UNIVERSITY OF TURKU

FIBROBLAST GROWTH FACTOR RECEPTORS AND THEIR INHIBITORS IN PRECLINICAL MODELS OF BREAST CANCER AND BONE METASTASIS

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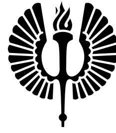

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ABSTRACT

Advanced, metastatic stages of breast cancer are associated with high morbidity and mortality, and there is a need to establish a proof-of-concept for novel compounds that can change this scheme. Fibroblast growth factors and their receptors (FGFRs) are altered in about 18% of breast cancers, resulting in malignant growth and resistance to conventional therapies. During the past few years many compounds inhibiting the activity of FGFRs have been discovered but not yet approved for breast cancer.

In this study, the effects of pharmacological inhibition of FGFRs by FGFR-selective and non-selective inhibitors on proliferation, apoptosis, migration, invasion and angiogenesis were evaluated by different *in vitro* and *in vivo* models, and in *ex vivo* explant cultures using clinical breast cancer tissue specimens.

FGFR1 was a major regulator of breast cancer growth. FGFR selective inhibitors were most effective in FGFR-amplified cells. FGFR inhibitors decreased proliferation of breast cancer cells, which was the major cause of growth inhibition. Migration and invasion were also inhibited, and induction of apoptosis was observed in certain experimental models. FGFR inhibitors decreased tumor growth in subcutaneous models, and also in a model mimicking growth of bone metastases *in vivo*. FGFR inhibitors did not have any harmful effects on healthy bone or on bone-forming osteoblasts.

In conclusion, FGFR inhibitors showed many anti-tumor effects in breast cancer. Many of these compounds are currently being evaluated in clinical trials.

KEYWORDS: breast cancer, bone metastasis, fibroblast growth factor receptor, drug discovery

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Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Edennyt, etäpesäkkeitä muodostava rintasyöpä lisää merkittävästi kuolleisuutta potilaissa, ja uusia tehokkaita lääkkeitä sen hoitamiseen tarvitaan kipeästi. Noin 18 %:ssa rintasyövistä havaitaan fibroblastikasvutekijöissä ja niiden reseptoreissa (fibroblast growth factor receptor, FGFR) geenimuutoksia, jotka johtavat syövän kehittymiseen ja lopulta lääkeresistenssiin. Viime vuosina on kehitetty useita FGFR:iin vaikuttavia lääkeaineita, mutta niitä ei ole vielä hyväksytty rintasyövän hoitoon.

Tässä tutkimuksessa selvitettiin FGFR-epäselektiivisten sekä FGFR-selektiivisten pienimolekyylisten inhibiittoreiden vaikutuksia solujen jakautumiseen, solukuolemaan, liikkumiseen, tunkeutumiseen ja verisuonten muodostukseen. Tutkimuksessa käytettiin erilaisia solu- ja tuumorimalleja, sekä potilaista saatuja rintasyövän kudosviljelmiä.

Tutkimuksessa FGFR1 osoittautui voimakkaaksi rintasyövän säätelijäksi. FGFR-selektiiviset inhibiittorit olivat erityisen tehokkaita malleissa, joissa oli FGFR geenimuutoksia. FGFR-inhibiittorit hidastivat ensisijaisesti syöpäsolujen jakautumista ja siten syövän kasvua. Lisäksi inhibiittorit vähensivät syöpäsolujen liikkumista ja tunkeutumista, sekä lisäsivät solukuolemaa tietyissä tutkimusmalleissa. FGFR inhibiittorit vähensivät syövän kasvua myös primäärituumorimallissa, sekä mallissa, jossa syöpäsolut kasvoivat luussa, vaikuttamatta kuitenkaan terveeseen luuhun tai luuta muodostaviin soluihin.

Yhteenvetona voidaan todeta, että FGFR inhibiittoreilla on monia syövän kasvua estäviä vaikutuksia. Tällä hetkellä näiden inhibittoreiden tehoa arvoidaan kliinisissä kokeissa.

AVAINSANAT: rintasyöpä, luuetäpesäke, fibroblastikasvutekijäreseptori, lääkekehitys

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Abbreviations

AFF3	AF4/FMR2 family member 3
AHCYL1	Putative adenosylhomocysteinase 2
AKT	Protein kinase B
AR	Androgen receptor
ATP	Adenosine triphosphate
BICC	Protein K
BMC	Bone mineral content
BMD	Bone mineral density
CD	Cluster of differentiation
c-Kit	Proto-oncogene c-Kit
CTC	Circulating tumor cells
ctDNA	Circulating tumor DNA
CTGF	Connective tissue growth factor
CXCL	C-X-C motif chemokine
CXCR2	CXC chemokine receptors
CYP	Cytochrome P450
CYR61	Cysteine rich angiogenic inducer 61
DTC	Disseminated tumor cell
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
Fbxw7	F-box/WD repeat-containing protein 7
FGF	Fibroblast growth factor
FGFBP	Fibroblast growth factor binding protein
FGFR	Fibroblast growth factor receptor
FLT3	Fms like tyrosine kinase 3
FLRT	Fibronectin-leucine-rich transmembrane protein
FRS2	Fibroblast growth factor receptor substrate 2
GLI2	Zinc finger protein GLI2
GRB2	Growth factor receptor-bound protein 2
HER	Human epidermal growth factor receptor

HIF-1	Hypoxia inducible factor 1
HSPG	Heparan sulfate proteoglycan
IGF	Insulin-like growth factor
IL	Interleukin
JNK	c-Jun N-terminal kinases
Ki67	Antigen KI-67
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid –derived suppressor cell
MET	Mesenchymal-to-epithelial transition
mTOR	Mammalian target of rapamycin
MMP	Matrix metalloproteinase
OPG	Osteoprotegerin
OPN	Osteopontin
PANX1	Pannexin 1
PARP	Poly (ADP-ribose) polymerase
PDGF	Platelet-derived growth factor
PHH3	Phospho-histone H3
PI3K	Phosphoinositide 3-kinases
PINP	N-terminal propeptide of type I procollagen
PLC	Phospholipase C
PR	Progesterone receptor
PTHrP	Parathyroid hormone-related peptide
P38	Protein 38
P53	Protein 53
RAF1	RAF proto-oncogene serine/threonine-protein kinase
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rb	Retinoblastoma protein
RFS1	Remodeling and spacing factor 1
RKT	Receptor tyrosine kinase
SEF	Similar Expression to FGFs
SERM	Selective estrogen receptor modulator
Snail	Zinc finger protein SNAI1
SOS1	Son of sevenless homolog 1
SPRY	Sprouty
STAT	Signal transducer and activator of transcription
TACC1	Transforming acidic coiled-coil-containing protein
TGFβ	Transforming growth factor beta
TK	Tyrosine kinase

TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TRACP 5b	Tartrate-resistant acid phosphatase 5b
TRV4	Transient receptor potential vanilloid subtype 4
VEGFR	Vascular endothelial growth factor receptor

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Tarkkonen KM, Nilsson EM, Kähkönen TE, Dey JH, Heikkilä JE, Tuomela JM, Liu Q, Hynes NE, Härkönen PL. Differential roles of fibroblast growth factor receptors (FGFR) 1, 2 and 3 in the regulation of S115 breast cancer cell growth. *PlosONE.* 2012. DOI:10.1371/journal.pone.0049970.

II Kähkönen TE, Toriseva M, Virta A-R, Maher A, Eigeliene N, Petruk N, Kaivola J, Boström P, Koskivuo I, Nees M, Tuomela J, Ivaska KK and Härkönen PL. Comparison of the effects of three investigational FGFR inhibitors, TKI258, BGJ398 and AZD4547, on breast cancer cells in 2D and 3D cultures and on breast cancer tissue in explant cultures. Manuscript submitted.

III Kähkönen TE, Tuomela JM, Grönroos TJ, Halleen JM, Ivaska KK, Härkönen PL. Dovitinib dilactic acid reduces tumor growth and tumor-induced bone changes in an experimental breast cancer bone growth model. *J Bone Oncol.* 2019. 16:100232. doi:10.1016/j.jbo.2019.100232.

IV Kähkönen TE, Ivaska KK, Jiang M, Büki KG, Väänänen HK, Härkönen PL. Role of fibroblast growth factor receptors (FGFR) and FGFR like-1 (FGFRL1) in mesenchymal stromal cell differentiation to osteoblasts and adipocytes. *Mol Cell Endocrinol.* 2017. doi: 10.1016/j.mce.2017.09.015.

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1 Introduction

Breast cancer is the most common cancer in women, counting approximately 30% of all cancers. In Finland, 4742 new breast cancer diagnoses were made during the years 2012-2016 according to Nordic Cancer Registry. Majority of the diagnoses are made when the cancer is so called primary breast cancer, affecting only the breast where the malignant growth is originating. Primary breast cancer can be sufficiently treated, and the 1-year and 5-year survival rates are relatively good. One year after the diagnosis about 97%, and after 5 years about 90% of the patients are alive.

However, this situation dramatically changes when the disease relapses and metastases, tumor cells originating from the breast that are growing in distant sites of the body, are observed. In breast cancer, the majority of metastases are formed to skeleton. Bone metastases are difficult to treat and they decrease the 5-year survival to 27%. Development of targeted therapies has improved the situation, but bone metastases are still described as incurable. Therefore, novel treatments that can also inhibit tumor growth at metastatic locations are urgently needed.

In this thesis, breast cancer and bone metastases with respect to current treatment options are discussed. This thesis evaluates the role of fibroblast growth factor receptors (FGFRs) in the regulation of breast cancer growth in both *in vitro* cell culture models and *in vivo* tumor models. The effects of investigational FGFR inhibitors were evaluated in *in vitro* cell culture models, *in vivo* primary and bone metastasis models, and *ex vivo* human breast cancer tissue explant cultures to demonstrate if they would provide a potential treatment option also for advanced breast cancer.

2 Review of the Literature

2.1 Breast cancer

2.1.1 Subtypes and molecular characteristics

Breast cancer consists of several subtypes depending on the origin of malignant growth and molecular characteristics of the tumor. Based on tumor origin, breast cancers can be divided into two main categories of ductal (75 – 80 %) and lobular (10 – 15 %) carcinomas. Ductal carcinomas originate from the breast ducts and lobular carcinomas from the breast lobules. Other subtypes are tubular (Limaiem et al., 2019), medullary (Romaniuk et al., 2015) and mucinous carcinomas (Lei et al., 2016), but these types are more uncommon and have distinctive clinical features (Huovinen 2013, Dieci et al., 2014).

Characterization of breast cancer is based on so called TNM staging system, which was first published in 1959 and thereafter renewed several times (Sawaki et al., 2018). In this staging system 'T' refers to tumor size, 'N' the number of lymph nodes with tumorous growth, and 'M' the number of observed metastases (Sawaki et al., 2018, Jung et al., 2015). The staging system is used to predict prognosis and assist in treatment planning (Sawaki et al., 2018, Jung et al., 2015, Yoon et al., 2019). In the past few years this staging system was updated to include "I" indicating immunological scoring, which evaluates immune cell infiltration into the tumor (Galon et al., 2014, Galon et al., 2012) and is used as a basis for treating patients with immunogenic tumors with immunomodulators (Criscitiello et al., 2015).

Current classification of breast cancer to several subgroups is based on different pathological features, clinical outcome, and expression of well-known molecular markers (Dai et al., 2015, Prat et al., 2015). These markers include tumor histological grade, proliferation rate, and estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu/erbB2) status (Yersal 2014, Dai et al., 2015, Yoon et al., 2019, Robertson et al., 2018). Current breast cancer subtypes are summarized in Table 1 below.

Table 1. Summary of breast cancer subtypes, molecular markers, histological and proliferation rate, and predicted outcome.

Tumor type	Sub-type	Molecular profile			Histo-logical grade	Prolife-ration rate	Predicted outcome	Location of metastases
Luminal	A	ER+	PR+	HER2-	low	low	good	skeleton
	B	ER+	PR+	HER-	high	high	medium	skeleton
HER2+		ER-	PR-	HER+	high	high	poor	brain and soft tissues
Basal, TNBC		ER-	PR-	HER2-	high	high	poor	lungs and skeleton
Normal		ER+	PR+	HER2-	low	low	medium	-

Abbreviations: ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2, TNBC = triple-negative breast cancer. The table is based on the following publications: Huovinen 2013, Dieci, et al., 2014.

Luminal-type breast cancers are the most common, counting about 75% of breast tumors (Yersal et al., 2014, Turner 2008b, Li et al., 2015). They are ER positive and express many luminal cell markers such as cytokeratines (Yersal 2014, Dai et al., 2015). These tumors can further be divided into two subgroups of A and B. Luminal A breast tumors are most common and count over 50% of all breast cancers (Yersal et al., 2014, Dai et al., 2015, Li et al., 2015). They usually have low histological grade and patients with these tumors have good prognosis (Yersal et al., 2014, Dai et al., 2015, Li et al., 2015). Relapse rate is low and metastases are most commonly observed in the skeleton (Yersal et al., 2014). Fifty to twenty percent of breast cancers are of luminal B tumors (Ades et al., 2014, Li et al., 2015). They are usually more aggressive than luminal A tumors and have higher histological grades and proliferation rates (Yersal et al., 2014, Dai et al., 2015, Ades et al., 2014). Also, the relapse rates are higher and survival rates lower (Yersal et al., 2014, Dai et al., 2015, Ades et al., 2014). The main difference between the luminal A and B tumors is the higher proliferation rate in A tumors, which can be explained by upregulation of proliferation associated genes (Yersal 2014, Dai et al., 2015, Ades et al., 2014).

Fifty to twenty percent of breast cancers are HER2 positive (Asif et al., 2016). HER2 positivity correlates with high proliferation rate, aggressive disease and poor clinical outcome (Yersal et al., 2014, Li et al., 2015, Asif et al., 2016). This may be partly due to mutation in the tumor suppressor p53, which occurs in about 40% of HER2 positive tumors. HER2 positivity is also linked to genes controlling invasion and metastasis (Yersal et al 2014, Dai et al., 2015). Metastases of this tumor type are usually formed to brain or soft tissues (Yersal et al., 2014).

Depending on the cohort, 8 – 37% of breast cancers are basal type (Yersal et al., 2014, Li et al., 2015, Prat et al., 2015, Lee et al., 2018). Basal-type breast cancers are ER, PR and HER2 negative and are often referred as triple-negative breast cancer (TNBC). These tumors have frequently mutation in p53 and retinoblastoma (Rb) pathways (Yersal et al., 2014). TNBC is an aggressive subtype of tumors with poor prognosis and high incidence of metastasis to skeleton and lungs (Yersal et al., 2014, Dai et al., 2015, Tseng et al., 2013).

Normal-type breast cancers count 5 – 10% of breast tumors, and the predicted outcome in patients is relatively good (Yersal et al 2014, Prat et al., 2015, Russnes et al., 2017). The tumors express a large number of adipose tissue -related genes and are somewhere in between luminal A and B tumors (Yersal et al., 2014, Russnes et al., 2017).

Even though tumor characterization is well established and widely used, it may be inefficient in some cases. This is because the characterization does not always provide enough information for personalized medicine as many histologically similar tumors may behave differently (Yersal et al., 2014, Badve et al., 2015). This problem is a result of tumor heterogeneity caused by variation in marker expressions both inter- and intratumorally (Song et al., 2016), and especially in metastases growing in different locations of the body (Yang et al., 2017, Roulot et al., 2016, Ellsworth et al., 2017). New biomarkers such as microRNAs (McGuire et al., 2015, Bertoli et al., 2015), extracellular vesicles (Sadovska et al., 2015), circulating tumor DNAs (ctDNA) (De Mattos-Arruda et al., 2016, Wang et al., 2017), and epigenetic modulators (Basse et al., 2015) are under investigation to be used for identifying patients that are likely to respond to certain treatments (Dai et al., 2015, Roulot et al., 2016).

2.1.2 Metastatic process

Recurrence of breast cancer can occur in three ways: local recurrence of the previously operated breast, in the other breast, or formation of metastases. Metastases are tumors that are growing in a location of the body that is not directly linked to the primary tumor (Lehti et al., 2012, Eccles et al., 2013, Scully et al., 2012). The word metastasis comes from the Latin and means ‘to change place’. The site for metastasis formation can in some cases be explained by the anatomical location and the vasculature near the primary tumor. For example, in the case of breast cancer through vena cava superior the cells have an easy access to lungs. However, this is not true for all tumors, and many tumors have a distinct pattern of metastasis formation. This was observed by Paget in 1889 when he came up with the well acknowledged seed-and-soil –theory (Eccles et al., 2013). According to Paget’s theory, all seeds (cancer cells) need sufficient and favorable growing conditions

(soil) to form metastases (Kozlowski et al., 2015, Lorusso et al., 2012). Also, breast cancer has its favorite 'soils' that are bone, lungs, liver, abdominal cavity with its lymph nodes, and brain (Huovinen 2013, Eccles et al., 2013). This cell seeding to certain tissue microenvironments was later confirmed by the expression of chemokines or other molecular markers in the tumor cells and in the cells of the metastatic microenvironment. For example, HER2 expressing tumors metastasize to brain where its ligands heregulin and neuregulins are highly expressed, and tumors expressing the chemokine receptor CXCR2 metastasize to lungs where its ligand CXCL12 is highly expressed (Kozlowski et al., 2015). Additionally, osteoblast precursor cells secrete CXCL12, and the interactions between CXCL12 and CXCR4 contribute to formation of bone metastases (Devignes et al., 2018). Overall, bone is the most preferred site of metastasis in breast cancer, counting about 70 – 80% of all breast cancer metastases. Bone metastases are discussed separately in chapter 2.1.3.

The metastatic process can be divided into several critical steps that the tumor cells need in order to develop clinically detectable metastases. These steps are: a) angiogenesis – and lymphangiogenesis; b) intravasation into the circulation; c) survival in the circulation; d) extravasation to the new tissue microenvironment; e) modulation of metastatic microenvironment to enable tumor growth, and; f) metastatic growth and tumor-induced changes in the metastatic microenvironment (Lehti et al., 2012, Scully et al., 2012). These steps are discussed in more detail in the following paragraphs.

a. Angiogenesis – and lymphangiogenesis

Tumors that are over 2 cm in diameter have a higher recurrence rate, and the possibility of micrometatasis already at diagnosis is greater. This can be explained by the increasing need for nutrients in the growing tumor (Scully et al., 2012). The tumor growth makes the tumor microenvironment hypoxic, leading to production of angiogenic factors (Bielenberg et al., 2015). Tumor secreted angiogenic factors include for example hypoxia inducible factor 1 (HIF-1), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) that can recruit endothelial cells to migrate towards the tumor (Bielenberg et al., 2015, Scully et al., 2012). The endothelial cells secrete many enzymes that help them to migrate towards the tumor, and the migrated endothelial cells finally form a lumen to allow blood flow in the newly formed vessels (Bielenberg et al., 2015). The larger the tumor grows the more nutrients it needs, which then increases blood vessel formation and the chance of cancer cells to escape from the primary tumor (Bielenberg et al., 2015).

b. Intravasation in the circulation

Intravasation is the process where cancer cells detach from surrounding tumor cells and break the basement membrane to enter the blood vessels (Bielenberg et al., 2015). This process requires the cancer cells of epithelial origin to obtain mesenchymal cell phenotype, and this process is called epithelial-to-mesenchymal transition (EMT) (Bong et al., 2017). EMT is mediated by many factors including tumor necrosis factor alpha (TNFα), insulin-like growth factor (IGF) and epidermal growth factor (EGF) (Khalid et al., 2015, Bong et al., 2017, Tan et al., 2015). In the process, cancer cells acquire mesenchymal cell properties to be able to migrate, invade and survive in vasculature (Khalid et al., 2015). Migration and invasion of cancer cells is regulated by loss of E-cadherin and increased expression of N-cadherin, which regulate cell-cell junctions in epithelial and stromal cells, respectively (Scully et al., 2012). This change in expression also results in morphological changes towards spindle-like cells that are able to invade through the basal membrane (Bong et al., 2017). Adherence of tumor cells to extracellular matrix is mediated by integrins, and later increased production of matrix metalloproteinases (MMPs) helps the cells to break the basal membrane (Scully et al., 2012). This process can also be enhanced by stromal cells. For example, a decrease in fibroblast-produced CXCL12 increases vascular permeability by decreasing endothelial cell junctions (Ahirwar et al., 2018). When the cancer cells enter the circulation, they are called circulating tumor cells (CTCs) (Bidard et al., 2016). In patients, CTCs are detected in circulation very early in the disease, and they are a prognostic factor both in early stage and metastatic breast cancer in predicting metastasis occurrence and their response to therapies, respectively (Bidard et al., 2016).

c. Survival in the circulation

Cancer cell survival in the vasculature is a crucial characteristic for metastasis formation (Scully et al., 2012, Kozlowski et al., 2015). Blood circulation is a hostile environment for CTCs and majority of them die shortly after intravasation (Bielenberg et al., 2015, Cominetti et al., 2019). The mechanism of cancer cell survival in vasculature is not well understood. The CTCs can undergo apoptosis due to high pressure that they encounter, and one suggested mechanism for surviving the pressure is increased release of ATP from mechanosensitive pannexin-1 (PANX1) channels that disables the cells to undergo apoptosis (Furlow et al., 2015). CTCs are also actively killed by natural killer (NK) cells (Bielenberg et al., 2015, Cominetti et al., 2019), but they are to some extent protected by platelets (Bielenberg et al., 2015). Also, the survival is enhanced when the CTCs stick together as aggregates or form aggregates for example with lymphocytes (Lehti et al., 2012, Kozlowski et al., 2015).

The bigger the glum is, the more likely it is to stuck in the capillary, allowing the CTCs to access the stroma.

d. Extravasation to the new tissue microenvironment

Extravasation is the process where the CTCs enter the stroma. Extravasation occurs through endothelial wall by loosening endothelial cell junctions (Lehti et al., 2012, Scully et al., 2012, Bielenberg et al., 2015). Then the tumor cells need their invasive properties and proteolytic enzymes to alter their surroundings to support the growth of metastasis in the new tissue microenvironment (Lehti et al., 2012, Scully et al., 2012). Mediators of extravasation are not completely understood. Some studies indicate that for example cysteine rich angiogenic inducer 61 (CYR61) is associated with aggressive disease and silencing of the gene blocks extravasation of TNBC cells (Huang et al., 2017). Another factor observed to inhibit invasion and extravasation, but not proliferation and growth, is transient receptor potential vanilloid subtype 4 (TRV4) (Lee et al., 2016).

e. Modulation of metastatic microenvironment to enable tumor growth

After extravasation, the cells are called disseminated tumor cells (DTCs). In patients, the number of DTCs alone is not a prognostic factor for distant recurrence free survival, but when combined with the number of CTCs it provides a prognostic factor for overall survival (Magbanua et al., 2019). Once disseminated, the tumor cells encounter another hostile microenvironment and in order to grow they need to adopt to this new microenvironment. In fact, many DTCs enter apoptosis or are eliminated by immune cells (Bielenberg et al., 2015). To prevent this, DTCs can enter a dormant, non-proliferative state where they are protected from elimination by apoptosis and are also resistant to many therapies (Bielenberg et al., 2015). Many factors such as F-box/WD repeat-containing protein 7 (Fbxw7) and IL-6 cytokine leukemia inhibitory factor (LIF) can keep tumor cells dormant (Shimizu et al., 2019, Johnson et al., 2016). Deletion of Fbxw7 forces DTCs to proliferate and sensitizes them to chemotherapy (Shimizu et al., 2019). For growth, tumor cells need their epithelial cell properties again, and for that they undergo a reverse process called mesenchymal-to-epithelial transition (MET) (Cominetti et al., 2019). This process then allows tumor cells to proliferate and to form secondary tumors (Cominetti et al., 2019).

The last step of growth of metastases and tumor-induced changes in the metastatic microenvironment are discussed in the next chapter in the concept of bone metastasis.

2.1.3 Bone metastases

Skeleton is the most common site for metastasis in breast cancer (Rose et al., 2006, Sowder et al., 2019, Wang et al., 2017). Bone metastases are most commonly formed to spine, ribs, pelvis, proximal femur, and also to the skull (Weidle et al., 2016, Rose et al., 2006). At physiological state, bone turnover is regulated by the activity of bone forming osteoblasts and bone resorbing osteoclasts. Osteoblasts are differentiated from mesenchymal stromal cells (MSCs) and their differentiation is regulated by many factors that are also active in cancer such as FGF/FGFR pathway (Marie et al., 2012). Osteoclasts are differentiated from hematopoietic stem cells (HSCs), and increased number and activity of osteoclasts causes osteolytic bone changes in breast cancer that are described later in this chapter (Johnson et al., 2019).

Bone microenvironment is in many ways a unique and favorable soil for metastasis. Once the CTCs arrive to bone they occupy the hematopoietic stem cell niches that support DTCs, and in these niches the cells can stay dormant from months to decades (Sowder et al., 2019, Weidle et al., 2016). The reason for the long dormancy is not fully known but long adaptation to the new microenvironment has been suggested (Weidle et al., 2016, Neves-E-Castro 2006). The reasons why tumor cells favor bone microenvironment may be related to interactions of tumor cells with bone-derived cells such as hematopoietic and mesenchymal stem cells, endothelial cells, bone-forming osteoblasts and bone-resorbing osteoclasts, and bone marrow adipocytes that are present during the metastatic process (Sowder et al., 2019, Rossnagl et al., 2018). Furthermore, bone is a fertile microenvironment for tumor to grow as it contains many growth factors and cytokines (Sowder et al., 2019). Bone marrow is also a hypoxic microenvironment (Sowder et al., 2019), and hypoxia is known to regulate tumor growth, migration, invasion, formation of metastases, tumor dormancy in bone, and also resistance to therapies (Butturini et al., 2019), but also pathways that regulate bone resorption and formation such as the receptor activator of nuclear factor kappa-B (RANK)/RANK ligand (RANKL) -pathway (Gilkes 2016).

In breast cancer, bone metastases are usually classified as osteolytic where the bone is degraded faster than new bone is formed, resulting in pathological bone loss (Weidle et al., 2016, Rose 2006). In some cases also osteosclerotic lesions with increased bone mass are observed (Weidle et al., 2016, Makhoul et al., 2016). Usually bone metastases are mixed, including both types of lesions in the same patient, but the metastases are classified based on the dominant process. Development of osteolytic metastases is a consequence of increased number and/or activity of osteoclasts. This happens through tumor cells acting in bone to modulate the microenvironment to support their growth (Rucci et al., 2018). First factors characterized in this process were parathyroid hormone-related peptide (PTHrP) (Guise et al., 2002) and TGFβ, which can modulate PTHrP secretion (Yin et al.,

1999). PTHrP increases the expression of RANKL in osteoblasts, which then binds to its receptor RANK in osteoclasts, leading to increased bone resorption (Rucci et al., 2018). Osteoblasts also secrete other factors such as IL-6 and TNFα that increase the differentiation and activity of osteoclasts (Rucci, et al., 2018). Also, breast cancer cells inhibit osteoblast differentiation through RUNX2 mediated pathways and increase expression of sclerostin, which further contributes to osteolytic phenotype (Mendoza-Villanueva et al., 2011). When osteoclasts degrade bone, many growth factors such as TGFβ, FGFs, IGFs and PDGF are released from bone matrix and further promote tumor growth in bone metastases (Rucci et al., 2018). Tumor cells can also promote differentiation of osteoclast precursor cells directly (Rucci et al., 2018). This well described phenomenon is called the vicious cycle of bone metastasis (Weidle et al., 2016, Makhoul et al., 2016, Johnson et al., 2018, Guise et al., 1996). Additionally, immune cells can contribute to tumor growth in bone in the vicious cycle (Owen et al., 2019). Breast cancer cells also express so called osteolytic drivers that increase cancer-induced bone degradation (Johnson et al., 2018, Brook et al., 2018), including for example the transcription factor zinc finger protein GLI2 (GLI2), connective tissue growth factor (CTGF) (Johnson et al., 2017), the cytokine IL-11 (Liang, Ma et al., 2019), and many more (Awolaran et al., 2016). Clinically, problems especially in osteolytic bone metastases include bone-related pain and fractures, hypercalcemia and spinal cord and nerve compression, which decrease the quality of life of the patients (Weidle et al., 2016).

2.2 Breast cancer treatment

Primary care for breast cancer is surgery (Turner et al., 2008a, Kumar et al., 2013). When possible, parts of breast can be saved in so called conserving surgery (Turner et al., 2008a) that is accompanied by post-operative radiation or chemotherapy, and/or hormonal or HER2 targeting treatments when applicable (Turner et al., 2008b). For example, post-operative radiation can reduce the risk for local recurrence by about 20% (Turner et al., 2008a). Also, post-operative chemotherapy should be given early to improve the survival of patients with aggressive tumor subtypes (Yu et al., 2017). In some cases radiation or cytotoxic treatment can also be applied in a neoadjuvant setting prior to surgery (Eccles et al., 2013), which decreases tumor volume and helps surgical removal of the tumor (Al-Hilli et al., 2016). There is no direct consensus about the best timing for surgery after neoadjuvant chemotherapy (Al-Hilli et al., 2016). All surgeries are accompanied with sentinel lymph node examination to identify metastases in the lymph nodes (Kumar et al., 2013). If found positive, axillary lymph node dissection is performed, but whether this improves the survival is still questionable (de Boniface et al., 2017).

Radiation therapy was first tried on breast cancer patients in the 1930s with promising results (Akram et al., 2012). After that, radiation therapy has evolved and currently it is applied as a 3D Conformal Radiotherapy (3DCRT) where the radiation is directed to the tumor, minimizing the exposure to healthy tissue (Akram et al., 2012).

Chemotherapy is a possibility for treatment of several breast cancer types. The anthracyclines doxorubicin or epirubicin were the first treatment options started in the 1980s that decreased breast cancer mortality by 20 – 38% (Turner et al., 2008b, Greene et al., 2015). Later anthracyclines were combined with the taxanes docetaxel or paclitaxel, and the combinations further reduced mortality by 15% (Turner et al., 2008b, Sparano 2000, Saloustros et al., 2008). Docetaxel has become a standard therapy for patients who have failed while on anthracycline therapy (Nabholtz et al., 2005). However, not all women benefit from chemotherapy, and the current challenge is how to identify the responders (Turner et al., 2008b). Currently, Oncictype DM, MammaPrint and H/I tests are used to predict the responses to chemotherapy (Turner et al., 2008b), and further personalized medicine approached using gene expression profiles are being developed (Yu et al., 2017).

Hormonal therapies are applied for patients with hormone receptor positive breast cancer. There are two classes of hormonal therapies, anti-hormonal therapy (tamoxifen or fulvestrant) and aromatase inhibitors (letrozole, anastozol, or exemestan). Anti-hormonal therapies block the estrogen receptor and estrogen mediated actions (Turner et al., 2008b). Aromatase inhibitors block the actions of an aromatase enzyme that converts androgens to estrogens (Turner et al., 2008b). The decision of which hormonal therapy the patients receive is based on the age of the patient. Premenopausal women typically receive tamoxifen as single agent or combined with chemotherapy, and postmenopausal women receive aromatase inhibitors (Turner et al., 2008b, Draganescu et al., 2017). Tamoxifen reduces the risk of cancer recurrence by 40% and mortality by 30% (Turner et al., 2008b, Draganescu et al., 2017). In postmenopausal women aromatase inhibitors are superior compared to tamoxifen, increasing disease-free survival by 13 – 40% (Turner et al., 2008b). A meta-analysis comparing the effects of tamoxifen and aromatase inhibitors showed that aromatase inhibitors were more effective in preventing relapses in the first 4 years of follow-up, and mortality was also lower during a 10 year follow-up time (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2015). However, the individual variation in response to aromatase inhibitors has been a problem, and CYP19A1 polymorphism and mutations in ESR1 have been suggested to predict the responses (Hamadeh et al., 2018).

Patients with HER2 overexpressing tumors can be treated with HER2 targeting therapies (trastuzumab or lapatinib) (Figueroa-Magalhaes et al., 2014). Trastuzumab treatment reduces the risk of relapse by 35 – 52% (Turner et al., 2008b). Trastuzumab

can be used as a first-line treatment as monotherapy or combined with antracyclines (Figueroa-Magalhaes et al., 2014). Also, the new generation HER2/HER3 antibody pertuzumab can be used especially on patients who have relapsed on trastuzumab treatment (Figueroa-Magalhaes et al., 2014).

Treatment of breast cancer will be decided individually, but the treatment plan is based on the tumor subtype. Luminal A and B tumors can be treated with hormonal therapy because they express ER and PR and are less sensitive to chemotherapy (Turner et al., 2008b, Diessner et al., 2016, Dai et al., 2015). In fact, survival of luminal A breast cancer patients was not increased when treated with combination of hormonal and chemotherapy (Diessner et al., 2016). Luminal B and HER2 overexpressing breast tumors can be treated with HER2 targeted therapies, and with hormonal therapy (Turner et al., 2008b). However, luminal B tumors have lower expression of ER and PR and therefore may not respond to hormonal therapies similarly than luminal A tumors (Ades et al., 2014). These tumors are also sensitive to chemotherapy (Turner et al., 2008b), and combination treatment of hormonal therapy and chemotherapy is beneficial (Dai et al., 2015). Some data suggests that treatment with the vascular endothelial growth factor A (VEGF-A) antibody bevacizumab or the mTOR-inhibitor everolimus could be also beneficial for patients with luminal breast cancers (Dai et al., 2015). Additonally, everolimus can overcome the resistance to hormonal therapies (Draganescu et al., 2017). HER2 positive cancers are treated with HER2 targeting therapies combined with chemotherapy (Figueroa-Magalhaes et al., 2014). TNBCs are sensitive to chemotherapy but not to hormonal or other targeted therapies (Turner et al., 2008b). In fact, TNBCs are aggressive and difficult to treat (Jhan et al., 2017, Yao et al., 2017). New treatment options for TNBC are under development, including AR targeting compounds, PARP1, mTOR, SRC, vascular epithelial growth factor receptor (VEGFR), FGFR inhibitors, and many more (Lee et al., 2018, Yao et al., 2017, Jhan et al., 2017). Immunotherapies, agents that activate the patient's own immune system to fight cancer, are also widely studied in different breast cancer subtypes (Nathan et al., 2018).

2.2.1 Treatment of bone metastases

Bone metastases are a vast clinical problem. Over 30% of breast cancer patients are in a risk of recurrence, which is typically observed as development of metastatic disease (Gluck 2007). There is no complete cure for metastatic cancer, but the increase in progression-free survival can prolong the time for development of metastases (Gluck 2007, Shen et al., 2017).

If the patient has received a good response to primary tumor treatment, the same treatment can be continued with metastases. Surgery is mainly palliative but some

studies also indicate a survival benefit of surgery in metastatic breast cancer (Teshome 2018). Radiation therapy can be directed to tumors growing in metastatic locations, including bone, where surgery is not an option (Akram et al., 2012). When appropriate, hormonal and HER2 targeting agents are widely used in metastatic patients. A single agent chemotherapy is usually enough to provide control over metastasis growth, but in many cases multiple chemotherapeutics are combined, which may lead to over-treatment and higher incidence of adverse effects (Senkus et al., 2017). An important aspect in treatment of bone metastases includes treatment of cancer-induced bone pain and skeletal health (Gnant et al., 2010). Also, therapies with anti-metastatic properties are being developed (Anderson et al., 2019).

Besides tumor-targeting agents, bone metastases are treated with bone-targeting agents that decrease tumor-induced changes in bone (Salmen et al., 2015, Holen et al., 2010). In breast cancer, these changes are mainly osteolytic and the bone targeted therapies are used to prevent cancer-induced bone loss (Salmen et al., 2015, Luftner et al., 2018). Bisphosphonates are a class of compounds that inhibit osteoclasts and induce their apoptosis, preventing bone loss (Weidle et al., 2016, Makhoul et al., 2016). Bisphosphonates can be divided into two groups: non-nitrogen-containing (eg. clodronate) and nitrogen containing (eg. zoledronic acid) (Weidle et al., 2016). Non-nitrogen containing bisphosphonates are converted to ATP analogs that accumulate in osteoclasts, while nitrogen containing bisphosphonates affect the mevalonate pathway and prevent Ras, Rho and Rab signaling in osteoclasts (Weidle et al., 2016). In general, bisphosphonates delay the occurrence of skeletal-related events, such as fractures, in breast cancer patients (Weidle et al., 2016, Salmen et al., 2015). In fact, bisphosphonates are associated with a decreased risk for developing bone metastases, but they have no effect on disease-free survival (O'Carrigan et al., 2017). Furthermore, they increase the time to first skeletal related event. Some differences in response to zoledronic acid have been observed in preclinical pre- and postmenopausal bone metastasis models (Ottewell et al., 2014). In the postmenopausal model, a higher incidence of bone metastasis was observed, and tumor growth was prevented by zoledronic acid, while it had no effect in the premenopausal model. Adjuvant bisphosphonate treatment is currently recommended for postmenopausal women with high risk of developing bone metastases (Hadji et al., 2016).

Denosumab is a fully humanized monoclonal antibody that binds to RANKL, preventing activation of osteoclasts (Weidle et al., 2016). Denosumab is effective in preventing skeletal effects in patients with bone metastases (Weidle et al., 2016). Denosumab delays the time to occurrence of first skeletal related event over zoledronic acid by 22% (Stopeck et al., 2010, O'Carrigan et al., 2017), and increases the quality of life of the patients (Martin et al., 2012).

Some cancer therapies have harmful effects on the skeleton. This is referred to as cancer treatment-induced bone loss (CTIBL) (D'Oronzo et al., 2015). This syndrome is caused by many treatments such as hormonal therapies, chemotherapies, and some tyrosine kinase inhibitors (D'Oronzo et al., 2015). It is also a well acknowledged problem with aromatase inhibitors and has led to their combination with anti-resorptive compounds such as denosumab (Makhoul et al., 2016, Abdel-Rahman 2016).

2.3 Fibroblast growth factor receptors

2.3.1 FGFR family

FGFs and FGFRs are important for many biological functions. They regulate embryo- and morphogenesis, homeostasis, wound healing, angiogenesis, and in cancer proliferation, survival, differentiation, migration, invasion and apoptosis (Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010, Perez-Garcia et al., 2018, Andre, Cortes 2015, Ornitz et al., 2015b).

There are altogether 18 FGFs, FGF1-10 and FGF16-23 (Perez-Garcia et al., 2018). FGFs are further divided to subfamilies of FGF1 (including FGF1 and FGF2), FGF4 (FGF4 – FGF6), FGF7, (FGF3, FGF7, FGF10, and FGF22), FGF9 (FGF9, FGF16, and FGF22), FGF8 (FGF8, FGF17, and FGF18), FGF15/19 (FGF15/19, FGF21, and FGF23), and FGF11 (FGF11 – FGF14) (Porta et al., 2017). The classification to subfamilies is based on sequence similarity and biological function (Ornitz et al., 2015b). FGF1, FGF4, FGF7, FGF8 and FGF9 family members are canonical or paracrine, FGF11 family members are intracellular, and FGF15/19 family members are endocrine FGFs (Ornitz et al., 2015b, Porta et al., 2017).

Canonical FGFs bind to heparan sulfate proteoglycan (HSPG) (Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010, Perez-Garcia et al., 2018, Ornitz et al., 2015b). HSPGs binds to both the ligand and the receptor, and stabilize the ligand-receptor -complex by binding to the cell membrane (Beenken et al., 2009, Turner et al., 2010, Wesche et al., 2011). HSPG also stabilizes FGFs by binding the ligands and thus avoiding their degradation (Beenken et al., 2009, Turner et al., 2010). Additionally, FGFs can be bound to FGF- binding proteins (FGFBP) where they are stored in inactive state before released and delivered to HSPG (Beenken et al., 2009, Ornitz et al., 2015b). Endocrine FGFs bind to α- or β-Klotho and intracellular FGFs interact via voltage gated sodium channels (Porta et al., 2017, Ornitz et al., 2015b).

FGFR family is composed of four receptor tyrosine kinases (FGFR1-4) that are encoded by four different genes (Andre et al., 2015, Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010, Ornitz et al., 2015b). Additionally, there is a fifth FGFR, FGFR like 1 (FGFRL1, or FGFR5) that has structural similarities with other

FGFRs but lacks the intracellular tyrosine kinase (TK) domain (Perez-Garcia et al., 2018, Turner et al., 2010, Ornitz et al., 2015a). Due to alternative splicing, FGFR1-3 are present in two variants (FGFRIIIb and –c isoforms) (Beenken et al., 2009, Ornitz et al., 2015b). In general, b isoforms are expressed in epithelial cells and c isoforms in mesenchymal cells (Beenken et al., 2009, Ornitz et al., 2015b). FGFs are produced in epithelial or mesenchymal cells and can activate the receptor in the opposing cells (epithelial to mesenchymal signaling or vice versa) (Beenken et al., 2009, Ornitz et al., 2015b). There are some exceptions to this as some FGFs, for example FGF1, can bind to both isoforms of the FGFRs (Beenken et al., 2009).

FGFRs compose of an extracellular domain, a ligand-binding domain, a transmembrane domain and an intracellular domain that mediates the downstream effects of FGFR activation (Andre et al., 2015, Perez-Garcia et al., 2018). FGFs bind to an immunoglobulin (Ig)-like part of the extracellular domain. There are three Ig-like parts (IgI, IgII or IgIII, named also D1-D3) in FGFRs, and an acid box between the IgI and IgII that is important for receptor autoinhibition (Perez-Garcia et al., 2018, Beenken et al., 2009). IgII and IgIII are the sites for FGF ligand binding (Perez-Garcia et al., 2018). Upon binding of the ligand, APT in the ligand binding domain is replaced by a higher binging affinity FGF, which then causes the two FGFRs to dimerize, and this allows the two TK domains to be in a close proximity for transphosphorylation (Ornitz et al., 2015a, Perez-Garcia et al., 2018). After transphosphorylation of the TK domains, two main intracellular signaling proteins FGFR substrate 2 (FRS2) and phospholipase C (PLC or FRS1) are activated (Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010). FRS2 activation promotes downstream signaling through RAS–mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)–AKT pathways that regulate cell proliferation, differentiation and survival (Beenken, Mohammadi 2009, Turner, N., Grose 2010, Perez-Garcia, Munoz-Couselo et al., 2018). These pathways lead to activation of a further pathway including for example JNK and p38 (Ornitz et al., 2015a). Activation of PLCγ releases calcium ions from the intracellular compartment and thus activates calcium-dependent signaling, affecting cell motility (Perez-Garcia et al., 2018). FGFRs also mediate gene expression by activating Signal Transducers and Activators of Transcription (STAT) pathway, mainly STAT1, -3 and -5, via PLCγ pathway (Ornitz et al., 2015a).

FGFR signaling can be inhibited by sprout-family members (Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010). This inhibition happens indirectly by altering growth factor receptor-bound protein 2 (GRB2), SOS1 or RAF1 proteins (Beenken et al., 2009). Other modulators are also present, such as MAPK phosphatase 3 (MPK3), a universal inhibitor of receptor tyrosine kinases that acts in FGF-pathway by dephosphorylating ERK (Beenken et al., 2009, Turner et al., 2010). Similar expression to FGFs (SEF) can also antagonize FGF-signaling through

modulation of MAPK pathway (Ornitz et al., 2015a, Turner et al., 2010). In contrast to inhibitory proteins, fibronectin-leucine-rich transmembrane protein family members (FLRT1 – FLRT3) are positive regulators of FGFR signaling that can enhance the FGF-mediated signaling (Wesche et al., 2011). A simplified image of the FGFR dimer and the pathways it activates is presented in the Figure 1.

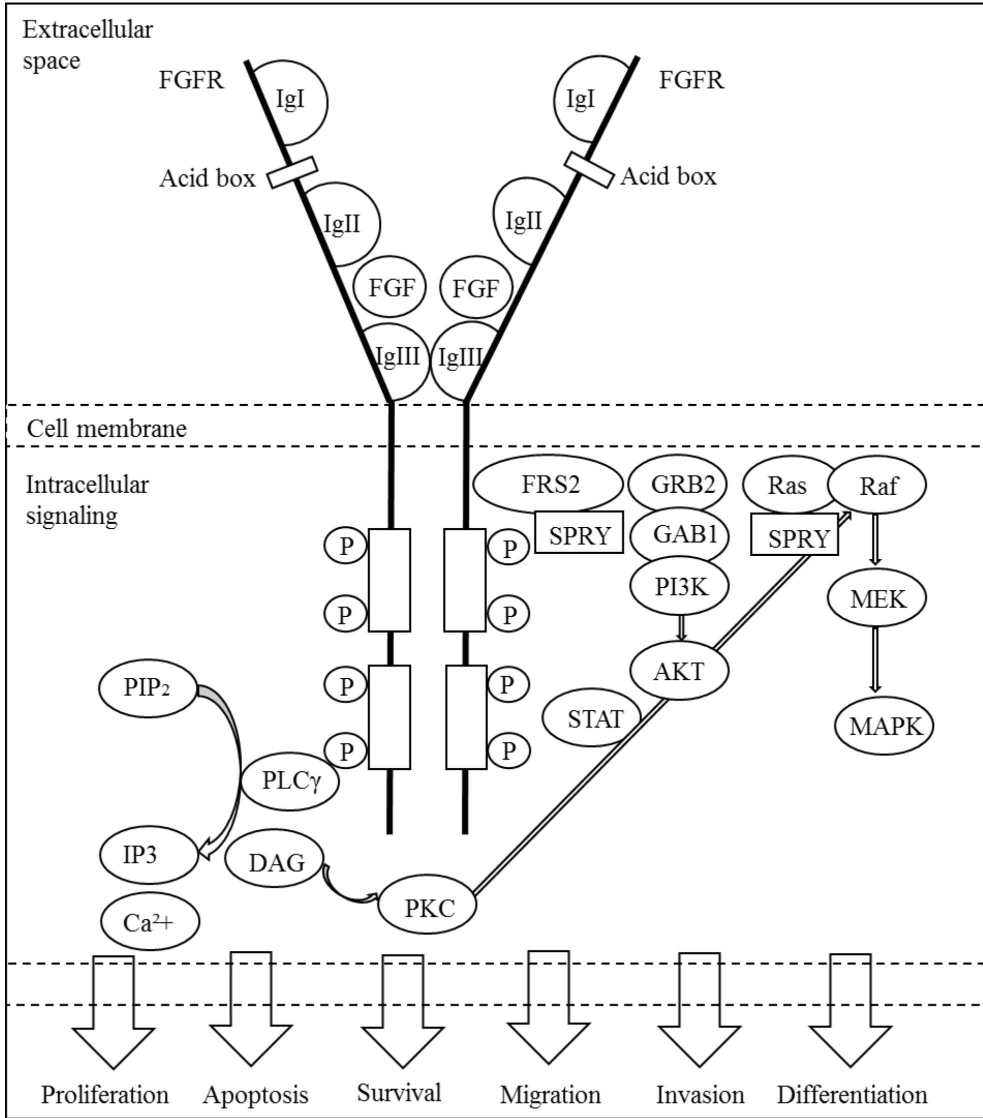

Figure 1. Basic structure of FGFR dimer and summary of FGFR signaling. AKT = protein kinase B, $Ca^{2}+$ = calcium ion, DAG = phosphatic acid, FGF = fibroblast growth factor, FGFR = fibroblast growth factor receptor, FRS2 = fibroblast growth factor receptor substrate 2, GAB1 = GRB2-associated-binding protein, GRB2 = Growth factor receptor-bound protein 2, Ig = immunoglobulin, IP3 = inositol trisphosphate, MAPK = mitogen-activated protein kinase, MEK = mitogen-activated protein kinase kinase, PIP_2 = phosphatidylinositol 4,5-bisphosphate, PI3K = phosphoinositide 3-kinases, PKC = protein kinase C, PLCγ = phospholipase gamma, Raf = RAF proto-oncogene, Ras = Ras GTPase, SPRY = sprouty protein. The figure is based on the following publications: Andre et al., 2015, Beenken et al., 2009, Ornitz et al., 2015a, Turner et al., 2010, Ornitz et al., 2015b.

2.3.2 FGFR genetic alterations in breast cancer

The FGF/FGFR pathway is often dysregulated in cancer (Perez-Garcia et al., 2018, Babina et al., 2017, Helsten et al., 2015, Parish et al., 2015, Dienstmann et al., 2014), and elevated or activated FGFR expression is associated with increased risk of breast cancer (Andre et al., 2015, Babina et al., 2017, De Luca et al., 2017, Fearon et al., 2013). A meta-analysis was conducted to evaluate the expression of receptor tyrosine kinases (RTKs) in breast cancer and their effect on survival (Templeton et al., 2014). The RTKs with worst prognosis were FGFR2 and FGFR3, followed by epidermal growth factor receptor (EGFR) (Templeton et al., 2014). FGFRs are proto-oncogenes that can be activated in cancer by gene amplifications (66%), mutations including single nucleotide polymorphisms (26%), and chromosomal translocations and gene-fusions (8% of the alterations) (Katoh et al., 2014, Perez-Garcia et al., 2018, Andre et al., 2015, Parker et al., 2014). In breast cancer, about 18% of patients have one of these aberrations in FGFR genes (Helsten et al., 2016). The frequency of alterations in FGFR1 – 4 are 3.5%, 1.5%, 2% and 0.5%, respectively (Helsten et al., 2016). Furthermore, about 5% of patients have multiple aberrations (Porta et al., 2017), and some patients may also have alterations in the downstream signaling molecules from FGFRs such as AKT, PI3K and PLCγ (Mikhaylenko et al., 2018). In fact, changes in FGFR1 and FGFR2 are considered as important drivers of oncogenesis (Yersal et al., 2014, Turner et al., 2010). These mutations usually lead to constitutively active receptors and ligand-independent or prolonged signaling by increasing receptor affinity to its ligands, affecting the hydrogen bonds that keep the receptor in active conformation, deleting of the last exon that results in a truncated, active receptor, and inducing changes in the ATP-binding pocket (Turner et al., 2010, Brooks et al., 2012, Mikhaylenko et al., 2018).

The increased signaling can also occur via loss of negative regulators (Brooks et al., 2012). For example, loss of SPRY1, SPRY2 and SEFs have been observed in breast cancer (Brooks et al., 2012). Gene fusions where TK domain of FGFR is fused with another gene can also result in constitutively active receptors (Parker et al., 2014). Furthermore, these fusion receptors are not negatively regulated or do not undergo lysosomal degradation leading to increased signaling (Wesche et al., 2011). FGFs are also overexpressed in breast cancer. FGF1 and FGF2 are expressed in almost all breast tumors (Penault-Llorca et al., 1995, Parish et al., 2015, Slattery et al., 2013, Giulianelli et al., 2019, Todorovic-Rakovic et al., 2017), while expression of FGF5, FGF6, FGF7 and FGF9 are more case restricted (Penault-Llorca et al., 1995, Parish et al., 2015). In addition, FGF8 is an oncogene (Mattila et al., 2007) that increases angiogenesis (Mattila et al., 2001) and migration and invasion of breast cancer cells (Ruohola et al., 2001). Increased expression or activity of FGFs can cause hyper-activation of signaling pathways leading to development of cancer, increased tumor growth due to increased proliferation and/or pro-survival signals by

activation of anti-apoptotic pathways of PI3K/AKT or STAT signaling, development of resistance to therapies, and formation of metastases (Perez-Garcia et al., 2018, Andre et al., 2015, Turner et al., 2010). As an example, FGF2 produced by stromal cells can activate malignant epithelial cells in both auto- and paracrine manner (Jain et al., 2012). Many tumors with FGFR mutations have mutations also in FGFR-related signaling pathways (Turner et al., 2010).

Even though FGFRs are one of the most commonly altered genes in cancer, it is still not clear whether they can solely cause the malignant transformation of epithelial cells. In rodent models, FGFR alterations have been seen to cause malignant growth of mammary epithelial cells. In humans, the tumors are examined late when typically there are multiple mutations present. It is possible that also in humans, malignant growth can be initiated by FGFR alterations, but the tumors soon acquire multiple mutations to enable rapid growth. Interestingly, FGFRs are important for the skeletal development and mutations in FGFR1 – 3 lead to skeletal dwarfism, craniosynostosis, and skeletal overgrowth syndromes (Ornitz et al.,2015). These skeletal defects are most common disorders caused by FGFR alterations.

This thesis concentrates on FGFRs in breast cancer, but FGFRs are also altered in many other cancers such as cholangiocarcinoma, urothelial, ovarian and squamous cell lung cancer (Porta et al., 2017, Helsten et al., 2016). Of these, several FGFR inhibitors are in pivotal, phase III clinical trials in cholangiocarcinoma. Alterations in different FGFRs in breast cancer are discussed in the following chapters, showing examples on how altered FGFR signaling can affect tumor growth.

2.3.3 FGFR1 alterations

FGFR1 is one on the most commonly amplified genes in breast cancer (Jain et al., 2012, Perez-Garcia et al., 2018). About 7.5 – 17% of all breast cancer patients have FGFR1 amplification in their tumors (Andre et al., 2015, Katoh et al., 2014, Turner et al., 2010). FGFR1 amplifications are common in luminal B breast cancers, being found in 16 – 27% of patients (Turner et al., 2010, Yersal et al., 2014, Ornitz et al., 2015a, Turner et al., 2010, Perez-Garcia et al., 2018, Piasecka et al., 2019, Shi et al., 2016). FGFR1 amplifications are also observed in other breast cancer subtypes. In ER+ breast cancers they are independent predictors of poor overall survival (Andre et al., 2015, Jain et al., 2012, Turner et al., 2010). FGFR1 gene (8p11.23) is also co-amplified with other genes. For example, co-amplifications of FGFR1 and CyclinD1 decrease survival of patients (Andre et al., 2015, Jain et al., 2012). Other co-amplification are found in the chromosome 11 that contains for example CyclinD1, FGF3, FGF4 and FGF19 (Perez-Garcia et al., 2018). FGFR1 may also be exclusively amplified with some genes. For example, FGFR1 and HER2 amplifications are usually not observed together in a tumor, suggesting that these pathways are

competing (Turner et al., 2010). However, when FGFR1 and HER1/2 amplification are observed in the same tumor, these patients have more distant metastases, recurrence and decreased disease-free survival (Chen et al., 2018). Furthermore, FGFR1 suppresses PR expression and FGFR1 tumors are likely to be PR negative (Turner et al., 2010). Some FGFR1 amplifications are also reported in TNBC (Andre et al., 2015, Jain et al., 2012, Turner et al., 2010) where FGFR1 is associated with poor overall survival (Cheng et al., 2015).

Besides amplifications, point mutations such as S125L (Andre et al., 2015) and single nucleotide polymorphism (SNPs) rs17182023, rs17175624 and rs10958704 (Wu et al., 2018) have been observed in FGFR1. FGFR1 and transforming acidic coiled-coil containing protein 1 (TACC1) fusions have been found in breast cancer, leading to continuously active receptor (Ornitz et al., 2015a).

Many breast cancer cell lines have elevated FGFR1 mRNA expression (Andre et al., 2015). FGFR1 may be important also in non-amplified cancers because it downregulates D-type cyclins, which increases proliferation (Turner et al., 2010). FGFR1 may also promote development of breast cancer (Perez-Garcia et al., 2018). This is supported by the findings from FGFR1 transgenic mice, where FGFR1 activation leads to epithelial cell proliferation and invasive breast lesions (Jain et al., 2012, Welm et al., 2002). Also, pharmacologically induced FGFR1 activation induces malignant changes in mammary epithelium, including invasive lesions and vascular branching (Welm et al., 2002, Turner et al., 2010). These findings suggest that FGFR1 is an important mediator in the malignant transformation of mammary epithelium.

2.3.4 FGFR2 alterations

Genome-wide association studies have identified FGFR2 as a susceptibility gene for breast cancer (Turner et al., 2010, Agarwal et al., 2014). Increased expression of FGFR2 is a prognostic factor for breast cancer that predicts poor overall and disease-free survival in patients (Andre et al., 2015).

FGFR2 gene (10q26.13) is amplified only in about 1% of all breast cancers (Andre et al., 2015, Katoh et al., 2014, Perez-Garcia et al., 2018). It is most commonly amplified in TNBC, where it is found in about 4% of tumors (Andre et al., 2015, Katoh et al., 2014, Jain, Turner 2012, Perez-Garcia, Munoz-Couselo et al., 2018). TNBC tumors are sensitive to FGFR-inhibition both *in vitro* and *in vivo* (Jain et al., 2012). FGFR2 gene fusion with Putative adenosylhomocysteinase 2 (AHCYL1), bicc family RNA binding protein 1 (BICC1), AF4/FMR2 family member 3 (AFF3), and TACC3 have been found in breast cancer (Ornitz et al., 2015a). Also FGFR2 copy number variations (CNVs) are present in breast cancer (Reintjes et al., 2013).

Mutations are more common in FGFR2 gene compared to other genetic alterations in breast cancer, and they are most common in ER+ breast cancer. Furthermore, SNPs in FGFR2 increase especially the risk for ER+ breast cancer (Turner et al., 2010). FGFR2 SNPs (rs2981582, rs1219648, rs2420946 and rs2981579) are associated with increased breast cancer risk especially in ER and/or PR positive cancers (Andre et al., 2015, Jain et al., 2012). Of these mutations, rs10736303 is located in ER binding site (Katoh et al., 2014). Also, a point mutation (R203C) has been found in FGFR2 (Andre et al., 2015). Interestingly, FGFR2 mutations have also been found in BRCA2 mutated breast cancers (Beenken et al., 2009), and in some cases BRCA2 mutation overlaps with FGFR2 rs2981575 (Katoh et al., 2014). Additionally, an association has been identified between FGFR2 (rs2981582 C>T) and MAP3K1 (rs889312 A>C) gene polymorphisms in breast cancer (Dankova et al., 2017). Two meta-analyses evaluated the association between FGFR2 SNPs and breast cancer risk (Cui et al., 2016, Zhang et al., 2017). In one analysis, 53 studies were enrolled and they included 23 variants in FGFR2, and the 10 most common of them (rs1078806, rs11200014, rs1219648, rs2420946, rs2981578, rs2981579, rs2981582, rs3135718, rs10736303, and rs3750817) were all associated with breast cancer risk (Cui et al., 2016). The other meta-analysis evaluated FGFR2 polymorphism (rs2981582, rs2420946 and rs2981578) on breast cancer risk from 35 studies, and the results showed that all these variants were associated with an increased risk of developing breast cancer (Zhang et al., 2017). The mutations in FGFR2 gene and their association to breast cancer are summarized in the review by Lei and Deng (Lei et al., 2017).

Besides genetic alterations, FGFR2 is overexpressed in about 5 – 10% of breast cancers (Reintjes et al., 2013). FGFR2 amplification has been observed in breast cancer cell lines MFM223 and SUM52PE, where the survival of the cells depends on FGFR2 expression and kinase activations (Andre et al., 2015). Amplification in cell lines led to ligand-independent constitutively activated receptor (Jain et al., 2012). In addition, *in vitro* studies have shown that FGFR2 activated by FGF7 can induce resistance to tamoxifen by inducing changes in ER phosphorylation, ubiquitination and degradation, leading to resistance to endocrine therapies in patients (Turczyk et al., 2017). Furthermore, FGFR2 counteracts estrogen effects on breast cancer by affecting ESR1 and partly inducing estrogen-independent growth (Campbell et al., 2016). Also, studies in transgenic mice have shown that FGFR2 b-isoform regulates breast morphogenesis (Jain et al., 2012).

2.3.5 FGFR3 alterations

FGFR3 expression in breast cancer cells is observed in about 40% of invasive breast cancers, and to correlate with decreased overall survival (Kuroso et al., 2010).

FGFR3 is amplified in less than 1% of breast cancers (Perez-Garcia et al., 2018). A FGFR3-TACC fusion is described in TNBC (Shaver et al., 2016). Mutations in FGFR3 are associated with breast cancer risk in patients with BRCA1/2 mutation, whereas mutations in FGFR3 are uncommon in BRCA1/2-negative patients with hereditary breast cancer (Bergman et al., 2009). SNP rs743682 in FGFR3 is associated with breast cancer risk (Agarwal et al., 2014).

Increased expression of FGFR3 is observed in tamoxifen-resistant breast tumors (Tomlinson et al., 2012). This finding was also verified *in vitro* by a constitutively active mutant of FGFR3 expressed in MCF7 breast cancer cells, where the activation reduced sensitivity to tamoxifen, followed by activation of MAPK, PI3K and PLCγ pathways (Tomlinson et al., 2012). Studies on breast cancer samples and xenografts with tumors expressing FGFR2 and FGFR3 show correlation with hormonal status, indicating that these receptors are important in regulating hormone-responsive breast cancers (Cerliani et al., 2012).

2.3.6 FGFR4 alterations

FGFR4 gene (5q35.2) amplifications are found in 2 – 10% of breast cancers, and mainly in ER and PR positive breast cancers (Andre et al., 2015, Ornitz et al., 2015a, Perez-Garcia et al., 2018). However, elevated mRNA expression of FGFR4 is found in about 30% of breast cancers (Andre et al., 2015, Perez-Garcia et al., 2018), and FGFR4 expression correlates with HER2 expression (Andre et al., 2015, Cerliani et al., 2012). In recurrent breast cancer, increased expression of FGFR4 correlates with tamoxifen insensitivity (Beenken et al., 2009), and the expression is increased in distant metastasis in invasive lobular carcinoma (Levine et al., 2019).

A SNP that leads to long-lasting activity of the receptor has been found in the transmembrane domain of FGFR4 gene (Andre et al., 2015, Jain et al., 2012). This change increases cell motility *in vitro* and tumor formation and progression of the disease *in vivo* (Andre et al., 2015). An activating point mutation in FGFR4 (Y367C) results in constituently active receptor and activates MAPK-signaling pathway in MDA-MB-453 cells (Andre et al., 2015). Another point mutation in FGFR4 (G388R) is linked to poor prognosis in breast cancer (Turner et al., 2010).

2.4 Targeting FGFRs in breast cancer

2.4.1 Development of FGFR targeting drugs

As seen from the paragraphs above, FGFRs are altered in cancer, which also increases the sensitivity of these tumors to FGFR inhibition. FGFR aberrations may increase tumor growth, induce resistance to therapies such as hormonal and targeted

therapies, and decrease survival of patients (Perez-Garcia et al., 2018, Hierro et al., 2015). Additionally, targeting FGFR-pathway could be beneficial for so-called "two-sided" inhibition, which means that such inhibitors could inhibit the cross-talk between stromal and tumor cells (Giacomini et al., 2016). This has made FGFRs a possible target for drug discovery. The FGF/FGFR pathway can be targeted by different approaches. These include inhibition of a) ligand binding by FGF ligand traps (for example FP-1039), b) the FGFR extracellular domain by antibodies (for example GP369 and MGFR1877S), or c) intracellular activity by small molecule inhibitors (examples in the following chapters) (Perez-Garcia et al., 2018, Porta et al., 2017, Dieci et al., 2013). Ligand traps are small molecule mimetics that have FGF binding/neutralizing capacity (Presta et al., 2017). Antibodies can have two main mechanisms of action. They either block ligand binding or prevent receptor dimerization, both resulting in inhibition of receptor activation (Porta et al., 2017). The mechanism of action for small molecule inhibitors is discussed separately later. One additional mechanism for possible inhibitory compounds would be to block effector proteins of the FGF/FGFR pathway, but this approach has resulted in major adverse effects because many of these proteins are involved in fundamental processes also in non-malignant cells (Perez-Garcia et al., 2018).

Currently available small molecule FGFR inhibitors can be divided into non-FGFR selective inhibitors (dovitinib, nintedanib, ponatinib, and lucitanib) and FGFR selective inhibitors (BGJ398, AZD4547, PD173074, and JNJ-42756493) (Porta et al., 2017). Non-selective FGFR inhibitors target also other receptor tyrosine kinases with structural similarity such as VEGFR and PDGFR, whereas FGFR selective inhibitors only target FGFRs. FGFR inhibitors and their target receptors are summarized Perez-Garcia and co-workers (Perez-Garcia et al., 2018). The benefit of non-selective inhibitors is a broad inhibition profile of kinases with similar function than FGFRs. For example, a non-selective FGFR inhibitor that also inhibits VEGFR could have a better efficacy in inhibiting angiogenesis (Porta et al., 2017). However, these inhibitors typically have lower binding affinity to FGFRs and increase the adverse effects in patients due to wider inhibitor profile (Porta et al., 2017). Additionally, a second generation of FGFR inhibitors have been developed, including for example the irreversible inhibitors 2 (FIIN-2) and 3 (FIIN-3) (Tan et al., 2014). FIIN2 and -3 inhibit the proliferation of cells dependent upon the gatekeeper mutations of FGFR1 or FGFR2, which can overcome the resistance of first-generation FGFR inhibitors (Tan et al., 2014).

FGFRs share a large structural similarity, but specific FGFRs can be targeted by selective antibodies. With small molecule inhibitors selective targeting is challenging and for that there are no inhibitors that would specifically inhibit only one of the FGFRs (Lei et al., 2017). It is still possible to develop inhibitors with higher binding affinity for example to FGFR1 or FGFR2 (Lei et al., 2017). However,

FGFR4 is structurally different from the other receptors, and typically inhibitors have a lower binding affinity to FGFR4 (Jain et al. 2012). However, FGFR4 specific inhibitors have also been developed, such as FGF401 (Weiss et al., 2019) and BLU9931 (Hagel et al., 2015).

2.4.2 FGFR inhibitors investigated in breast cancer

Examples of FGFR inhibitors and antibodies currently investigated for breast cancer are briefly described in this chapter. Of these, PD173074, TKI258 (dovitinib), BGJ398, and AZD4547 are described in more detail in the upcoming chapters as they have been studied in this thesis project. Ongoing clinical trials for these compounds are summarized in Table 2 and by summarized in Sobhani and co-workers (Sobhani et al., 2018). Additionally, FGFR inhibitors have been studied in other cancers with FGFR alterations (Holmstrom et al., 2019).

Nintedanib is a non-selective FGFR inhibitor that targets FGFR1-3, VEGFR1-3, PDGFR and Flt3 (Porta et al., 2017). It has shown promising results with a variety of tumors including TNBC in preclinical models (Liu et al., 2017, Reguera-Nunez et al., 2019), and also potential anti-tumor effects in combination with programmed death ligand 1 (PD-L1) antibody (Reguera-Nuñez et al., 2019). Phase 0/I clinical trial has been reported (Quintela-Fandino et al., 2019), and further clinical trials are ongoing for breast cancer.

Ponatinib is a non-selective FGFR inhibitor targeting Bcr-Abl, VEGFRs, FGFRs, TIE2 and Flt3 (Porta et al., 2017). It has shown efficacy in breast cancer cell lines having FGFR1 – FGFR4 alterations, and it has the potency to inhibit all four FGFRs. Because ponatinib targets Bcr-Abl kinase it has been widely studied in hematological cancers, but recently, results have been published also in solid tumors (Musumeci et al., 2018, Bauer et al., 2018). Ponatinib is currently studied in clinical trials in solid tumors with FGFR alterations (Porta et al., 2017).

Lucitanib (E-3810) inhibits FGFR1/2, VEGFR1-3 and PDGFRα/β, and is currently undergoing phase I/II clinical trial for breast cancer, including metastatic breast cancer (Porta et al., 2017, Criscitiello et al., 2015, Andre et al., 2015, Soria et al., 2014). Preclinical data suggests antitumor efficacy in a FGFR1 and FGFR2 amplified xenograft model, partially mediated by inhibition of angiogenesis (Perez-Garcia et al., 2018, Guffanti et al., 2017), and tumor regression in combination with paclitaxel in a TNBC model (Andre et al., 2015, Bello et al., 2013). A phase Ib study evaluated the maximum tolerated dose and safety profile of lucitanib in metastatic breast cancer (Campone et al., 2019). In breast cancer patients with FGFR aberrations, the objective response rate was 50% and progression free survival was increased by about 40 weeks (Porta et al., 2017). Phase II clinical trials are currently

ongoing with metastatic breast cancer patients (Porta et al., 2017, Hui et al., 2018, Campone et al., 2018).

Erdafitinib (JNJ 42756493) is an oral pan-FGFR inhibitor targeting FGFR1 – FGFR4 (Porta et al., 2017, Perez-Garcia et al., 2018, Andre et al., 2015). It has anti-tumor effects in FGFR wild-type and FGFR2 amplified cells *in vitro* and *in vivo*, where it induces cell death and decreases cell survival (Porta et al., 2017, Karkera et al., 2017). It is currently in phase I clinical trial for advanced solid tumors with promising results. Forty percent of patients had stable disease, including patients with FGFR1 amplified breast cancer (Porta et al., 2017, Perez-Garcia et al., 2018). Erdafitinib (Balversa) was earlier this year approved by FDA for treatment of metastatic bladder cancer patients with genetic alterations in FGFR2 (Marandino et al., 2019).

Table 2. Current status of FGFR inhibitors TKI258, BGJ398 and AZD4547 in clinical development for breast cancer, either ongoing or completed. Data obtained from clinicaltrials.gov on July 20, 2019.

FGFR inhibitor	Condition	Title	Compound(s)	Clinicaltrials.gov identifier
TKI258	Metastatic breast cancer	Safety and efficacy of TKI258 in FGFR1 Amplified and Non-amplified Metastatic HER2 Negative Breast Cancer	TKI258	NCT00958971
	Breast cancer	TKI258 for Metastatic Inflammatory Breast Cancer Patients	TKI258	NCT01262027
BGJ398	Advanced/ metastatic solid tumors	Phase 1b Trial of BGJ398/BYL719 in Solid Tumors	BGJ398	NCT01928459
AZD4547	Breast cancer	AZD4547 & Anastrozole or Letrozole (NSAIs) in ER+ Breast Cancer Patients Who Have Progressed on NSAIs (RADICAL)	AZD4547 and anastrozole or letrozole	NCT01791985
	ER positive breast cancer	Safety and Efficacy of AZD4547 in Combination With Fulvestrant vs. Fulvestrant Alone in ER+ Breast Cancer Patients	AZD4547 and Exemestane or Fulvestrant	NCT01202591

2.4.3 PD173074

PD173074 is among the first of first-generation FGFR inhibitors. It inhibits mainly FGFR1 and FGFR3 (Andre et al., 2015). It has been studied in many breast cancer cells lines where it inhibited FGFR mediated signaling and resulted in many anti-tumor effects.

In a FGFR expressing syngeneic tumor model, PD173074 decreased tumor growth and induced apoptosis in a dose-dependent manner (Andre et al., 2015, Ye et al., 2014). In PD743074 treated tumors, increased expression of the pro-apoptotic protein survivin and increased ratio of Bax/Bcl-2 were observed, explaining the increase in apoptosis (Ye et al., 2014). Additionally, PD173074 induced anoikis in a FGFR1-amplified cell model (Chen et al., 2016) and decreased microvessel density, which could contribute to the decreased tumor growth (Ye et al., 2014). PD173074 blocks migration and invasion *in vitro,* which led to decreased formation of lung metastases *in vivo*. Supporting these findings, PD173074 treatment is associated with morphological changes in EMT cell lines expressing FGFR1, including spindle- to cobble stone -like change in shape (Nguyen et al., 2013). Also, expression of MMPs and Snail1 and 2 that are involved in EMT, are decreased with treatment of PD173074, indicating that PD173074 could inhibit EMT and prevent formation of metastases (Nguyen et al., 2013). Interestingly, PD173074 is also associated with increased number of CD4+ and CD8+ T cells in the spleen and tumors (Andre et al., 2015, Ye et al., 2014), and decreases the number of myeloid-derived suppressor cells (MDSCs) indicating that the compound has also immunological effects (Ye et al., 2014).

PD173074 has also been studied in basal-type breast cancer models. About half of basal-type breast cancer cell lines and breast cancers express FGF2 (Sharpe et al., 2011). Silencing of FGF2 from these cell lines results in decreased FGFR-mediated signaling and decreased proliferation, suggesting autocrine signaling of FGF2 (Sharpe et al., 2011). This signaling is blocked by PD173074, which reduces tumor growth in a xenograft model (Andre et al., 2015, Sharpe et al., 2011).

Generally, TNBC cell lines are more sensitive to FGFR inhibition than other breast cancer cell lines (Sharpe et al., 2011). In TNBC cell lines, PD173074 inhibits MAPK and PI3K/AKT signaling (Andre et al., 2015, Koziczak et al., 2004). PD173074 decreases proliferation in TNBC cell lines and the effect is more profound in anchorage-independent 3D culture conditions (Sharpe et al., 2011). PD173074 causes cell cycle arrest to G1 state, downregulates D1 and D2 cyclin expression, and inhibits cyclin D to cdk4 activity (Andre et al., 2015, Sharpe et al., 2011, Koziczak et al., 2004). These results show that D cyclins are important in FGFR-driven cancers (Koziczak et al., 2004).

In a HER2 positive model that was induced to become resistant to HER2 targeted therapies, a loss of HER2 expression and an amplification in FGFR2 were observed

(Azuma et al., 2011). This change in expression sensitized the cells to PD173074, and the cells were 10 000 times more sensitive to the compound compared to parental cells. PD173074 decreased phosphorylation of FGFR2 in the model, and induced apoptosis *in vitro*.

Despite its many potential anti-tumor effects, PD173074 is not undergoing clinical development, and it has been replaced with second-generation FGFR inhibitors. It is mostly used in preclinical studies for its high potential to target FGFR1.

2.4.4 TKI258 (dovitinib)

Dovitinib is an oral non-selective FGFR inhibitor that binds to FGFR1, VEGFR1 – VEGFR3, c-KIT, fms-related tyrosine kinase 3 (FLT3) and PDGFRb (Andre et al., 2015, Porta et al., 2017, Perez-Garcia et al., 2018). Additionally, some activity against FGFR2 and FGFR3 have been observed in preclinical models (Porta et al., 2017).

In 4T1 cell line with constitutively active FGFR-pathways and 67NR cell line with FGFR2 and FGFR3 expression, dovitinib decreases phosphorylation of FRS2 and phosphorylation of downstream proteins of ERK1/2, AKT, and PLCγ (Andre et al., 2015, Dey et al., 2010, Porta et al., 2017). Additionally, dovitinib decreases phosphorylation of FRS2 and ERK/MAPK in FGFR1- and FGFR2-amplified cell lines (Andre et al., 2015). This inhibition in FGFR-mediated signaling blocks proliferation and growth of the cells (Andre et al., 2015, Dey et al., 2010). Additionally, dovitinib increases apoptosis in the 4T1 model by blocking the PI3K/AKT pathway (Dey et al., 2010). Also, the effects of dovitinib have been studied in co-culture models where no effects on cancer-associated fibroblasts (CAFs) were observed (Dittmer et al., 2011).

Dovitinib prevented FGFR-mediated signaling by blocking FRS2 and ERK1/2 signaling in *in vivo* tumor models (Andre 2015). Only one dose of TKI258 rapidly lowered FRS2 phosphorylation and ERK1/2 and AKT activity in the tumors (Dey et al., 2010). Dovitinib decreased tumor growth in 4T1, 67NR, and in FGFR1- and FGFR2-amplified models (Andre et al., 2015, Dey et al., 2010, Andre et al., 2013). This anti-tumor activity was mediated through direct inhibition of FGFR and PDGFR and partially caused by inhibition of angiogenesis (Dieci et al., 2013). No effects were observed for dovitinib in models with no changes in FGFR pathways, such as the MMTV-PyMT breast cancer model (Hernandez-Agudo et al., 2016). Importantly, long-term treatment of dovitinib decreased lung metastases in 4T1 and 67NR tumor-bearing mice (Andre et al., 2015, Dey et al., 2010), which could be mediated through downregulation of MMPs (Dey et al., 2010). Additionally, dovitinib inhibits CAF-induced invasion by inhibiting secretion of CCL2, CCL5 and

VEGF, and the PI3K/Akt/mTOR signaling pathway (Zang et al., 2015). When dovitinib was combined with the PI3K/mTOR inhibitor NVP-BEZ235 or the pan-ErbB inhibitor AEE788 in the 4T1 and 67NR breast cancer models, it blocked the FGFR/FRS2/ERK and PI3K/Akt/mTOR pathways, further inhibited tumor growth, and blocked formation of lung metastases (Andre et al., 2015, Issa et al., 2013).

Dovitinib has proceeded to phase II clinical trials in breast cancer. It was studied as a single agent in HER2 negative metastatic patients with amplification of FGFR1 (Perez-Garcia et al., 2018, Andre et al., 2015, Andre et al., 2013). Dovitinib was given orally as a dose of 500 mg/d (5-days-on/2-days-off) in 28-day cycles. Twenty five percent of *FGFR1*-amplified patients experienced either partial response or stable disease for more than 6 months (Dieci et al., 2013, Andre et al., 2013). Despite of these promising results, the trial did not meet pre-defined efficacy endpoints (Andre et al., 2015, Andre et al., 2013). The study revealed that amplifications in FGFR2 and FGF3 might be beneficial in evaluating who will benefit from FGFR-targeted treatments (Dieci et al., 2013). This study led to another study in HER2 negative and hormone receptor positive metastatic breast cancer in combination with fulvestrant (Dieci et al., 2013). The median progression free survival was 10.9 months in dovitinib treated patients vs 5.5 months in patients treated with placebo (Musolino et al., 2017). Dovitinib is also evaluated in a phase II study in patients with metastatic FGFR1 amplified and HER2 negative inflammatory breast cancer, and in a phaseI/II study of dovitinib in combination with aromatase inhibitors in patients with metastatic breast cancer with resistance to prior aromatase inhibitor therapy (Dieci et al., 2013, Andre et al., 2015).

Dovitinib treatment is associated with adverse effects, including diarrhea, nausea, vomiting and fatigue that affect about 90% of the patients (Porta et al., 2017). Other adverse effects include cardiovascular events, asthenia, gastrointestinal disorders, abnormal values in liver function tests, and lymphopenia (Perez-Garcia et al., 2018, Dieci et al., 2013, Musolino et al., 2017). Interestingly, hyperphosphatemia, which is a common off-target effect for FGFR inhibitors, was not observed in dovitinib treated patients (Dieci et al., 2013). Despite of these adverse effects, dovitinib is considerably well-tolerated in heavily pre-treated patients (Andre, Cortes 2015), also as a combination partner with other therapies (Musolino et al., 2017).

2.4.5 BGJ398 (infigratinib)

BGJ398 is an oral selective pan-FGFR tyrosine kinase inhibitor that also inhibits FGFR1 – FGFR3 (Dieci et al., 2013). BGJ398 decreases activation of FGFR1 and phosphorylation of MEK/ERK signaling in FGFR1-amplified breast cancer cells (Golfmann et al., 2018). MCF10A cells with higher FGFR1β levels are more

sensitive to BGJ398 compared to the same cells with high FGFR1α levels (Zhao et al., 2019). Only cells with FGFR1β-expression had enhanced cell growth and motility in the model. Expression of FGFR1β is also dominant in a metastatic model where pharmacological inhibition of FGFR1 by BGJ398 decreases lung metastases (Wendt et al., 2014).

BGJ398 decreases proliferation (Perez-Garcia et al., 2018) and the number of lung metastases (Sahores et al., 2018) in breast cancer models with FGFR expression. Treatment with BGJ398 leads to rapid tumor regression in a transplantable FGFR1 mammary tumor model, leaving a non-palpable mass of dormant tumor cells organized into a luminal and basal epithelial layer similar to the normal mammary gland, but surrounded by dense stroma with decreased levels of MDSCs and tumor vasculature (Holdman et al., 2015). When the treatments were stopped, the tumors recurred in 1 to 4 months. The recurrent tumors displayed dense stroma with increased collagen, tenascin-C expression, and MDSC infiltration (Holdman et al., 2015). Activation of the EGFR pathway was observed in recurrent tumors, and inhibition of EGFR with lapatinib in combination with BGJ398 resulted in a significant delay in tumor recurrence accompanied by reduced stroma (Holdman et al., 2015). Treatment of mice with bone metastases with BGJ398 led to reduced osteoclast activity and bone destruction, demonstrating that tumor cell -derived FGFs enhance osteoclast function and contribute to the formation of metastatic lesions in the model (Aukes et al., 2017).

BGJ398 has been studied in a phase I trial. It was evaluated at doses from 5 to 150 mg/d once daily and also 50 mg twice daily given continuously in 28-day cycles for patients with advanced solid tumors with *FGFR* alterations (Perez-Garcia et al., 2018). In later clinical trials, 10 of 32 patients (31%) treated with BGJ398 had stable disease. Additionally, 26 breast cancer patients with pre- and post-treatment target lesion measurements had tumor reduction (Perez-Garcia et al., 2018).

In general, BGJ398 is well tolerated and common adverse events include hyperphosphatemia (82.5%), constipation (50.9%), decreased appetite (45.6%), stomatitis (45.6%), and also diarrhea, fatigue, and nausea (Perez-Garcia et al., 2018). Other adverse-effects including increase in aspartate aminotransferase and alanine aminotransferase levels, hyperphosphatemia and corneal toxicity (Perez-Garcia et al., 2018) require interruptions in the dosing schedule to improve tolerability, and for this the patients are treated with phosphate binders and diuretics (Perez-Garcia et al., 2018, Dieci et al., 2013). Increased serum phosphate levels can be used as a biomarker of FGFR inhibition in humans (Wu et al., 2013, Wöhler et al., 2013).

2.4.6 AZD4547

AZD4547 is an oral FGFR selective inhibitor for FGFR1 – FGFR3 (Porta et al., 2017, Gavine et al., 2012, Perez-Garcia et al., 2018, Andre et al., 2015). It inhibits FGFR downstream signaling pathway and induces cytotoxic and cytostatic effects (Porta et al., 2017). It has a strong activity against FGFR1, FGFR2, FGFR3, FRS2, and PLCγ (Gavine et al., 2012, Porta et al., 2017). AZD4547 inhibits recombinant FGFR kinase activity *in vitro* and suppresses FGFR signaling and growth in tumor cell lines expressing FGFR (Gavine et al., 2012).

AZD4547 has potent anti-proliferative effects and suppresses FGFR/RTK signaling in HER2 overexpressing human breast cancer cells (Zhao et al., 2017). To study the effects of AZD4547 on mammary development in mammary epithelial cell (MEC) populations, MMTV-ErbB2 transgenic mice were administered AZD4547 for 10 weeks during the 'risk window' for mammary tumor development (Zhao et al., 2017). AZD4547 inhibited ductal branching and MEC proliferation *in vivo*, supporting the *in vitro* finding on anti-proliferative properties. AZD4547 downregulated RTK, mTOR, and Wnt/β-catenin signaling pathways in premalignant mammary tissues (Zhao et al., 2017).

AZD4547 has anti-proliferative effects in a wide range of FGFR-dependent cell lines and xenografts models (Perez-Garcia et al., 2018). AZD4547 suppresses FGFR signaling and growth in tumor cell lines with deregulated FGFR expression (Dieci et al., 2013). In a FGFR3-driven human tumor xenograft model, oral administration of AZD4547 was well tolerated and resulted in potent antitumor activity (Dieci et al., 2013). In a syngeneic 4T1 model, AZD4547 decreased tumor growth, blocked migration and invasion, induced apoptosis, and decreased formation of lung metastases (Liu et al., 2014). AZD4547 also increased the number of CD4+ and CD8+ T cells and decreased the number of MDSCs in peripheral blood and spleens. AZD4547 was also studied in a TNBC bone metastasis model, where it decreased tumor-induced osteolytic lesions and suppressed osteoclastogenesis (Kang et al., 2019).

AZD4547 is currently evaluated in clinical trials. In a phase II study, patients with FGFR1-amplified HER2 negative breast cancer had a 12.5% response rate (Porta et al., 2017). In another phase II trial, treatment of patients with FGFR amplified tumors with 80 mg of AZD4547 twice daily in a 2-weeks-on/1-week-off schedule or continuously, 1/8 (12.5 %) of the patients had partial response (Perez-Garcia et al., 2018). AZD4547 is being studied in combination with letrozole or anastrozole in patients with ER positive tumors who have relapsed on the treatment (Andre et al., 2015). AZD4547 is generally well tolerated, with the most common adverse events reported being fatigue, hyperphosphatemia, mucositis, nausea, and nail changes (Perez-Garcia et al., 2018). AZD4547 is one of promising compounds

for cancers with FGFR alterations. However, more studies are needed to determine the patient population who will benefit from the treatment (Porta et al., 2017).

3 Aims

The main objectives of this thesis were to assess the role of FGFRs in breast cancer, and to study the effects FGFR inhibitors in primary and bone metastatic disease, and in osteoblasts, using preclinical models.

Specific aims were:

1. To study the role of FGFRs in the regulation of breast cancer growth
2. To study the effects of FGFR inhibitors in 2D and 3D breast cancer cell culture models and in human breast cancer tissue explants
3. To study the effects of one FGFR inhibitor, dovitinib, in an experimental breast cancer bone growth model in vivo
4. To study the role of FGFRs in the differentiation of mesenchymal stromal cells to osteoblasts

4 Materials and Methods

4.1 FGFR inhibitors

PD173074 was a gift from Pfizer Pharmaceuticals. DMSO-stocks of TKI258 (S2769), BGJ398 (S2183) and AZD4547 (S2801) were purchased from Selleck Chemicals. For the in vivo study, TKI258 was ordered as a powder, dissolved in sterile water, filtered and stored at -20^{0}C in single-use aliquots.

4.2 Cell culture

4.2.1 Cell lines (I–IV)

The following cell lines were used: Shionogi 115 (S115, I), MCF-7 (II), MDA-MB-231(SA) (II), MFM223 (II and III), MSC#6 and MSC#22 (IV). S115 and MCF-7 cells were purchased from American Type Culture Collection and MFM223 cells from Sigma-Aldrich. MDA-MB-231(SA) cells were a gift from Dr. Theresa Guise from University of Texas Health Science Center at San Antonio. Immortalized mouse mesenchymal stromal cell lines, MSC#6 and MSC#22, were established and are reported in subproject IV. The culture conditions are described in the respective research articles.

4.2.2 Transfections (I)

Transfections of the S115 cell line is described in detail in subproject I. Briefly, lentiviral transfection technique using pLKO.1 lentiviral vectors for FGFR1 and FGFR2 siRNAs for were used for S115 cells. The siRNA constructs were a gift from Dr. Nancy Hynes from University of Basel. The cells were incubated with multiplicity of infection medium with lentiviral constructs for 6 h. The medium was replaced with normal culture medium containing puromycin, a selection antibiotic. Puromycin-resistant pools of cells were used in further studies.

4.2.3 Osteoblast differentiation (IV)

Immortalized mouse mesenchymal stromal cell lines MSC#6 and MCS#22 were established and are reported in subproject IV.MSC#6 cells were cultured in osteoblast differentiation medium containing 15% iFBS, sodium-β-glycerophosphate and ascorbic acid in αMEM. The differentiation period was 2 weeks, and half of the medium was replaced with fresh medium every 3-4 days during the differentiation process.

4.3 In vitro models

4.3.1 Proliferation and viability (I–II)

Several proliferation assays were used. 3(H)-thymidine incorporation assay is described in subproject I and IncuCyte proliferation assay in subproject II. Briefly, in 3(H)-thymidine incorporation assay the incorporation of 3(H)-thymidine to newly synthetized DNA was measured as β radiation emitted from the hydrogen. In IncuCyte assay, proliferation was measured as increase in confluency in the wells by real-time imaging.

4.3.2 Migration and invasion (II)

IncuCyte migration and invasion assays were used as instructed by the manufacturer. Briefly, in migration assay the cells were allowed to migrate on plastic, and in invasion assay the cells were embedded between two layers of rat tail collagen I matrix. The wound closure was imaged and analyzed with IncyCyte ZOOM analysis software.

4.3.3 Organoid cultures (II)

The method for 3D organotypic cultures has been previously described (Harma, Virtanen et al., 2010). Briefly, the cells were seeded between two layers of matrigel to 30% confluency, with the aim to have organoids formed from single cells. The organoids were allowed to form for 2-3 days and thereafter treated for 7-9 days. At the end of the experiment the spheroids were stained with calcein/EthD1 (live/dead cells, respectively) and imaged with a confocal microscope. The images were analyzed with AMIDA software to obtain morphological and quantitative data (Härmä et al., 2014).

4.4 In vivo models

4.4.1 Ethical statement (I–III)

Animal experiments were carried out at the Central Research Laboratory of the University of Turku. Ethical approval for animal studies was given by the local ethics committee (license number: 2008-05531(I) and 3908/04.10.03/2011 (III)).

Human breast cancer tissue samples were obtained from Department of Plastic and General Surgery at Turku University Hospital (Turku, Finland). The study was carried out with approval from the Ethics Committee of the Hospital district of Southwestern Finland and a written consent from the patients (§279, 9/2001).

4.4.2 Subcutaneous tumor model (I)

Male athymic nude mice (Envigo) were implanted with 10 mg testosterone releasing pellets (Innovative Research of America) before injection of 1x106 S115 cells into the lower back of the mice. Tumor growth was followed twice a week by caliper measurements until study termination at 4 weeks.

4.4.3 Intratibial model (III)

Female athymic nude mice (Envigo) were inoculated with 5x105 MFM223 cells into the bone marrow of right proximal tibia. TKI258 treatment was started at 4 weeks and the study was terminated at 9 weeks.

4.4.4 Ex vivo explant cultures (II)

The method to culture human breast tissue explant has been described (Eigeliene et al., 2006). The cultures were treated with FGFR inhibitors for 7 days. At the endpoint, tissues were fixed in 10% neutral buffered formalin and proceeded to further analysis.

4.5 Analysis methods

4.5.1 qRT-PCR (I–IV)

mRNA was extracted from the cells using RNeasy kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthetized with Oligo-dT -primers using Maxima RT enzyme (Thermo Fisher Scientific). For qRT-PCR, DyNAmo HS SYBR Green qPCR kit (Thermo Fisher Scientific) with gene-specific primers (see original

articles I-IV) was used. The samples were run with CFX96 qPCR system (Bio-Rad) and the results were analyzed using ΔΔCt-method.

4.5.2 Western blot (I, II and IV)

Protein samples were run on 10% SDS-PAGE gel, transferred to Ultra Cruz nitrocellulose (Santa Cruz Biotech) or PVDF membranes (Millipore), and incubated with primary antibodies at (see original articles I, II and IV) +4°C o/n. The emitted fluorescence (Li-Cor Odyssey® CLx imaging system) or chemiluminescence (ECL method, GE Healthcare) were detected to Kodak films (PerkinElmer).

4.5.3 Immunohistochemistry (I–III)

Sections were stained with hematoxylin and eosin (HE) or with primary antibodies (for proliferation: Ki67 and/or PHH3, for apoptosis: TUNEL, for vasculature: PECAM-1) at $+4^{0}$C o/n, and secondary antibody for 1 h at room temperature (see original articles I, II and III for details). The chromogenic peroxidase-based staining with diaminobenzene (Vector Laboratories) was used with counterstaining with Mayer's hematoxylin.

Sections were scanned with a digital slide scanner (Pannoramic 250 Slide Scanner, 3DHistech). For quantifying proliferation results, the number of PHH3 positive cells per section from the subcutaneous tumors were counted from 5 – 15 fields of view. From explant cultures, the number of Ki67 positive cells were quantified from cancerous area and adjusted to the total number of tumor cells in the sections. For quantifying apoptosis, the number of fluorescent cells from TUNEL staining were counted. For quantifying vascularization, the length of PECAM-1 positive capillaries were analyzed from 3 fields of view per tumor. All analyses were performed with Image J program.

4.5.4 Bone biomarkers (III)

Mouse serum samples were analyzed for tartrate-resistant acid phosphatase 5b (TRACP5b, IDS) as a marker of osteoclast number (Alatalo et al., 2000) and procollagen I N-terminal propeptide (PINP, IDS) as a marker of bone formation (Hale et al., 2007). The assays were performed according to the manufacturer’s instructions.

4.6 Imaging

4.6.1 X-ray imaging (III)

Mice were imaged with X-ray (Faxitron) using settings of 34 kV for 7 seconds. The images were analyzed with Image J software by quantifying the area of osteolytic lesions in bone.

4.6.2 PET imaging (III)

Positron Emission Tomography and CT (PET/CT, Inveon) imaging was performed with 5 MBq of ^{18}F-FDG to visualize metabolic activity of intratibial tumors 2 h after injection. PET/CT images were analyzed to obtain metabolically active tumor volume.

4.6.3 pQCT imaging (III)

Formalin-fixed tumor-bearing tibias were analyzed with peripheral quantitative computed tomography (pQCT, Norland Stratec). Scannings were performed at the area of trabecular bone 0.7–1 mm below the growth plate to obtain values for trabecular and cortical bone mineral density (BMD) and bone mineral content (BMC).

4.6.4 Statistics (I–IV)

Statistical analysis was performed with GraphPad Prism software (versions 6 and 7). All in vitro experiments were repeated at least two times, and the number of replicates was at least 3 in each experiment. All in vivo studies were performed two separate times. All experiments were analyzed separately and the results of one representative experiment are presented. Statistical methods used for each analysis are detailed in the figure legends or statistics chapter in the research articles (see original articles I-IV).

5 Results

5.1 FGFR expression in breast cancer cells and patient samples

FGFRs were differentially expressed in the breast cancer cell lines used in the study. Analysis of mRNA levels by qPCR in S115 cells showed highest expression of FGFR1, followed by FGFR2 and FGFR3 (I, Figure 1). FGFR4 mRNA was not expressed in these cells. MCF-7 cells expressed FGFR4, FGFR1, FGFR2 and FGFR3 mRNA in the order of magnitude (II, Figure 1). MDA-MB-231(SA) expressed high levels of FGFR4 and FGFR1 mRNA, whereas the expression of FGFR2 and FGFR3 mRNA were low (II, Figure 1). MFM223 cells have FGFR1 and FGFR2 gene amplifications (Turner et al., 2010), and it was confirmed that MFM223 cells expressed high levels of FGFR2, FGFR4 and FGFR1 mRNA (II, Figure 1). Additionally, increased levels of mRNA of one or several FGFRs in 7/10 samples was observed in a small set of human breast cancer tissue samples (II, Figure 5). In these samples, FGFR3 and FGFR4 were expressed at the highest levels.

5.2 Differential effects of FGFRs in breast cancer

The roles of FGFR1 and FGFR2 in the regulation of growth of S115 cells was studied. For this, S115 cells were silenced for either FGFR1 or FGFR2 using lentiviral transfections to establish stable cell lines with decreased expression of the FGFRs. The established cell lines had low FGFR1 (FGFR1 silencing) and low FGFR2 (FGFR2 silencing) mRNA and protein levels, respectively (I, Figure 1). As a result of FGFR2 silencing, FGFR1 expression was increased, indicating mutual regulation between these receptors. Silencing FGFR1 did not change the expression of the other FGFRs.

FGFR2 silenced cells (named hereafter FGFR1highFGFR2low, named shR2 in subproject I) had increased proliferation compared to FGFR1low (named shR1 in subproject I) and control (with FGFR levels similar to parental S115 cells, named shLacZ in subproject I) cells, as evaluated by 3(H)-thymidine incorporation assay (I, Figure 2). FGFR1highFGFR2low cells also had increased expression of cell cycle regulatory proteins cyclin D1 and B1, as studied by western blotting (I, Figure 2).

FGFR1highFGFR2low cells formed larger tumors in vivo in a subcutaneous model compared to tumors formed from FGFR1low or from control cells (I, Figure 3).

5.3 Inhibition profile of FGFR inhibitors and their effects on intracellular signaling

The effects of TKI258, BGJ398 and AZD4547 on inhibition of FGFR-mediated intracellular signaling in FGFR amplified and non-amplified breast cancer cells were studied by western blotting. TKI258 decreased phosphorylation of FRS2 and ERK at 1 nM concentration in FGFR-amplified cells (II, Figure 1). The effects were not so clear on non-FGFR amplified cells with EKR, whereas the decrease in pFRS2 was observed in non FGFR-amplified cells at 1-10 nM concentrations. BGJ398 decreased pFRS2 and pERK at 1 nM concentration in FGFR-amplified cells (II, Figure 1). Non-FGFR amplified cells responded differentially to BGJ398, as MCF-7 cells had decreased phosphorylation at 100 nM, whereas no effects were observed in MDA-MB-231(SA) cells at any concentrations tested. AZD4547 was equally effective in decreasing pFRS2 and pERK in FGFR amplified cells. In non-FGFR amplified cells, a decrease was observed at 100 nM concentration in MCF-7 cells (II, Figure 1), and no effects were observed in MDA-MB-231(SA) cells.

5.4 FGFRs differentially regulate proliferation and growth of breast cancer cells

PD173074 decreased the proliferation in FGF8b-treated S115 cells in 3(H)-thymidine incorporation assay, which occurred regardless of the FGFR silencing in the cell lines (I, Figure 2). TKI258 decreased proliferation of FGFR amplified MFM-223 cells at 100 nM concentration, as analyzed by kinetic proliferation with the IncuCyte assay (II, Figure 2). Proliferation was decreased in MCF-7 cells at 10 µM and MDA-MB-231(SA) cells at 5 µM concentration. BG398 decreased proliferation of FGFR amplified cells at 10 nM concentration (II, Figure 2). In MCF-7 cells, proliferation was decreased at 1 µM, and in MDA-MB-231(SA) cells at 5 µM concentration. AZD4547 decreased proliferation of FGFR amplified cells at 10 µM concentration (II, Figure 2). Decreased proliferation was observed at 5 µM and 10 µM concentrations in MCF-7 and MDA-MB-231(SA) cells, respectively.

FGFR amplified cells formed less organized and irregular-shape organoids compared to organoids formed from MCF-7 and MDA-MB-231(SA) cells in a 3D organotypic culture model (II, Figure 3). TKI258, BGJ398 and AZD4547decreased the growth of FGFR amplified organoids at 1 nM concentration (II, Figure 3). Decreased growth of MCF-7 and MDA-MB-231(SA) organoids was observed at 5 µM and 1 µM concentrations of TKI258, respectively (II, Figure 3). BGJ398

decreased growth of MCF-7 and MDA-MB-231(SA) organoids at 10 µM and 5 µM concentrations, respectively (II, Figure 3). AZD4547 was equally effective in organoids formed from non-FGFR amplified cells, and decreased growth was observed at 5 µM concentration (II, Figure 3).

PD173074 treatment decreased the proliferation and growth of FGFR1highFGFR2low S115 subcutaneous tumors compared to vehicle treated mice (I, Figure 3). In the intratibial tumor model, TKI258 induced a trend towards decreased tumor volume and metabolic activity evaluated by ^{18}F-FDG PET imaging at endpoint (III, Figure 1). However, no changes were observed in proliferation rate in the tumors evaluated by PHH3 IHC (III, Figure 1). A trend towards decreased proliferation was observed in human breast cancer explant cultures when treated with FGFR inhibitors (II, Figure 5). As the number of samples was very limited, these results can only be considered as preliminary.

5.5 FGFR inhibitors do not induce apoptosis

No increase in apoptosis was observed in vitro in monolayer cultures with FGFR inhibitors (II), or in FGFR-amplified organoids (II, Figure 3). In organoids formed from MCF-7 and MDA-MB-231(SA) cells, increased amount of dead cells quantified from live/dead cell stainings were observed with BGJ398 and AZD4547 at 1 to 5 µM concentrations (II, Figure 3). TKI258 increased the amount of dead cells only in organoids formed from MDA-MB-231(SA) cells (II, Figure 3). PD173074 treatment slightly increased the number of dead cells in tumors with FGFR1highFGFR2low expression in the S115 tumor model (I, Figure 5).

5.6 Other potential effects of FGFR inhibitors: angiogenesis, migration and invasion

No effects were observed on angiogenesis in FGFR1highFGFR2low S115 tumors treated with PD173074 when quantitating the length of blood vessels in the tumors (I, Figure 4). TKI258 decreased migration of FGFR-amplified cells at 1 µM, and BGJ398 and AZD4547 at 100 nM concentrations in a wound healing assay (II, Figure 4). TKI258 decreased migration of MDA-MB-231(SA) cells at 5 µM concentration, and no effects were observed with FGFR-selective inhibitors. On the contrary, all inhibitors decreased invasion of MDA-MB-231(SA) cells at 5 µM concentration (II, Figure 4).

5.7 The effects of FGFR inhibitors on bone and osteoblast differentiation

In the intratibial bone growth model, TKI258 decreased cancer-induced bone lesions evaluated by X-ray imaging (III, Figure 2). Correspondingly, total BMC and cortical BMC and BMD were higher in the tumor-bearing tibia in TKI258 treated mice compared to mice treated with vehicle (III, Figure 2). Additionally, the systemic level of osteoclasts was lower and bone formation was higher, as evaluated by TRACP 5b and PINP measurements in serum samples, respectively (III, Figure 2). No effects on osteoclast or osteoblast numbers were observed in healthy bone surface evaluated by histology (III, Supplement I).

FGFRs are expressed in MSCs and their expression is changed during the differentiation to osteoblasts (IV, Figure 2). Expression of FGFR2 and FGFRL1 is increased during osteoblast differentiation, and silencing their expression inhibits the differentiation (IV, Figure 3 and Figure 4). This indicates that FGFR2 and FGFRL1 are needed for MSC differentiation to osteoblasts. However, treatment of MSCs, pre-osteoblasts, or mature osteoblasts in vitro with PD173073 at different stages of differentiation, or continuously during the differentiation process, had no effects on the differentiation when evaluated by expression of osteoblast marker genes (IV, Figure 3).

5.8 Conclusions

The results of this thesis study demonstrate that mRNA expression of FGFRs is increased in many breast cancer cell lines and also in breast cancer tissue samples obtained from patients. FGFR1 is a major regulator of breast cancer growth in S115 cells *in vitro* and *in vivo*, whereas FGFR2 may mediate growth opposing signals in breast cancer cells. FGFR-amplified cells were about 50 times more sensitive to FGFR inhibition than non-FGFR amplified cells, and non-FGFR amplified cells responded differentially to FGFR inhibition.

FGFR-amplified cells are most sensitive to FGFR-selective inhibitors BGJ398 and AZD4547 compared to the non-selective FGFR inhibitor TKI258. FGFR-amplified cells seemed to be more sensitive to FGFR inhibition in organoid cultures compared to traditional monolayer cultures. The preliminary results obtained from breast cancer explant cultures are in line with the findings from the other assays.

FGFR inhibitors do not induce major apoptosis, and growth regulation of the inhibitors comes mainly through decreased proliferation. No effects on angiogenesis were observed with the FGFR inhibitors used, and the FGFR inhibitors had differential effects on migration and invasion of breast cancer cells. FGFR inhibitors decreased tumor growth and tumor-induced effects on bone. No adverse effects were observed on bone cells.

As a summary, the main effect of FGFR inhibitors was to decrease proliferation. This was observed *in vitro* and *in vivo*, and preliminarily verified with *ex vivo* explant cultures. Induction of apoptosis was only seen in organoid cultures, and some evidence of decreased migration and invasion was observed. No direct effects on bone cells were observed, which suggests that FGFRs inhibitors have no harmful effects on bone cells. The simplified key findings are summarized in Table 3 below.

Table 3. Summary of the FGFR inhibitors used in the study and simplified results of their effects on various parameters studied. "↓" = inhibition/decrease, "-" = no effects, "NA" = data not available.

	PD173074	**TKI258**	**BGJ398**	**AZD4547**
Target IC_{50} (nM)	FGFR1 25nM VEGFR 200nM	FLT3 1nM c-Kit 2nM FGFR 1 8nM VEGFR 3 8nM FGFR 3 9nM	FGFR1 0.9nM FGFR3 1.0nM FGFR2 1.4nM FGFR4 60nM	FGFR1 0.2nM FGFR3 1.8nM FGFR2 2.5nM KDR 24nM
FGFR signaling	↓	↓	↓	↓
Proliferation *in vitro* (2D)	↓	↓	↓	↓
Proliferation *in vitro* (3D)	NA	↓	↓	↓
Proliferation *ex vivo*	NA	↓	↓	↓
Proliferation *in vivo* (s.c. model)	↓	NA	NA	NA
Proliferation *in vivo* (i.t. model)	NA	↓	NA	NA
Apoptosis *in vitro* (2D)	NA	-	-	-
Apoptosis *in vitro* (3D)	NA	↓	↓	↓
Apoptosis *in vivo*	-	NA	NA	NA
Migration	NA	↓	↓	↓
Invasion	NA	↓	-	-
Bone cells *in vitro*	-	NA	NA	NA
Bone *in vivo*	NA	-	NA	NA

6 Discussion

FGFRs or FGFR pathway is altered in about 18% of breast cancers (Helsten et al., 2016). Currently, many FGFR inhibitors have ongoing clinical investigations in breast cancer patients with FGFR gene alterations. In this thesis we evaluated the role of FGFRs in breast cancer and studied the effects of four FGFR inhibitors in *in vitro*, *in vivo* and *ex vivo* breast cancer models.

FGFR1 is the most commonly amplified FGFR receptor in breast cancer (Jain et al., 2012, Perez-Garcia et al., 2018) and in this thesis work, FGFR1 was observed to be the major regulator among FGFRs for promoting breast cancer cell growth, and increased proliferation and upregulation of Cyclin D1 was observed with increased expression of FGFR1. This has also been observed by others, and co-amplification of FGFR1 and CyclinD1 has been identified to decrease survival of breast cancer patients (Andre et al., 2015, Jain et al., 2012). Our results show that silencing of FGFR1 from mouse breast cancer cells strongly decreased proliferation. In the silencing experiments we also observed a regulation between FGFR1 and FGFR2. Silencing of FGFR2 increased the expression of FGFR1. To our knowledge, the mechanism of this regulation has not been clarified but it could be related for example to changes in the FGFR feedback system. However, this would suggest that low FGFR2 expression in tumors would be associated with increased expression of FGFR1. Thus, targeting FGFR2 could result in increased expression of FGFR1 and escape from FGFR2-targeted therapies, but this remains to be evaluated in future studies. A similar observation was made in breast tumors where increased expression of FGFR3 mediated the resistance to endocrine therapy (Tomlinson et al., 2012).

Many breast cancer cell lines express FGFRs, but only a few of currently available cell lines have FGFR gene alterations (Turner et al., 2010). In this thesis work we used FGFR1 and FGFR2 amplified cell lines and two cell lines that do not have genetic alterations in FGFR genes. Our results show that FGFR amplified cells are about 50 times more sensitive to FGFR inhibition than non-amplified cells. However, cell lines without FGFR gene alterations can also respond to FGFR inhibition, but they need higher concentrations for inhibition of FGFR mediated signaling and proliferation. This is also observed in patients, as good responses for single agent FGFR inhibitors are typically observed in patients with high levels of

FGFR alterations. In general, patients have a moderate levels of FGFR amplifications, and a model with high level of amplified genes may not be predictive to a larger population of patients. Our results with non-FGFR amplified cell lines show that patients with no amplifications but FGFR driven or FGFR growth stimulated cancers may respond to FGFR inhibitor treatment, especially in combination with other therapies (Perez-Garcia et al., 2018).

FGFR drug discovery has resulted in both FGFR selective and non-selective inhibitors. Due to a structural similarity of FGFRs to many other RTKs, many non-selective FGFR inhibitors also target for example VEGFR. Targeting especially VEGFR is considered as an advantage, as dual-targeting of pathways responsible for angiogenesis could lead to better therapeutic efficacy. It is difficult to evaluate whether the FGFR selective or non-selective inhibitors have better efficacy, and most likely this will be case sensitive. Additionally, new FGFR targeting compounds such as second class reversible inhibitors have been developed (Farrell et al., 2018). In this thesis work we evaluated for the first time the effects of the non-selective FGFR inhibitors PD173074 and TKI258, and the selective FGFR inhibitors BGJ398 and AZD4547 in breast cancer cells. When comparing the non-selective and selective FGFR inhibitors, the selective FGFR inhibitors BGJ398 and AZD4547 were more effective in breast cancer cells but overall the inhibitors induced similar effects with same concentrations.

The effects of TKI258, BGJ398 and AZD4547 on FGFR-mediated intracellular signaling in FGFR amplified and non-amplified breast cancer cells were studied in this thesis work. As expected, FGFR amplified cell lines were more sensitive to inhibition of FGFR-mediated signaling, and decreased proliferation was also observed at lower concentrations. In fact, the decrease in proliferation seemed to be the major cause of growth regression by the inhibitors. Only some signs of increased apoptosis were observed in 3D culture models, but not in traditional 2D monolayer cultures. The observed apoptosis in low nanomolar levels in the spheroids can be due to unspecific instead of FGFR-related effects. For example, a recent study showed that expression of certain kinases can determine the sensitivity to an FGFR inhibitor in an FGFR2 amplified gastric cancer model (Chen et al., 2019). The induction of apoptosis was the major difference observed in the results between 2D and 3D culture models in this study. Another difference was the sensitivity of the assays. All the cell lines were more sensitive to FGFR inhibition induced decrease to proliferation in 3D cultures compared to 2D culture conditions. This difference in sensitivity and expression of molecular markers has also been reported by others (Breslin et al., 2016, Imamura et al., 2015). In *in vivo* models, a trend towards increased apoptosis was observed. Others have reported increased apoptosis *in vivo* with PD173073 (Ye et al., 2014) and TKI258 (Dey et al., 2010). This thesis work showed that FGFR inhibitors have differential effects on migration and invasion of

breast cancer cells. Others have reported that PD173074 decreases both migration and invasion, which is supportive of the findings of this thesis work (Penault-Llorca et al., 1995). However, inhibition of migration and invasion is also reported for AZD4547, which is contradictory to our finding (Liu et al., 2014), but this could be due to differences in the assays used.

Many cancer treatments induce adverse-effects in the skeleton (D'Oronzo et al., 2015). This is also a concern for FGFR inhibitors, as FGFRs are widely expressed in and regulate many stromal cells, including bone cells (Katoh 2016, Ornitz et al., 2015a), and FGFR germline mutations induce skeletal syndromes (Marie et al., 2012). In this thesis work, no harmful bone effects were observed in mice treated with FGFR inhibitors, and also no effects were observed for the FGFR inhibitors on osteoblasts, suggesting that FGFR inhibitors do not have harmful effects on the skeleton at least in these experimental settings. However, a limitation of these experiments was the use of only one concentration of compounds that was sufficient to show the desired effects but did not allow to study potentially observable adverse effects at higher doses. Others have shown that FGFR inhibitors can decrease osteoclastogenesis and tumor-induces osteolysis similarly to our findings (Kang et al., 2019).

Selecting the right patient population is important in clinical trials. A biomarker for determining FGFR status would help to evaluate clinical responses and to identify the optimal patient population that would benefit most from FGFR targeted therapies (Andre et al., 2015, De Luca et al., 2017). These approaches include for example PCR and *in situ* hybridization methods (Andre et al., 2013). This would also be important for predicting adverse effects of FGFR inhibitors. FGFR selective inhibitors usually have higher binding affinity to FGFRs and less adverse effects compared to non-selective inhibitors with wider range of kinase inhibition and off-target toxicities. To study inhibitor effects and inhibitor engagement with the target, serum FGF23 and phosphate levels can be used as biomarkes of treatment response (Wu et al., 2013, Wöhler et al., 2013).

Resistance is a problem with many, if not all targeted therapies (Criscitiello et al., 2015). FGFRs have been observed to mediate resistance to conventional therapies (Dieci et al., 2013), and also to HER2 and VEGF targeting therapies (Andre et al., 2015). In the case of HER2, the resistance could be overcome by dual treatment of FGFR and HER2 inhibition (Andre et al., 2015). FGFR4 expression increases for example in models that are resistant to chemotherapy, and the high expression of FGFR4 correlates with poor clinical benefit and shortened progression free survival (Andre et al., 2015). This demonstrates the importance of evaluating FGFR4 levels and developing new compounds that would also target FGFR4.

In conclusion, we showed in this thesis that FGFRs are potential drug targets and regulators of growth and progression of breast cancer. Especially FGFR1 is an

important regulator of breast cancer growth, and targeting FGFRs with selective and non-selective FGFR inhibitors induced mainly decreased proliferation of breast cancer cells and tumors. Importantly, this thesis showed that FGFR inhibitors did not have unwanted effects in the skeleton, even though FGFR are important regulators in bone. Clinical trials currently evaluate FGFR inhibitors as single agents and in combination with several other therapies in breast cancer patients. There are some challenges also described in this thesis such as patient selection, development of resistance, and adverse effects that may hinder this development. However, FGFR drug development has been promising and the approval of the first FGFR targeting compound erdafitinib for advanced bladder cancer will hopefully fasten the development in breast cancer.

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