TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

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INHIBITION OF FIBROBLAST GROWTH FACTOR RECEPTOR IN FGFR3:TACC3 FUSION POSITIVE GLIOBLASTOMA CELLS

Master of Science thesis

Examiner: prof. Minna Kellomäki
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ABSTRACT

SONJA MÄNTYLÄ: Inhibition of fibroblast growth factor receptor in FGFR3:TACC3 fusion positive glioblastoma cells
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Glioblastoma multiforme is the most common of adult primary brain tumors. It is a highly aggressive form of diffuse gliomas and with current treatments median life expectancy of patients with glioblastoma is approximately 14 months. Resent research of genetic and molecular changes in glioblastoma has provided new insight on disease initiation and progression. Fibroblast growth factor receptor 3 gene fusion with transforming acidic coiled coil 3 (FGFR3:TACC3) was found in subset of glioblastomas and has been shown to increase proliferation and anchorage independent growth of tumor cells. It is known to be mutually exclusive with other growth factor receptor alterations so it seems to be providing alternative pathway for tumor formation. However, FGFR3:TACC3 is usually not one of the initiating alterations in glioblastoma. Inhibition of FGFR in fusion positive tumors has shown promising results in vitro and in preclinical studies.

The aim of this study was compare cellular mechanisms between SNB19 glioblastoma cells that had been stably transfected with FGFR3:TACC3 fusion vector, FGFR3 vector or empty plasmid vector. Proliferation, migration, colony formation and survival of these overexpressed cell lines were compared. Effect of pan-FGFR tyrosine kinase inhibitor JNJ-42165279 to same cellular mechanisms in these different overexpression cell lines was also assessed. Furthermore, downstream effects of FGFR3:TACC3 and FGFR3 expressing cells to inhibition and stimulation with fibroblast growth factor was analyzed at protein level.

In these experiments, FGFR3:TACC3 overexpressed cells showed increased proliferation and FGFR3 overexpressed cells decreased proliferation compared to the control cells. FGFR3 expressing cells had also lowered colony forming capability. Inhibition of FGFR in these overexpressing cells showed decrease of proliferation in fusion positive cells. Inhibition had no significant effects on other cellular mechanisms. Phosphorylation of FGFR was decreased by inhibition in both FGFR3:TACC3 and FGFR3 overexpressed cell lines.

TIIVISTELMÄ

SONJA MÄNTYLÄ: Fibroblastikasvutekijäreseptorin inhibointi FGFR3:TACC3 fuusio positiivisissa glioblastoma soluissa
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Glioblastoma multiforme on yleisin aikuisilla esiintyvä primaarinen aivokasvain. Se on hyvin aggressiivinen diffuusi glioman muoto ja nykyisillä hoitomenetelmillä glioblastoma potilaiden elinajanodotteen mediaani on noin 14 kuukautta. Tutkimus glioblastoman geneettisistä ja molekulaarisista muutoksista on tuonut uutta tietoa taudin synnystä ja etenemisestä. Fibroblastikasvutekijäreseptori 3 geenin fuusion TACC3:n kanssa löydettiin osasta glioblastomia ja sen on näytetty lisäävän solujen proliferaatiota sekä kasvua alustalla, johon ne eivät voi kiinnittyä. Sen on näytetty esiintyvän kasvaimissa, joissa ei ole muiden kasvutekijä reseptoreiden muutoksia, joten se vaikuttaa tarjoavan vaihtoehtoisen reitin kasvaimen muodostukselle. FGFR:n inhibointi fuusio positiivisissa kasvaimissa on osoittanut lupaavia tuloksia sekä *in vitro*, että alustavissa kliinisissä kokeissa.

Tämän tutkimuksen tavoite oli tutkia eroja SNB19 glioblastoma solulinjalla, johon oli transfektoitu FGFR3:TACC3 vektori, FGFR3 vektori ja tyhjä plasmidi vektori. Solulinjojen proliferaatiota, migraatiota, kolonnien muodostus kyvyä ja selviytymistä vertailtiin. Pan-FGFR tyrosiini kinaasi inhibiittorin JNJ-42165279 vaikutusta samoihin solumekanismeihin arvoitiin myös eri yliekspressiosolulinjoilla. Lisäksi inhiboitujen ja fibroblasti kasvutekijällä stimuloitujen solujen FGFR3:TACC3 ja FGFR3 ilmentymisen vaikutuksia signalointi reitteihin yritettiin selvittää proteiini tasolla.

Näissä tutkimuksissa havaittiin FGFR3:TACC3 yliekspressiosolujen kasvavan nopeammin ja FGFR3 yliekspressiosolujen puolestaan kasvavan hitaammin kontrollisoluihin verrattuna. FGFR3 ilmentävillä soluilla oli myös alhaisempi kolonnien muodostus kyky. FGFR:n inhibointi yliekspressiosolulinjoilla hidasti fuusio positiivisten solujen kasvua. Inhibointi ei vaikuttanut merkittävästi muihin tutkittuihin solumekanismeihin. FGFR:n fosforylaatio väheni inhibition vaikutuksesta sekä FGFR3:TACC3, että FGFR3 yliekspressio solulinjoilla.

PREFACE

Thesis was done for the Computational Biology group led by Professor Matti Nykter at Institute of Biosciences and Medical Technology (BioMediTech) at the University of Tampere. Thesis was part of a larger brain tumor research project "Utilizing patient-derived cells for optimizing FGFR inhibitor treatment response in aggressive brain tumors" and was funded by Sohlberg Foundation. All the laboratory work was done at close collaboration with Molecular Biology of Prostate Cancer group led by professor Tapio Visakorpi and with PELICAN – personalized cancer medicine group led by professor G. Steven Bova.

I would like to thank professor Matti Nykter for this great opportunity to finish my studies and do my thesis in his group. I also wish to thank my supervisor professor Kirsi Granberg for all the valuable advices and excellent guidance during my thesis process. Gratitude for the practical part in laboratory belong especially to our groups laboratory technician Paula Kosonen as well as other members of our laboratory group.

Lastly, I want to thank my family for the support during the whole thesis process. Special thanks to my husband for the encouragement during the writing process.

Tampere, 22.11.2017

Sonja Mäntylä

CONTENTS

LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
ATP	Adenosine triphosphate
AKT	Protein kinase B
BSA	Bovine serum albumin
CNS	Central nervous system
CDKN2A	Cyclin dependent kinase inhibitor 2A
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFRL1	Fibroblast growth factor receptor like 1
FRS2	Fibroblast growth factor receptor substrate 2
GAB1	GRB associated binding protein 1
GBM	Glioblastoma multiforme
GRB2	Growth factor receptor-bound 2
HRP	Horseradish peroxidase
HSPG	Heparin sulfate proteoglycans
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry
LOH 10q	Loss of heterozygosity 10q
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphoinositide 3-kinase
$PI(3,4,5)P_3$	Phosphatidylinositol(3,4,5)-triphosphate
PM	Phospholipid membrane
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
PVDF	Polyvinylidene fluoride
RAS	Ras sarcoma protein
RB	Retinoblastoma
RT	Room temperature
SDS	Sodium dodecyl sulfate
SNB19	Glioblastoma cell line
SOS	Sons of sevenles
TACC3	Transforming acidic coiled-coil containing protein 3
TCGA	The Cancer Genome Atlas
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1. INTRODUCTION

Glioblastoma (GBM, *Glioblastoma multiforme*) is the most malignant and most frequently diagnosed primary brain tumor in adult patients. GBM is a diffuse glioma that can infiltrate the surrounding tissues. Most of the GBM are primary tumors but it can also develop from a lower grade glioma. (Wilson et al. 2014) Prevalence of GBM in Europe and North America is approximately 3-4 new patients for 100 000 people in one year (Louis et al. 2007). Usually patients suffering from GBM are between ages 45 to 75, but it can be found at any age. It is more common on males, but is found in both genders. (Ray 2010)

Standard treatment for glioblastoma includes surgery, radiation and chemotherapy, but even with treatment the survival rate of patients is only approximately 14 months (Stupp et al., 2009). Unsuccessful treatment of GBM is due to the high proliferation capability, invasive nature and multiple forms it can take. Research on genetic and molecular alterations resulting in formation and progression of GBM is hopefully in the future leading to specific targeted therapies as a treatment for this devastating disease. (Marumoto & Saya, 2012)

Fusion genes are hybrids of two rearranged genes that have fused together. Fibroblast growth factor receptor fusions have been found from many cancers including GBM, prostate, lung, breast and bladder cancer. FGFR can fuse with different partner genes creating multiple different fusion genes in different cancers. FGFR3:TACC3 (TACC, *transforming acidic coiled-coil containing protein 3*) fusion was found in approximately 5 % of GBMs (Dienstmann et al., 2014). In earlier studies this fusion has been shown to be oncogenic since it is increasing proliferation and anchorage independent growth of a tumor cells as well as promoting cell transformation and increasing aneuploidy (Parker et al., 2013; Singh et al., 2012).

Inhibition of FGFR3 in fusion positive glioblastoma is interesting treatment approach, since normal brain has low expression of FGFR3. Therefore, inhibition of FGFR3 through cerebral fluid might cause only few neurological side effects. (Parker, Engels, Annala, & Zhang, 2014) Several small molecule tyrosine kinase inhibitors for FGFR inhibition have been developed and they have shown significant responses in fusion positive tumors at animal studies and preliminary clinical trials (Di Stefano et al., 2015; Tabernero et al., 2015).

Purpose of this study was to evaluate effects of overexpression of FGFR3:TACC3 and FGFR3 on cellular mechanisms in glioblastoma cell line. Proliferation, migration, colony formation and survival of these cells was assessed with different functional tests *in vitro*. Furthermore, inhibition of FGFR and its effect on cellular mechanisms was investigated.

Aim was to see whether our cell model behave similarly as those studied earlier by Parker et al (2013) and to evaluate if these cells could be used as a model for FGFR3:TACC3 fusion. Also, we wanted to gain better understanding on oncogenic effect of FGFR3:TACC3 fusion and on cellular responses that are induced by the inhibition of FGFR3 in fusion positive cells. Earlier studies have shown fusion to be promoting cell proliferation and anchorage independent growth, but other cell mechanism that might be enhanced by fusion have not yet been evaluated.

2. GLIOBLASTOMA

Cancer formation is believed to be process with multiple steps that includes several oncogenic alterations that drive transformation of normal cells into more malignant cell. Tumorigenesis begins when a single cell starts to divide abnormally due to changes in its DNA and forms a population of tumor cells. Tumor progression continues when cells go through additional genetic alterations and some cells gain selective advantage with superior growth, survival and invasion abilities. (Cooper 2000)

Hanahan & Weinberg (2000) suggested that similar molecular mechanisms lead to progression of cancer and listed hallmarks of cancer similar to all cancer types. These hallmarks are shown in Figure 1. According to their research most if not all cancer cells can produce their own growth signals, avoid growth-inhibitory signals from suppressor cells, acquire resistance to apoptotic signals, replicate endlessly, develop angiogenesis inducing capabilities and invade other tissues and therefore form metastases and these were referred as hallmarks of cancer. (Hanahan & Weinberg, 2000)

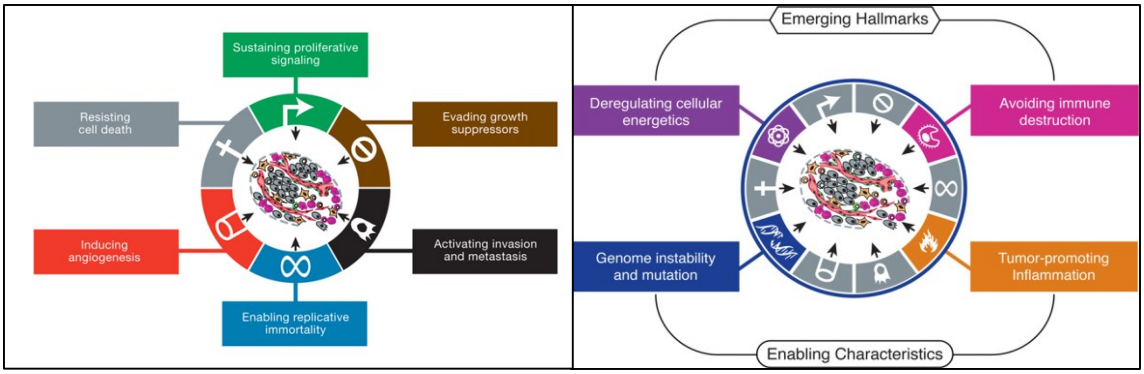

Figure 1. *Hallmarks of cancer suggested in 2000 (left image) and additional emerging hallmarks of cancer suggested in 2011 with addition of two enabling characteristics (right image) (Hanahan & Weinberg, 2011)*

Later due to a progress in research Hanahan & Weinberg (2011) added two additional hallmarks; cancer cells can alter cellular metabolism to support cancer proliferation and escape the effects of immune defense. Furthermore, they named two characteristics that enable and facilitate these hallmarks. Genomic instability allows mutations to alter cells more easily and inflammation promoted by tumor cells can support hallmark abilities. (Hanahan & Weinberg, 2011)

2.1 Cells of central nervous system

Brain tissue consist of neurons, which convey electrical stimuli to the target cells and glial cells, which are supporting cells of the nervous system. Neurons consist of dendrites,

soma and axon. Dendrites collect the signal from other neurons or cells and pass the signal to the cell body soma, from where one axon delivers the signal further to the central nervous system (CNS, *central nervous system*). Glial cells have many important functions in the nervous system. Glial cells release gliotransmitters that participate in modulation of synaptic efficiency of neurons and neuronal excitability (Newman, 2015). They work as supporting cells of the CNS, help to speed up the signal delivery and maintain the homeostasis of the brain. (Brodal 2004)

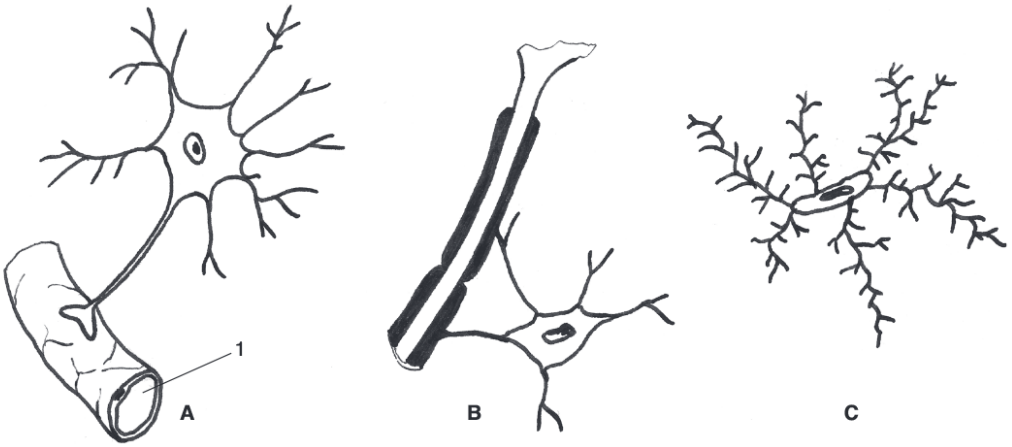

Figure 1. *Glial cells of central nervous system A. Astrocyte B. Oligodendrocyte C. Microglia. Number 1 shows the capillary of the brain. (Valenta et al., 2012)*

Glial cells are divided to three groups: astroglia or astrocytes, oligodendroglia or oligodendrocytes and microglia (Figure 1). Some infrequent types of glial cells can also be found in the CNS: ependyma cells covering the cavities of CNS, Müller cells found at the retina, Bergman cells found from the cerebellum and pituicytes found from the pituitary gland (Brodal 2004). Astrocytes and oligodendrocytes are large cells that arise from the neural tube. Astrocytes work as insulators of specific neurons. They also form the blood-brain barrier and take part to the regulation of different functions in the brain. Oligodendrocytes create myelin sheets around axons thus working as insulators around axons. Microglia are smaller cells that work as macrophages of CNS and are especially important after trauma or during diseases of CNS. (Valenta et al. 2012)

2.2 Formation of Glioblastoma Multiforme

Gliomas are primary tumors found in CNS that resemble glial cells. There are two types of gliomas; diffuse gliomas have ability to infiltrate other parts of the brain tissue and circumscribed growth gliomas do not have that ability. (Aldape et al. 2015) Diffuse gliomas can be classified by their histology and cells of origin as astrocytomas, oligodendrogliomas and oligoastrocytomas. Usually they consist of cells that resemble different types of CNS cells. They are the most common types of tumors in CNS. (Marumoto & Saya 2012)

Gliomas are graded according the World Health Organization (WHO) grading system to grades I–IV depending on how malignant tumor is (Marumoto & Saya 2012). Glioblastoma is the most malignant form of diffuse gliomas and it is graded as a grade IV tumor. (Louis et al. 2007). Grade IV tumors are the most aggressive form of brain tumors and they have the ability to invade different parts of the brain, which is why they are difficult to remove by surgery. Also grade II and III tumors are diffusive and can infiltrate surrounding tissues. Grade I tumors are much less aggressive than other grades. (Ray 2010, Chapter 2) In 2016 WHO updated their grading system by adding some tumor type specific molecular changes seen in brain tumors to the classification. WHO grading system for selected tumors of CNS is shown in table 1. (Louis et al., 2016)

Table 1. *WHO grading system for selected tumors of CNS (Louis et al., 2016)*

WHO grades of select CNS tumours	
Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	II
Anaplastic astrocytoma, IDH-mutant	III
Glioblastoma, IDH-wildtype	IV
Glioblastoma, IDH-mutant	IV
Diffuse midline glioma, H3 K27M–mutant	IV
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	II
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	III
Other astrocytic tumours	
Pilocytic astrocytoma	I
Subependymal giant cell astrocytoma	I
Pleomorphic xanthoastrocytoma	II
Anaplastic pleomorphic xanthoastrocytoma	III
Ependymal tumours	
Subependymoma	I
Myxopapillary ependymoma	I
Ependymoma	II
Ependymoma, *RELA* fusion–positive	II or III
Anaplastic ependymoma	III
Other gliomas	
Angiocentric glioma	I
Chordoid glioma of third ventricle	II
Choroid plexus tumours	
Choroid plexus papilloma	I
Atypical choroid plexus papilloma	II
Choroid plexus carcinoma	III
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	I
Gangliocytoma	I
Ganglioglioma	I
Anaplastic ganglioglioma	III
Dysplastic gangliocytoma of cerebellum (Lhermitte–Duclos)	I
Desmoplastic infantile astrocytoma and ganglioglioma	I
Papillary glioneuronal tumour	I
Rosette-forming glioneuronal tumour	I
Central neurocytoma	II
Extraventricular neurocytoma	II
Cerebellar liponeurocytoma	II
Tumours of the pineal region	
Pineocytoma	I
Pineal parenchymal tumour of intermediate differentiation	II or III
Pineoblastoma	IV
Papillary tumour of the pineal region	II or III
Embryonal tumours	
Medulloblastoma (all subtypes)	IV
Embryonal tumour with multilayered rosettes, C19MC-altered	IV
Medulloepithelioma	IV
CNS embryonal tumour, NOS	IV
Atypical teratoid/rhabdoid tumour	IV
CNS embryonal tumour with rhabdoid features	IV
Tumours of the cranial and paraspinal nerves	
Schwannoma	I
Neurofibroma	I
Perineurioma	I
Malignant peripheral nerve sheath tumour (MPNST)	II, III or IV
Meningiomas	
Meningioma	I
Atypical meningioma	II
Anaplastic (malignant) meningioma	III
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma	I, II or III
Haemangioblastoma	I
Tumours of the sellar region	
Craniopharyngioma	I
Granular cell tumour	I
Pituicytoma	I
Spindle cell oncocytoma	I

There are two forms of GBM, primary and secondary form. Primary form is more general with older patients and secondary with under 45-year-old patients. Primary GBM is more common since approximately 90 % of GBM are primary. (Wilson et al. 2014) Secondary GBM develops from lower grade diffuse astrocytomas and primary tumor occurs *de novo* meaning there is no earlier symptoms or sign of lower grade glioma (Marumoto & Saya 2012). Classifying and treatment of GBM is challenging because it can take many different forms like small cell, large cell, spindle cell or gemistocytic forms. (Ray 2010)

2.3 GBM treatment and prevalence

Glioblastoma is aggressive tumor with high vascular proliferation, necrosis and pleomorphism (cell sizes and shapes may vary) (Marumoto & Saya 2012). Standard treatment for GBM is first surgery and then radiation therapy alone or together with chemotherapy, usually temozolomide (Nicolaidis 2015). Despite the treatment, patient survival with GBM is low and median life expectancy after the diagnosis is 12 to 14 months. GBM has a poor response on chemotherapy. All grade II–IV brain tumors including GBM are diffusive by nature that makes them impossible to get the whole tumor removed with surgery (Aldape et al. 2015). Often tumor is located in the areas of brain that control speech, motor functions or senses, which makes the surgery even more difficult (Davis, 2016). Combination treatment with radiation and temozolomide has shown significantly better survival results, with median of 2,5 months longer survival (Stupp et al., 2005). Radiation combined with temozolomide is therefore widely used all over the world.

Other therapies including radioimmunotherapy, iodine-125 brachytherapy, stereotactic radiosurgery and hyperfractionation have also been tried as a treatment for GBM, but results of these treatments have not shown better survival of the patients. Treatments targeting different molecular changes like inhibitors of tyrosine kinases and signal transduction are in clinical trials but also these have failed to provide significant survival benefit in GBM patients. (Davis, 2016)

3. MOLECULAR MECHANISM OF GBM

Gliomagenesis is derived by many molecular and genetic changes that will alter signaling pathways leading to the growth and progression of glioblastoma. Also, some epigenetic changes have been observed in glioblastoma and DNA methylation is most studied of those changes. Different changes in methylation of genes related to the control of cell cycle, suppression of tumor progression, repairing DNA and inhibiting apoptosis for example have been observed. Of genetic changes, alterations of growth factor tyrosine kinase pathways are one of the key factors in gliomagenesis. (Crespo et al., 2015) Some of the genetic alterations frequently seen in GBM are introduced in this chapter.

3.1 Overexpression of growth factor receptors in GBM

Epidermal growth factor receptor (EGFR, *epidermal growth factor receptor*) is expressed in brain during the whole brain development taking part in proliferation, differentiation, migration and survival of cells of the CNS (Nicholas et al., 2006). EGFR gene is frequently amplified, mutated or rearranged in GBM. Highly observed activity of EGFR signaling pathway can be caused by overexpression of receptor and its ligand or by constitutive activation of a receptor even without the ligand binding due to mutation or by genomic amplification of EGFR (Huang, Xu, & White, 2009). These will lead to uncontrolled phosphorylation of EGFR making it oncogenic in gliomas. (Hatanpaa et al., 2010)

Platelet derived growth factor receptor (PDGFR, *platelet derived growth factor receptor*) and its ligand are often overexpressed in GBM. Alterations of PDGFR are believed to be early events in gliomagenesis followed by changes in other parts of the signaling pathways. (Crespo et al., 2015) Amplifications and rearrangements of PDGFR as well as overexpression of its ligand have been characterized by the Cancer Genome Atlas (TCGA, *the Cancer Genome Atlas*) (Verhaak et al., 2010). Results of animal studies have suggested that alteration of PDGFR could be oncogenic driver for converting neural stem cells to tumor cells (Rheinbay et al., 2013).

Tyrosine protein kinase MET, (MET, *tyrosine protein kinase*) also known as hepatocyte growth factor receptor is also frequently or overexpressed in GBM and also in many other cancers. MET overexpression is suggested to promote malignancy of GBM and to promote the cancer progression if EGFR is inhibited which could explain treatment resistance of GBM to inhibition (Xu et al., 2012). MET overexpression is also found to be corresponding to aggressiveness of gliomas since it is only seen in higher grade gliomas and GBM. (Kwak et al., 2015) Amplifications of MET are also frequently seen in glioblastoma. Most of the tumors harboring MET amplification are shown to harbor also EGFR alterations. (Brennan et al., 2013)

Fibroblast growth factor receptor (FGFR) is a tyrosine kinase receptor that participates to cell proliferation, angiogenesis, migration and survival. FGFRs have recently been uncovered as oncogenic drivers in many cancers and are studied as a potential target for inhibition therapies. Subset of GBMs was found to harbor fusion of FGFR1 or FGFR3 with transforming acidic coiled coil (TACC, *transforming acidic coiled coil*). (Dienstmann et al., 2014) Structure and signaling of FGFR and its fusion are explained more specifically in chapter 4.

3.2 IDH mutation in GBM

Isocitrate Dehydrogenases (IDH) are family of isoenzymes IDH1, IDH2 and IDH3 that are encoded by five genes. IDH3 is found from the matrix of mitochondria and catalyzes transformation of isocitrate to α-ketoglutarate (α-KG, *α-ketoglutarate*) in citric acid cycle, converting NAD+ (NAD, *Nicotinamide adenine dinucleotide*) to NADH at the same time. While IDH3 reactions are irreversible, IDH1 and IDH2 catalyze reversible oxidative decarboxylation reactions outside of citric acid cycle. They are also transforming isocitrate to α-KG producing NADPH (NADPH, *Nicotinamide adenine dinucleotide phosphate*) from NADP+ at the same time. IDH1 is located in peroxisomes found from cytoplasm and its expression is highest in liver cells. It takes part in glucose sensing and metabolism of lipids. IDH2 is found from mitochondria, where it regulates citric acid cycle. It is highly expressed in muscle and heart tissue as well as activated lymphocytes. IDHs are also thought to maintain redox state in the cells and therefore defending cells against oxidative stress. (Cohen, Holmen, & Colman, 2013; Reitman & Yan, 2010)

IDH1/2 are known to be mutated in most of the low-grade gliomas but also in secondary higher-grade gliomas. Amino acid residue 132 is mutated in all IDH1 mutations in gliomas and approximately 85 % of those contain a heterozygous missense mutation changing arginine to histidine. The mutation prevents IDH1 binding to isocitrate, because mutation happens in the active site of the enzyme. When isocitrate cannot bind to IDH1, levels of α-KG and NADPH are reduced in the cells and cells are more sensitive to effects of reactive oxygen species. (Cohen et al., 2013) IDH2 mutation happens at residue 172 exactly like mutation at residue 132 in IDH1. Mutation of residue 140 has also been found in IDH2, but it has not been seen in gliomas. (Reitman & Yan, 2010)

IDH1 and IDH2 mutations are mutually exclusive in gliomas and they are seen early on in tumor progression. Approximately 80 % of grade II-III gliomas and secondary GBMs harbor IDH1 mutation, some are harboring IDH2 mutation and small subset that harbor wild type IDH (Cohen et al., 2013). It has been shown that while mutation of IDH is reducing α-KG in the cells it is also producing more 2-hydroxyglutarate (2-HG). It is now thought that productions of 2-HG could be the initiating oncogenic incident. (Yen & Schenkein, 2011)

IDH1 mutation is more common in GBM than IDH2. IDH1 mutation is often found in secondary GBM and usually not found in primary GBM Therefore, mutation on IDH1 is shown to be reliable genetic marker for secondary glioblastoma since in 95% of the patient's mutation status of IDH1 corresponds to clinical diagnosis. (Nobusawa et al., 2009; Oh et al., 2016) In the treatment of gliomas IDH mutation is important prognostic factor since lower grade gliomas that harbor wild type IDH are behaving almost as aggressively as GBMs. Tumors with IDH mutation show better response to chemotherapy than IDH wild type tumors, so mutation is also thought to be predictive treatment marker. (Karsy et al., 2017)

In 2016 WHO classification system of brain tumors was updated so that the clinical evaluation of tumors previously done histologically should now include molecular changes seen in tumors. IDH mutation is one of the main molecular markers for classification of gliomas and its expression is histologically evaluated in all tumor samples. Figure 2 shows diagram for classification of gliomas, where IDH status is the defining the characterization. (Louis et al., 2016)

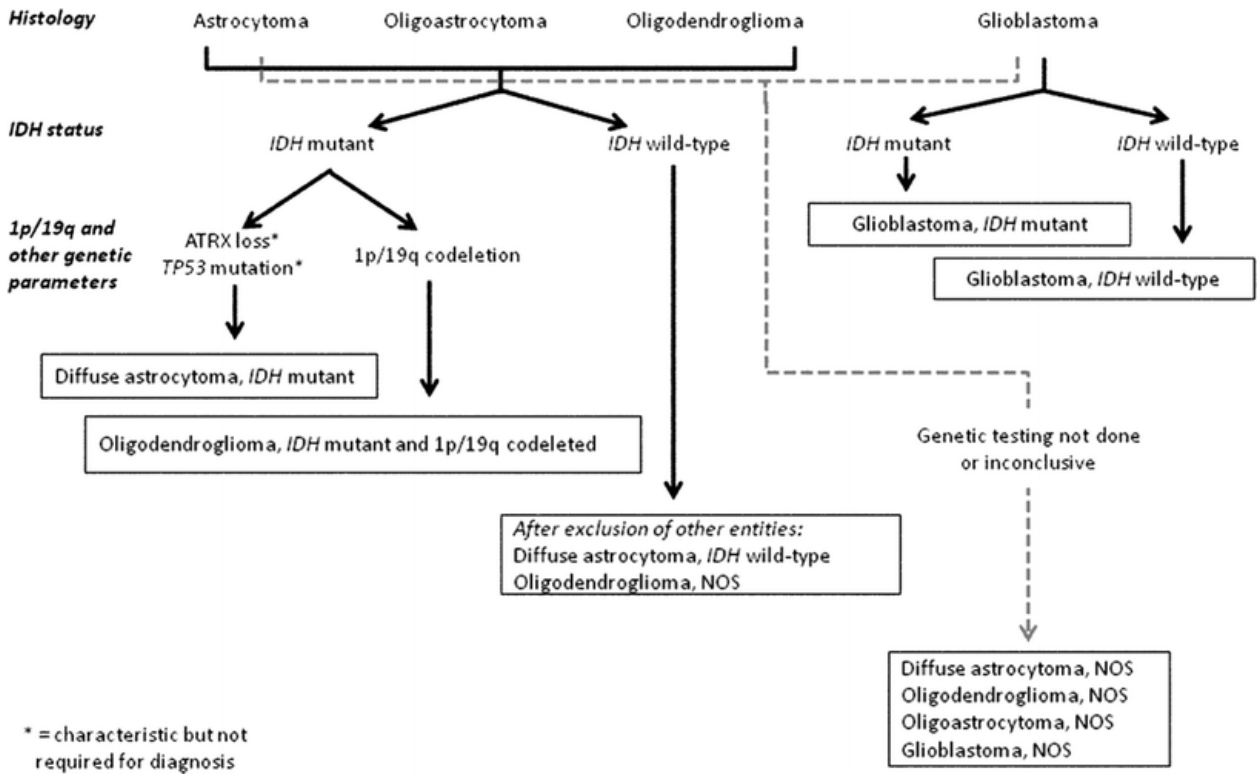

Figure 2. *Classification of gliomas after 2016 update on WHO classification (Louis et al, 2016).*

IDH mutation has shown to be mutually exclusive with FGFR3:TACC3 fusion. Fusion seems to be present only in subset of lower grade gliomas with IDH wild type as well as

IDH wild type GBMs. Brain tumors with IDH wild type have significantly poorer prognosis than IDH mutated tumors suggesting that FGFR3:TACC3 fusion might be one cause increased tumor aggressiveness. (Di Stefano et al., 2015)

3.3 Other genetic alterations frequently seen in GBM

Phosphatase and Tensin Homolog Deleted from Chromosome 10 (PTEN, *Phosphatase and Tensin Homolog Deleted from Chromosome 10*) is a tumor suppressor gene that is mutated in many different cancer types. It is more frequently mutated in higher grade tumors including GBM. PTEN does not need to be mutated at the initiation of tumor but is thought to be important factor in progression of tumor to more malignant form. PTEN is known to be negative regulator of PI3K signaling pathway and wild type PTEN has the ability to suppress tumor progression of glioma cells (Fan et al., 2002). In GBM mutation of PTEN and loss of heterozygosity of chromosome 10 (LOH10q, *loss of heterozygosity 10q*) that will alter PTEN have been seen. PTEN also takes part to G1 cell cycle arrest and apoptosis in GBM. (Koul, 2008)

Tumor suppressor gene retinoblastoma (RB, *retinoblastoma*) has central role as a cell cycle regulation taking part to G1 checkpoint. RB and its downstream pathway are also participating differentiation, proliferation, apoptosis, maintaining chromosomal stability and regulating cell cycle arrest. It can bind to various transcription factors inhibiting or promoting the transcription. RB also participates on control of enzymes that remodel chromatin structure and regulate expressions of target genes. (Burkhart & Sage, 2008) RB gene alterations in GBM include mutations or deletions of gene as well as methylation of promoters and alterations on regulators of RB pathway (Crespo et al., 2015).

Tumor protein 53 (TP53, *tumor protein 53*) is a transcription factor that binds to DNA activating transcription or modulating other transcription proteins. It is affecting many cellular processes including cell cycle regulation, cell death, differentiation and cell response to damages in DNA (Shangary & Wang, 2009). It can promote DNA repair during cellular stress or promote cell death if damage is impossible to repair. This way TP53 prevents cells with damaged DNA from dividing inhibiting tumor formation and progression. (Crespo et al., 2015) Amplification and mutation of TP53 are seen in GBM as well as overexpression of its negative regulator MDM2 (E3 ubiquitin protein ligase) and some other alterations that will inhibit the activity of TP53. Some genetic alteration of 53 or a member of its downstream pathway has been detected by TCGA at 87 % of glioblastomas (TCGA Research Network, 2008).

Cyclin dependent kinase inhibitor 2A (CDKN2A, *cyclin dependent kinase inhibitor 2A*) is a tumor suppressor gene that is frequently mutated in different cancers. CDKN2A encodes tumor suppressor protein p16 and takes part to cell cycle control in the RB pathway. (Harland, M. 2012) Homozygous deletion of CDKN2A is seen in approximately 50 % of glioblastomas. (TCGA Research Network, 2008). Loss of CDKN2A leads to increased

proliferation and disrupts the control mechanisms of apoptosis. It is mainly seen in higher grade (III-IV) gliomas and is suggested to be one reason for more aggressive tumor formation. (Reiss et al., 2015)

3.4 Molecularly different primary and secondary GBM

Histologically primary and secondary GBMs are very similar but they have some genetic and molecular differences. Many genetic alterations can be found on both types of GBMs, but some are more common in one type than the others. Figure 3 summarizes some alterations on both types of GBMs. Isocitrate dehydrogenase (IDH, *isocitrate dehydrogenase)* mutation is one of the main mutations separating primary and secondary GBMs since it is mainly present in secondary glioblastoma (Louis et al., 2016). IDH wild type GBMs are mostly exhibiting genetic and molecular changes that are known in primary glioblastoma. Gain of chromosome 7 and loss of chromosome 10 as well as EGFR amplification or mutation are commonly seen in primary GBM (Aldape et al., 2015). PTEN, that is also localized in 10q is frequently lost or mutated in primary GBM (Crespo et al., 2015). Other important characteristics for primary GBM are homozygous deletion of CDK2N2A and CDKN2B. (Marumoto & Saya 2012; Wilson et al. 2014) Figure 3 lists some mutations that are commonly seen in primary and secondary GBMs.

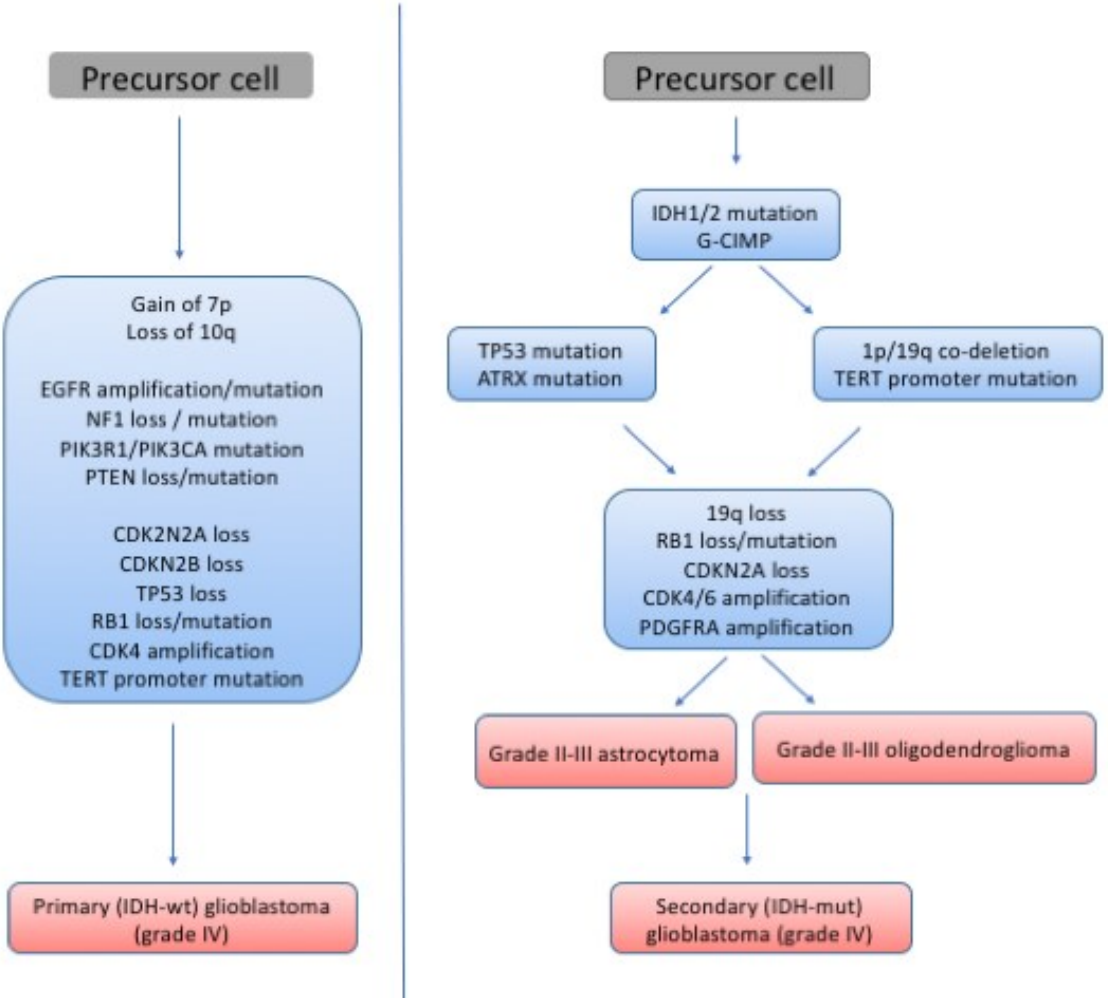

Figure 3. *IDH1/2 mutation is one of the most important mutation separating primary and secondary GBMs. Other commonly seen mutations in IDH wild type and IDH1/2 mutated GBMs are also shown. Modified from* (Aldape et al., 2015)

For IDH1/2 mutant glioblastoma, first alteration after IDH mutation is hypermethylation of CpG islands (G-CIMP). After that there seems to be two alternative pathways for secondary glioblastoma development. Subset of secondary GBMs harbor mutations of TP53 and ATRX and other subset show co-deletion of 1p and 19q as well as TERT mutations. (Aldape et al., 2015) Other common alterations in secondary GBM are 19q loss (meaning the loss of chromosome 19) (Figure 3) and LOHs of 13q and 22q (Wilson et al. 2014; Crespo et al., 2015).

3.5 Different subtypes based on molecular markers

Different classification of GBM based on genetic and molecular changes has been suggested (Crespo et al., 2012; Freije et al., 2004; Phillips et al., 2006). Studies have aimed

on finding alterations that could also provide knowledge of disease progression and clinical outcome. Most comprehensive analysis on genetic alterations was collected by TCGA. They published mRNA data from 206 GBMs and sequencing data of over 600 genes from 91 GBMs that showed alterations of tyrosine kinase receptor pathways on 88 % of GBM, TP53 signaling pathway alterations on 87 % of GBM and alterations on RB pathway on 78 % of GBM. (TCGA Research Network, 2008)

According to TCGA data GBMs were divided to four different subtypes based on their genetic alterations and changes in the copy numbers: classical, mesenchymal, proneuronal and neural type. Classical subtype is characterized by some of the most common genetic alterations in GBM. EGRF amplification was seen in 95 % of classical subtype GBMs, 93 % showed chromosome 7 amplifications and deletions on chromosome 10 and 95 % of samples harbored homozygous deletion at Ink4a/ARF locus. Mesenchymal subtype is identified with high expression on CHI3L1 and MET as well as NF1 mutation or deletion. Mesenchymal subtype seems to be expressing more Schwann cell markers and microglial markers than other subtypes. Proneural subtype harbors more alterations on PDGFRA as well as IDH1 and TP53 mutations. These are same markers that have been seen in secondary glioblastoma, and most of the secondary GBM are classified as proneural subtype. Neural subtype expresses highly some neuronal markers like NEFL, GABRA1, SYT1 and SLC12A5. Neural subtype has closest expression pattern to normal tissue in the brain. These subtypes also seem to be evaluating clinical outcome and response of GBM to treatment. (Verhaak et al., 2010)

DNA methylation alterations are widely present in gliomas and glioma-CpG Island Methylator Phenotype (G-CIMP) has been characterized in subset of gliomas (Noushmehr et al., 2010). Ceccarelli et al. (2016) performed large genomic analysis of diffuse gliomas and suggested updating the subtyping of gliomas based on their IDH mutation status and G-CIMP profile. Many different classifications for subtyping gliomas has been suggested and their relevance has also been widely questioned. More research is still needed for clear understanding of molecular alterations defining gliomas.

4. FUNCTION OF FGFR AND ITS MUTATION

Signaling pathways of fibroblast growth factor receptor (FGFR) have proven their importance in proliferation, migration, angiogenesis and survival of cells. FGFR pathway can have supplemental effect on vascular endothelial growth factor (VEGF, *vascular endothelial growth factor*) and platelet derived growth factor (PDGF, *platelet derived growth factor*) pathways. (Lieu et al. 2011) Aberrations of FGFR have also shown to act as initiating oncogenes in some cancers. Gene amplification, mutations, translocations leading to fusion genes, alternative splicing of FGFR and overexpression of fibroblast growth factor (FGF, *fibroblast growth factor*) have been observed in different cancers including breast-, lung-, gastric- and ovarian cancer and glioblastoma. (Dienstmann et al. 2014)

4.1 FGFR/FGF signaling pathway

FGFRs include four transmembrane tyrosine kinase receptors that bind FGF glycoproteins. There is also fifth receptor called FGFRL1 (FGFRL1, *Fibroblast growth factor receptor like one*) that can also bind FGF, but it doesn't have the tyrosine kinase domain and therefore participates to different cell functions (Dieci et al. 2013). FGFRs are formed of three different domains: extracellular-, transmembrane- and cytoplasmic domain. Ligand binding happens in the extracellular domain that consists of immunoglobulin parts (IgI, IgII, IgIII) and acidic box formed by eight acidic residues (Figure 4). IgII and IgIII are mainly responsible for ligand binding and IgI and acidic box produce auto-inhibitory functions of the receptor. Due to the alternative splicing IgIII can also take isoforms IgIIIb and IgIIIc that can bind different forms of ligands. Transmembrane- and cytoplasmic domains are comprised of juxta-membrane- and split kinase domains and a tail of carboxy terminal. (Dienstmann et al. 2014)

FGFs are signaling molecules and their family consist of 22 members that have similar molecular structure. They are divided to hormone-like FGFs and canonical FGFs (Dieci et al. 2013). They are mostly secreted out of the cells, but transportation of FGF-1 and FGF-2 is not yet understood. FGFs have interactions with heparin sulfate proteoglycans (HSPG, *heparin sulfate proteoglycans*) at the cell surface that control the binding of them to FGFR (Figure 4). (Lieu et al. 2011) FGFs take part in embryogenesis by taking part on regulation of cell migration and differentiation as well as proliferation. In developed tissues FGF participates in the regulation of nervous system, tissue and wound repair and angiogenesis during tumor formation. (Eswarakumar, Lax, & Schlessinger, 2005)

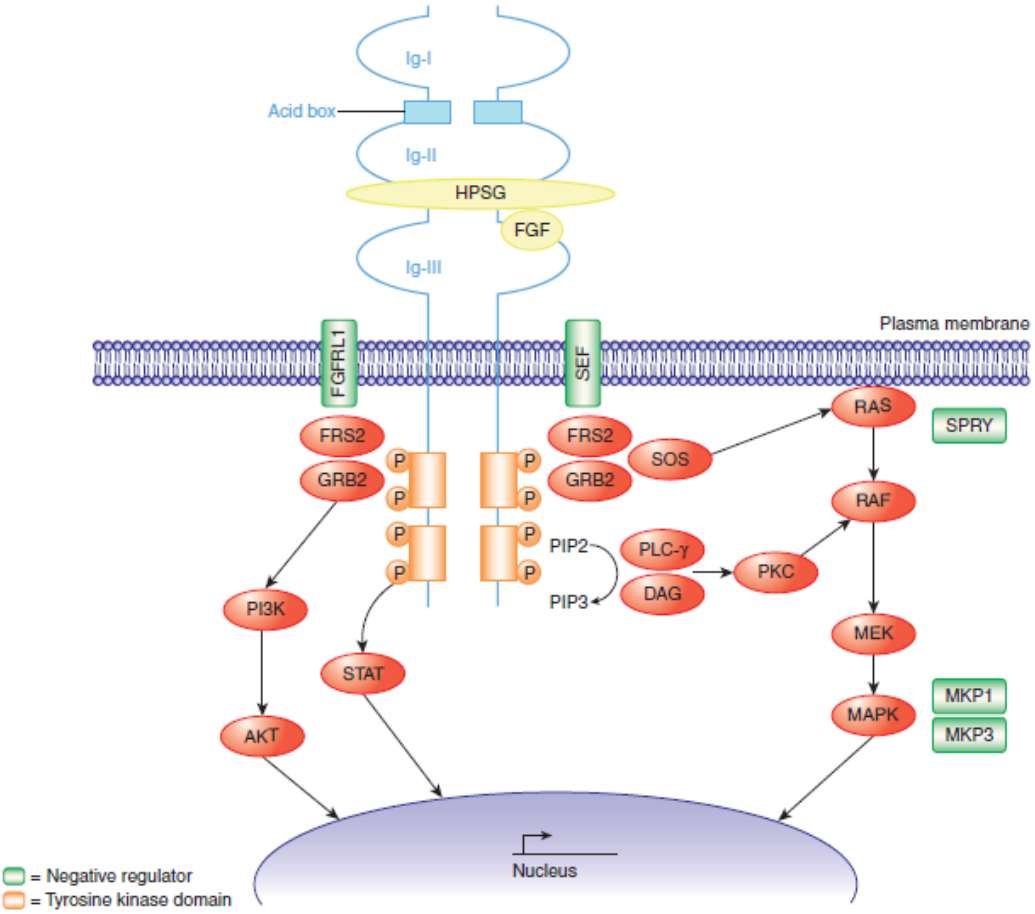

Figure 4. *Structure of FGFR and its downstream signaling pathways RAS/MAPK and PI3K. MAPK phosphatases 1 and 3 (MKP1, MPK3), FGFRL1, Spotry proteins (SPRY) and similar expression to FGF (SEF) can work as a negative feedback for FGFR downstream signaling. (Image from article Dieci et al. 2013)*

Figure 4 represents activation of FGFR and its downstream signaling pathways. Once the FGFR is activated it phosphorylates FGFR substrate 2 (FRS2, *fibroblast growth factor receptor substrate 2*) and growth factor receptor-bound 2 (GRB2, *growth factor receptor-bound 2*). Those activate Ras Sarcoma (RAS) G-protein that will send a signal to the nucleus with the help of mitogen-activated protein kinase (MAPK, *mitogen activated protein kinase*) pathway. Another signaling route activated by FGFR is phosphoinositide 3-kinase (PI3K, *phosphoinositide 3-kinase*) pathway. Phospholipase C-y (PLC-y) and signal transducer and activator of transcription (STAT) can also be activated by FGFR without the help of FRS2 (Ornitz & Itoh, 2015). They can start the cascade leading also to MAPK pathway. (Dieci et al. 2013) FRS2 also takes part to negative regulation of FGFR signaling therefore balancing translation of FGF signal. E3 Ubiquitin ligase protein Cbl can form complex with GRB2 bound to FRS2. This results in ubiquitination of both FGFR and FRS2 blocking the signaling of FGF. (Eswarakumar, Lax, & Schlessinger, 2005).

4.1.1 RAS/MAPK pathway (ERK)

After autophosphorylation of FGFR, adaptor protein GRB2 binds to FRS2 and then links RAS/MAPK signaling pathway to FGFR with the help of guanine nucleotide releasing factor sons of sevenles (SOS, *sons of sevenles*) protein. GRB2 bind to FRS2 with its SH2 domain and to SOS protein with SH3 domain. (Kouhara et al., 1997) SOS binds to RAS creating the activation of a protein kinase cascade that finally proceeds to the activation of MAP kinase. First RAS binds to a threonine/serine kinase RAF (RAF, *rapidly accelerated fibrosarcoma*), which then binds and phosphorylates mitogen activated protein kinase (MEK, mitogen activated protein kinase kinase). Finally, MAPK is phosphorylated by MEK. When MEK binds to MAPK it exposes tyrosine residue that is not exposed in inactive form of MAPK. MEK phosphorylates that tyrosine and also threonine residue close by. These phosphorylated amino acids change the conformation of MAPK so that ATP (ATP, *adenosine triphosphate*) is not able to bind to catalytic site and specific binding sites for substrates are formed. (Lodish et al., 2000)

MAPKs are serine/threonine kinases that convert extracellular signals to different cellular responses. There are several different MAPK pathways that together regulate metabolism, mitosis, motility, apoptosis, survival and differentiation. Extracellular signal regulated kinases 1 and 2 (ERK, *extracellular signal regulated kinase*), c-Jun amino N-terminal kinases 1,2 and 3 (JNK 1,2 and 3) p38 isoforms and ERK5 are categorized as conventional MAPKs. Unconventional MAPKs comprise of ERK 3, 4 and 7 and nemo like kinase (NLK). ERK1/2 are activated by cell surface growth factor receptors and it has an important role in regulation of proliferation. MAPK family members further activate MAPK activated protein kinases which is a family of 11 members. Activation of those will finally result in responses on proliferation, differentiation, stress and inflammation for example. (Cargnello & Roux, 2011)

4.1.2 PI3K pathway

Another important downstream signaling pathway of FGFR is PI3K pathway that is controlling cells proliferation and survival of cells by blocking apoptosis. After phosphorylation of FGFR and FRS2 and GRB2 binding, GRB2 binds to PI3K with the help of GRB associated binding protein 1 (GAB1, *GRB associated binding protein 1*). (Ornitz & Itoh, 2015) PI3Ks are lipid kinases and they participate for example to cell survival, proliferation and differentiation via different signaling molecules. PI3Ks generate phospholipids called phosphatidylinositol (3,4,5)-triphosphate ($PI(3,4,5)P_3$, *phosphatidylinositol (3,4,5) - triphosphate*), which will then activate protein kinase B (AKT, *protein kinase B*). AKT is a serine/threonine kinase that is found in three different isoforms, AKT1, 2 and 3. AKT can then phosphorylate many different proteins like glycogen synthase kinase 3 (GSK3), the forkhead family of transcriptome factors (FOXOs) and mechanistic target of rapamycin (mTOR, *mechanistic target of rapamycin*). These proteins can regulate processes like

protein synthesis, metabolism and survival. MTOR is strongly associated with cell proliferation, since it is regulating availability of nutrients, energy and nutrient levels and mitogenic signals. (Liu et al., 2009)

PTEN, an important tumor suppressor gene that is mutated in many cancers including glioblastomas, is regulating PI3K pathway activity in normal cells. PTEN can work as a tyrosine phosphatase as well as lipid phosphatase in cells. It is capable of eliminating 3' phosphate from PI(3,4,5)P3 changing it to PI(4,5)P2 and therefore antagonizing activation of AKT on PI3K pathway. AKT participates in regulation of tumor growth and angiogenesis as well as cell survival (Koul, 2008). Loss of tumor suppressor PTEN is leading to cancer through uncontrolled signaling of PI3K pathway. (Liu et al., 2009)

4.2 FGFR3:TACC3 Fusion

Fusion genes are hybrid genes of two genes that have rearranged and fused together. They are found in many cancer and can work as oncogenic drivers or produce oncogenic fusion proteins in cancers (Dienstmann et al., 2014). FGFR3 fusion with TACC3 (TACC3, *transforming acidic coiled-coil containing protein 3*) was first found in glioblastoma and later also in bladder cancer (Parker et al., 2013; Singh et al., 2012; Williams et al., 2013). Since then other FGFR fusions have been found also in lung, thyroid, oral and prostate cancers. (Parker et al., 2014)

Fusion genes can be formed by intrachromosomal or interchromosomal rearrangements meaning that genes are originally located in same chromosome or different chromosomes. Tandem duplication and deletion are intrachromosomal fusion formation methods. Fusion of FGFR3 and TACC3 happens by intrachromosomal tandem duplication on 4p16.3 which means that before the rearrangement TACC3 is located upstream of FGFR3 but after fusion their order is reversed (Figure 5). Deletion happens when part of the DNA between two genes is deleted. This way rearrangement of two fused genes stays same as it was before the fusion. Translocation is an interchromosomal mechanism that happens when double stranded DNA break occurs and strands are rearranged and joined together differently than before the break. (Parker et al., 2014)

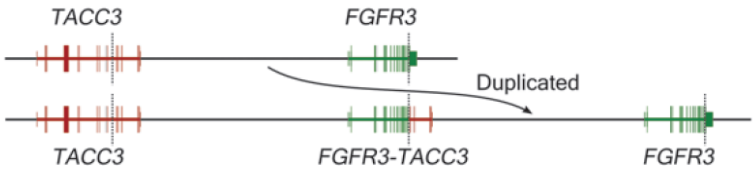

Figure 5. *Formation of tandem duplication on FGFR3:TACC3* (Parker et al., 2013).

Fusion happens primarily so that IIIc isoform of FGFR3 breaks at exon 18 fusing with smaller portion of TACC3 that can break at different exons. Different types of FGFR3 fusions with TACC3 are shown in Figure 6. However, diversity between breakpoints in

different tumors has been shown and length changes on FGFR3 side have also been seen. Some fusion samples are shown to be formed so that breakpoint is situated downstream of FGFR3. Two genes are transcribed as so called read through transcripts and then through splicing terminal exon of FGFR3 is removed. This will lead to transcript of two genes fused together (Akiva et al., 2005). FGFR3:TACC3 fusion leads to the loss of 3'UTR of FGFR3. Usually in normal brain and in GBM without fusion expression of FGFR3 is low due to a negative control of miR-99a binding to the UTR region. miR-99a is regulating FGFR3 expression and this is why in GBMs with FGFR3:TACC3 fusion FGFR3 expression is enhanced. (Parker et al., 2013; Parker et al., 2014)

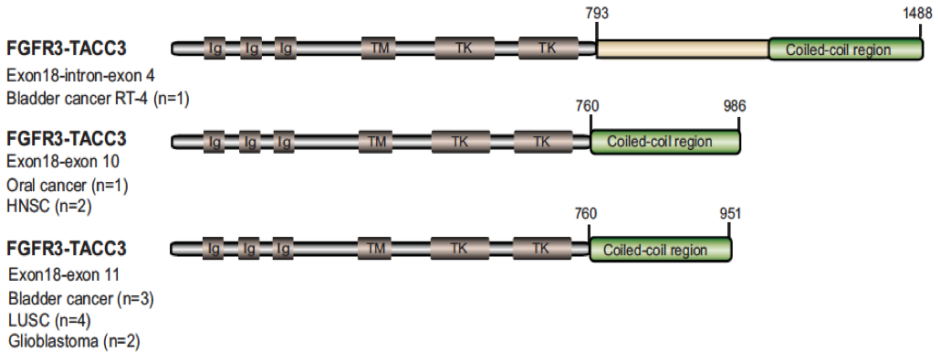

Figure 6. *Different ways FGFR3 can fuse with TACC3* (Wu et al., 2013).

Like discussed in chapter 2.3, in GBM genes PDGFRA, MET and EGFR are often amplified. However, it has been proven that FGFR3-TACC3 fusion is mutually exclusive for these amplifications as well as alterations of TP53. Furthermore, FGFR3:TACC3 fusion seems to be expressed only in IDH wild type tumors (Di Stefano et al., 2015). These findings suggest FGFR3:TACC3 fusion might be initiating factor for GBM providing alternative pathway for tumor formation. In cellular level FGFR3:TACC3 fusion is promoting proliferative capability and anchorage independent growth of the cells therefore accelerating tumor progression compared to wild type FGFR3. Fusion has also shown to promote cell transformation and to increase aneuploidy. (Parker et al., 2013; Singh et al., 2012)

FGFR3:TACC3 fusion positive glioblastomas are more often found on women and they seem to be more aggressive than fusion negative tumors. FGFR3:TACC3 fusion can be detected from tumor samples with FGFR3 recognizing antibody. Immunohistochemical staining of FGFR3 in tumor samples have been heterogeneous, showing that not all the cells in fusion positive GBM tumors express FGFR3:TACC3 fusion but some of the cells are negative for this fusion. (Granberg et al., 2017)

5. TARGETING FGFR3:TACC3 FUSION WITH INHIBITOR

Expression of FGFR3 is low on normal brain cells. As discussed in chapter 3.2 loss of UTR in FGFR3:TACC3 fusion leads to higher expressions of FGFR3 in fusion positive cells suggesting that inhibition of FGFR3 might cause smaller neurological effects due to lower impact of inhibition on normal cells. Development of inhibitors that could cross the blood brain barrier is challenging and administration straight to the cerebral spinal fluid could be the more effective for brain tumor treatment. This way inhibitor could avoid contact with other organs with higher levels of FGFR3. (Parker et al., 2014)

5.1 Anti FGFR drug for FGFR signaling inhibition

Different drugs are being developed for inhibition of FGFR. Small molecule tyrosine kinase inhibitors are already in clinical trials and commercially available. Monoclonal anti-FGFR antibodies and drugs that can trap FGF are also being developed. Tyrosine kinase inhibitors are targeting FGFRs intracellular tyrosine kinases. There are reversible inhibitors that occupy the ATP binding site of the tyrosine kinase and irreversible covalently binding inhibitors that bind to a cysteine residue of the tyrosine kinase (Katoh 2016). There are inhibitors available that are highly selective for FGFR but also those that are selective for also other tyrosine kinases like vascular endothelial growth factor receptor (VEGFR, *vascular endothelial growth factor receptor*) kinases. Multitarget inhibitors have shown better antitumor activities than selective inhibitors, but they have more VEGF related toxicity issues. Selective inhibitors have only FGFR related toxicity issues, which would make them a better candidate for combination treatments with other medicines. (Dieci et al. 2013)

FGF-signaling takes part on tumor development in many ways. FGFs take part in paracrine signaling, angiogenesis and immune evasion of tumor microenvironment. Studies have suggested that besides inhibiting effect on overexpression of FGFR in cancer cells, inhibitors could have effect on regulation of tumor microenvironment as well. Based on recent findings FGF signaling has also been suggested to be one reason for development of drug resistance in various cancers. Therefore, FGFR inhibitors are studied to block tumor progression in FGF altered tumors, to target angiogenesis in different types of cancers and to prevent resistance that cancer cells seem to acquire for targeted therapies. (Dieci et al. 2013; Katoh 2016)

5.2 Targeting FGFR3 with JNJ-inhibitor

JNJ-42756493 is orally administrated pan-FGFR tyrosine kinase inhibitor. It targets all members of the FGFR family and has low activity on other kinases like VEGFR. It inhibits phosphorylation of tyrosine kinases of FGFR by targeting their intracellular ATP-binding site. Its effective on concentrations at nanomolar level and it has a half-life of 50–60 h. (Tabarnero et al., 2015) Orally administered JNJ-42756493 has high tissue distribution to lung, kidney and liver tissues, but distribution to brain across blood-brain barrier is not well known. (Perera et al., 2014)

JNJ-42756493 has been studied *in vitro* in FGFR signaling dependent engineered and tumor cell lines where it has shown inhibition of FGFR signaling and proliferation. Cells take inhibitor into lysosomal compartment rapidly and possibly due to its slow release from those compartments it inhibits FGF signaling for prolonged times. Furthermore, JNJ-42756493 has shown tumor inhibitory effects both in patient derived cells and *in vitro* cell line established FGFR signaling dependent xenograft models. It is shown to decrease phosphorylation of FGFR end ERK which could lead to observed antitumor activity. (Perera et al., 2017; Tabernero et al., 2015)

JNJ-42756493 is currently on clinical phase II safety evaluation studies. Phase I studies have shown no toxicities that are related to VEGFR, like proteinuria or hypertension. In high doses, it will cause FGFR related hyperphosphatemia, but with lower or intermitted doses toxicity has been manageable. It has also shown preliminary tumor inhibitory results in patients diagnosed with FGFR alterations in their solid tumors. (Tabernero et al., 2015)

Di Stefano et al. studied JNJ-42756493 inhibition in FGFR3:TACC3 cells in vivo and vitro showing that cells with FGFR3:TACC3 fusion were highly sensitive to inhibition in contrast to cells with inactivated fusion or empty vector. Antitumor results were also seen on xenograft model harboring FGFR3:TACC3 fusion. They evaluated effects of orally administrated JNJ-42756493 inhibitor on FGFR3:TACC3 with two patients harboring the fusion. Both patients showed clinical improvement after JNJ-42756493 treatment as well as tumor size reduction. Both patient showed some toxicity symptoms; hyperphosphatemia, asthenia, dry mouth, and nail changes for example. However, all the symptoms were mild and manageable. (Di Stefano et al., 2015)

6. INTRODUCTION TO PROTEIN DETECTION

Western blotting is often used in cell and molecular biology for detecting specific proteins and their expression levels from cell samples containing mixture of different protein (Mahmood & Yang 2012). Other way to detect antibody depended proteins and their localization in cells samples is immunohistochemistry (Duraiyan et al. 2012). In this work, expression of FGFR3 and different downstream pathways of FGFR3:TACC3 fusion were evaluated using Western blotting method. Also, number of transfection positive cells was estimated using immunohistochemical staining methods. Principles of these methods are explained in this chapter.

6.1 Western blotting method

Western blotting is a method to separate and identify wanted proteins from complex mixtures of different proteins. First proteins are separated using SDS-PAGE (*polyacrylamide gel electrophoresis*, PAGE; *sodium dodecyl sulfate*, SDS), which is a protocol to separate different proteins according to their size, conformation and charge using electric field. Proteins are denatured with SDS before the run to make them negatively charged. Due to the negative charge, they will move towards the positive electrode. Smaller proteins move faster than larger proteins making separation by size possible. (Mahmood & Yang 2012)

In electrophoretic transfer proteins are transferred from the gel to the membrane using electric voltage as a driving force. Membrane will work as a solid support for the protein bands during detection. During the transfer membrane is placed between the gel and a positive electrode so that the electric field will move proteins from the gel to the membrane. Two different kind of membranes are available, nitrocellulose and PVDF (PVDF, *polyvinylidene fluoride*) membranes. Nitrocellulose membrane has high affinity for protein but it is more brittle than PVDF. PVDF membranes give better support for proteins but have higher background staining. (Mahmood & Yang 2012)

Membrane needs to be blocked before detection to prevent nonspecific binding of antibodies. Blocking will reduce background staining of the membrane. Blocking is usually done with 5 % BSA (BSA, *bovine serum albumin*) or nonfat milk. Detection of proteins of interest is done with protein specific primary antibodies and secondary label antibody with enzyme like HRP (HRP, *horseradish peroxidase*). Enzyme produces a signal that can be detected on a film. Protein bands can be evaluated and the thickness of the band relates to the amount of the protein in the sample. (Mahmood & Yang 2012)

6.2 Fast immunohistochemical staining

Immunohistochemistry (IHC, *immunohistochemistry*) is a technique to detect antibody specific antigens from tissue or cell samples. IHC can be used for example to detect over-expressed or tumor specific antigens in diagnosis of cancer. Tissue or cell samples are first fixed to preserve the cell and tissue structure and prevent the degradation (Alturkistani et al. 2016). Then tissue sections or cell samples are incubated in antigen specific primary antibody after which detection is done using label antibody linked with enzyme, fluorescent dye or some other detection molecule. Results can be visualized with light microscope or fluorescent microscope depending on used detection molecule. (Duraiyan et al. 2012)

Fast immunohistochemical staining protocols have been developed where antibodies are incubated in higher temperatures for faster reactions. These methods can be used during operations to detect specific tumor markers for example. (Kämmerer et al., 2001; Richter et al., 1999) For this study, protocol used was designed for FGFR3 antibody at 41.5 °C.

Staining in this study was done enzymatically using HRP enzyme and DAB (3,3 Diaminobenzidine) substrate. Enzyme linked to antibody is reacted with substrate to achieve color that can be visualized (Nakane & Pierce 1967). DAB is a chromogen typically used as a substrate for HRP enzymes. DAB creates dark brown staining that can be detected with light microscope. Hematoxylin is commonly used in histology as a counterstain for different stains like DAB and is bind to nuclei and nucleic acids of cells dying them blue. (Boster 2017)

7. INTRODUCTION FOR *IN VITRO* FUNCTIONAL TEST

Cell culture is easy and cost-effective way to study cellular mechanisms of different tissues and effects of different drugs on those mechanisms. In this study, different functional tests for cell lines were performed to assess differences between FGFR3:TACC3 fusion and FGFR3 overexpressed cell-lines as well as control cell lines. This chapter explains the basic principles behind those functional tests.

7.1 Viability and proliferation

There are several different methods to assess cell proliferation, viability and cytotoxicity. Most commonly used methods are created multi-well plate systems and can be measured with plate reader. Protease activity assays, tetrazolium reduction and reassuring reduction are the most common assays and are based on enzymatic or metabolic activity in the cells. In this study, we used tetrazolium reduction assay with MTT (MTT, *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) to detect the number of viable cells. (Riss et al., 2004; Stockert et al., 2012)

MTT is cell permeable and inside the cells NAD(P)H dependent oxidoreductase enzymes are converting MTT to a purple colored formazan product. Dead cells don't have this ability therefore making it possible to evaluate only number of viable cells. Inside the cells formazan stays as an insoluble substance until it is solubilized to stabile color. For instance, acidified isopropanol, SDS, dimethyl sulfoxide (DMSO, *dimethyl sulfoxide*) and dimethylformamide can be used to solubilize formazan. Purple formazan product has an absorbance maximum around 570 nm which can be measured with plate reader. Absorbance is dependent on the number of viable cells as well as their metabolic activity. Also, concentration of MTT and incubation time can have effect on the absorbance. (Riss et al., 2004)

There are some culturing conditions that may affect cells capability to reduce MTT to formazan. Changes in pH and loss of some nutrients on cells may reduce the ability as well as confluence of the cells. If cell growth becomes contact inhibited, cells may slow down their metabolism slowing down also the reduction of MTT. (Riss et al., 2004)

7.2 Colony Forming

Colony forming (or colonogenic) assay has been used over 50 years to determine differences between cell-lines or differently treated cells in their reproductive viability (Rafehi

et al., 2011). Assay is basically testing the number of cells in population capable to undergo unlimited division, so it evaluates single cells ability to form a colony. In all cell population only a fraction of cells has the ability to form colonies. Colony needs to have at least 50 cells to be included. (Franken et al., 2006)

Basically, cells are seeded as a single cell suspension to a plate where they will attach and start to divide. They can be treated differently to evaluate effect of radiation or cytotoxic agents for example. They are grown for 1–3 weeks after which formed colonies are stained and calculated. (Rafehi et al., 2011)

7.3 Migration of the cells

Migration is a cell mechanism that includes movement in any direction resulting in new position of the cell in tissues (Gotsulyak et al., 2014). Migration happens all the time, during embryonic development, wound healing and in our immune system for example. Migration plays also important role in cancer since migration of endothelium is a beginning of blood vessel formation in tumor tissue. Furthermore, cancer metastases are initially formed by tumor cells migrating to circulation and from there to new tissues. (Horwitz & Webb, 2003)

Wound healing assay is based on a scratch made on a confluent monolayer of cells. Scratch is imaged right after it is made and then after certain incubation time, when cells have started to migrate and fill the scratch. Assay measures and compares cells ability to migrate and fill the scratch. (Justus et al., 2014)

7.4 Apoptosis and necrosis

Apoptosis is a well-organized event in cells that leads to a morphological and biochemical changes and finally to cell death. Apoptosis is characterized by chromatin condensation, reduction in cell volume, nuclear fragmentation and membrane blebbing for instance (Atale et al., 2014). Apoptosis is essential for maintaining homeostasis in organisms as well as regulating some physiological processes. In normal cells PI3K pathway promotes cell growth and blocks apoptosis. Because in many cancers cell PI3K pathway is over activated it leads to inhibition of apoptosis and even higher proliferation of cancer cells. (Kalimuthu & Se-Kwon, 2013)

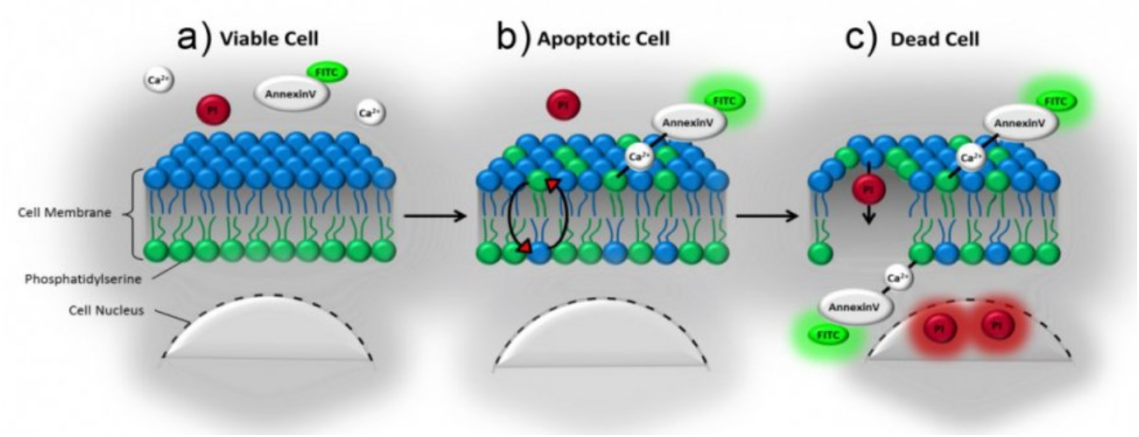

Figure 7. *Annexin V can bind to apoptotic cell that has phosphatidyl serine residues exposed to the outer leaflet of plasma membrane. In dead cells PM has already disrupted and PI can enter cell nucleus staining the cells. (Synentec, 2016)*

Annexin V – FITCH (fluorescein isothiocyanate) and PI (propidium iodide) double staining is a way to detect apoptotic cells from necrotic and viable cells by the differences in the permeability and integrity of their cell membrane (Rieger et al., 2011). Method of Annexin V – FITCH and PI binding on viable, apoptotic and necrotic cells are shown in figure 7. Loss of phospholipid membrane (PM, *phospholipid membrane*) asymmetry is one of the early apoptotic events in cells. Phosphatidyl serine residues are flipped to the outer leaflet of the PM and annexin V can interact with those residues in the presence of Ca^{2+}. In viable cells PM is intact and phospahtidyl serine located in the inner leaflet of PM therefore making it impossible for annexin V to bind. PI is red fluorescent dye that binds to DNA. It can only stain dead cells that have already disrupted PM so it can penetrate PM and transport to nucleus. Therefore, cells in apoptosis are positive for Annexin V staining, but negative for PI staining. Necrotic cells on the other hand are positive for both stains. Viable cells with intact PM are negative for Annexin V and PI. (Atale et al., 2014)

7.5 SNB19 Glioblastoma cell line

SNB19 glioblastoma cell line was used for all the experiments in this study. SNB19 cell line was derived in 1980 from a 47-year-old male patient from surgically removed tissue sample of his left parieto-occipital glioblastoma multiforme tumor. This cell line has maintained its transformation and differentiation capabilities as well as tumorigenesis even after 13 years of culture and over 200 passages. SNB19 cell line is adherent morphologically fibroblast like cell line that proliferates fast, has ability for anchorage independent growth and expresses high level of proteins. These cells have doubling time around 52 h, saturation density $7{,}8 \times 10^4$ cells/cm^2 and their colony forming efficiency in soft agar is 41,5 % (Gross et al., 1988). SNB19 cells can develop in vivo tumors that resemble the original tumor even after a long culture times and show all the same properties as GBM shows in vivo. (Welch et al., 1995)

8. MATERIALS AND METHODS

Glioblastoma SNB19 cell line had been transfected with vector containing either FGFR3:TACC3 fusion, FGFR3 or empty vector. Cells transfected with empty vector were used as control cells. Transfection had been done with the antibiotic resistant selectable marker. Then cells had been cultured with selection antibiotic (Geneticin, Gibco), so that unsuccessfully transfected or transiently transfected cells would die and stably transfected cells would survive. Stably transfected clones had been selected as colonies and transferred as single cells to be cultured in selection media to ensure their resistance to antibiotic. This stable transfection method includes transfected DNA to the cells genome making the transfection long lasting. Transfection efficiency was evaluated with Western blotting and immunohistochemically. Growth, survival, migration and invasion capabilities of the chosen cells were studied with functional testing and response of the cells to FGFR inhibitor and FGF stimulation was assessed.

8.1 Cell-line analysis using Western blot method

Western blotting protocol was used to analyze SNB19 cell-lines of which five had been transfected with FGFR3 containing vector, five with FGFR3:TACC3 containing vector, and eight controls that had been transfected with empty vector.

8.1.1 SDS-PAGE protocol

First four gels for SDS-PAGE were prepared. 10 % and 8 % gels were used for the running gel and 5 % gel for the stacking gel. Ingredients and their volumes are represented in table 2.

Table 2. *Ingredients and volumes used to prepare SDS-page gels*

Ingredient	Running gel 10%	Running gel 8%	Stacking gel 5%
Acrylamide (30 %) / 0,8 % Bis-Acrylamide (ml)	10	8	2,5
Water (ml)	12,2	14,2	10,3
1,5 M Tris-HCl, pH 8.8 + 0,4 % SDS (ml)	7,5	7,5	-
0,5 M Tris-HCl, pH 6.8 + 0,4 % SDS (ml)	-	-	1,9
10 % APS (µl)	300	300	160
TEMED (µl)	20	20	10

First 10 % running gel layers, next 8 % gel layers and finally at the top 5 % stacking gel layers were casted. Into the stacking gel layer combs were pushed to make the wells for samples. Between every layer, gels were allowed to solidify for 45 min. Gels were left to set on the fridge overnight.

Next day samples were diluted to H_20 so that each sample had the concentration of 1 mg/ml. Protein levels had been measured prior to these Western experiments using DC Protein Assay –kit (Cat#5000112, Biorad). 4xsamplebuffer was added to the samples and then they were boiled in 91 ºC for 5–10 min. Gels were placed to a chamber where 1 x SDS Running buffer was added. Samples were pipetted to the wells in a gel. Gels were run first for 10–15 min at 100 V and when the samples had reached the boarder of running gel at 120 V approximately for 2 h.

8.1.2 Western blot protocol

Proteins were transferred from gels to membrane using process called electroblotting, which is explained in chapter 6.1. Voltage used for electroblotting was 100 V for 1 h 30min and buffer was 1 x transfer buffer in 20 % methanol. Then membranes were cut in three pieces so different pieces could be processed with right antibodies and different proteins could be detected. Membranes were washed ones. All the washes in the protocol were done using TBST (Tris-buffered saline + 0.1 % Tween 20). Membranes were blocked using 5 % non-fat milk in TBST solution for 1h and then washed again. Primary

antibodies (FGFR3, pAKT and pERK) were diluted to blocking buffer and added to samples and incubated overnight +4 ºC. Then membranes were washed three times 5–10 min and incubated in secondary antibodies for 1 h in RT (*room temperature,* RT). Antibodies and their dilution ratios for different proteins are listed in the table 3.

Table 3. *Antibodies and their dilution ratios used to detect sizes of different proteins*

Protein detected	Primary antibody	Dilution ratio	Secondary antibody	Dilution ratio
FGFR3	(B-9) SC-13121 Lot #B0414 Mouse monoclonal IgG, Santa Cruz Biotechnology	1:300	Polyclonal Rabbit anti-mouse Immunoglobulins/HRP Lot 00055754, Dako	1:1000
pAKT	(D9E) XP Rabbit mAb Lot. 16 #4060L, Cell Signaling Technology	1:1000	Polyclonal Swine anti-rabbit Immunoglobulins/HRP, Dako	1:2000
AKT	AKT Rabbit AB Lot 27 9272S, Cell Signaling Technology	1:1000	Polyclonal Swine anti-Rabbit Immunoglobulins /HRP Lot00093964, Dako	1:2000
pERK	P-p44/42 MAPK (T202/Y204) XP(R) Rabbit mAb Lot 12 4370S, Cell Signaling Technology	1:2000	Polyclonal Swine anti-rabbit Immunoglobulins/HRP, Dako	1:2000
ERK	P44/42 MAPK (ERK1/2) 137F5 Rabbit mAb Lot 14, Cell Signaling Technology	1:500	Polyclonal Swine anti-Rabbit immunoglobulins/HRP, Dako	1:2000
GAPDH	GAPDH (H-18) SC-20357 Lot#B2814 Goat polyclonal IgG, Santa Cruz Biotechnology	1:500	Polyclonal Rabbit anti-Goat Immuno-glonulins/HRP Lot 00035737, Dako	1:1000

After secondary antibodies membranes were washed again three times. For the detection ESL solution was prepared (50 % Western blotting luminol reagent A and 50 % Western blotting luminol reagent B, Immuno Cruz) and pipetted 1ml on top of every membrane. Then membranes were moved to Kodak Biomax Cassette and filmed in the dark room using AGFA CP1000 film processor.

After first detection, total amount of AKT and ERK were detected from membranes using AKT and ERK specific antibodies (Table 3). Primary antibodies were incubated on membranes again overnight in +4 ºC and secondary antibodies in RT for 1 h. Washing steps and detection of proteins were repeated same way as earlier.

Lastly GAPDH that is used as a loading control in this experiment was detected from the membrane. Antibodies are again listed in table 3, and incubations, washes and detection were done similarly.

8.2 Staining of stable cell lines

Slides were made of each cell sample and were stained with Fast IHC (immunohistochemical) staining protocol. First slides were fixed for 2 min in acetone at -20 ºC. Staining was done with protocol presented in table 4.

Table 4. *Fast IHC Staining protocol for SNB19 cell lines. All the steps were done on JL-stainer heat plate at 41.5 ºC.*

Step	Reagent	Time
Wash	TBS (Tris-buffered saline)	10 s
Peroxidase blocking	H_2O_2 (Hydrogen Peroxide Solution 3 %, Sigma Aldrich 88597)	1 min
Wash	0,5 % TBST (0,5 % Tween 20 in Tris-buffered saline)	10 s
Primary antibody	FGFR3 (sc-13121, SCBT) 1:100 diluted in Dako antibody diluent	5 min
Wash	0,5 % TBST	10 s
Secondary Antibody	Immunodiagnostics N-Histofine Simple Stain MAX PO (MULTI)	3 min
Wash	0,5 % TBST	10 s
Staining	DAB (ImmPACT DAB Peroxidase Substrate Kit, Vector) 1:50	1,5 min
Wash	H_2O	10 s
Staining	hematoxylin	10 s
Wash	0,5 % TBST	10 s
Wash	H_20	2 x 10 s

After staining, slides were dehydrated (2 min in 70 % ethanol, 2 min in 94 % ethanol, 2 min in absolute ethanol and 2 x 10 min in xylene) and mounted. Slides were imaged with Olympus BX41 light microscope and MShot digital imaging system. Cell samples were stained with same procedure again after 5 weeks of culture to assess whether the expression is stable in the cells. Results of that staining are presented in Appendix 1.

8.3 Functional tests of stabile cell lines

Based on Western blotting and Fast IHC staining four cell lines were selected for further studies (FGFR3:TACC3, FGFR3 and two control cell-lines). Proliferation, colony formation, migration and invasion of these cells were evaluated. Response of inhibitor on these features were also assessed. Cells were cultured in DMEM F-12 Nutrient mixture (Gibco) containing 10 % fetal bovine serum (FBS, *fetal bovine serum*, Sigma Alldrich), 1 % glutamax (Gibco, 35050-061) and 900 µg/ml Geneticin (Gibco) if not otherwise mentioned.

8.3.1 Proliferation studies with MTT viability assay

Cells were plated on 96-well plates 300 cells/plate. Cells were calculated using Bürker chamber. Plates were incubated overnight. Cell proliferation was studied during seven days of culture, so that the viability of cells was measured every other day starting from the day after cells were plated. Effects of JNJ-42165279 inhibitor on proliferation was studied so that 10 nM, 200 nM or 1 µM inhibitor media was added to the cells the day after they were plated. For control cells, fresh media containing 0,01 % DMSO (Dimethyl Sulfoxide, Sigma) was added. Cells were cultured 7 days and media was changed once after 4 days.

Measurements were done with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). MTT was diluted to cell media (1:10) and added to the cells and cells were incubated for 3 h. Cells were centrifuged for 5 min with 300*g in RT. MTT reagent was removed and 150 µl DMSO was pipetted to the wells. Plates were then shaken for 5–10 min and absorbances were measured with Perkin Elmer UV/VIS Envision. Both experiments were done four times to verify the results.

8.3.2 Colony forming

Cells were plated on 6-well plates 300 cells/well. 1 µM or 200 nM inhibitor media (JNJ-42165279) was added on half of the wells. Other half were used as controls for inhibition and were treated with normal media containing 0,01 % of DMSO. Cells were incubated for 10 days and both medias were changed once after five days of incubation. Cells were stained with 0,5 % Crystal Violet in 20 % methanol. Cells were imaged and colonies were counted with ImageJ. Colony formation assay was repeated four times to ensure the results.

8.3.3 Migration

Cells were plated on 12-well plates so that the cell layer was confluent the next day (180 000 cell/well for FGFR3:TACC3, FGFR3 and control 5 and 160 000 cells/well for

control 2). Wound was made to the cell layer with 1 ml pipet tip. Media was changed 6h before wound making so that half of the wells had 1 µM inhibitor media (JNJ-42165279) and other half had control media containing 0,01 % DMSO. Wounds were imaged right after at 0 h and again after 21 h of incubation with Zeiss Axio Vert A1 microscope. Images were analyzed using ImageJ wound healing tool to calculate the area of wound. Wound healing assay was repeated four times.

8.3.4 Survival assay

Survival of cells was studied with Annexin V – FITCH kit (Milteney Biotec, 130-092-052). Cells were plated 100 000 cells/well to 6-well plate and incubated over night at 37 ºC. 1 µM JNJ-42165279 media or 0,01 % DMSO as a control was changed to the cells. Cells were left to incubate for 24 h and collected from wells with 0,05 % EDTA. Cells from every two wells were combined as one sample.

Staining of the cells was done according to manufacturer's instructions (Milteney Biotec, Annexin V – FITCH kit). Shortly, cells were first washed with 1 x binding buffer and centrifuged at 300*g for 10 min. Supernatant was aspirated and cells were suspended at 100 µl of binding buffer. Annexin V – FITCH was mixed to the cell suspension and solution was incubated 15 min in the dark at RT. Cells were then washed again with binding buffer and centrifuged for 10 min at 300*g. Supernatant was removed and cells were resuspended at 500 µl of binding buffer. PI stain was added to samples just before analyzing the samples with BD Accuri C6 flow cytometry. One negative sample without the stains was made for each cell sample. Also, one sample with only Annexin V – FITCH and one sample with only PI stain was made to balance the colors for analysis. Survival assay was done twice, first without inhibition and then with inhibition and control. Survival assay was repeated twice, first without inhibition and then with inhibition treated samples.

8.4 FGF and inhibitor response of fusion cells

Cells' response to JNJ-42165279 inhibitor and FGF (Fibroblast growth factor) stimulation on protein level was studied with FGFR3:TACC3, FGFR3 and control cell line. Protein samples were extracted from cell lines that were incubated in presence of the inhibitor or the growth factor. Differences in protein levels was evaluated with Western blotting.

8.4.1 Cell response to FGF stimulation

Cells were plated 10 cm plates 200 000 cells/plate. They were incubated overnight and starved for 48 h (culture without any growth factors in DMEM F-12). DMEM-F12 containing 0,85 µg/ml FGF (Human FGF-basic, Cat# 100-18B, Peprotech) was added to

cells. Protein samples were collected 30 min, 2 h and 24 h after addition of FGF. Also control samples, where no FGF was added were collected.

8.4.2 Cell response to JNJ-42165279 inhibitor

Cells were plated 10 cm plates 150 000 cells/plate. They were incubated 48 h. Media containing 1 µM JNJ-42165279 was added on inhibited cells and for control cells normal media was changed. Protein samples for inhibited and control cells were collected 30 min, 2 h, and 24 h after media change.

8.4.3 Protein extraction and detection

Protein samples were collected to RIPA buffer containing 1x protein phosphatase. Samples were sonicated for 7 min (30 s on/off) and centrifuged 2 min at 10 000*g. Protein concentrations of samples were measured with DC Protein Assay –kit (Cat# 5000112, Biorad) according to manufacturer's protocol. Briefly, samples and BSA protein standards were pipetted to 96-well plate 5 µl/well. 20 µl reagent S was added to every ml of reagent A. 25 µl of mixture was pipetted to the wells. Then 200 µl of reagent B was pipetted to the wells. Absorbances were measured with Perkin Elmer UV/VIS Envision. Sample concentrations were evaluated in relation to the concentrations of BSA standards. Proteins were detected with Western blotting like was done in chapter 4.1. Blocking the nonspecific bonding of antibodies was done with 2,5 % milk + 2,5 % BSA mixture for phosphorylated FRFR membrane and with 5 % milk for other proteins. Phosphorylated FGFR was detected from membranes to evaluate the effects of inhibition and stimulation on phosphorylation. Phosphorylated STAT3 and total amount of STAT3 were detected to see the effects of inhibition and stimulation on JAKK/STAT downstream pathway. Antibodies used to detect these proteins are listed in table 5.

Table 5. *Antibodies and their dilution ratios used to detect phosphorylated FGFR and STAT3 and total amount of STAT3.*

Protein detected	Primary antibody	Dilution ratio	Secondary antibody	Dilution ratio
pFGFR3	pFGF receptor (Y653/Y654) (55H2) Mouse mAb, Cell Signaling Technology	1:500	Polyclonal Rabbit anti-mouse Immunoglobulins/HRP Lot 00055754, Dako	1:1000
pSTAT3	P-STAT3 (Y705) (D3A7) XP(R) Rabbit mAB #9145S, Cell Signaling Technology	1:2000	Polyclonal Swine anti-rabbit Immunoglobulins/HRP, Dako	1:2000
STAT3	STAT3 (124H6) Mouse mAb #9139, Cell Signaling Technology	1:1000	Polyclonal Rabbit anti-mouse Immunoglobulins/HRP Lot 00055754, Dako	1:1000

Furthermore, total amount of FGFR3 and other downstream signaling pathways related to FGFR were evaluated with detection of phosphorylated ERK and AKT. Total amounts of ERK and AKT were also detected. As a loading control for the experiment GAPDH was detected. Antibodies used for FGFR3, pAKT, AKT, pERK, ERK and GAPDH detection were same as in chapter 4.1 and are listed in table 3.

9. RESULTS

Glioblastoma cell line (SNB19) was stably transfected with a vector containing FGFR3:TACC3 fusion, FGFR3 or an empty vector. Purpose was to evaluate differences between cell models expressing FGFR3:TACC3 fusion and normal FGFR3. Cells with empty vector were used as a control.

9.1 Selection of cell lines with high proportion of FGFR3 positive cells

Five cells lines with FGFR3:TACC3 transfection, five cell lines with FGFR3 transfection and eight control cell-lines with empty vector were analyzed first using Western blotting. Proteins detected from membranes were FGFR3, AKT and ERK. Also, phosphorylation levels of proteins ERK and AKT were evaluated. As a loading control for this experiment levels of GAPDH protein were detected. Results are shown in figure 8.

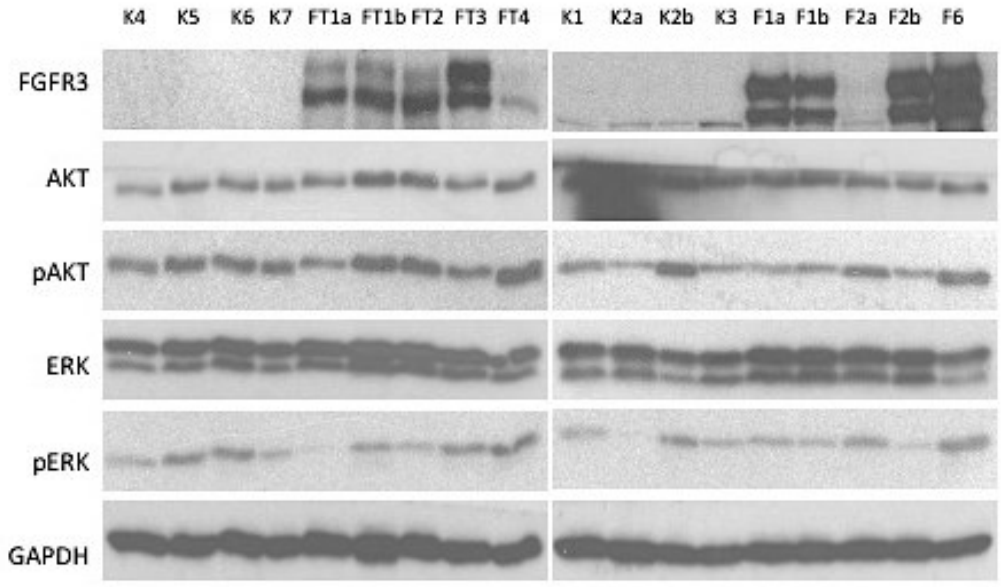

Figure 8. *Image panel shows detection of FGFR3, AKT and ERK proteins in all the samples. Also amount of phosphorylated ERK and AKT are shown. GAPDH is shown as a loading control.*

Samples FT4 and F2a were discarded since FGFR3 expression was low or nonexistent in these samples. Rest of the samples were then analyzed with fast IHC staining. Cells expressing FGFR3 stained brown and cells that did not express FGFR3 stained blue. Figure

9 shows staining of control cells and figure 10 staining of fusion transfected cells and FGFR3 transfected cells.

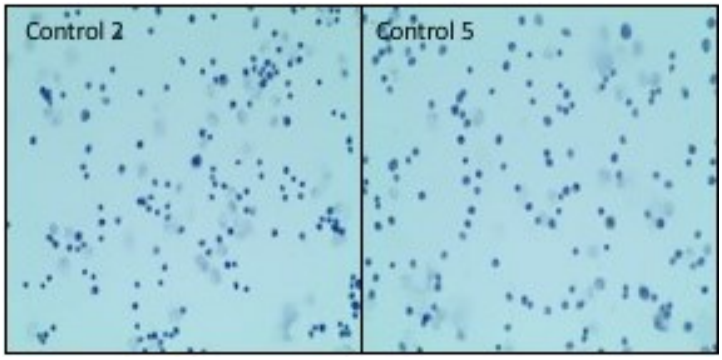

Figure 9. *Control cells stained with fast IHC staining for FGFR3.*

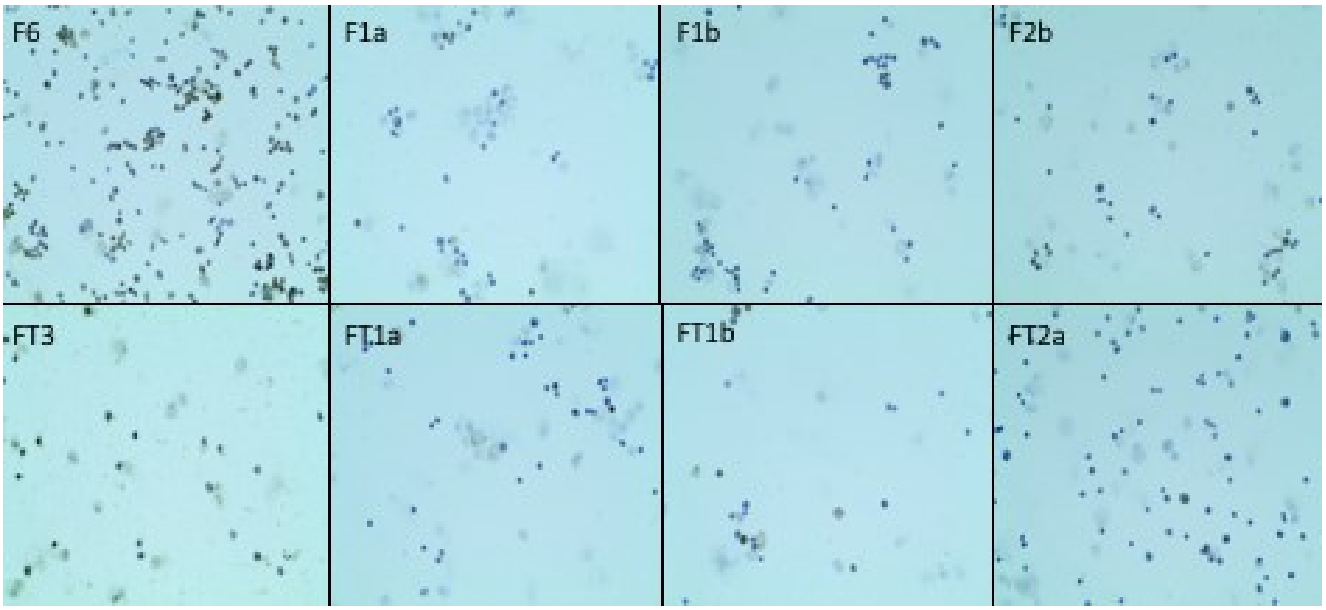

Figure 10. *Upper row shows the FGFR3 transfected cells and lower row FGFR3:TACC3 transfected cells stained with fast IHC staining for FGFR3.*

Stained slides were analyzed and number of FGFR3 positive cells was evaluated with light microscope Olympus BX41 light microscope. Percentages of FGFR3 positive cells in samples is shown in figure 11.

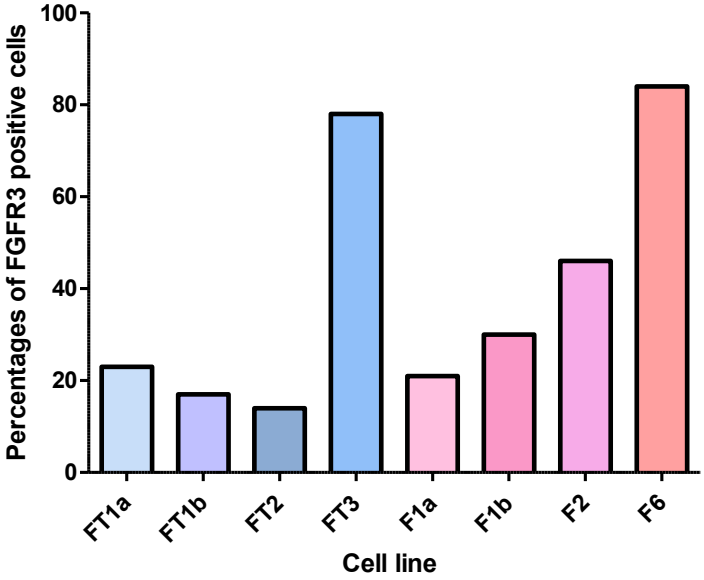

Figure 11. *Percentages of cells expressing FGFR3 in samples determined by immunohistochemical staining.*

Samples F6 and FT3 were selected for further studies, since these samples had over 70 % of FGFR3-staining positive cells. Percentage of FGFR3 positive cells in sample F2 was little under 50 % and in other cell lines under 30 %. All the other samples were discarded from the studies because the results would have not been reliable with them. Cells were stably transfected and clones were selected with selection antibiotic. This should provide cell lines with high expression of wanted protein. However, we noticed that during long time culture, over-expression of FGFR3 and FGFR3:TACC3 is lost in the cells. Appendix 1 shows images of cells cultured for 5 weeks and stained with IHC staining. Also percentages of FGFR3 positive cells were calculated and especially FGFR3:TACC3 expressing line had significantly lower amount of FGFR3 positive cells after 5 weeks of culture than they had after couple days of culture. Stable transfection and selection takes long culturing times and this could be the one reason why expression of FGFR is lower on some cell lines.

9.2 Proliferation capability of cells

Proliferation of cells was assessed using MTT-viability assay. Measurements were done every other day starting on first day after plating and ending on seventh day from plating. Absorbances were measured with Perkin Elmer UV/VIS Envision. Figure 12 shows relative absorbances in proportion to first day absorbances. Values in all of the experiments are mean values of parallel samples and their standard deviations are also shown in figures.

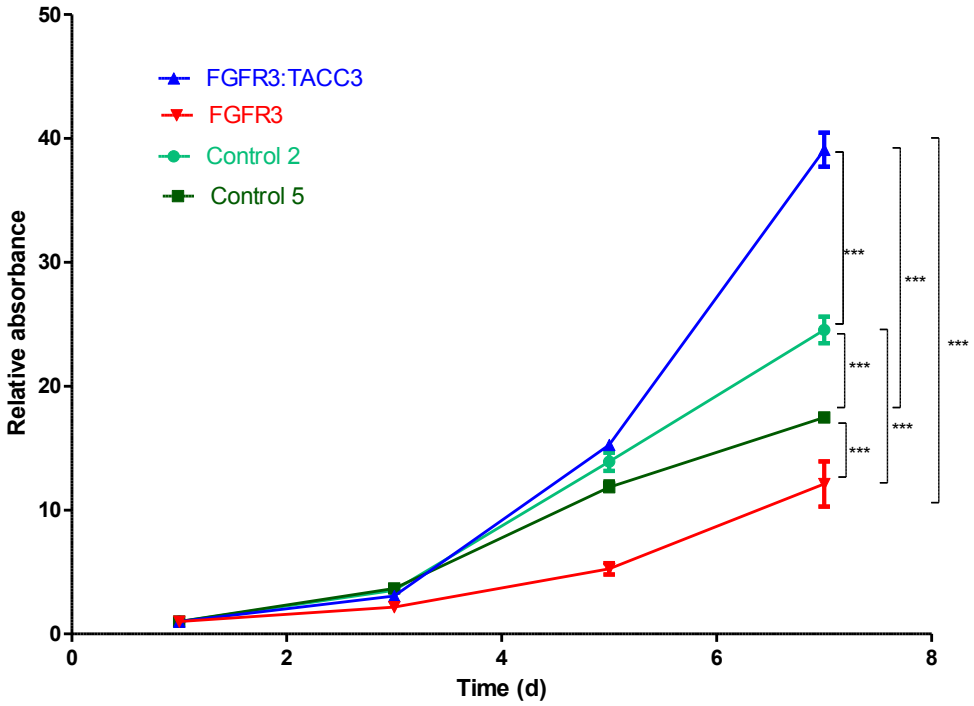

Figure 12. *Proliferation of FGFR3:TACC3, FGFR3 and control cells measured with MTT-viability assay. Absorbance values are mean values of parallel samples. Standard deviations of samples are also shown. Significant differences between cell lines are marked (two-way ANOVA, ***P<0,001.*

FGFR3:TACC3 cells were proliferating fastest and FGFR3 cells proliferate slower than control cell lines. There is a significant difference seen between two control cell lines as well. Effects of FGFR inhibition with JNJ-42165279 inhibitor on cell proliferation was also assessed. Cells were cultured 7 days after which viability of the cells was measured with MTT-assay. Figure 13 shows relative absorbance of parallel samples for inhibited and non-inhibited cells.

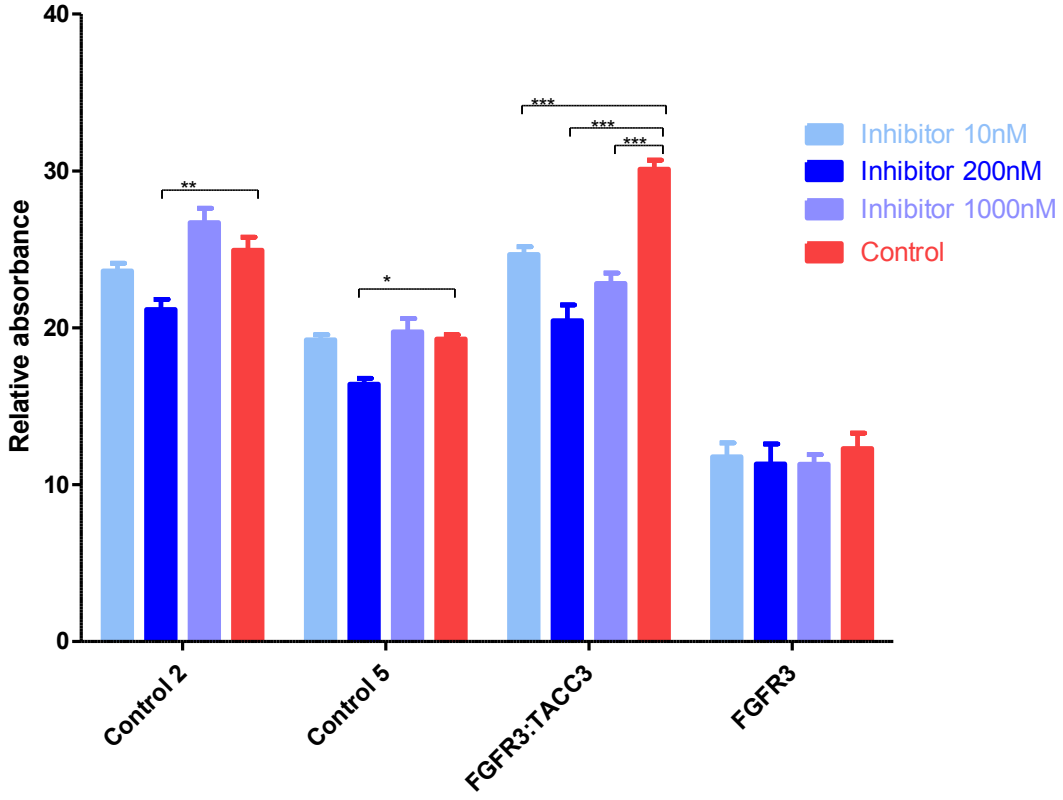

Figure 13. *Proliferation of FGFR3:TACC3, FGFR3 and control cells without inhibitor and with different concentrations of inhibitor (10nM, 200nM, 1000nM). Absorbance values are mean values of parallel samples. Standard deviations of samples are also shown. Significant differences are shown (two-way ANOVA, *P<0,05, **P<0,005, ***P< 0,001)*

Inhibitor has significant effect on FGFR3:TACC3 proliferation on all concentrations. For control cells, effect of inhibitor on proliferation is seen with 200 nM inhibitor concentration. However, effect of inhibition seen on control cells is lower than effect seen on fusion positive cells. For FGFR3 cells, no significant effect of inhibition to proliferation is seen.

9.3 Colony formation of cells

Ability of single cell to form colony was studied with colony formation assay. Figure 14 shows panel of images with one example well containing each cell-types in different concentrations of JNJ-42165279 inhibitor and in control media.

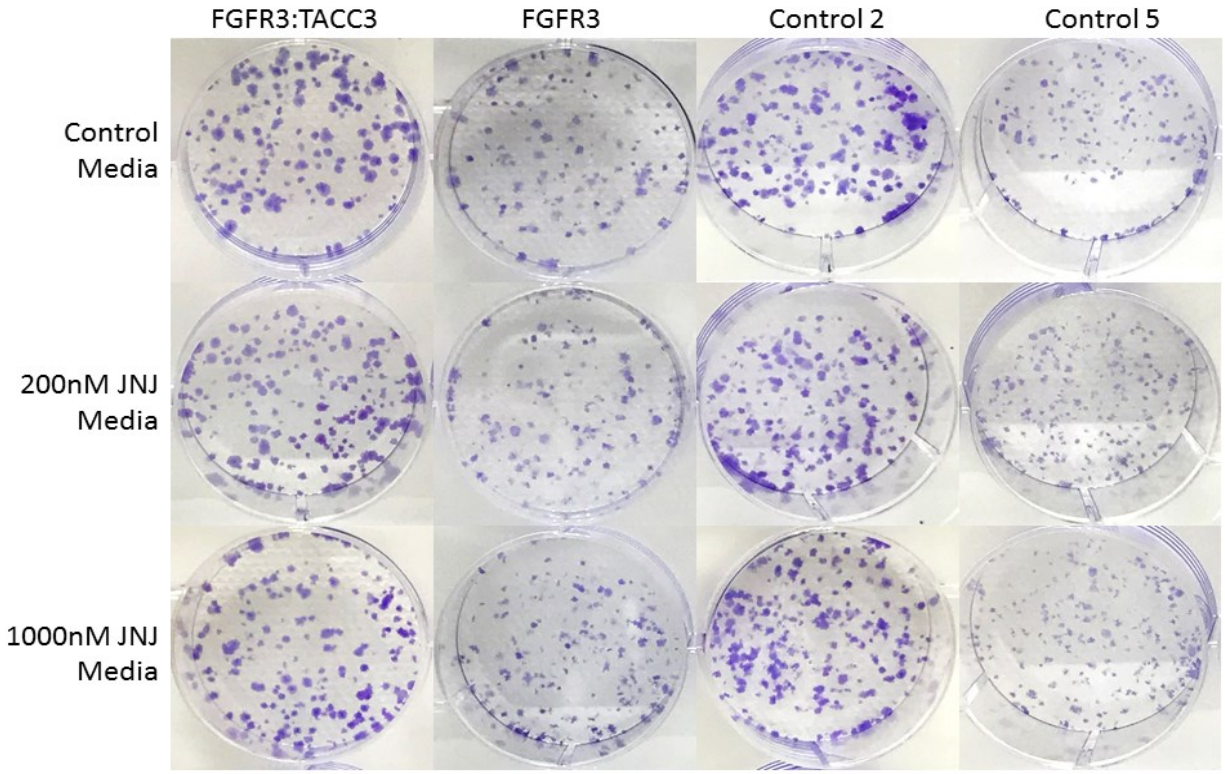

Figure 14. *Colonies formed by FGFR3:TACC3, FGFR3 and control cells with and without inhibitor.*

Colonies were calculated with ImageJ and figure 15 shows the mean value of number of colonies for FGFR3:TACC3 and FGFR3 expressing cells as well as control cells with 1000nM and 200nM JNJ-42165279 inhibitor and with control media.

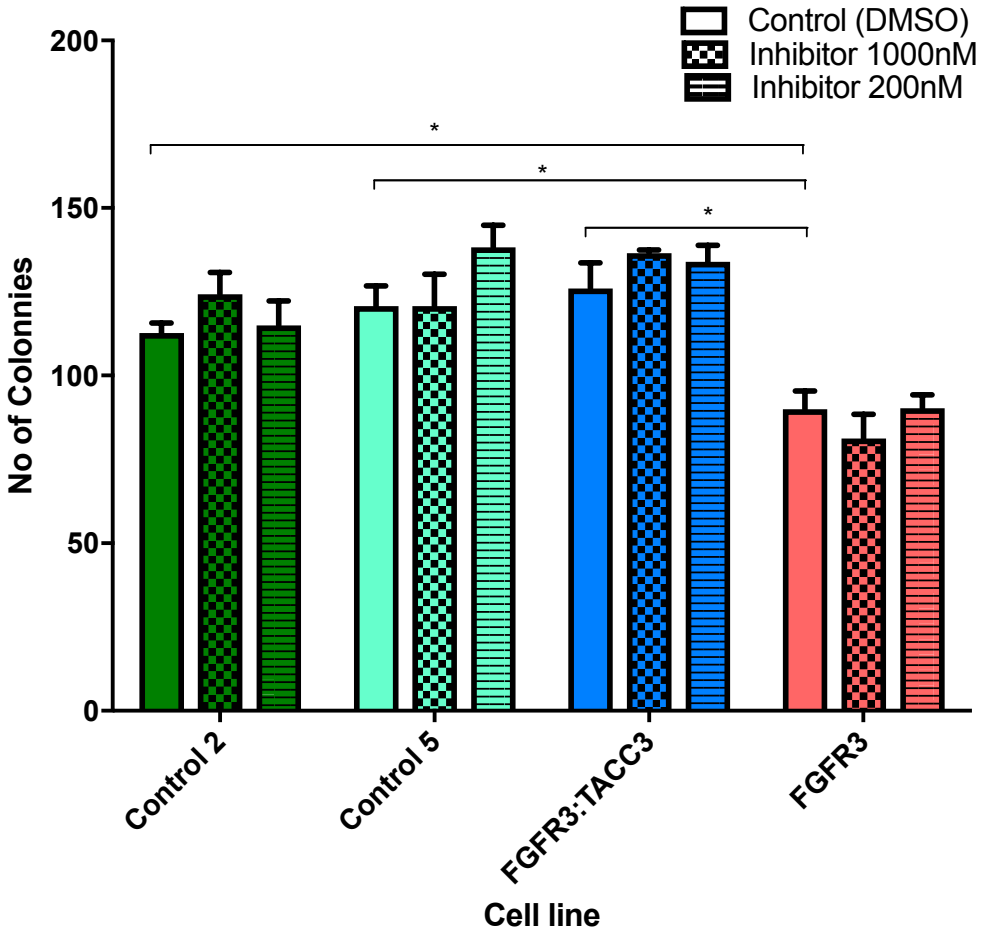

Figure 15. *Number of colonies formed by different cell types (FGFR3:TACC3, FGFR3 or control) in control media and with 200nM and 1000nM JNJ-42165279 inhibitor. Values are means of parallel samples. Standard deviations of samples are also shown. Significant differences are shown (two-way ANOVA, *P<0,05).*

There were no significant differences between different inhibitor concentration treated cells and control cells. FGFR3 expressing cells have lower colony formation capability than other of cells, but no significant differences were seen between fusion and control cells.

9.4 Migration

Cell migration was studied with wound healing assay. Cells were imaged right after the wound was made and again after 21h incubation in 37 °C. Images of cells with 1000nM JNJ-42165279 inhibitor and with control media are collected to figure 16.

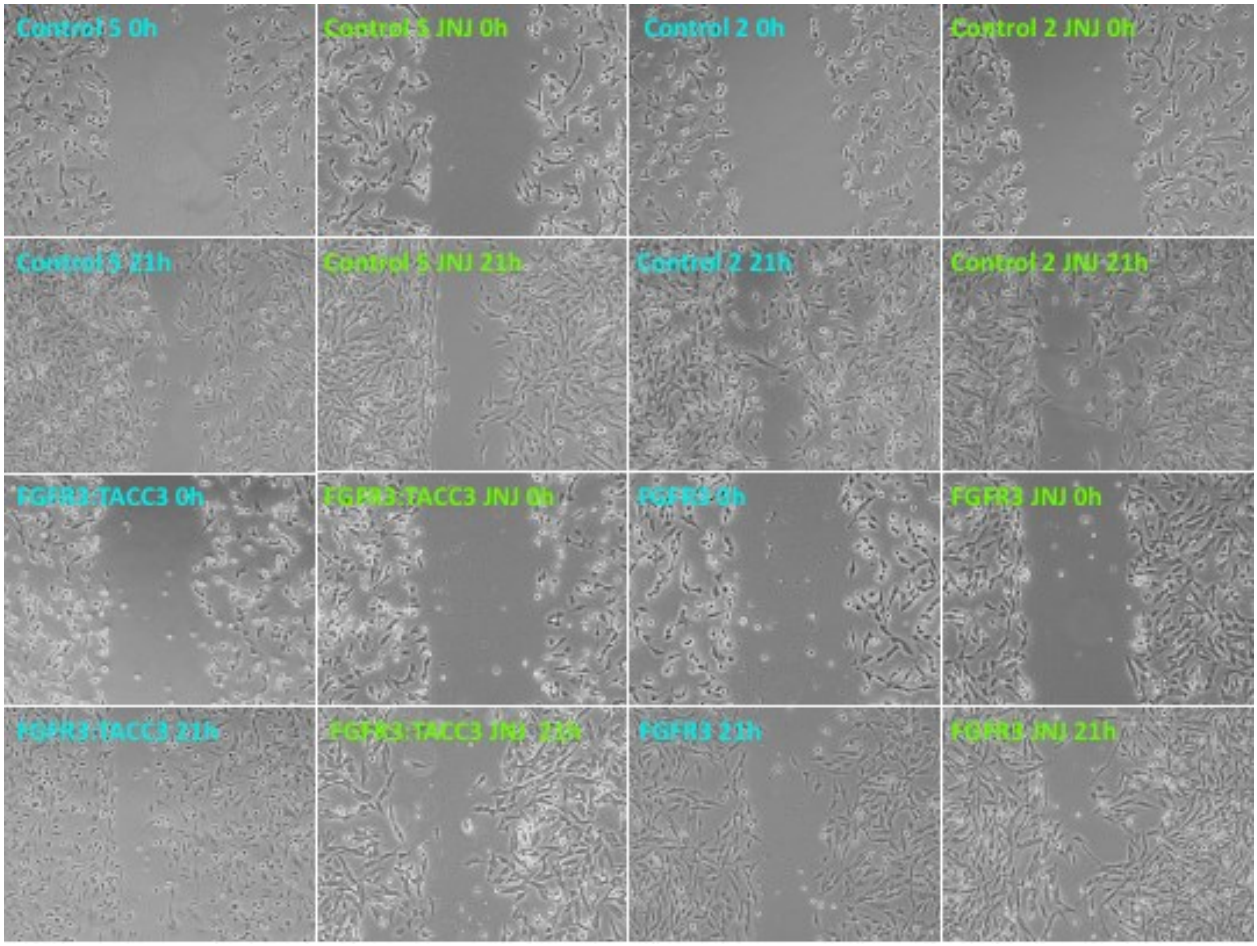

Figure 16. *Images of different cells (FGFR3:TACC3, FGFR3 and controls 2 and 5) with and without inhibitor right after wound was made and after 21h incubation (5x). Inhibitor treated cells are marked green and with control media treated cells blue.*

Images were analyzed using ImageJ wound healing tool. Percentage of area loss was calculated and mean values of those percentages are presented in figure 17.

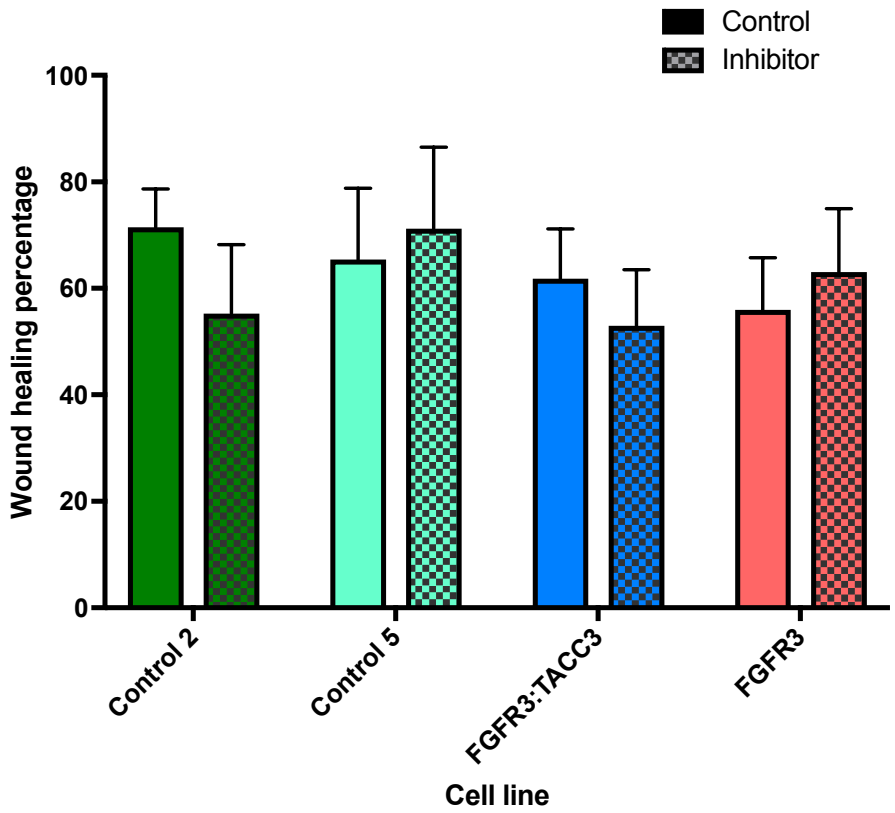

Figure 17. *Percentage of wound healing meaning the wound area loss after 21h incubation with FGFR3:TACC3, FGFR3 and control cells. Values are means of parallel samples. Standard deviations of samples are also shown. No significant differences were seen (two-way ANOVA)*

There were no significant differences between migration of different cell lines. For fusion positive cells, some decreased effect on inhibition to the migration was seen but it was not statistically significant. Same effect was seen in control 2 cell line. For FGFR3 and control 5, inhibition showed no decrease of migration.

9.5 Survival of the cells

Survival of cells was studied with Annexin V FITCH and PI staining and the samples were analyzed with BD Accuri Flow cytometer. Cells were divided to viable, apoptotic and necrotic cells according to the staining and percentages of each group is shown in the figure 18.

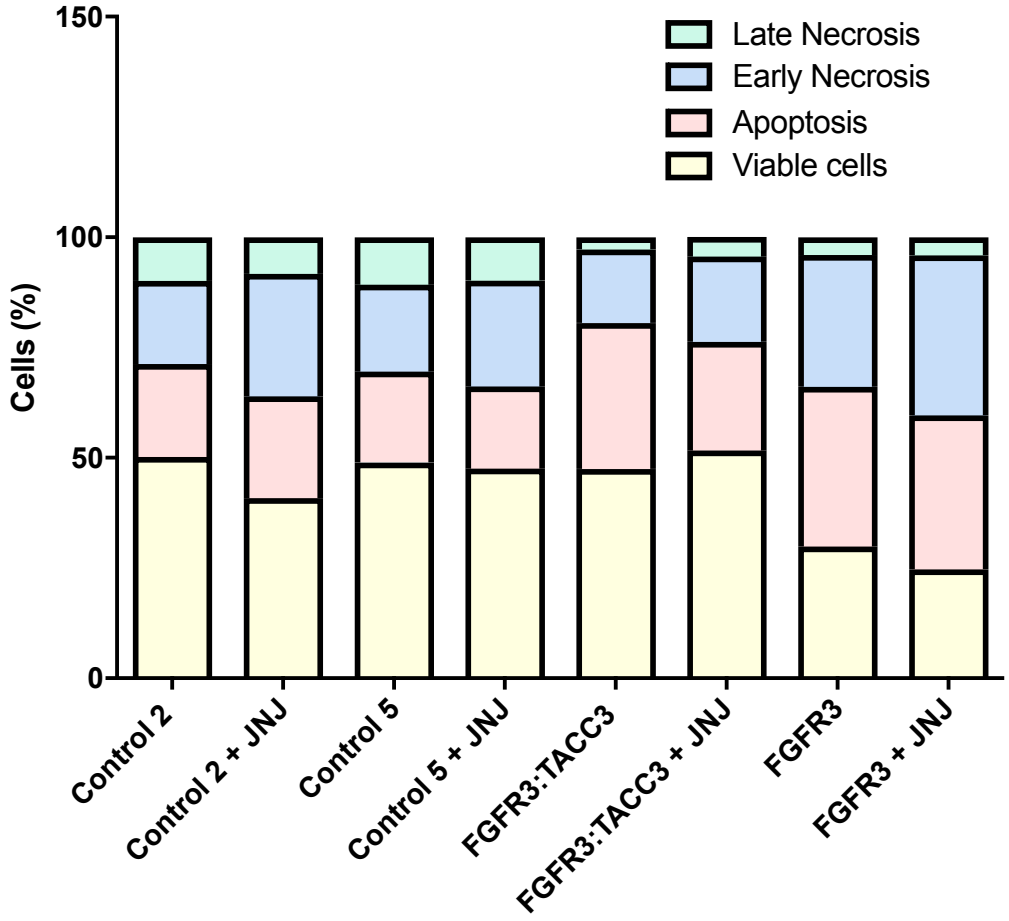

Figure 18. *Percentages of viable, apoptotic, early necrotic and late necrotic cells in samples treated with 1000nM inhibition media or control media.*

Percentage of viable cells is lower with FGFR3 overexpressed cells than fusion or control cells. Percentage of early necrotic cells is on the other hand higher with FGFR3 cells. Percentage of apoptotic cells is similar on FGFR3 overexpressed cells and FGFR3:TACC3 overexpressed cells, but differs from percentage of apoptotic cells in both controls. All the control cell samples seem to have higher percentage of late necrotic cells than fusion or FGFR3.

Inhibition treatment seem to be slightly lowering percentage of viable cells in FGFR3 and control 2 cells, however change is small so might not be significant. Furthermore, percentage of early necrotic cells is higher on those same samples as well as for control 5, suggesting that inhibition is decreasing survival by inducing necrosis on some FGFR3 and both control samples. Percentage of viable cells is increased and apoptotic cells decreased in FGFR3:TACC3 fusion cells with inhibition, suggesting that inhibition would prolong survival of fusion positive cells. Survival assay was done twice, but only once with inhibitor treated samples, so some inhibitor effects on cell survival can be normal variation in the cell behavior.

9.6 Cell response to FGF stimulation

Growth factor stimulation of different cell lines was assessed on protein level with Western blotting, where expressions and phosphorylation levels of FGFR3 related downstream proteins were detected. For FGF stimulation experiment cells were first starved for 48h without any growth factors and then stimulated with FGF. Figure 19A shows effects of starvation at 0h samples and FGF stimulation on cells and their downstream proteins after 30min, 2h and 24h of FGF addition. Intensities of bands were calculated with Image J and figure 19B shows bar plots of intensities of different proteins compared to GAPDH control.

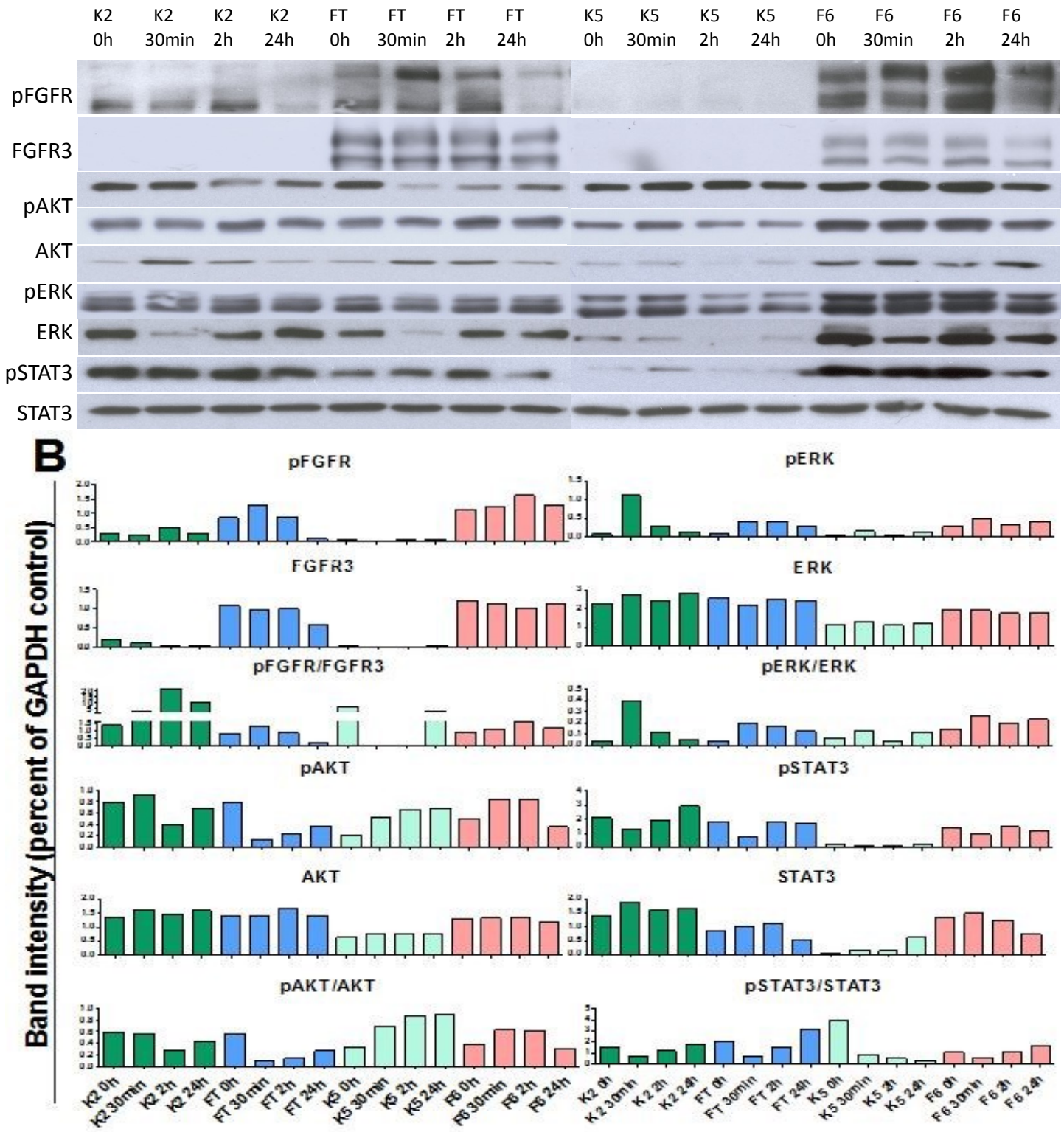

Figure 19. *Expressions of proteins from samples that were first starved (0h sample) and then stimulated different times (30min, 2h and 24h) with FGF(A). Band intensities as a percent of GAPDH control and relation of phosphorylated form to un-phosphorylated form are shown in (B).*

In FGFR3 and FGFR3:TACC3 cells, phosphorylation of FGFR goes up when cells are stimulated with FGF-2. After 24h stimulation phosphorylation will go down even lower than in starved samples. Effect is seen more clearly in FGFR3:TACC3 cells. FGFR3 cells have overall stronger phosphorylation expression than fusion cells. Some phosphorylation is seen also in control number 2. Blot showing relation of pFGFR and FGFR3 is not relevant for control samples since they have so low expression of FGFR3. Minos phosphorylation seen in controls could be caused by some other FGF receptor. Intensities of phosphorylated FGFR calculated with Image J include both glycosylated and non-glycosylated form of FGFR.

Some activation patterns for FGF stimulation can be seen for downstream signaling pathways as well. Phosphorylation of AKT goes down in FGFR3:TACC3 cells and up in FGFR3 cells when they are stimulated. Phosphorylation of ERK has similar pattern in FGFR3:TACC3 cells and control 2 cells; when stimulated, phosphorylation increases and returns back down after 24h. Control 2 has an air bubble at 30 min time point in total expression of ERK, which will lead to a lowered intensity of ERK in analysis. This then leads to increased intensity of pERK/ERK, so true effect seen on control 2 is lower than effect seen on plot. Phosphorylation of ERK is overall higher on FGFR3 wild type cells than other cells. Phosphorylation of ERK increases in FGFR3 cells after stimulation and stays up after 24h of stimulation. Control 5 does not have any significant changes in ERK phosphorylation. STAT3 phosphorylation goes down when FGFR3:TACC3, FGFR3 and control 2 cells are stimulated with FGF and returns back up after 2 h incubation. In control 5 cells total STAT3 expression is really low, so its phosphorylation pattern difficult to determine. However, it seems that STAT3 phosphorylation also decreases when stimulated.

9.7 Cell response to inhibition

Cells response to inhibition was studied with 30min, 2h and 24h inhibitor treatment with JNJ-42165279 inhibitor and compared to control cells treated with normal media. Phosphorylate FGFR and downstream protein AKT, and ERK and their phosphorylated forms were detected. GAPDH was used as a loading control. Figure 20A shows expression of these proteins detected with Western blotting. Intensities of bands were calculated with Image J and figure 20B shows bar plots of intensities of different proteins compared to GAPDH control.

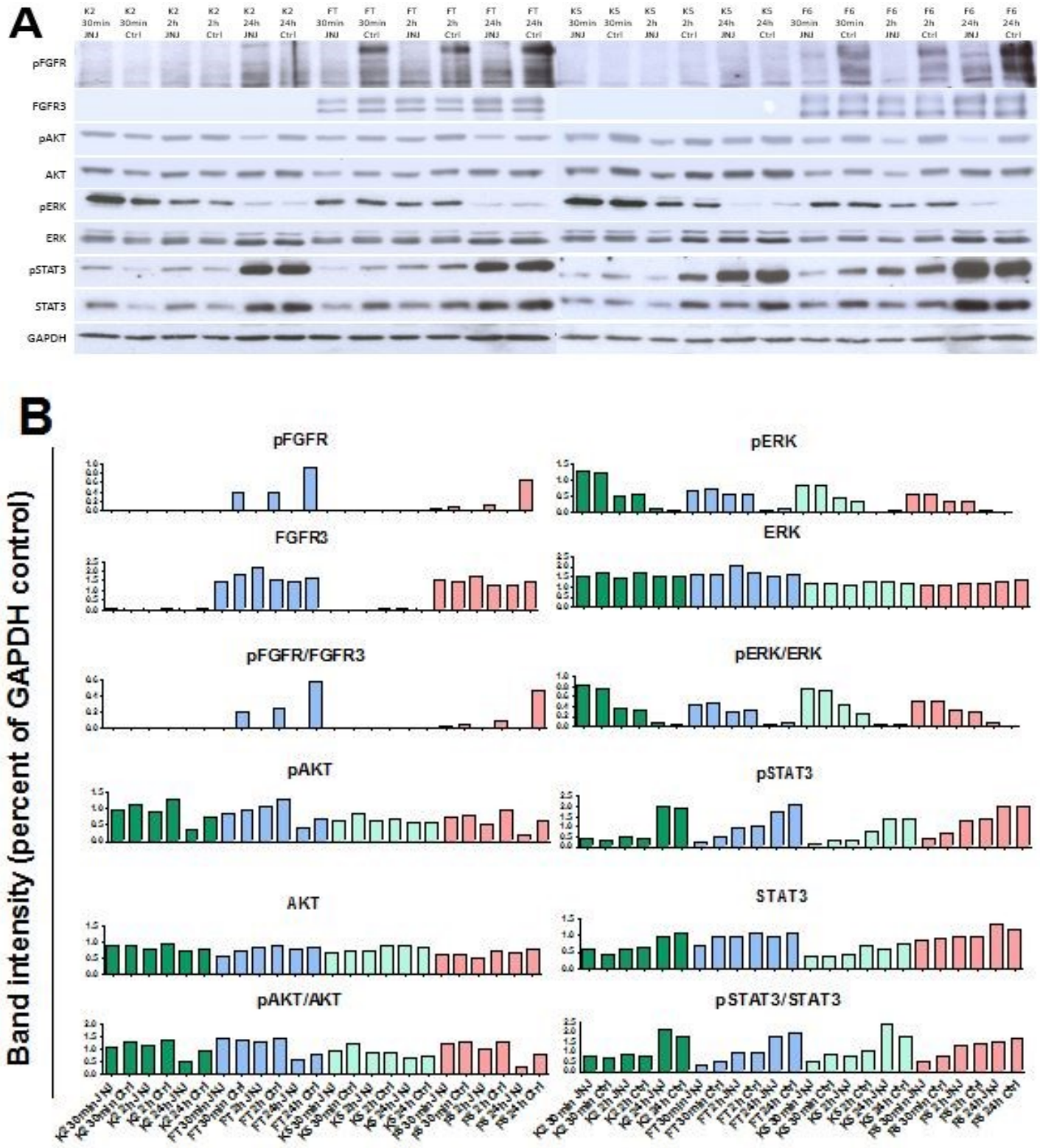

Figure 20. *Proteins from inhibitor treated and control samples after 30min, 2h and 24h treatment (A). Band intensities as a percent of GAPDH control and relation of phosphorylated form to un-phosphorylated form are shown in (B).*

Inhibition of FGFR in fusion and FGFR3 overexpressed cell lines seem to lower phosphorylation of FGFR, since all JNJ-42165279 treated FGFR3:TACC3 and FGFR3 treated cell lines have lowered FGFR phosphorylation expression compared to same time point control treated cells.

Inhibition of cells does not seem to have clear effect on downstream signaling. AKT phosphorylation has decreased expression at 24h time point for control treated versus inhibited cells on FGFR3:TACC3, FGFR3 and control 2 cell lines. Overall phosphorylation of AKT is slightly decreasing during time. Furthermore, phosphorylation of ERK seem to lower as time passes on all cell lines and it is on its lowest at 24h time point in both control treated and inhibited cells. Slight decreasing effect of inhibition can be seen on pERK/ERK on FGFR3:TACC3 cells but not on other cells. STAT3 phosphorylation on the other hand seem to be increasing during the incubation, as it is on its highest at 24h time point. However, looks like total amount of STAT3 is also increasing in the cells during that 24h incubation.

10. DISCUSSION

In this study, FGFR3:TACC3 expressed cell line was compared to FGFR3 expressing and control cells lines. Stably transfected overexpression cell lines were used to compare those differences. Previous studies have shown FGFR3:TACC3 fusion to increase proliferation and anchorage independent growth of the cells (Parker et al., 2013). Aim was to see if our cell models behave similarly and could be used as a model for FGFR3:TACC3 fusion and also to evaluate effects FGFR3:TACC3 overexpression on other cell characteristics. In addition, inhibition of FGFR in these overexpressed cell lines with JNJ-42756493 inhibitor was studied. Preliminary clinical trials with JNJ-42756493 inhibitor have shown promising results with reduction of tumor size and improving clinical outcome (Di Stefano et al., 2015).

10.1 Expression of FGFR3:TACC3 is increasing proliferation of the cells

This study showed that FGFR3:TACC3 expressing cells are proliferating faster than control cells (Figure 12). This same effect was shown earlier by Parker et al. (2013). This result suggests that our stably transfected fusion positive cells behave like was expected and can be used as an *in vitro* model for FGFR3:TACC3 fusion in glioblastoma. Increased proliferation of FGFR3:TACC3 fusion positive cells suggests that fusion is somehow over-activating tyrosine kinase receptor. This is seen in Western results, where phosphorylation of FGFR is seen mainly in over-expressed samples and only small phosphorylation is seen in control samples (Figures 19 and 20).

Two main downstream pathways of FGFR are MAPK and PIK3 signaling pathways, of which MAPK signaling pathway is mainly responsible for proliferation of the cells. PI3K is responsible for avoiding apoptosis, but the signaling pathways do have some cross talk and for example in migration, both signaling pathways are activated. (Wesche, Haglund, & Haugsten, 2011) Fusion has clear effect on proliferation, but not on other cellular mechanisms suggesting that at least MAPK pathways is activated by FGFR3:TACC3. In fact, Western blotting results in this study showed increased phosphorylation of ERK and decreased expression of phosphorylated AKT in FGFR3:TACC3 cells after stimulation with FGF. Same increase in phosphorylation of ERK was seen in FGFR3 cells and control 2 cells, however control cells had air bubble in the ERK band at 30 min time point (Figure 19a), which has lowered the intensity value of total ERK. This will lead to increased value of pERK/ERK at that time point, so difference seen in ERK phosphorylation on control 2 is not as significant on reality as it shows in the intensity plot (Figure 19b). Decrease in AKT phosphorylation was only seen in fusion positive samples. Overall expression level of pERK was higher in FGFR3 expressing cells.

No significant difference was seen in colony formation and migration between fusion positive and control cells. Figure 18 shows that FGFR3:TACC3 cells have higher percentage of apoptotic cells than other samples and percentage of necrotic cells is lower. Number of viable cells in FGFR3:TACC3 cells is similar with two control samples and higher than in FGFR3 cells. Apoptosis is determined as a programmed process where cell goes to a highly regulated and controlled cell death whereas necrosis is more passive process of cell death activated by outer signals. However, in the absence of phagocytic cells that are present in the tissues, apoptotic cells will start to lose their membrane integrity and become necrotic (Fink & Cookson, 2005). In our survival experiments, necrotic cells are therefore a combination of cells that have started as necrotic cells and cells that have first gone through apoptosis and then proceeded to necrotic stage. This suggests that fusion positive cells have more active control mechanisms, and more cells are going through controlled apoptosis and cells are reaching the necrotic stage slower than in other samples. PI3K pathway is mainly responsible of avoiding apoptosis in the cells (Wesche et al., 2011). In fusion positive cells phosphorylation of AKT is highest after starvation and decreased after stimulation with FGF (Figure 19), which could mean that in stressful situations FGFR3:TACC3 is avoiding apoptosis well. Control 2 had slightly similar activation pattern of pAKT, but FGFR3 cells and control 5 cells had opposite effect. Overall level of AKT phosphorylation was highest in FGFR3 cells.

Fusion protein is formed so that kinase domain of an FGFR3 is fused together with dimerization domain of TACC3. Because the fusion happens in intracellular side of the cell membrane, normal down-regulation of receptors, that happens in the cell surface is evaded and this will result in on-going signaling with lowered negative feedback. (Wesche et al., 2011) It is possible that FGFR3:TACC3 fusion has also other downstream effects that are not dependent on the tyrosine kinase activity. One interesting effect of fusion was found on the study of Sarkar et al. (2017) that focused on FGFR3's fusion partner TACC3. TACC3 is an important regulator of mitotic spindle on cell division. TACC3 domain of FGFR3:TACC3 fusion is shown to recruit endogenous TACC3 from the mitotic spindle causing reduction in normal TACC3 levels and dysregulation of mitotic spindle. This will lead to mitotic defects in cancer cells and this mechanism is suggested to work together with FGFR3:TACC3 signaling affecting the oncogenic nature of the fusion. It is unclear how this affects tumor progression, but it is possible that mitotic defects caused by the fusion could be one reason why FGFR inhibitors have been seen in clinical trials to gain treatment resistance. (Sarkar, Ryan, & Royle, 2017)

10.2 FGFR3 expressing cells have lowered proliferation capability

In this study FGFR3 over-expressing cells showed lowered proliferation compared to control and fusion positive cells (Figure 12). Furthermore, results show that colony formation capability is lowered with FGFR3 over-expression (Figure 15). Survival studies

showed that FGFR3 has higher percentage of apoptotic and necrotic cells than other cell types (Fig. 18). According to these results it seems like glioblastoma cells expressing FGFR3 do not survive as well as other cell lines. Parker et al (2013) showed in their *in vivo* mice studies that mice injected with FGFR3 expressing cells had similar survival than mice injected with cells transfected with empty vector, however mice injected with high-expressing FGFR3:TACC3 clone had significantly lowered survival time. Our *in vitro* proliferation results are in line with these results shown earlier.

Parker et al. (2013) studied also in vitro proliferation of transfected cells and showed increased proliferation with wild type FGFR3 compared to control cells. This is controversial result compared to our proliferation results, where wild type FGFR3 was suppressing proliferation. It needs to be considered, that cell clone itself is proliferating slower and FGFR3 over-expression is not the reason for our obtained results. Our results show also clear differences between cell characteristics of two controls in almost all experiments. If the two clones of control cells that are transfected similarly with empty vector, are behaving so differently, it is challenging to draw conclusions on behavior of FGFR3 and FGFR3:TACC3 over-expressed clones. Results in this study do show some evidence on oncogenic nature of FGFR3:TACC3 and suppressive nature of FGFR3. However, it needs to be considered how much of these behaviors can be explained with original cell clones just behaving differently. There might be some other mechanism than FGFR3 or FGFR3:TACC3 over-expression, which is affecting cell mechanisms of different clones.

In Western blotting results, wild type FGFR3 shows clearly higher activation of downstream signaling pathways than other cell lines (Figure 19). FGFR3 expressing cells also seem to be active regardless of the starvation. Controls 2 and 5 were selected to the experiments because control 2 resembles FGFR3:TACC3 and is proliferating faster. Control 5 on the other hand is proliferating slower and resembles more FGFR3. In contrast to FGFR3, control 5 clone has low overall expression of pERK and pSTAT3. Low downstream activation might explain slower proliferation of this clone. FGFR3 expressing clone is proliferating slowly even though its downstream proteins are expressed well which could suggest that higher downstream activation seen in FGFR3 cell line is FGFR3 over-expression mediated.

10.3 Inhibition of FGFR in FGFR3:TACC3 cells is decreasing proliferation

Inhibition of FGFR3 showed lowered proliferation on FGFR3:TACC3 fusion cells but not with other cells (Figure 13). Our experiments also showed minor effect of inhibition on proliferation of control cell lines. Inhibition had conflicting results on FGFR3:TACC3 and FGFR3 cells survival. Inhibition seems to be slightly lowering number of viable cells in FGFR3 and control 2 cell lines but not with FGFR3:TACC3 cells. This is counter effect to that seen in viability studies, where inhibition lowered number of viable cells in

FGFR3:TACC3 over-expressing cells, but not with FGFR3 cells. This could mean that inhibition is targeting also FGFR3, but proliferation is so much slower that significant differences of inhibition on proliferation is not seen in MTT viability experiment. MTT viability assay measures also more cell activity than number of cells. FGFR3:TACC3 cells might have more active metabolism, since they are proliferating faster and inhibition can be targeting something in the metabolism that is not as active on FGFR3 expressing cells. Inhibition was added to the cells only 24h prior to analysis, so clear effect on cell survival might not been seen yet in that incubation time. Furthermore, this assay was done only once with inhibition, so effect seen might only be normal variation of cells.

Early clinical trials have shown that fusion positive tumors respond to inhibitor treatment with partially shrinking, but during the treatment tumors gain treatment resistance and restart the tumor progression (Di Stefano et al., 2015; Dienstmann et al., 2014; Perera et al., 2014). Furthermore, it has been shown that fusion positive tumors have heterogeneous FGFR3 expression. All the cells in fusion positive GBM tumors do not express FGFR3:TACC3 fusion but there are also cells in the tumors that are negative for this fusion. (Granberg et al., 2017). Portions of fusion negative cells in tumor might explain effects seen in preclinical trials with fusion positive tumors treated with FGFR inhibitor. Cell models used in this experiment have also some amount of fusion negative cells in them. This could have effect on inhibitor response as well.

Clear decreasing effect of inhibition on FGFR phosphorylation was seen with both FGFR3 and FGFR3:TACC3 cells (Figure 20). Control cells without overexpression had none or only very low phosphorylation of FGFR. FGF stimulation and JNJ-42756493 inhibition have opposite effects on FGFR phosphorylation on overexpressed cells, since stimulation is increasing phosphorylation and inhibition is decreasing phosphorylation of FGFR.

In FGF simulation experiment some FGFR phosphorylation was seen also in non-glycosylated FGFR on control 2. FGFR3 expression was not seen in control 2, so this suggest that some other FGFR might be present in this control sample. Since JNJ-42756493 inhibitor is not specific for FGFR3 but is targeting all FGFRs, it is possible that it will have effect on controls as well, if the control cells express some other FGFR. This could explain variation in inhibitory results in this study and also differences between behavior of two controls. Therefore, we are planning to check expression of other FGFRs (FGFR1, 2, 4 and FGFRL1) from all the cell lines. Phosphorylation of FGFR seen on control samples in FGF stimulation experiment proposed that there might be phosphorylation of FGFR in inhibitor response experiment as well, even though figure 19 showed no clear changes in phosphorylation level of control samples. Appendix 2 shows longer exposure for pFGFR detection in control cell lines and band intensities for control samples measured with Image J. Expression of pFGFR is so low on control cells that even with longer exposure effects of inhibition are challenging to asses, but it seems like phosphorylation of FGFR in control samples does not seem to be lowered due to inhibition.

Inhibition had slightly decreasing effect on phosphorylation of AKT in FGFR3:TACC3, FGFR3 and control 2 samples in 2h and in 24h time points (Figure 20). Activation pattern in all other downstream targets is similar for inhibitor treated versus control treated samples. Phosphorylation of ERK is decreasing during time and phosphorylation of STAT3 increasing during time. Because differences between control and inhibitor treated cells is not seen, media change that was done at the beginning of the experiment at 0 h time point is probably causing these seen changes in ERK and STAT3 phosphorylation.

Despite the clear decrease in FGFR phosphorylation due to inhibition seen in this experiment on both FGFR3 and FGFR3:TACC3 over-expressed cell lines, effect of inhibition on cellular mechanisms was small. Downstream signaling expression is not highly changed due to inhibition, which is suggesting that cells are using other tyrosine kinase receptors to control downstream signaling so inhibition does not have that much effect on them. Crosstalk between different tyrosine kinase receptors is seen in many different studies and can be facilitated through downstream signaling without affecting receptors themselves.

Cell lines used in these experiments were stable overexpression cell lines of FGFR3:TACC3 fusion and FGFR3, so their expression in cells is overemphasized in these lines. Inhibition of these overexpression proteins may not be as efficient as it could be in primary cell lines. Furthermore, SNB19 cell line is commercial cell line that has been cultured for long times. Cell line has developed quite much since the time it was harvested from patient in 1980 (chapter 7.5). This cell line has probably stabilized signaling routes that are optimal for culture conditions and continuous stimulation with serum. They may have higher expression of other tyrosine kinase receptors and their downstream targets and may not resemble GBM tumor as well as primary cell lines would. This could be the reason of only minor effects that were seen in downstream signaling due to inhibition. Effects of inhibition seen in these overexpression cell lines will be validated with primary cell lines later.

10.4 Experimental aspects

Most of the experiments were repeated at least three times. There was a slight variance seen between parallel experiments suggesting that experimental protocols were not standardized enough. In proliferation assay, outermost wells had often lower absorbance than other wells. Cells were divided to multi-well plate using multi-well pipet, so it is possible that multi pipet has some differences between tips and the outermost tips don't take enough liquid in. Cells can also be unevenly distributed in the solution, even though they were mixed between the pipetting. Both of these would cause variations in the cell number in parallel wells and standard deviations might be higher. However, procedures were done similarly to all cell lines, so it could be assumed that error is similar in all the cell lines.

MTT is widely used for the cell viability studies, but it has some issues that are questioned. It is known to cause cytotoxicity and it is not known how the incubation times in MTT are affecting cell viability on different cell clones. MTT assay has also multiple steps, because it needs to be solubilized after incubation. Multiple steps increase the possibility of errors in pipetting and removal of fluids. Issues in MTT assay include the fact that it reflects more to on cell metabolism of viable cells than on number of viable cells. For instance, cell confluence can have effect on cell metabolism and cause errors in the absorbance values. (Riss et al., 2004; Riss, 2014) In these experiments cells were seeded so that they would not reach the full confluence, but FGFR3:TACC3 fusion was proliferating fastest, so it reached more confluent monolayer than others.

In colony formation assay, cells are diluted to very low concentration so they can be divided to wells as a single cells suspension. When working with very low concentrations, it is possible that cells in suspension pipetted to the wells are not divided equally and number of cells in the well can therefore vary. When wanted cell number on wells is so low, even some small differences between wells equals significant percentage in difference. Furthermore, some air bubbles in media can move cells to cluster in a certain part of the well closer to each other before they have attached. If the cells are not properly attached as single cells, their signaling with each other may affect colony formation.

Parallel experiments also showed that SNB19 cells are not behaving similarly in every experiment. This was seen especially in wound healing assay, where results varied a lot depending on confluence of cells at the beginning of the experiment. Even though same number of cells was seeded to parallel wells, cells would cluster to the edges or in the middle of the plate. Cells also migrate actively after plating, which also affects their position and confluence. Therefore, overall confluence was not same in all parallel wells. Cells are using autocrine and paracrine signaling to affect the behavior of themselves or neighboring cells. Signaling efficiency is dependent on cell confluence, since more confluent are affected more by paracrine signaling. (Alberts, Wilson, & Hunt, 2014) Another important cell signaling method in cell culture is signaling through cell-cell junctions. Cells will communicate with neighboring cells with small molecules like ions and secondary messengers that move through narrow pores between two cells called gap junctions. Confluence of cells is increasing cell signaling also via these gap junctions. (Alberts, 2009) These different confluence dependent signals have great effect on proliferation and migrations of the cells. Differences in confluence between parallel wells was compensated by plating more cells on those lines that did not seem to be as confluent at the beginning of wound healing assay, but even with compensation some differences were seen.

Stable transfection had been used for the cell lines in this experiment to introduce gene into the cell for long time. But even with selection antibiotic, expression of over-expressed gene can be lost from the cells over long culturing times (Appendix 1). Chromatin

modifications in DNA can affect expression of integrated protein in different clones (Dalton & Barton, 2014). Furthermore, it is known that even stably transfected cell lines have instability of the integrated protein, meaning that its expression is lost in the cells. Instability can be caused by instability of the genome and for example some genes encoding integrated protein can be lost. Furthermore, cells that are not expressing integrated genome can have growth advantage over those that express the gene. (Barnes et al., 2003). It is also possible that only section marker has integrated to the genome, but studied gene has not. These aspects can explain lower expression of FGFR3 in other cell clones (Fig 11).

Results of wound healing assay was analyzed using Image J wound healing tool. This tool would recognize the edges of the scratch and measure the area of scratch. SNB19 cells are very transparent and when they had migrated, different cell clones had very different morphologies. Appendix 3 shows wound healing images at 21h time point for all the cell lines. FGFR3 expressing clone is spreading more widely to the platform than FGFR3:TACC3 expressing clone. Also some of the cells in the images are difficult to detect from the platform due to their transparency. These aspects were affecting the Image J wound heling tool so that it could not always separate edges of the cells, from the bottom of the plate creating possible errors in image analysis. If error was seen in the analysis, analysis was repeated by reducing transparency of cells with enhancing contrast of the picture or by measuring scratch area by hand drawn edges. This potentially reduced the error caused by Image J analysis.

11. CONCLUSION

To sum up, FGFR3:TACC3 over-expressed clone was proliferating faster and FGFR3 slower than control cells. Lower colony formation capability was also seen in FGFR3 expressing cells. These results are consistent with the suggestion that fusion might be increasing proliferation in GBM and wild type FGFR3 could be suppressive in cells. Inhibition was suppressing growth of FGFR3:TACC3 cell line. Observed effect of inhibition on other cell mechanisms, however, were small and inconsistent. Western blotting showed clear decrease in phosphorylation of FGFR after inhibition in fusion positive and wild type FGFR cells and some minor effects on downstream signaling were also seen. Based on these results it is challenging to draw conclusions on inhibition mechanism in GBM cells, but it seems likely that effect of inhibition *in vitro* is somehow compensated in the cells. Cell lines used in this experiment were overexpression cell line of FGFR3:TACC3 and FGFR3, which may affect results seen in inhibition efficiency. More information is needed on how inhibition is affecting fusion positive cells and whether cells will counterbalance inhibition of FGFR signaling with some alternative signaling methods.

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APPENDIX 1: AFFECTS OF CULUTURE TIME ON PERCENTAGE OF FGFR3 POSITIVE CELLS IN SAMPLES

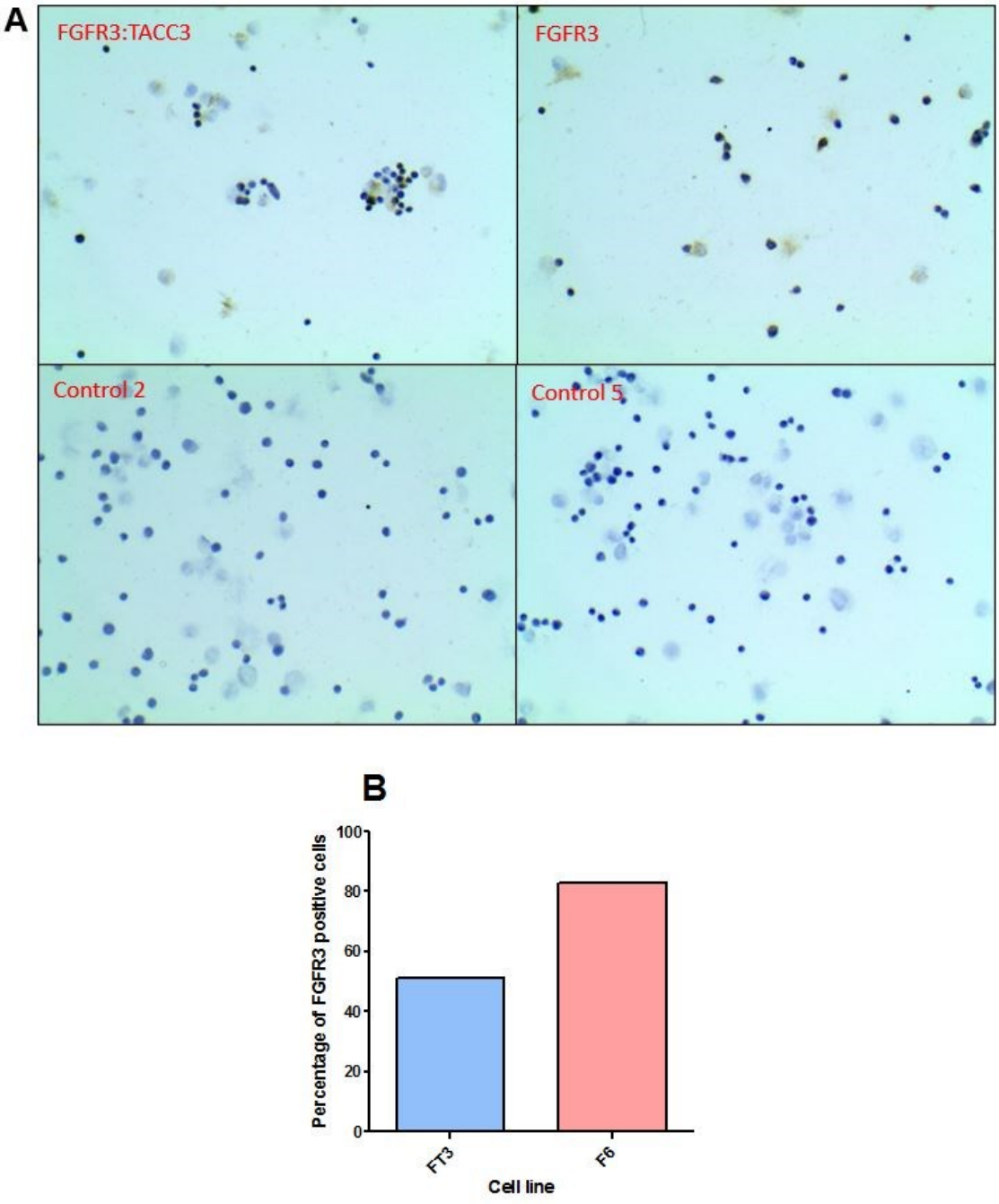

Figure 1. *Percentages of FGFR3 positive cells detected with immunohisto-chemical staining after 5 weeks of culturing (A). Percentages of FGFR3 positive cells calculated with microscope in over-expressed cell lines (B).*

APPENDIX 2: AFFECTS OF INHIBITION ON FGFR PHOSHORYLATION ON CONTROL SAMPLES

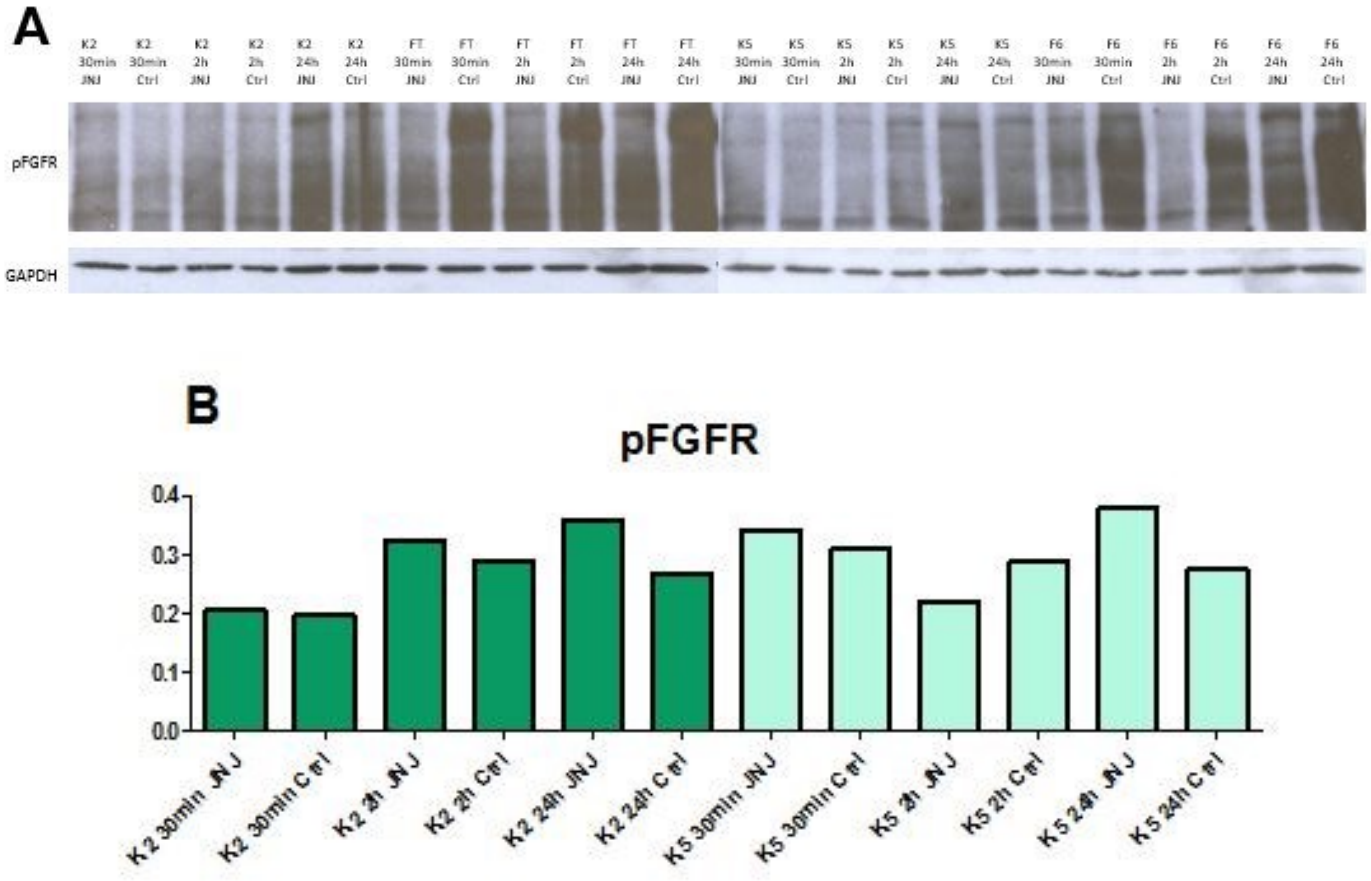

Figure 2. *Long exposure Western image of phosphorylated FGFR from inhibitor treated and control samples after 30min, 2h and 24h treatment and loading control GAPDH (A). Band intensities of a two control samples as a percent of GAPDH control (B). Intensities are measured as a mean gray value of bands. Since FGFR3:TACC3 and FGFR3 expressing control treated cells have so high expression with this long exposure, their intensity values were excluded from B.*

APPENDIX 3: MORPHOLOGY OF THE CELLS

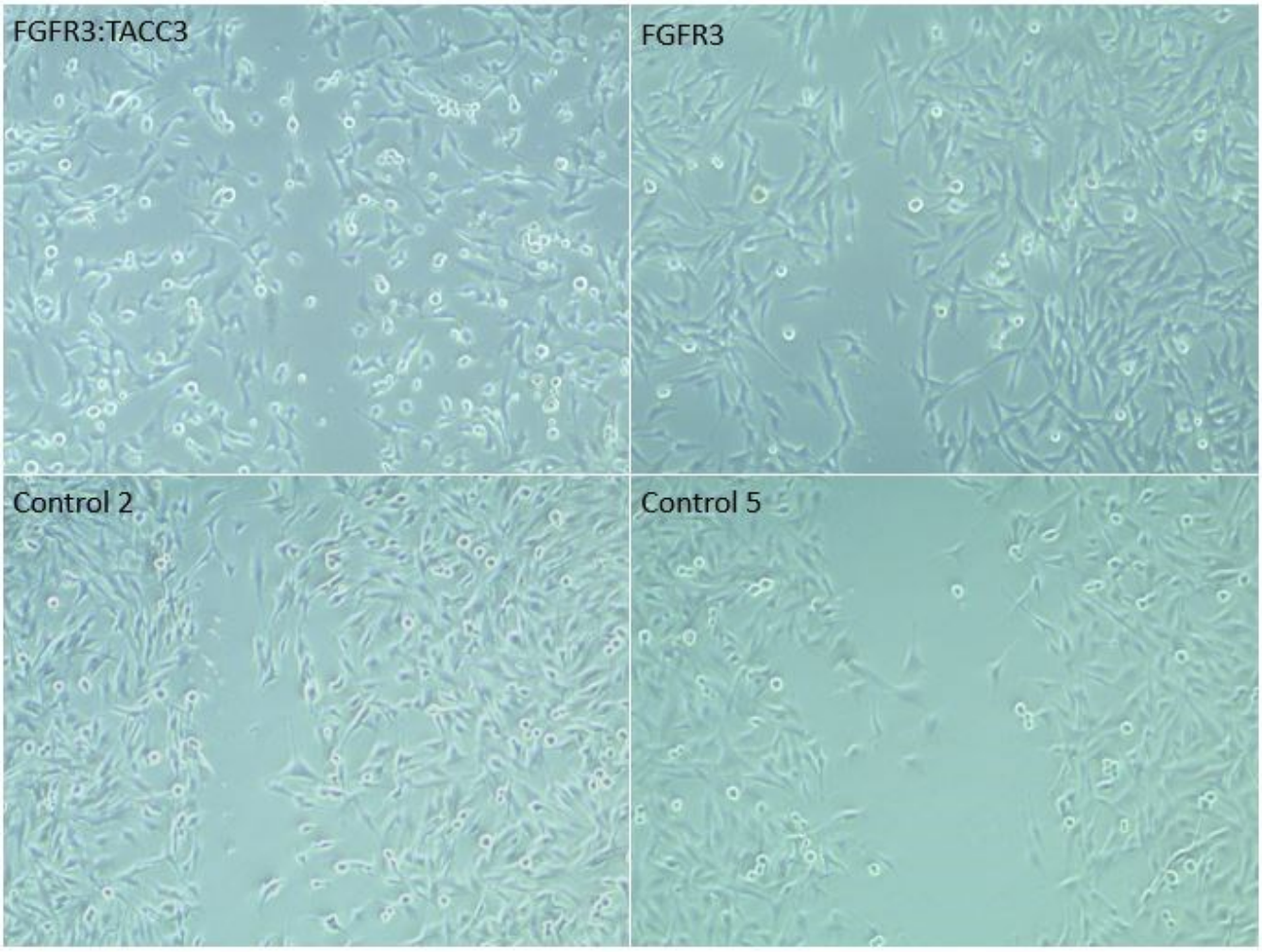

Figure 3. *Wound healing images of all cell lines at 21h time point. Some differences between morphology of the cell is shown. Especially FGFR3 expressing cells are spreading more widely to the surface, than FGFR3:TACC3 expressing cells. All images were plated 160 000 cell/well and magnification of microscope on images is 5x.*