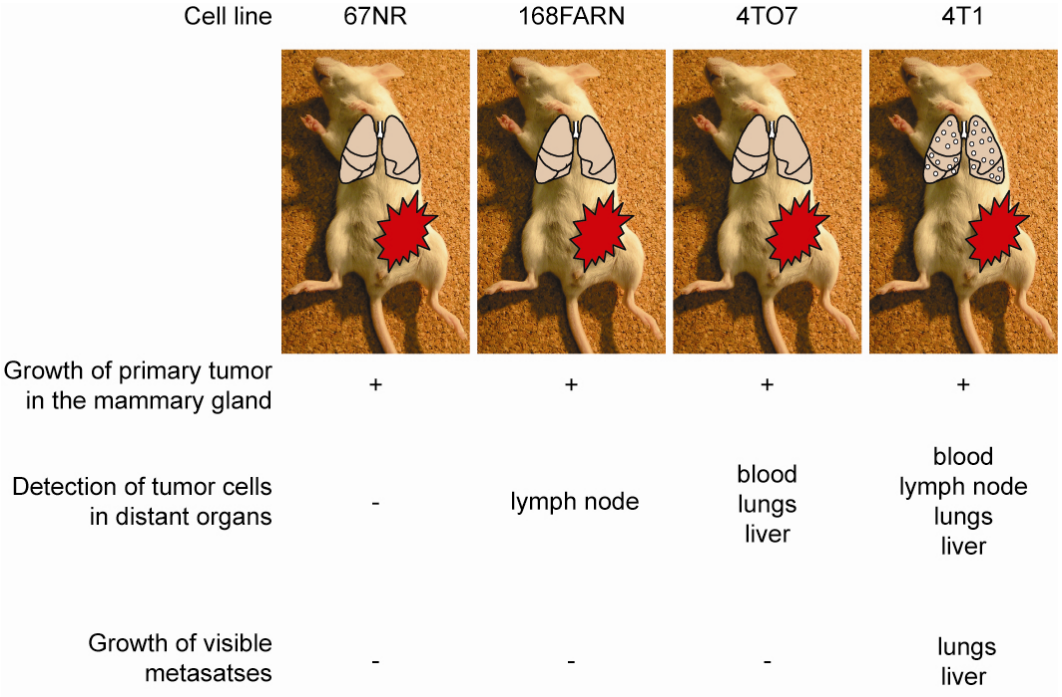


Figure 5-7 Tumorigenic and metastatic properties of 4T1, 4TO7, 168FARN and 67NR

The 67NR line is non metastatic and appears to be unable to intravastate as they were never detected in the circulation. The non metastatic line 168FARN cells spread through the lymphatics and clonogenic cells can be recovered from lymph nodes. The line 4TO7 is able to complete all steps of the metastatic process except the final one that consists of growing as metastatic nodules in distant organs. The 4T1 line is highly metastatic and forms visible lung metastases after injection in the mammary gland.

First we examined the expression of FGFRs by RT-PCR and western blot in these four mouse mammary carcinoma cell lines. 4T1, 4TO7 and 168FARN cells express FGFR1, FGFR2 and FGFR3, with 168FARN having a lower amount of FGFR3. FGFR1 was not detected in the 67NR (Figure 5-8 A and B). FGFR4 was not detected in any of these cell lines. Expression of FGF1 and FGF2, which are the ligands with the broadest activity towards the several isoforms of FGFRs was investigated and FGF1 was detected in 4T1, 4TO7 and 67NR, but not in 168FARN cells. In contrast, the 168FARN were the only ones were expression of FGF2 was detected (Figure 5-8 C).

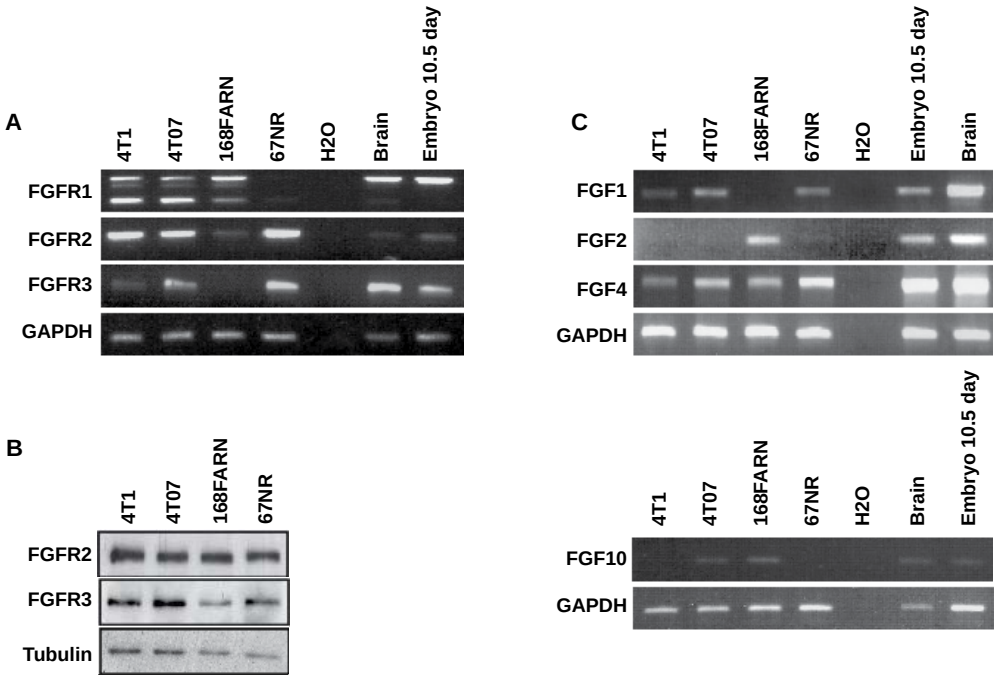


Figure 5-8 Expression of FGFRs and ligands in 4T1, 4TO7, 168FARN and 67NR cells

A, Semi-quantitative RT-PCR for FGFRs, performed on cDNA from 4T1, 4TO7, 168FARN and 67NR cells grown in full medium. GAPDH was used as loading control, cDNA from brain and embryo were used as positive controls and H_2O as negative control. *B*, Lysates of cells growing in full medium were probed with specific FGFR2 and FGFR3 antibodies. Tubulin was used as a loading control. *C*, Semi-quantitative RT-PCR for different FGFs, performed on cDNA from 4T1, 4TO7, 168FARN and 67NR cells grown in full medium. GAPDH was used as loading control, cDNA from brain and embryo were used as positive controls and H_2O as negative control.

The 4T1, 4TO7, 168FARN and 67NR cell lines were isolated from a spontaneous mammary carcinoma arising in a BALC/c mouse that was foster-nursed by a C_3H female, a mouse strain that harbors endogenous milk-borne mouse mammary tumor virus (MMTV). We therefore

investigated whether these cells have high expression of *FGF3*, *FGF4*, *FGF8* and *FGF10* that are known to be insertional hot spot for the MMTV provirus (Mattila & Harkonen, 2007; Peters et al., 1989; Theodorou et al., 2004; Theodorou et al., 2007). FGF4 was the only ligand detected in the four cell lines, suggesting that its expression could be caused by MMTV insertion in the primary tumor from which the four cell lines were derived (Figure 5-8 C upper panel). In addition, we detected expression of FGF10 in 4TO7 and 168FARN cells (Figure 5-8 C lower panel). FGF8 was not detected in the tumor cell lines and the PCR for FGF3 never gave a signal in the positive control (data not shown). As co-expression of FGF receptors and FGFs was observed in the four cell lines, we tested the hypothesis that these mammary tumor cell lines have autocrine FGFR activity.

The activity of signaling proteins downstream of FGFR was measured in lysates of cells grown in full medium or serum-starved with or without FGF2 stimulation (Figure 5-9). FRS2 is an adaptor protein that gets tyrosine phosphorylated by active FGFRs and links these receptors to various pathways (Kouhara et al., 1997; Ong et al., 2000). The level of Ptyr196-FRS2, a docking site for Grb2 was high in the four cell lines. Furthermore, high level of P-ERK1/2 was detected and with the exception of the 168FARN, P-PLCγ was high in all the cell lines (Figure 5-9). Serum deprivation had little to no effects in 4T1, 4TO7 and 67NR. However following serum starvation, the 168FARN showed a marked decrease on ERK1/2 activity. In addition, 168FARN cells were the only ones that showed consistent increased ERK1/2 activity 5 and 15 minutes after FGF2 stimulation and increased PLCγ activity upon FGF2 addition (Figure 5-9). Taken together, these results provide strong evidence supporting the hypothesis that 4T1, 4TO7, 168FARN and 67NR cells possess autocrine FGFR activity that can even be stimulated in the 168FARN.

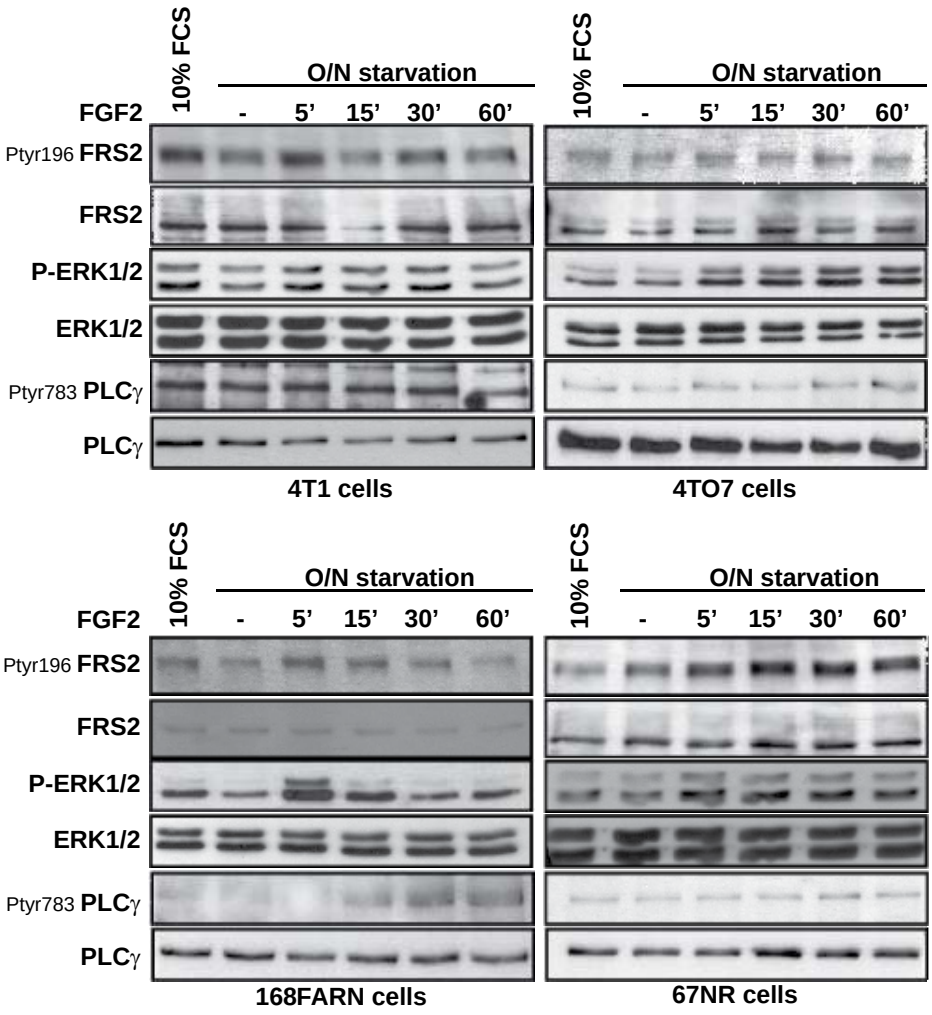


Figure 5-9 Active FGFR signaling in mouse mammary carcinoma cell lines

Lysates were prepared from cells in full serum or serum starved and stimulated for the indicated times with FGF2. Immunoblot analyses were performed with the indicated antibodies.

To investigate the intracellular pathways controlled by FGFRs, we used TKI258, a selective FGFR TKI (Lee et al., 2005; Renhowe et al., 2009; Sarker et al., 2008). As shown in Figure 5-1, TKI258 treatment of 4T1 and 67NR cells blocks several signaling pathway. Here we extended this analysis to the 4TO7 and 168FARN cells and observed that TKI258 treatment decreases both, basal and FGF2 induced, Ptyr196-FRS2 and ERK1/2 activity in these two cell lines (Figure 5-10). Compared to control IPs, PLCγ IPs from lysate of inhibitor treated cells revealed lower Ptyr content (Figure 5-10). There was no effect of TKI258 on the constitutive activity of Stat3 (Figure 5-10), suggesting that an autocrine loop independent of FGFR keeps this pathway active. These results show that TKI258 has a strong effect on constitutive autocrine FGFR signaling in 4TO7

and 168FARN cells. In addition, TKI258 treatment of 168FARN cells prevents FGF2 induced activation of ERK1/2.

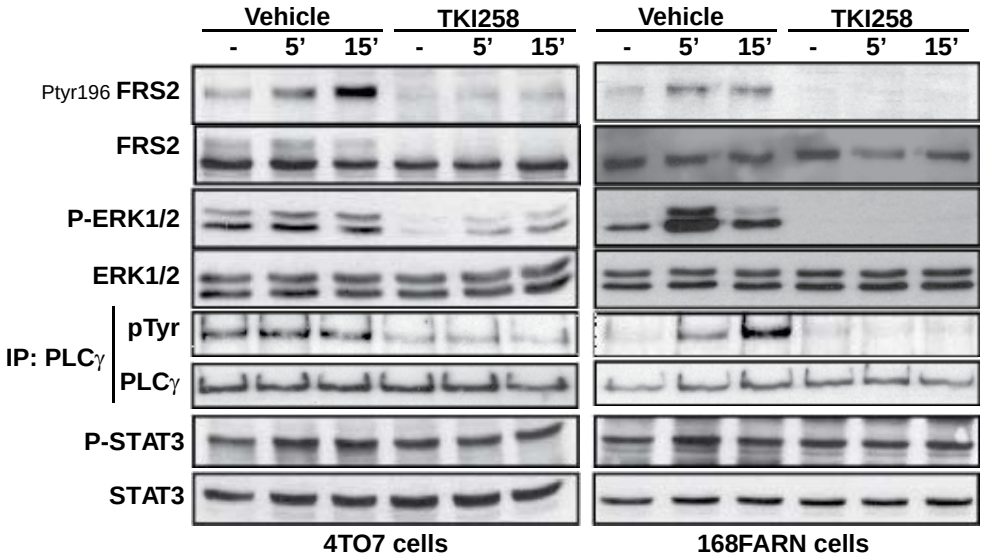


Figure 5-10 TKI258 blocks intracellular signaling in 4TO7 and 168FARN cells

Cultures were serum-starved over-night then pretreated with 1μM TKI258 or DMSO for 1 hour prior to stimulation with FGF2 for the indicated times. Lysates were prepared and immunoblot analyses were performed with the indicated antibodies. PLCγ IPs were probed for Ptyr content.

Next we tested the effects of FGFR inhibition on cell proliferation. Using BrdU incorporation to measure S-phase, we observed a dose-dependent decrease in proliferation with an 80% decrease at the highest dose in the 4T1 and a 50% to 60% decrease in the other cell lines (Figure 5-11 A). As already seen upon treatment with TKI258 in Figure 5-2, we observed a strong dose-dependent increase of cell death in 4T1 cells upon treatment with PD173074, another TKI selective for FGFRs. The effect of FGFR inhibition using PD173074 on 67NR cell death was minor (Figure 5-11 B black and white bars). Interestingly, we found that in the 4TO7 line, cell death was induced in a dose-dependent manner upon blockade of FGFRs, however the treatment had little to no effect on the 168FARN (Figure 5-11 B dark and light gray bars). These data show that blockade of autocrine FGFR activity has a strong impact on the proliferation of these four mammary cancer cell lines. In addition, we show that cell death is induced in 4T1 and 4TO7 cells in a dose dependent manner upon FGFR inhibition.

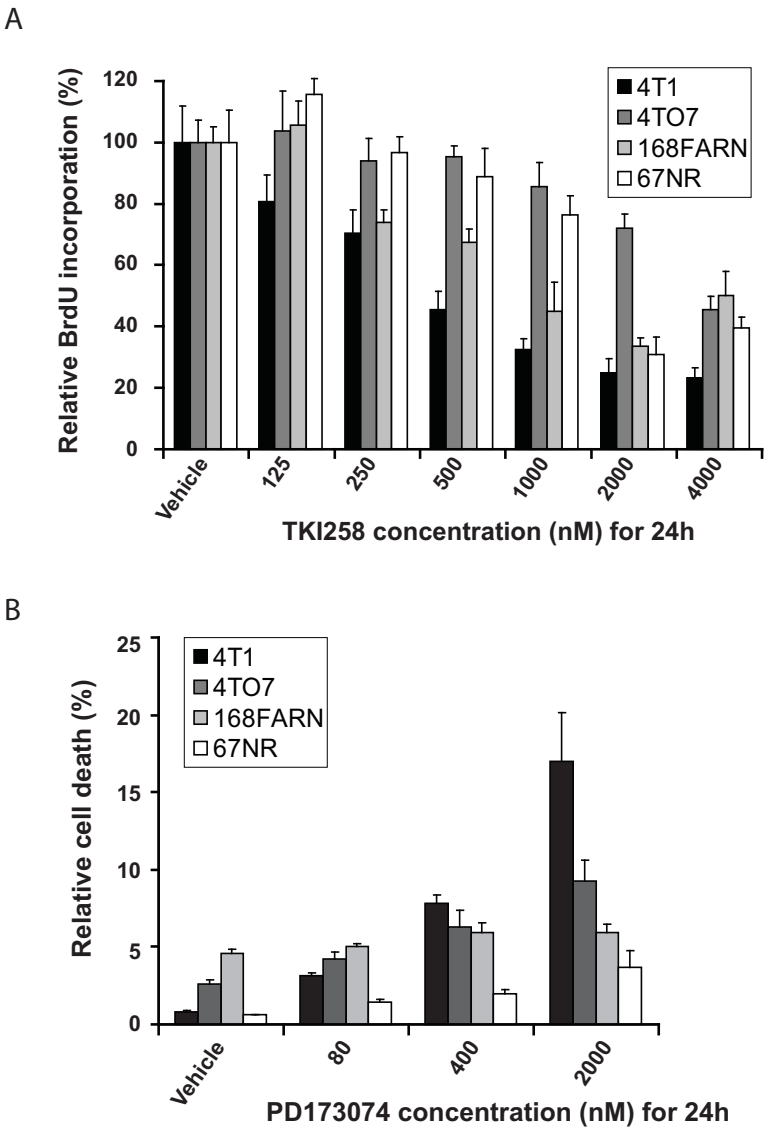


Figure 5-11 FGFR inhibition affects proliferation and survival in mammary carcinoma cell lines

A, Cells were treated for 24 hours with increasing concentration of TKI258; BrdU was added 2 hours before the end of the experiment. The percentage of incorporated BrdU relative to vehicle treated cells is plotted. *B*, Cells were treated 24 hours with the indicated concentration of PD173074 and cell death was detected with the YO-PRO assay and plotted relative to the vehicle-treated control.

5.2.2. Overexpression of FGFR1 in 67NR

In early mammalian development, FGFR1 is required for the expression of the transcription factor snail that downregulates the levels of E-cadherin, allowing proper gastrulation (Ciruna &

Rossant, 2001). The downregulation of E-cadherin is part of the EMT process that allows epithelial cells to change morphology and acquire motility and invasive phenotypes. EMT is believed to play an essential role in the early steps of metastatic dissemination of tumor cells.

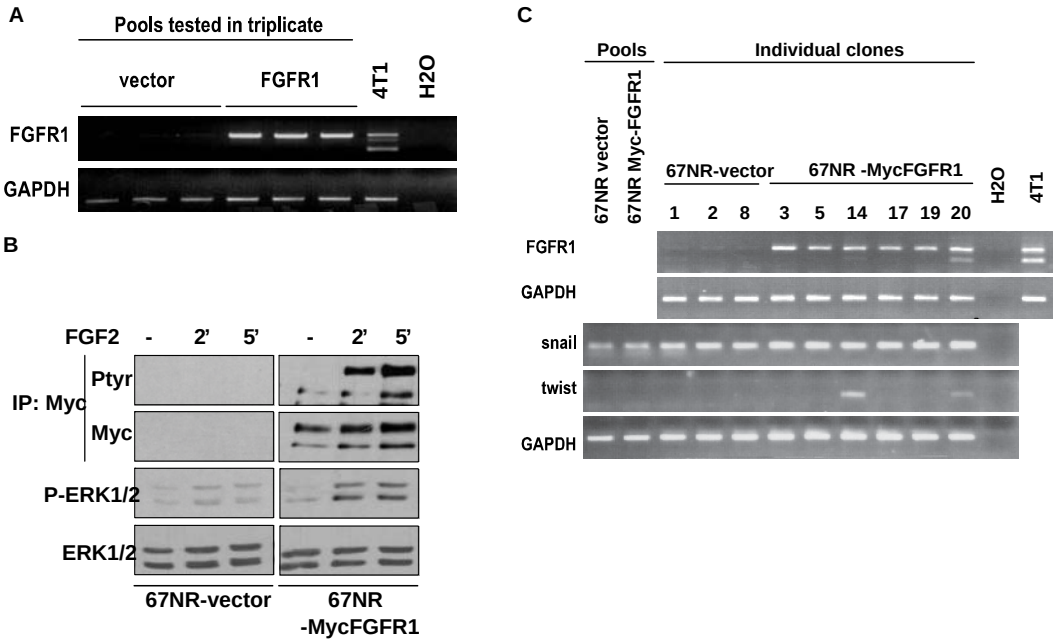


Figure 5-12 Ectopic expression of FGFR1 in 67NR

A, Semi-quantitative RT-PCR for FGFR1 in 67NR cells stably transfected with empty vector or Myc-FGFR1. 4T1 cells were used as positive control for detection of FGFR1 and H2O was used as negative control. *B*, Pool of cells expressing the empty vector or Myc-FGFR1 were starved over-night and stimulated with FGF2 for the indicated time. Myc-FGFR1 was IP with an antibody recognizing the Myc tag and probed with Ptyr to test the activity of FGFR1. Lysates were probed with the indicated antibodies to test for active pathway. *C*, Semi-quantitative RT-PCR for FGFR1, snail and twist in 67NR pools or clones stably transfected with empty vector or Myc-FGFR1. GAPDH was used as loading control.

As FGFR1 is not expressed in the non-metastatic 67NR cells, we ectopically expressed a myc-tagged FGFR1-IIIc in the 67NR cells and selected pools and clones. Correct expression of the construct was confirmed by RT-PCR (Figure 5-12 A and C, top two panels) and IP followed by western blot for the Myc-tag (Figure 5-12 B, second panels). Even though 67NR express FGF1 that has the property to activate all FGFRs, IP of the Myc-FGFR1 and immunoblot for Ptyr showed that the ectopic Myc-FGFR1 is not active in serum-starved cells. This result shows that in 67NR cells, ectopic FGFR1 is not activated by the endogenous FGF1, suggesting that either the level of ligand is too low or that these cells do not express the correct co-receptors that allows

formation of FGF1-FGFR1 complexes and activation of this receptor. However, Myc-FGFR1 gets phosphorylated on tyrosine residues following FGF2 stimulation of the cells (Figure 5-12 B top panel). In addition, FGF2 induced ERK1/2 activation was slightly higher in the cells expressing Myc-FGFR1 compared to control (Figure 5-12 B, bottom panel). The morphology of the 67NR cells was not affected by ectopic expression of FGFR1, suggesting that no EMT occurred. According to this, we did not detect changes in the expression of snail upon introduction of FGFR1 (Figure 5-12 C, third panel), but we found two clones (clone 14 and clone 20) that had detectable levels of twist, that is normally absent from 67NR cells (Figure 5-12 C, fourth panel) and that plays a role for EMT and invasion in 4T1 cells (Yang et al., 2004). Together these results show that ectopic FGFR1 is expressed in 67NR cells, but does not get activated by the endogenous FGFs. However Myc-FGFR1 gets tyrosine phosphorylated and activated upon stimulation of the cells with FGF2. Interestingly, we found that two clones expressing Myc-FGFR1 had expression of Twist that is normally not found in the 67NR cells.

Next we compared the tumor growth properties of 67NR-vector and 67NR-Myc-FGFR1 cells in BALB/c mice. First we injected pools of 67NR cells, transfected with empty vector or with the Myc-FGFR1 into the 4th mammary gland of mice and followed tumor growth (Figure 5-13 A). Expression of the ectopic Myc-FGFR1 in the tumors was confirmed by RT-PCR and western blot (Data not shown). However we did not observe significant differences in tumor size and histological quantification of the mitotic marker phospho-histone H3 (PHH3) didn't show increase in proliferation in tumors overexpressing Myc-FGFR1 compared to control tumors (Figure 5-13 B). Then, we investigated the properties of the different clones that we selected, to form tumors in the mice. We decided to pool three empty vector clones (clones 1, 2 and 8 from Figure 5-12 C), four clones expressing Myc-FGFR1 (clones 3, 5, 17 and 19 from Figure 5-12 C) and to keep as single clones the two that were expressing twist (clone 14 and clone 20 from Figure 5-12 C). Expression of Myc-FGFR1 was tested in whole cell lysates and after IP of the Myc-tagged FGFR1 in the newly made pools of clones (empty vector and Myc-FGFR1 expressing) and single clones (clone 14 and clone 20) (Figure 5-13 C). Clone 14 showed the highest expression of ectopic FGFR1, clone 20 had similar level than the pool of Myc-FGFR1 clones and no signal was detected on the empty vector pool of clones (Figure 5-13 C). We then injected these pools of clones and single clones into the 4th mammary gland of BALB/c mice and

followed tumor growth (Figure 5-13 D). The tumors onset was similar in the four groups, with tumors being palpable at day 7 post-injection. In addition, we did not detect significant changes in tumor size after 29 days of growth (Figure 5-13 D). These results show that ectopic expression of FGFR1 in 67NR cells does neither influence tumor growth nor proliferation of tumor cells. In addition, we found that the two clones expressing Twist did not have increased tumor growth when compared to empty vector or to 67NR-Myc-FGFR1 where Twist was not expressed.

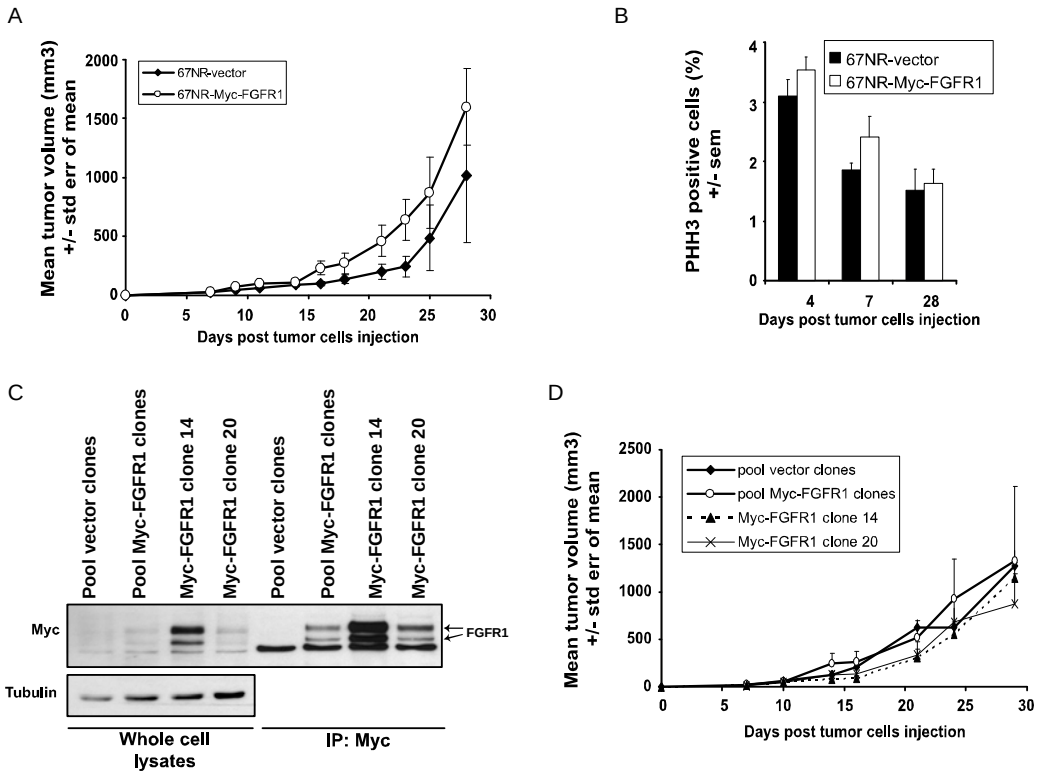


Figure 5-13 Effect of FGFR1 expression on tumor growth

A, 5x10^5 cells were injected in 50µl PBS into the 4th mammary gland of Balb/C mice. Tumor growth was measured three times a week using a caliper. *B*, 67NR-vector and 67NR-Myc-FGFR1 mammary tumors were harvested at the indicated days after injection and prepared for histology. Frozen sections were stained for phospho-histone H3 (PHH3). The number of mitotic cells was determined by counting PHH3 positive and total cells in each field and plotted as a %. *C*, three vector clones and four Myc-FGFR1 expressing clones were pooled together, whereas clones 14 and 20 were kept as clonal culture. Proteins were extracted from culture in serum condition and IP was performed with an anti-Myc antibody (9E10). Blots were performed with the 9E10 antibody to analyse Myc-FGFR1 expression and tubulin was used as loading control. *D*, 10^6 cells were injected in 100µl PBS into the 4th mammary gland of Balb/C mice. Tumor growth was measured using a caliper.

To analyze if ectopic expression of FGFR1 in 67NR was sufficient to render these cells metastatic, we aimed to detect disseminated 67NR cells in the lungs. To do this, we extracted RNA of lungs from tumor bearing mice 29 days after injection into the 4th mammary gland with: a) the pool of empty vector clones, b) the pool of Myc-FGFR1 clones, c) the clone 14 or d) the clone 20. Detection of metastatic cells in the lungs of the animals was performed via RT-PCR amplification of the specific transcript for transfected 67NR, the neomycin resistance gene NPT2 (Figure 5-14 A). Neither FGFR1 expressing cells (Pool Myc-FGFR1 clones, clone 14 or clone 20) nor empty vector pools could be detected in the lungs of the animals 29 days after injection into the mammary gland (Figure 5-14 A). This result suggests that, as already observed by Aslakson and Miller, the 67NR are not metastatic (Aslakson & Miller, 1992) and ectopic expression of FGFR1 does not change their invasive properties in vivo.

To confirm that the RT-PCR based technique was sensitive enough to detect metastatic cells in the lungs, we performed a second set of experiments, where we injected the pool of empty vector clones, the pool of Myc-FGFR1 clones, the clone 14 or the clone 20 directly into the blood stream of the animals via their tail vein. We collected the lungs and extracted lung RNA 14 days after injection and performed a NPT2 specific RT-PCR (Figure 5-14 B). Interestingly, we found that the 67NR-vector pool cells were able to colonize the lungs and form visible metastatic nodules after tail vein injection (Figure 5-14 C). Even though we observed different level of metastases formation and different amount of tumor cells were found in the lungs, all pools of tumor cells injected via the tail vein formed metastases in the lungs (Figure 5-14 B). These results show that the 67NR are capable to form metastatic nodules in the lungs when they are injected directly into the blood stream. This means that these cells are impaired in their early metastatic spread and are not able to escape from the primary tumor. FGFR1 expression did not influence the metastatic properties of these cells when injected into the fat pad or into the blood stream, showing that FGFR1 in this model is not required for metastasis formation.

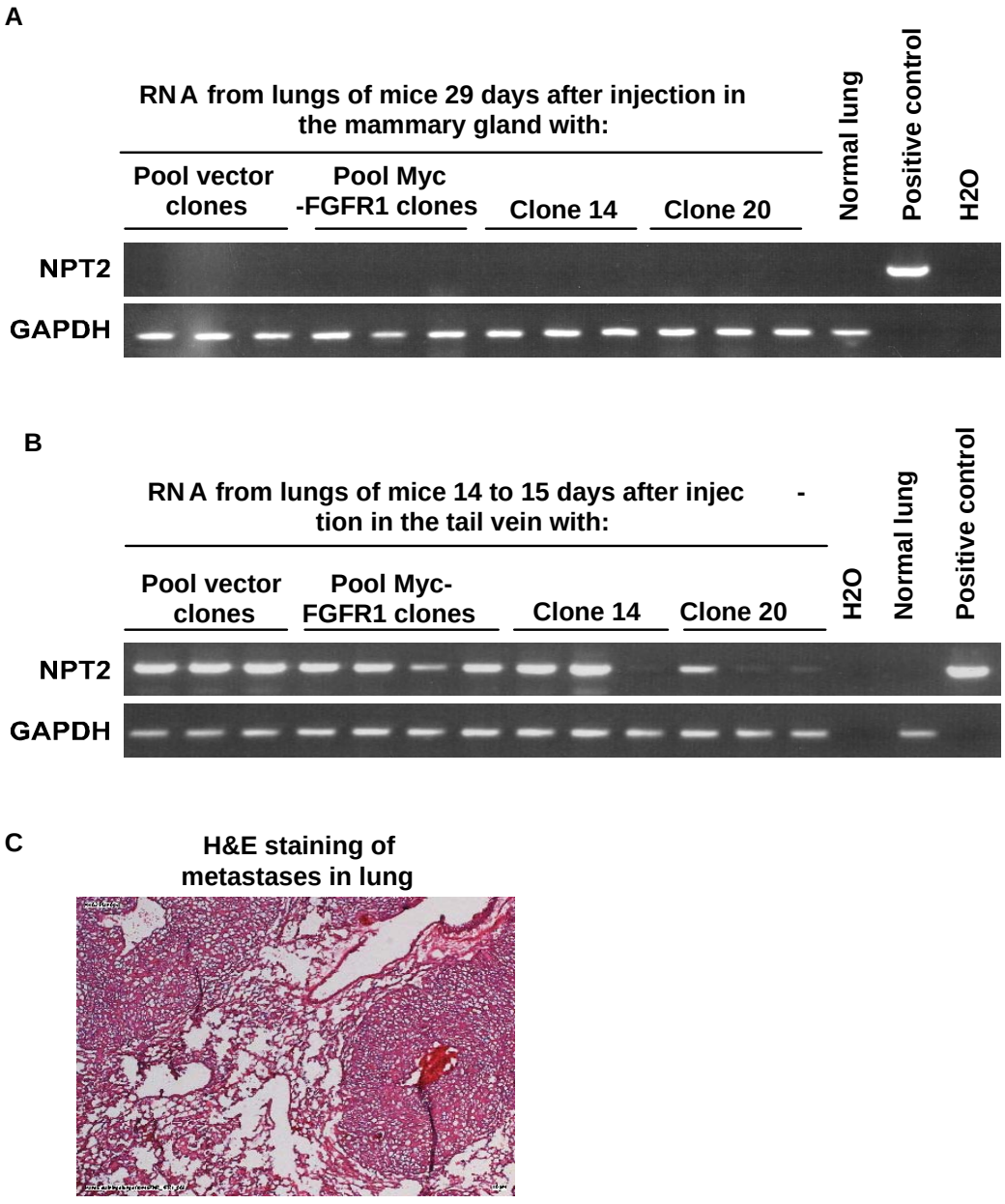


Figure 5-14 Metastatic properties of 67NR cells

A, Semi-quantitative RT-PCR performed on RNA from lungs of BALC/c mice 29 days after injection into the 4th mammary gland with: a) Pool of empty vector clones, b) Pool of Myc-FGFR1 expressing clones, c) Myc-FGFR1 and twist expressing clone 14, d) Myc-FGFR1 and twist expressing clone 20. GAPDH was used as a loading control, H2O and normal lung were used as negative controls, a vector containing the NPT2 gene was used as positive control. *B*, Semi-quantitative RT-PCR performed on RNA from lungs of Balb/C mice 14 to 15 days after injection into the tail vein with: a) Pool of empty vector clones, b) Pool of Myc-FGFR1 expressing clones, c) Myc-FGFR1 and twist expressing clone 14, d) Myc-FGFR1 and twist expressing clone 20. GAPDH was used as a loading control, H2O and normal lung were used as negative controls, a vector containing the NPT2 gene was used as positive control. *C*, Hematoxylin and Eosin (H&E) staining of lungs from a mouse injected in the tail vein with 67NR cells reveals the presence of macroscopic metastatic nodules.

5.2.3. Knock-down of FGFRs in 4T1 cells

As ectopic expression of FGFR1 did not change the metastatic properties of the 67NR, we decided to downregulate the expression of different FGFRs in metastatic 4T1 cells. In an attempt to uncover which of the 3 expressed FGFRs might be responsible for the tumor phenotype, we used a shRNA approach. We infected 4T1 cells with viral particles that contained the pLKO vector in which specific shRNA designed to individually silence members of the FGFR family or the LacZ control were cloned. We first tested the effects of the individual shRNA on the expression of FGFR1, FGFR2 and FGFR3. We found that the control cells stably expressing LacZ shRNA had variable amount of FGFR1, suggesting that 4T1 have different populations of cells with variable levels of FGFR1 expression. However, a strong decrease in FGFR1 mRNA was found in the cells expressing the shFGFR1-B and shFGFR1-D vectors (Figure 5-15 A). Downregulation of FGFR2 was not efficient using single shRNAs (Figure 5-15 B). We therefore tried to combine different shRNAs targeting FGFR2, but the downregulation was not more efficient than using single shRNAs (Data not shown). Two out of three shRNA targeting FGFR3 showed a 50% reduction in FGFR3 mRNA (Figure 5-15 C).

We then tested whether the cells with downregulated FGFRs exhibit a proliferation defect using BrdU incorporation (Figure 5-15 D). The proliferation of parental 4T1 was used as standard and we found that the control cells stably expressing LacZ shRNA showed some variation compared to the parental 4T1 (higher and lower proliferation rate for shRNA LacZ2 and shRNA LacZ3, respectively). The effect of FGFR downregulation led to a maximum of 20 to 30 % decrease of proliferation for cells stably expressing shRNA FGFR1-B, FGFR1-D, FGFR3-B and FGFR3-B+C (Figure 5-15 D black bars). Silencing FGFR2, even using combination of several shRNA, had no effect on cell proliferation. Overall downregulating single FGFRs, had minor effects on cell proliferation, when compared to the strong effect of blocking the activity of all three FGFRs using a TKI. Although not completely conclusive, these data suggest that all three FGFRs might contribute to the proliferative phenotype. Alternatively, the cell lines with the strongest down-regulation may not have proliferated and were lost during the selection process.

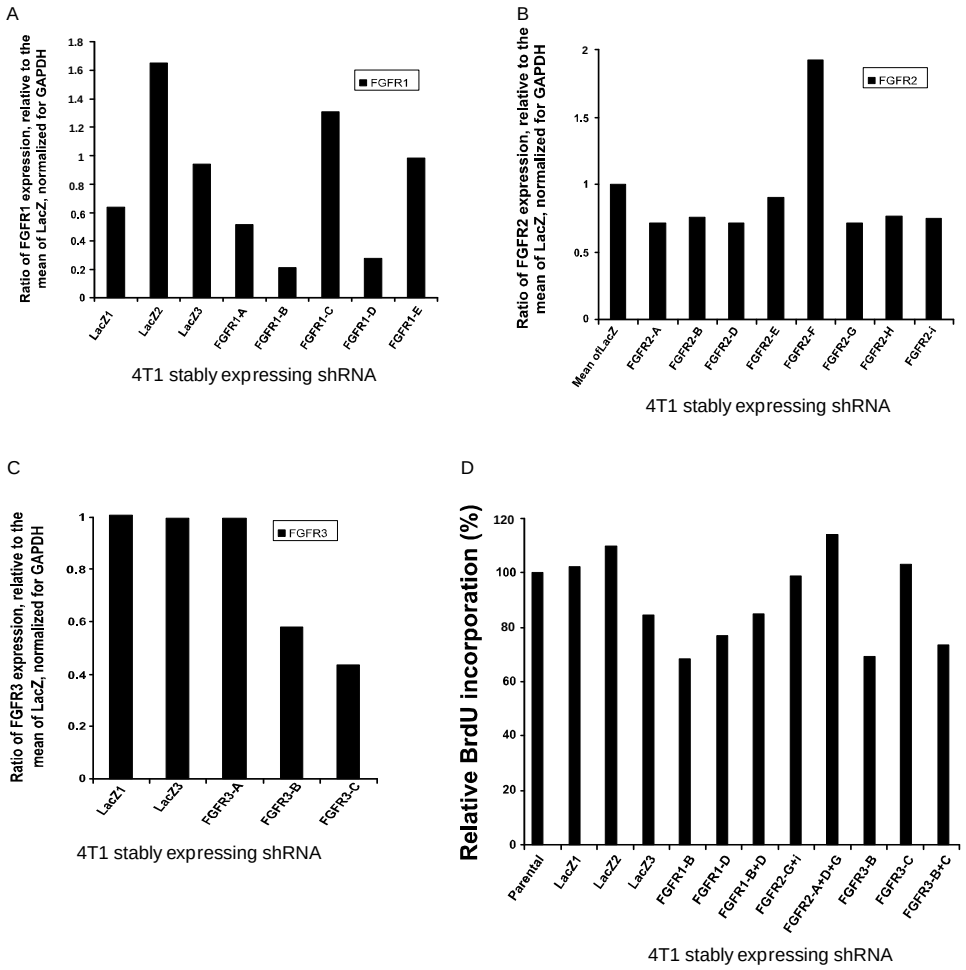


Figure 5-15 Stable downregulation of FGFRs using shRNA

A, *B* and *C*, 4T1 cells were infected with virus containing different shRNAs targeting FGFR1, FGFR2 or FGFR3. LacZ shRNA was used as a non targeting control shRNA. Stable pools of cells were selected and total RNA was extracted. Quantitative real-time RT-PCR was run on cDNA with primers specific for FGFR1 (*A*), FGFR2 (*B*) or FGFR3 (*C*). The results are shown as ratio of FGFR level compared to LacZ, normalized for GAPDH expression. *D*, Cells were starved over night and BrdU was added 2 hours before experiment-end. The percentage of incorporated BrdU relative to parental 4T1 cells is plotted.

As the constitutive expression of shRNA in 4T1 cells did not show strong effects on cell proliferation and survival, we decided to use an inducible shRNA approach. The 4T1 were infected with viruses containing the Tet-on-pLKO vector (Wiederschain et al., 2009), that allows doxycyclin induced expression of shRNA targeting FGFRs or LacZ (Figure 5-16).

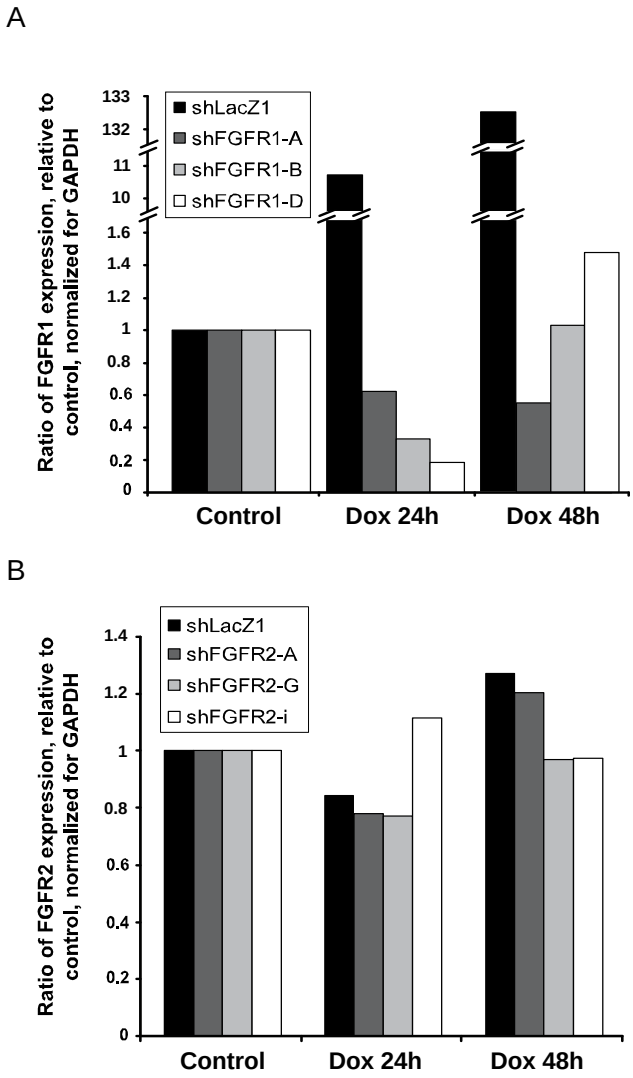


Figure 5-16 Inducible knock-down of FGFR1 and FGFR2 in 4T1 cells

A and *B*, 4T1 cells were infected with virus containing different inducible shRNAs targeting FGFR1, FGFR2. LacZ shRNA was used as a non targeting control shRNA. Stable pools of cells were selected and total RNA was extracted. Quantitative real-time RT-PCR was run on cDNA with primers specific for FGFR1 (*A*), FGFR2 (*B*). The results are shown as ratio of FGFR levels in doxycyclin treated samples compared to non treated cultures and normalized for GAPDH expression.

Interestingly, we observed that 4T1 cells stably expressing inducible shRNA targeting FGFR1 showed a decrease in mRNA for FGFR1 24 hours after treatment with doxycyclin, suggesting that the system is functional. However, 48 hours after treatment, only the cells with shFGFR1-A had low FGFR1 mRNA, whereas the expression of FGFR1 went back to the basal level in shFGFR1-B and was even higher than basal in shFGFR1-D (Figure 5-16 A). Another surprising

observation is the level of FGFR1 that strikingly increases upon induction of the LacZ1 shRNA (Figure 5-16 A, black bars). Two out of three inducible shRNA targeting FGFR2 (shFGFR2-A and shFGFR2-G) showed only a weak decrease in FGFR2 mRNA 24 hours after induction with doxycyclin and the level went back to the basal level 48 hours after treatment (Figure 5-16 B, dark and light grey bars), whereas shFGFR2-i did not decrease FGFR2 expression (Figure 5-16 B, white bars). Together these results show that downregulation of FGFRs in 4T1 cells is not achieved in a convincing manner using constitutive as well as inducible expression of shRNA. The reasons for these observations could be due to technical problems, as 4T1 cells are difficult to transfect or infect and they express relatively low levels of FGFRs, which render the detection of the receptors difficult.

5.2.4. Active Ras partially rescue TKI258 induced cell death

As showed in Figure 5-3, inhibition of MEK with UO126 impairs cell proliferation with no induction of cell death and blockade of PI3K using BEZ235 decreases cell number and affects cell survival. To further analyze the contribution of these different pathways to proliferation and survival of 4T1 cells, we expressed active AKT (Myr-AKT) (Figure 5-3) and observed that active AKT partially rescue TKI258 induced cell death. To investigate the contribution of the MAPK pathway, we stably expressed a constitutively active Ras (mutant V12), a wild-type Ras and the empty vector in 4T1 and selected pools of cells. Expression of the ectopic Ras proteins was confirmed by western blot using a Ras antibody as well as an antibody directed against the Ha tag of the constructs (Figure 5-17 A). The different pools were treated for 13 to 24 hours with TKI258 1μM and cell lysates were analyzed for ERK1/2 activity. Surprisingly, we did not detect an increase of the basal ERK1/2 activity upon expression of active Ras-V12 or WT-Ras in the 4T1, moreover, TKI258 induced decrease of ERK1/2 activity was not rescued by either of the Ras constructs (Figure 5-17 A). To confirm that the Ras-V12 mutant was functional, we transiently transfected HEK293 cells with this construct. We observed a strong increase of the basal ERK1/2 activity when the Ras-V12 was expressed (Figure 5-17 B), showing that active Ras construct activates the MAPK pathway in HEK293 cells. Then we examined the pools of 4T1 Ras-V12, WT-Ras and pLHCX cells for their sensitivity to TKI258. We found that 4T1 cells expressing active Ras were 4 times less sensitive to TKI258 induced cell death than cells expressing WT-Ras or empty vector (Figure 5-17 C). Together these results show that Ras-V12 is

constitutively active and induces ERK1/2 activity in HEK293 cells. However, in 4T1, ERK1/2 basal level seems to be independent of Ras-V12 and this construct did not rescue TKI258 induced blockade of ERK1/2 activity. In addition, ectopic expression of the mutant Ras partially rescues TKI258 decreased cell survival. Together, these results suggest that Ras-V12 partially overcome TKI258 induced cell death, through a mechanism that is independent of ERK1/2. These observations are interesting, as it has been reported that Ras signaling does not only activate ERK1/2 but as well PI3K and other downstream effectors (Kodaki et al., 1994). It would therefore be interesting to look at AKT activity in the V12-Ras 4T1 upon TKI258 treatment.

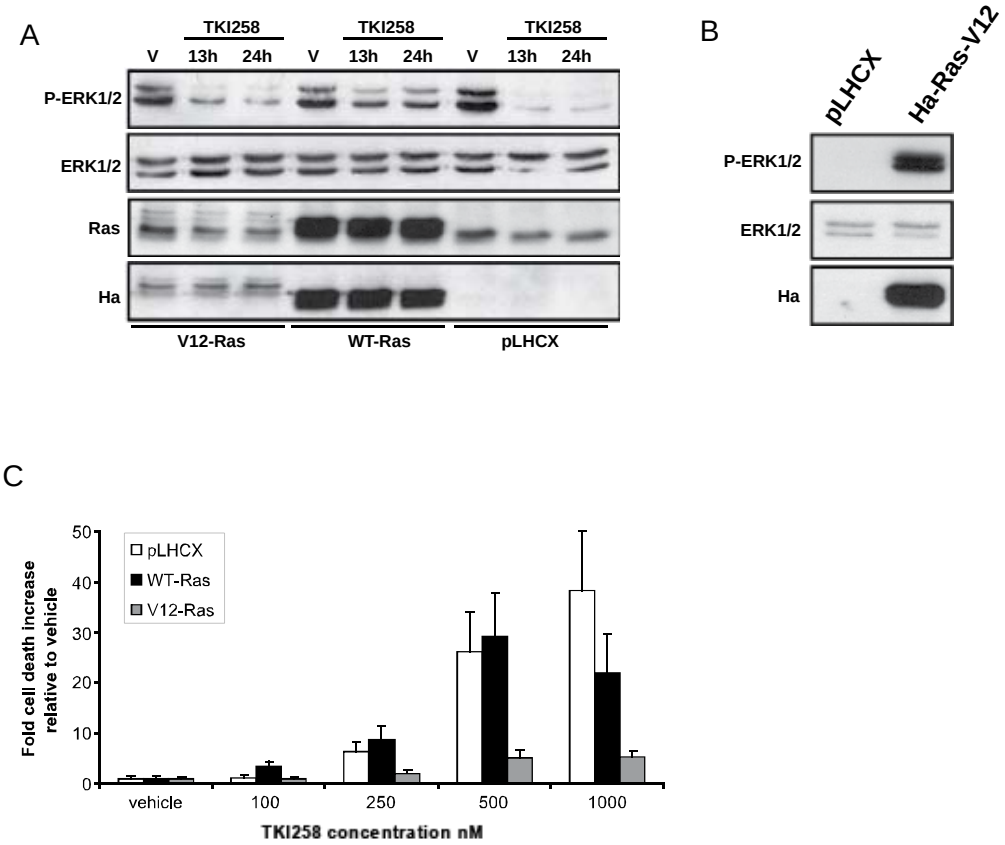


Figure 5-17 Active Ras partially rescue TKI258 induced cell death

A, Pools of 4T1 cells stably expressing Ras-V12, WT-Ras or pLHCX were treated with TKI258 for the indicated times. Cell lysates were probed with specific antibodies to test the expression of the constructs and ERK1/2 activity. *B*, HEK293 cells were transiently transfected with pLHCX or Ras-V12. Cell lysates were probed with antibodies to test the expression of the construct and ERK1/2 activity. *C*, Cells stably expressing vector control, WT-Ras or Ras-V12 were treated 24 hours with the indicated concentrations of TKI258 and the fold increase in cell death relative to vehicle treated cells was determined using the YO-PRO assay.

5.2.5. Combination of TKI258 with MEK and PI3K inhibitors

Interestingly, we found that TKI258 has a strong effect in vitro on 4T1 cells proliferation and survival, however it did not induce tumor shrinkage in the animals. Therefore we also investigated if blocking of FGFRs in combination with other inhibitors of downstream signaling pathways might have a stronger effect than TKI258 alone (Figure 5-18).

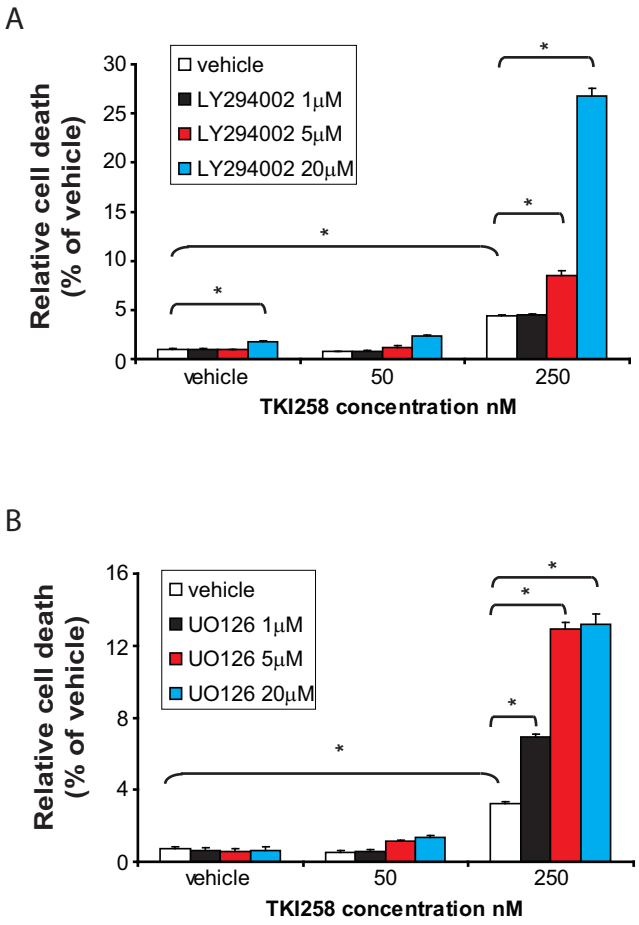


Figure 5-18 Combination of MEK and PI3K inhibitors with TKI258

A, 4T1 cells were treated with the indicated concentration of TKI258 and 0, 1, 5 or 20 μM of LY294002 (white, black, red and blue bars respectively). Cell death was analyzed after 24 hours of treatment using YO-PRO assay. Statistical analysis for each treatment and interaction of treatment combination was performed using two-way ANOVA: *, P<0.001 for TKI258 treatment, for LY294002 treatment and for interaction between TKI258 and LY294002. *B*, 4T1 cells were treated with the indicated concentration of TKI258 and 0, 1, 5 or 20 μM of UO126 (white, black, red and blue bars respectively). Cell death was analyzed after 24 hours of treatment using YO-PRO assay. Statistical analysis for each treatment and interaction of treatment combination was performed using two way ANOVA: *, P<0.001 for TKI258 treatment, and for interaction between TKI258 and UO126.

We examined effects of the MEK inhibitor UO126 and the PI3K inhibitor LY294002 on cell survival, alone or in combination with TKI258. Treatment of 4T1 cells with LY294002 at 20μM led to a slight but significant increase in cell death (Figure 5-18 A, compare white and blue bars in the vehicle treated cells), this correlates well with the data obtained in Figure 5-3 B, where an increase of apoptosis was observed in the 4T1 upon treatment with BEZ235. However we never detected cell death with UO126 at any tested concentration (Figure 5-18 B, compare white and blue bars in the vehicle treated cells). A four fold increase in cell death was detected in cultures treated with 250nM TKI258 as compared to control treatment (Figure 5-18 A and B, white bars). Interestingly, addition of sub-optimal doses of LY294002 (5μM) or UO126 (1 and 5μM) to 250nM TKI258 strongly potentiated TKI258 induced cell death in comparison to treatment with single agent (Figure 5-18 A and B, compare the white bar to black and red bars in TKI258 250nM treated cultures). Together these results suggest that targeting FGFR in combination with other inhibitors blocking key nodes in signaling pathways downstream of receptor tyrosine kinases, strongly potentiate mammary carcinoma cell death as compared to single treatment. It will be interesting in the future to test combination of treatments for their effects in vivo on tumor outgrowth

5.3. Materials and methods for supplemental experiments

Reagents, antibodies, plasmids, cell culture and viral infections

FGF2 was from Sigma-Aldrich (St. Louis, MO, USA); UO126 was from Promega (Madison, WI, USA), LY294002 was from cell signaling (Danvers, MA, USA), TKI258 was a gift from Drs. D. Graus-Porta and C. Garcia-Echeverria (NIBR, Basel, CH), PD173074 was a gift from Dr. P. Manley (NIBR, Basel, CH). The following antibodies were used: FGFR2 (3116), FGFR3 (3163), Ptyr^{196}-FRS2 (3864), Pthr^{202}/tyr^{204}-ERK1/2 (9101), ERK1/2 (9102), Ptyr^{705}-STAT3 (9131) and Pser^{10}-Histone H3 (9701) from Cell Signaling (Danvers, MA, USA); PLCγ (sc-81), FRS2 (sc-8318), Myc (9E10) and CyclinD1 (sc-20044) from Santa-cruz Biotechnology Inc. (Santa Cruz, CA, USA); alpha-tubulin from NeoMarkers (Fremont, CA, USA); STAT3 from BD transduction lab (San Jose, CA, USA); Ras and Ptyr^{783}-PLCγ from Upstate (Billerica, MA, USA); Ha (MMS-101P) from Covance, P-tyrosine (Ptyr) was detected with mAb 4G10 (gift from Dr. J. Mestan, NIBR, Basel). cDNA encoding Ha-HRas-V12 was a gift from Dr. M. Bentires-Alj (FMI, Basel). The Ha-HRas-WT was obtained by site directed mutagenesis of the V12 mutant using the

QuickChange XL Site-directed Mutagenesis Kit (Stratagene). pMIRB plasmid encoding Myc-FGFR1-IIIc was a gift from Dr. D. Ornitz (Washington University, St-Louis, MO, USA). pLKO vectors containing shRNA targeting FGFRs were from Sigma and the shRNA sequences were as follow:

shFGFR1-A:
CCGGCTTGCCTCTAAGAAGTGTATACTCGAGTATACACTTCTTAGAGGCAAGTTTTT
shFGFR1-B:
CCGGCCTGGAGCATCATAATGGATTCTCGAGAATCCATTATGATGCTCCAGGTTTTT
shFGFR1-C:
CCGGCGAGGATAACGTAATGAAGATCTCGAGATCTTCATTACGTTATCCTCGTTTTT
shFGFR1-D:
CCGGCATATCGACTACTACAAGAAACTCGAGTTTCTTGTAGTAGTCGATATGTTTTT
shFGFR1-E:
CCGGTGGAGTTAATACCACCGACAACTCGAGTTGTCGGTGGTATTAACTCCATTTTTG
shFGFR2-A:
CCGGGCACACACTTACAGAGCACAACTCGAGTTGTGCTCTGTAAGTGTGTGCTTTTT
shFGFR2-B:
CCGGGCCACCAACCAAATACCAAATCTCGAGATTTGGTATTTGGTTGGTGGCTTTTT
shFGFR2-D
CCGGCCTCTCTACGTCATAGTTGAACTCGAGTTCAACTATGACGTAGAGAGGTTTTT
shFGFR2-E:
CCGGGCCATCTCATCTGGAGATGATCTCGAGATCATCTCCAGATGAGATGGCTTTTT
shFGFR2-F:
CCGGCGAGTATGAGTTGCCAGAGGACTCGAGTCCTCTGGCAACTCATACTCGTTTTT
shFGFR2-G:
CCGGCGGGCAAGTAGTCATGGCTGACTCGAGTCAGCCATGACTACTTGCCCGTTTTT
shFGFR2-H:
CCGGGCCAGGGATATCAACAACATACTCGAGTATGTTGTTGATATCCCTGGCTTTTT
shFGFR2-i:
CCGGGCATCGCATTGGAGGCTATAACTCGAGTTATAGCCTCCAATGCGATGCTTTTT

shFGFR3-A:
CCGGGCGGGCAATTCTATTGGGTTTCTCGAGAAACCCAATAGAATTGCCCGCTTTTT
shFGFR3-B:
CCGGCCTTGGAATCTAACTCCTCTACTCGAGTAGAGGAGTTAGATTCCAAGGTTTTT
shFGFR3-C:
CCGGGCCACTTCAGTGTGCGTGTAACTCGAGTTACACGCACACTGAAGTGGCTTTTT

Forward and reverse oligos with the sequences of the described shRNA were ordered at Mycrosynth (Balgach, CH), annealed and cloned into the pLKO-Tet-on (Gift from Dr. D. Wiederschain, DMP, Cambridge, MA, USA) using the AgeI and EcroR1 restriction sites.

The 4T1, 4TO7, 168FARN and 67NR mouse mammary carcinoma cell lines, obtained from Dr. J. Yang (University of California, San Diego), EcoPack-293 (Clontech, Mountain View, CA, USA) and HEK293-T (from Dr. M. Bentires-Alj) were cultured in DMEM with 10% heat inactivated FBS (Sigma), supplemented with penicillin and streptomycin (Sigma).

Retroviruses production was performed by transient transfection of EcoPack-293 cells with 8µg of the following vectors: pLHCX2, Ha-WT-Ras or Ha-V12-Ras using Fugene (Roche, Basel Switzerland) with a ratio Fugene:DNA 1:6. Three days post transfection, viruses were collected, filtered and used immediately for over-night infection of 4T1 cells (6cm plates with polybrene at a final concentration of 8µg/ml). Selection of pools was performed with 500µg/ml hygromycin.

Lentiviruses production was performed by transient transfection of HEK293-T cells with 8µg of pLKO or pLKO-Tet-on, 0.4µg pHDM-tat1b, 0.4µg pHDM-HgPM2, 0.4µg pRC-CMV-RaII and 0.8µg pHDM-VSV-G using PEI (Polysciences, Eppelheim, Germany) with a ratio PEI:DNA 4:1, in serum-free media. One day after transfection, the media was changed to media containing 10% FBS and four days post transfection, supernatant were harvested, filtered and used for over-night infection on 4T1 cells (6cm plates with a final polybrene concentration of 8µg/ml). Selection of pools was performed with 10µg/ml puromycin.

Stable transfection of 67NR cells was performed with 8µg of pMIRB or Myc-FGFR1-pMIRB in 800µl of Optimem using 16µl of lipofectamine. One day after transfection, the medium was changed and selection of pools and clones started with 1mg/ml G418.

RNA extraction, RT-PCR and real-time PCR

RNA from growing parental lines (4T1, 4TO7, 168FARN or 67NR), from FGFR1 transfected 67NR, from pLKO transfected 4T1 or from pLKO-Tet-on transfected 4T1 treated with doxycylin for 24 or 48 hours was extracted using the Qiashredder and RNeasy Mini Kit coupled with RNase-free DNase set (Qiagen, Venlo, The Netherlands Qiagen) following the manufacturer's instructions. RNA from lungs was obtained from snap frozen pieces. RNA extraction was performed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNAs were washed using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase set (Qiagen). cDNA was obtained from 2.5µg of total RNA, using the Ready-to-go You-Prime First-Strand Beads kit (GE-healthcare, Little Chalfont, UK) with oligos-dT primers (Promega). Semi-quantitative PCR was performed as follow: 2µl of 10X Buffer (Roche), 0.2µl of Taq polymerase (5U/µl Roche), 0.4µl of 10mM dNTP mix (Roche), 0.1µl of each primer (100µM), 1µl of cDNA, filled to a final volume of 20µl with sterile H_2O. Thermal cycling reaction using an Icycler device (Bio-Rad, Reinach, Switzerland) were: 94°C for 2 min; followed by 25 to 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec for detection of FGFR1 and FGFR2: 96°C for 2 min; followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec for detection of FGFR3: 94°C for 5 min; followed by 35 or 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec for detection of FGF1 and FGF2 or FGF10 (Forward: CTGGAGATAACATCAGTGGAAATCG, Reverse: GAGCAGAGGTGTTTTTCCTTCTT): 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec for detection of FGF4: 96°C for 2 min; followed by 5 cycles of 96°C for 30 sec, 62°C for 30 sec (0.5°C decreases every cycles), 72°C for 35 sec followed by 25 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 35 sec for detection of Twist (Forward: ACATCGACTTCCTGTACCAGGTC, Reverse: AACAATGACATCTAGGTCTCCGG) and Snail (Forward: CTCTGAAGATGCACATCCGAA, Reverse: GGCTTCTCACCAGTGTGGGT): 95°C for 2 min; followed by 35 cycles of 95°C for 30 sec, 59.5°C for 30 sec, 72°C for 2 min for detection of NPT2 (Forward: TTGAGCCTGGCGAACAGTTCG, Reverse: GAGGCTATTCGGCTATGACTG). he amplified products were further extended by additional incubation at 72°C for 10 min. PCR products were then loaded on a 1% agarose gel containing ethidium bromide.

Quantitative RT-PCR was performed with ABI prism 7000 (Applied Biosystems, Austin, TX, USA) using Absolute QPCR SYBR Green ROX Mix (THERMO Scientific, Waltham, MA, USA) following the manufacturer's guidelines. All quantitations were normalized to GAPDH. If not specified in the text, primers for semi-quantitative PCR and real-time PCR were previously described (Kurosu et al., 2007; Ozawa et al., 1997).

Lysates, immunoprecipitations and western blot analysis

Whole cell lysates were extracted in NP-40 buffer (50mM Hepes (pH 7.4), 150mM NaCl, 25mM β-glycerophosphate, 25mM NaF, 5mM EGTA, 1mM EDTA, 15mM PPI and 1%NP40), supplemented with leupeptin (10μg/ml), aprotinin (10μg/ml), vanadate (2mM), DTT (1mM) and PMSF (1mM) and immunoblotted as previously described (Matsuda et al., 2009). Over-night serum-starved cultures were pre-treated for 60 minutes with 1μM TKI258 or DMSO then stimulated for the indicated time with 50ng/ml FGF2. Immunoprecipitations (IPs) were performed following standard procedures (Koziczak et al., 2004).

Proliferation and apoptosis assays

Antiproliferative effects of TKI258 were evaluated in 96-well plates over 24 hours using a bromodeoxyuridine (BrdU) ELISA kit (GE-healthcare, Little Chalfont, UK) as described (Sini et al., 2005). Antiproliferative effects of shRNA targeting FGFRs in pools of stably infected 4T1 cells were evaluated using BrdU ELISA kit. In brief, cells were seeded in 96-well plates for 24 to 48 hours and BrdU was added 2 hours before the end of the experiment. The cytotoxic effects of PD173074, were evaluated in 96-well plates 24 hours after treatment by measuring cell death using the YO-PRO assay (Invitrogen, Carlsbad, CA, USA) as described (Beuvink et al., 2005). The same assay was used to measure the rescue of TKI258 dose dependent induced cell death using control, WT-Ras or V12-Ras expressing 4T1 cells. Finally the effects of combining different concentrations of TKI258 with increasing concentrations of UO126 or LY294002 were tested on cell survival after 24 hours of treatment using the YO-PRO assay.

Injection into mammary gland

Animal experiments were done according to the Swiss guideline governing animal experimentation and approved by the Swiss veterinary authorities. Pools of 67NR cells ($5x10^5$

cells in 50µl PBS) expressing empty vector or Myc-FGFR1 were injected into the fourth mammary fat pad of 10 wk old BALB/c mice (RCC, Basel, Switzerland). Starting 7 days after injection, tumors were measured three times a week and volume was calculated using: height x ((diameter/2)^2 x π). Pooled empty vector clones, Myc-FGFR1 clones, clone 14 and clone 20 (10^6 cells in 100µl PBS) were injected into the fourth mammary gland of 10 wk old BALB/c mice (RCC, Basel, Switzerland) and tumor growth was followed as previously described. For experimental metastasis, pooled vector clones, Myc-FGFR1 clones, clone 14 and clone 20 (10^6 cells in 100µl PBS) were injected into tail veins. At the endpoint, mice were sacrificed and tumors and lungs were dissected. Pieces of lungs and tumors were snap frozen for RNA extract and prepared for immunohistochemistry.

Immunohistochemistry

Tumors and lungs were fixed 24 hours in PBS containing 4% paraformaldehyde then incubated 24 hours in PBS containing 15% sucrose. Fixed tissues were embedded in OCT media and frozen at -80°C. Immunohistochemistry was performed on 8 µm thick sections using the antibodies: phospho-histone H3 (Cell signaling). Stainings were carried out with the Discovery XT Staining Module (Ventana Medica Systems S.A.). Standard hematoxylin and eosin staining was performed on lungs to detect macro-metastases. Quantification of the phospho-Histone H3 positive cells was performed using image access software.

6. Discussion and outlook

Breast cancer is the most commonly occurring cancer among women; in 2009 it accounted for 27% of all newly diagnosed cancers. Despite improvement in techniques for detection of breast cancer and development of new targeted therapies, this cancer still ranks second as a cause of cancer death in women. The main reasons for this elevated mortality rate are a high risk of relapse and resistance to treatments. The contribution of fibroblast growth factors (FGF) signaling pathway to breast cancer was first reported in mice, where genomic integration of the mouse mammary tumor virus (MMTV) was shown to lead to overexpression of several *Fgf*s and subsequently to cause development of mammary carcinomas (Figure 6-1).

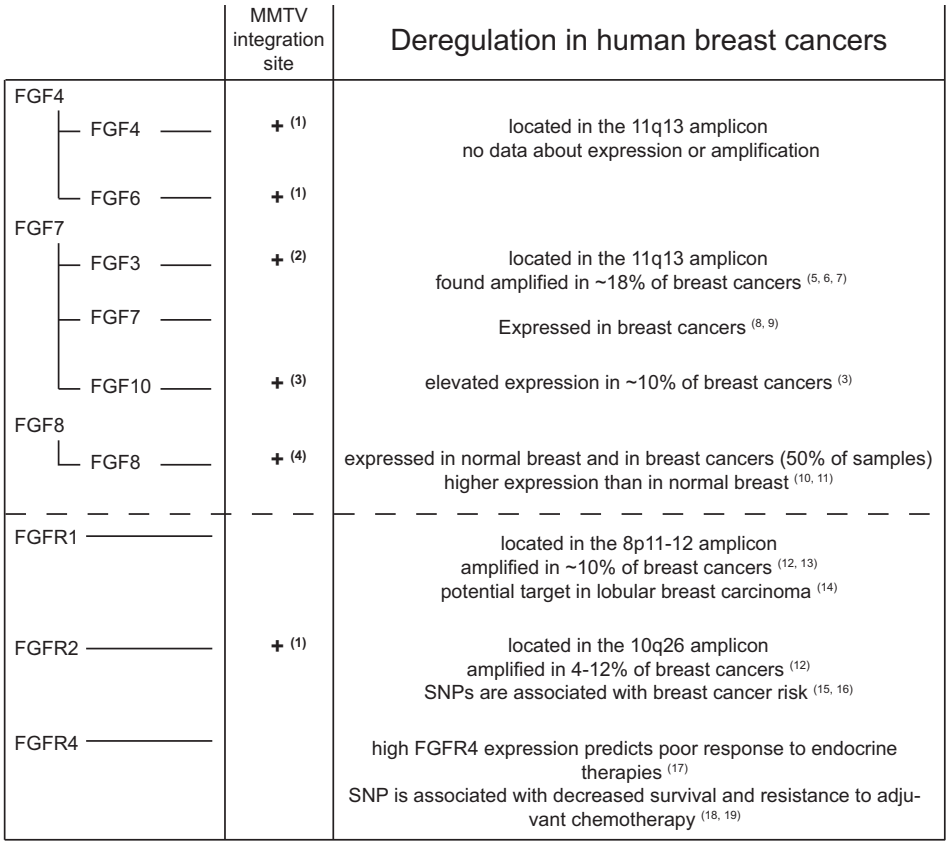


Figure 6-1 Deregulation of FGFR signaling in breast cancer

FGFs are common hot spots for integration of the MMTV provirus 1)(Theodorou et al., 2007), 2) (Peters et al., 1989), 3)(Theodorou et al., 2004), 4)(MacArthur et al., 1995). Deregulation of FGF signaling has been reported in human breast cancers 5)(Naidu et al., 2001), 6)(Fioravanti et al., 1997), 7)(Berns et al., 1995), 8)(Jacquemier et al., 1994), 9)(Bansal et al., 1997), 10)(Zammit et al., 2002), 11)(Marsh et al., 1999), 12)(Adnane et al., 1991), 13)(Ray et al., 2004), 14)(Reis-Filho et al., 2006), 15)(Hunter et al., 2007), 16)(Easton et al., 2007), 17)(Meijer et al., 2008), 18)(Bange et al., 2002), 19)(Thussbas et al., 2006).

In addition to its role in mouse mammary tumors, FGF signaling has been studied in human breast cancer. Aberrant expression of ligands and receptors due to amplification of genes were reported. Polymorphisms in the *FGFR2* gene have been linked with breast cancer and have been shown to deregulate the expression of this receptor (Figure 6-1). A polymorphism in *FGFR4* gene has been linked with decreased survival and resistance to adjuvant chemotherapy (Figure 6-1).

In this study we show that autocrine FGFR signaling can be blocked in vitro and in vivo using a tyrosine kinase inhibitor (TKI) that blocks FGFRs (TKI258) in models of breast cancer. We report that blockade of FGFRs affects cell proliferation as well as survival and strongly reduces tumor growth of orthotopically injected mammary carcinoma cells. In addition, we observed that upon treatment with TKI258, lung metastases were decreased in the 4T1 model. To understand how FGFR inhibition mediates these mechanisms, we analyzed differential gene expression from FGFR inhibitor treated 4T1 cells or 4T1 tumors, compared to controls. While comparing these lists of genes to publicly available breast cancer datasets, we found that genes downregulated by TKI258 were highly expressed in clusters of patients with poor prognostic. Therefore, it would be interesting to know if these cancers are driven by a receptor tyrosine kinase like FGFR, which could be targeted using a TKI.

Autocrine activation of FGFR signaling

We report in Figure 5-1 and Figure 5-9 that the four cell lines (4T1, 4TO7, 168FARN and 67NR) described by Aslakson and Miller (Aslakson & Miller, 1992) possess an autocrine active FGFR signaling. The activation of FGFRs is well understood and the interactions between the different ligands and receptors are well characterized (Figure 6-2). As FGF1 is the universal ligand for all FGFR isoforms, its expression was tested in the four cell lines and detected in 4T1, 4TO7 and 67NR but not in 168FARN. We first hypothesize that co-expression of FGF1 with several FGFRs in these lines might be the cause of the autocrine activity. However, data from Figure 5-12 contradict this hypothesis and show that at least in 67NR, the endogenous FGF1 is not sufficient to activate ectopic Myc-FGFR1. The mechanisms underlying secretion of FGF1 are not yet fully understood and it might well be that in these cells, FGF1 is not secreted and stays intracellular. In addition, the contribution of heparan sulfate proteoglycan (HSPG) as co-receptor for FGF is well

documented and one other possibility why endogenous FGF1 fails to activate ectopic FGFR1 might be that 67NR cells don't express the correct co-receptor for FGF1 at their surface.

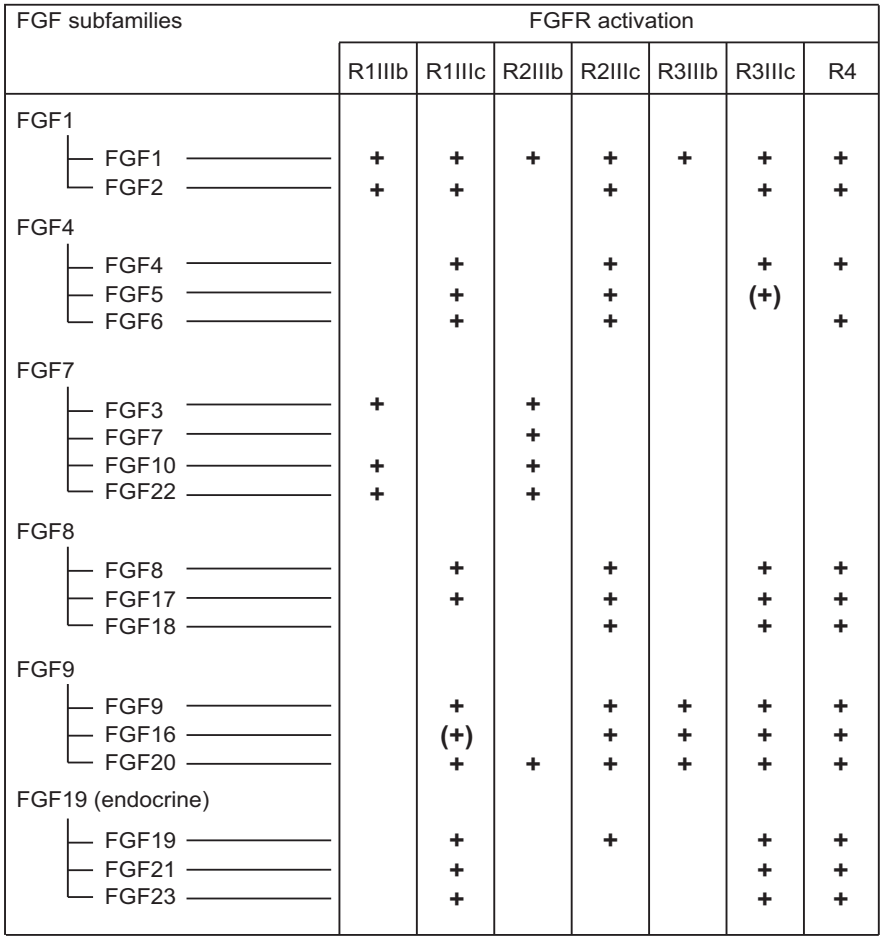


Figure 6-2 Ligand-receptor specificity of the FGF/FGFR family

Another possible mechanism leading to constitutive activation of RTK is via activating point mutations. We cloned FGFR1 from 4T1 cells and sequenced it, looking for activating mutations on this gene. No mutations were found on FGFR1, but as we did not sequence FGFR2 and FGFR3, we cannot exclude that these two receptors may be mutated and, therefore, constitutively activate the common downstream effectors of FGFR signaling (ERK, AKT or PLCγ). Interestingly, these four cell lines showed heterogeneity in their FGF expression. As these lines are all derived from the same tumor that appeared in a BALB/c mice that was foster-nursed by a C_3H female, a mouse strain that harbors endogenous milk-borne mouse mammary tumor virus

(MMTV), it is possible that different integration site of MMTV in the primary tumor led to different selection of the cell lines (two lines out of the four express FGF10). However we found that FGF4 or FGFR2, genes known to be overexpressed by MMTV insertion were detected in the four cell lines and therefore could be responsible for autocrine FGFR signaling (Figure 5-8).

Differential response to FGFR inhibitor

As these four cell lines displayed autocrine activation of FGFR signaling, we treated them with different TKIs (TKI258 or PD173074) to block FGFR activity. We found that treatment with the FGFR inhibitors strongly decreased ERK1/2 and PLCγ activity in all four cell lines (Figure 5-1 and Figure 5-10). Interestingly, we found that in the 4T1, the AKT pathway was also decreased when FGFR signaling was inhibited and expression of a constitutively Myristylated-AKT in 4T1 cells partially rescued TKI258 induced cell death (Figure 5-3). Cell survival was differentially affected in the four cell lines upon FGFR inhibition (Figure 5-2 and Figure 5-11). Therefore, it would be interesting to broaden the analysis of the effect of FGFR blockade on AKT activity to the other cell lines. It would particularly be interesting to see if a general conclusion can be made between modulation of AKT activity and induction of cell death upon FGFR blockade.

In addition to MAPK, AKT and PLCγ, we found that Stat3 and Src were constitutively active in these cell lines. It has been described in the literature that FGFRs modulate Stat3 and Src activation (Hart et al., 2000; Landgren et al., 1995; Zhan et al., 1994), however we did not see changes in the activity of these proteins upon inhibition of FGFRs in our experiments (Supplementary Fig.1 and Figure 5-10). These results suggest that other pathways are responsible for Stat3 or Src activity. As these molecules are known to contribute to the tumorigenic phenotype by regulating proliferation and/or migration of cells, it would be interesting to understand what keeps them active and what their contribution to the cancer phenotype is in an FGFR dependant model. Approaches using selective Src inhibitors, Jak inhibitor acting upstream of Stats or dominant-negative Stat molecules could be used to answer these questions.

In vivo effects of TKI258

Targeting FGFRs in 4T1 cells affects several mechanisms involved in cancer progression. In particular, we showed that 4T1 cells undergo apoptosis when treated with TKI258 even in the presence of serum (Figure 5-2). These observations suggest that blockade of FGFR signaling is

enough to induce cell death and that other growth factors found in the serum are not sufficient to prevent TKI258 induced apoptosis. Based on these in vitro results, we predicted that TKI258 treatment of 4T1 tumor-bearing mice would lead to tumor regression. Even though we observed decreased of tumor growth, we did not see tumor shrinkage upon prolonged treatment with TKI258 (Figure 5-4). Interestingly, after 14 days of treatment, tumors from the treated groups were growing with kinetics similar to the control tumors a few days after injection. These striking differences between in vitro and in vivo effects show that the tumor microenvironment strongly influences the response of tumor cells to an inhibitor such as TKI258.

To investigate the differences in TKI258-treated tumors compared to control, we performed a microarray analysis on tumors after 14 days of treatment. Interestingly, we found that genes coding for several ligands (Nrg4, Vegf-C, Pdgf-C) or even for a receptor tyrosine kinase (Egfr) were upregulated in the TKI258 treated group (data not shown). Upregulation of these ligands could lead to formation of new autocrine loop in the tumors, therefore activating tyrosine kinase receptors other that the ones efficiently blocked by TKI258 thus promoting growth even in the presence of the compound.

Using a combination of inhibitors in tissue culture, we investigated what intracellular pathways co-operate to promote cell survival. We found that treatment of 4T1 cells with TKI258 simultaneously with MEK or PI3K inhibitors showed at least an additive effect on cell death. These observations show that when used in combination with UO126 or BEZ235, we can decrease the dose of TKI258 required for induction of cell death from 250μM to 50μM (Figure 5-18). The next obvious experiment to perform is to compare in vivo the effect of the combination relative to each treatment. It would therefore be very interesting to see if we can induce regression of the tumors in vivo by using such a combination of inhibitors.

Angiogenesis is the process by which tumors acquire new blood vessels which supply the tumor with oxygen and nutrients required for growth. Targeting angiogenesis to inhibit tumor growth was proposed some decades ago (Folkman & Ingber, 1992) and nowadays, angiogenesis blocking-treatments are used as first line medication in certain cancers (colon cancer) (Alekshun & Garrett, 2005; Diaz-Rubio, 2004). The main target of antiangiogenic therapies is the VEGF signaling pathway that can be blocked either using an anti-VEGF antibody (Bevacizumab) or a

TKI blocking the VEGFRs (PTK787). As TKI258 targets VEGFRs, we compared the effects of blocking selectively VEGFRs using PTK787 and blocking both FGFRs and VEGFRs using TKI258 (Supplemental Figure 5). The results obtained showed that inhibition of VEGFR alone has no effect on tumor growth whereas TKI258 treatment leads to a strong reduction of tumor size compared to control. These results suggest that VEGFRs are not required for 4T1 tumor growth. We can therefore hypothesize that either 4T1 tumors use other angiogenic factors like FGF2 to recruit blood vessels and that they are independent of VEGFRs for proliferation.

Predicting resistance mechanisms

In order to predict resistance to FGFR inhibitors, we treated 4T1 cells with a sublethal dose of TKI258 or DMSO for 16 hours and analyzed changes in gene expression. This approach allowed us to detect genes that are upregulated upon FGFR inhibition and that could be involved in the activation of pathways conferring resistance to TKI258. We found that some genes known to regulate growth and proliferation were upregulated upon TKI258 treatment. Of special interest, were members of the PDGF/PDGFR signaling as well as members of the Stat transcription factor family (Figure 6-3).

It is surprising that PDGFR and PDGF ligands get upregulated as TKI258 blocks as well PDGFR. This result suggests that expression of PDGFR and PDGFs is regulated by a signaling pathway that is modulated by TKI258 treatment. However, we consider unlikely that this signaling pathway is involved in resistance to TKI258 treatment, because PDGFRs are as well blocked by this inhibitor. Interestingly we found that members of the Stat family were upregulated and they can modulate growth and survival of cancer cells. In addition it has been shown that in the 4T1 model, Stat3 is required for tumor formation in the mice (Ling & Arlinghaus, 2005). Further analyses investigating the contribution of PDGFR and Stats to TKI258 resistance mechanisms are warranted.

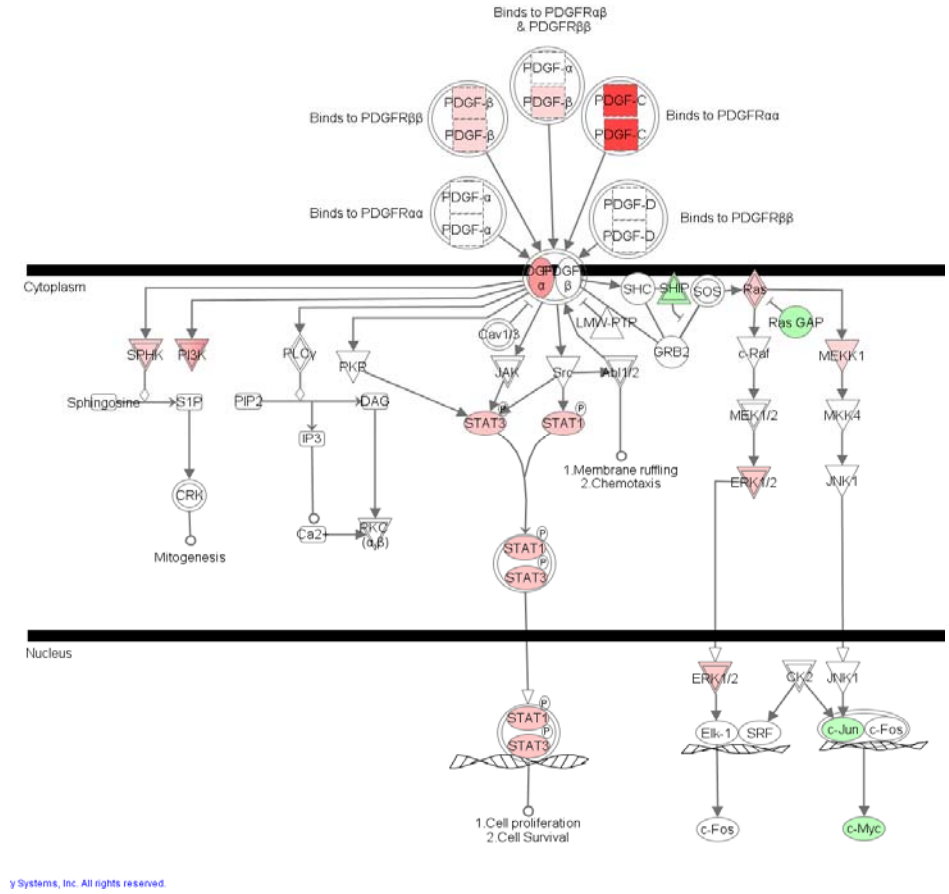


Figure 6-3 IPA of genes regulated in 4T1 cells after TKI258 treatment

In red, ingenuity pathway analysis of genes that are upregulated in 4T1 cells 16 hours after TKI258 treatment. In green genes that are downregulated upon FGFR inhibition.

TKI258 regulated genes identify two subgroups of breast cancer patients

We have shown in Figure 5-6 that genes regulated after TKI258 treatment of 4T1 cells identified one cluster (1A) of breast cancer patients and genes regulated after TKI258 treatment of tumor bearing mice identified two clusters (1B and 2B) of breast cancer patients. Interestingly, we found that patients from cluster 1A almost completely overlapped with the ones from cluster 2B. The particularity of these patients is that they have a worse prognosis compared to the general cohort and they have high expression of genes that were mainly downregulated after TKI258 treatment in vitro and in vivo. It would therefore be interesting to analyze if these patients have component or mutations that could drive an autocrine FGFR signaling or if any other TKI258 target (VEGFR, PDGFR or others) are active in the breast tumors of these patients. If this appears to be true we can hypothesize that these patients might benefit from TKI258 treatment.

The interpretation of the results concerning patients that belong to the cluster 1B and show a better prognosis than the general cohorts is more complex. These patients show high expression of genes that are mainly upregulated in the TKI258 treated tumor bearing mice. These patients probably don't have an active autocrine receptor tyrosine kinase signaling, as they already have high expression of genes that are upregulated upon inhibition of RTK with TKI258. This probably means that these patients would not respond to TKI258 treatment, and it would therefore be interesting to understand what the driving force of these cancers is in order to be able to give them the best treatment possible.

TKI258 in the clinics

TKI258 already went through phase I clinical trial in patients with advanced solid tumors (Sarker et al., 2008). The aim of this study was to determine the maximum tolerated dose and analyze the pharmacokinetics and pharmacodynamics of TKI258. Thirty-five patients were enrolled in this study and the results showed an acceptable safety profile for the patients. Furthermore, antitumor activity was observed in a patient with metastatic melanoma and in another one with an imatinib-refractory gastrointestinal stromal tumor. Since TKI258 is a rather well-tolerated compound that showed antitumor effects in advanced tumors, we can hypothesize that TKI258 will enter phase II clinical trials. It would therefore be interesting to see if selection of breast cancer patients with aberrant FGFR signaling like *FGFR1* amplification or *FGFR2* SNP will benefit more from this treatment than patient without deregulated FGFR signaling. However, the interesting inhibitory profile of TKI258, and specially the fact that it target angiogenesis by inhibiting VEGFR could make it an interesting anti-angiogenic drug even in cancer that are not FGFR driven.

In summary, we show that FGFR signaling contribute to several aspects of carcinogenesis, ranging from proliferation and cell survival, to modulation of pro-invasive phenotype. Furthermore, we show that targeting autocrine FGFR signaling in vivo in a breast cancer model has strong antitumor and antimetastatic effects. Finally we show that a subgroup of breast cancer patient, with high expression of a subset of genes regulated by TKI258, has a poor prognosis. Together, these interesting results strongly support investigating the role of FGFRs in breast cancer.

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8. Abbreviations

ACC2	acetyl-CoA carboxylase 2
ADHR	autosomal dominant hypophosphataemic rickets
aFGF	acidic fibroblast growth factor
AIGF	androgen-induced growth factor
ATP	adenosintriphosphate
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
Ca2+	calcium
Cbl	casitas B-lineage lymphoma
cKO	conditional knockout
CYP24	cytochrome P450, family 24
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1
Da	dalton
DAG	diacylglycerol
DMEM	dulbeccos modified eagles medium
Dox	doxycyclin
Dusp	dual specificity phosphatase
EGF(R)	epidermal growth factor (receptor)
EMS	8p11 myeloproliferative syndrome
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
ER	estrogen receptor
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF(R)	fibroblast growth factor (receptor)
FHF	fibroblast growth factor homology factors
FNIII	fibronectin type III
FRS2	fibroblast receptor substrate 2
FXR	fanesoid X receptor
Gab1	GRB2-associated binding protein 1
GAG	glycosaminoglycan
GAPDH	glycerhaldehyde-3-phosphate dehydrogenase
GDNF(R)	glial cell derived neurotrophic factor (receptor)
GEO	gene expression omnibus
GlcA	β-D-glucuronic acid
GlcN	D-glucosamine
GlcNAc	*N*-acetylated-glucosamine
$GlcNSO_3$	*N*-sulfated-glucosamines
GLUT1	glucose transporter member 1
Grb2	growth factor receptor-bound protein 2
GSEA	gene-set enrichment analysis
H&E	hematoxylin and eosin
hFGF	hormone-like fibroblast growth factor
HSPG	heparan sulfate proteoglycan
IdoA	α-L-iduronic acid
iFGF	intracellular fibroblast growth factor
Ig-like	immunoglogulin-like
IP	immunoprecipitation
IP3	inositoltriphosphate
kDa	kilo Dalton
KGF	keratinocyte growth factor
KO	knockout
LADD	lacrimo-auriculo-dento-digital
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MEK	cf: MAP2K
MKP	mitogen-activated protein kinase phosphatase
MMP	matrix metalloproteinase

MMTV	mouse mammary tumor virus
MPS	myeloproliferative syndrome
MS	mass spectrometry
Myr-AKT	Myristylated AKT
NCAM	neural cell adhesion molecule
NPT2a	Na^+-phosphate cotransporter type IIa
NPT2c	Na^+-phosphate cotransporter type IIc
O/N	over-night
PBS	phosphate buffered saline
PDGF(R)	platlet-derived growth factor (receptor)
PEG	polyethylene glycol
PEI	polyethylenimine
PG	proteoglycans
PH	pleckstrin homology
PHEX	phosphate regulating endopeptidase homolog, X-linked
PHH3	phospho-histone H3
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidyl-inositol-4,5-bisphosphate
PKC	protein kinase C
PLCγ	phospholipase Cγ
PN-1	protease nexin-1
PPAR	peroxisome proliferator-activated receptor
PTB	phosphotyrosine-binding domain
PTH	parathyroid hormone
PTK	protein tyrosine kinase
PTPN11	protein tyrosine phosphatase, non-receptor type 11
Ptyr	phosphor-tyrosine
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SCD1	stearoyl-CoA desaturase 1
Sef	similar expression to Fgf genes
SH2	Src homology 2
SH3	Src homology 3
Shb	Src homology 2 domain containing adaptor protein B
Shp2	cf: PTPN11
shRNA	short hairpin RNA
SM	sex myoblasts
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SOS	son of sevenless
SP	signal peptide
Stat	signal transducer and activator of transciption
TEB	terminal end bud
TFA	trifluoroacetic acid
TiO2	titanium dioxyde
TKI	tyrosine kinase inhibitor
TM	transmembrane
VEGF(R)	vasculat endothelial growth factor (receptor)
WCL	whole cell lysate

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10.Curriculum Vitae

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- Design, planning and execution of in vivo and in vitro pre-clinical experiments. Oral presentations and discussions of data for laboratory and department meetings. Oral presentations and poster presentation of results during international conferences. Writing of scientific papers. Teaching and correcting exercises of biology in English for a group of first year students.

04.-07.2005 — Department of Pharmacology and Toxicology, University of Lausanne, Switzerland

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