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Expression of Fibroblast Growth Factor 8 isoforms in the breast cancer cell line MDA-MB-231 and in malignant and non-malignant breast tissue

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Expression of Fibroblast Growth Factor 8 isoforms in the breast cancer cell line MDA-MB-231 and in malignant non-malignant breast tissue

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Resumo

O cancro da mama representa um quarto de todos os cancros diagnosticados em mulheres. O fator de crescimento fibroblástico 8 (FGF8) tem um papel importante na embriogénese e está envolvido no desenvolvimento e diferenciação de diferentes órgãos. Apesar de níveis baixos terem sido detetados em tecidos hormonais, foi descrito que o FGF8 é expresso em níveis elevados no cancro da mama.

Para avaliar os efeitos do FGF8 nas células de cancro da mama, foi estudada a proliferação e migração de células MDA-MB-231 transformadas com as isoformas a, b e e em comparação a células controlo. A expressão das isoformas de FGF8 em tecido mamário foi estudada por qPCR.

Os resultados mostraram níveis aumentados de células transformadas com isoformas de FGF8 quando comparadas com células controlo. A taxa de proliferação foi mais elevada em células transformadas com a isoforma e. Os resultados também demonstraram que a taxa de migração foi mais elevada em células MDA-MB-231 FGF8a quando comparada com células controlo.

Baixos níveis de FGF8b e FGF8e foram detetados num pequeno número de tecidos mamários malignos e não malignos. No entanto, a demonstração da expressão de FGF8b e e em tecido maligno e não maligno justifica futuros estudos para entender o papel das isoformas de FGF8 no cancro da mama. Devido à heterogeneidade do tecido mamário, mais amostras precisam de ser estudadas.

Palavras-chave: cancro da mama, qPCR, proliferação, migração

Abstract

Breast cancer represents a quarter of all cancers diagnosed in women. Fibroblast growth factor 8 (FGF8) plays an important role in embryogenesis and it is involved in the development and differentiation of different organs. The level of FGF8 in normal adult tissues is very low or non-detectable but it has been reported to be expressed at elevated levels in some malignancies including breast cancer.

To evaluate the effects of FGF8 on breast cancer cells, proliferation and migration of MDA-MB-231 cells transfected with the FGF8 isoforms a, b and e in comparison with control transfected cells were studied. The expression of the FGF8 isoforms breast tissue using qPCR was also studied.

The results showed an increased level of each FGF8 isoform in transfected cells compared to mock-transfected control cells. Rate of proliferation was higher in the cells transfected with FGF8e than in control cells. The results also demonstrated that the rate of migration was higher in MDA-MB-231 FGF8a cells than in control cells.

Expression of FGF8b and FGF8e was detected in a small number of breast cancer tissues and non-malignant breast tissues. However, demonstration of FGF8b and FGF8e expression in breast and breast cancer tissue warrants further studies to understand the role of the FGF8 isoforms in breast cancer. Due to the heterogeneity of breast cancer tissue, a large number of tumour samples would be needed for further analyses.

Keywords: breast cancer, proliferation, migration

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Index of abbreviations

aFGF	Acidic fibroblast growth factor (= fibroblast growth factor 1)
AIGF	Androgen-induced growth factor
AKT	Protein kinase B
bFGF	Basic fibroblast growth factor (= fibroblast growth factor 2)
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold value
DMEM	Dulbecco's modified eagle's medium
dNTPs	Deoxynucleotide triphosphates
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FGF	Fibroblast growth factor
FGF8	Fibroblast growth factor 8
FGFR	Fibroblast growth factor receptor
HBT	Human (non-malignant) breast tissue
HER2	Human epidermal growth factor receptor 2
HSPG	Heparin sulphate protein glycan
iFBS	Heat inactivated fetal bovine serum
Ig	Immunoglobulin
IGF-I	Insulin-like growth factor I
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
PBS	Phosphate buffered saline

PI3K	Phosphoinositide 3-kinases
PR	Progesterone receptor
qPCR	Real-time polymerase chain reaction
RAF	"Rapidly accelerated fibrosarcoma" serine/threonine kinase proto-oncogene
RAS	"Rat sarcoma" small GTPase signalling protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
STAT	Signal transducer and activator of transcription
TBE	Tris/Borate/EDTA
TBP	TATA box binding protein
TGFβ	Transforming growth factor β
VEGF	Vascular endothelial growth factor

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Chapter I – Review of the literature

i. Breast Cancer

Breast cancer is a major public health problem. It represents a quarter of all cancers diagnosed in women and is the leading cause of cancer death among women.[1] The age seems to be a major risk factor: 50% of patients are 65 years or more and 30% are more than 70 years.[2] It is predicted that by 2050 the global incidence of female breast cancer will reach approximately 3.2 million cases per year.[3]

1.1. Classification of breast cancers

Breast cancer can be characterized by different systems. The classifications aim at selecting the best treatment, which optimally can be related to the type of cancer. This classification is mostly made according to the histopathological type and grade, staging, and molecular expression patterns.[4]

In the histopathological classification breast cancers are divided in *in situ* carcinomas and invasive carcinomas. The *in situ* carcinomas can be divided into ductal or lobular carcinomas and they can develop into invasive breast cancer. Invasive carcinomas comprise subtypes such as ductal, lobular and tubular carcinomas. [5,6]

According to gene expression profiling, breast tumors can be classified into five types: luminal A, luminal B, HER2 positive, basal-like and normal-like.[7] Luminal A cancers are the most differentiated types. They are usually estrogen receptor (ER) and progesterone receptor (PR) positive and they express Ki67 (a cell marker associated with proliferation) at a low level, which all suggest the best prognosis for the patient. Luminal B cancers are also ER positive but present higher levels of Ki67 expression, and therefore the prognosis in this subgroup is slightly worse compared to luminal A.[8] Human epidermal growth factor receptor 2 (HER2) positive cancers occur in 15–20% of total breast cancers and is associated with shortened survival.[9,10] Basal-like carcinomas are also sometimes referred as triple negative (lacking receptors for oestrogen, progesterone or human epidermal growth factor 2, thus being ER-,PR-, HER2-) and they have an aggressive phenotype and poor outcome.[7,11] However, it has been shown that despite triple negative breast cancers and basal-like breast tumours share many characteristics, they are not synonyms.[12]

1.2. Regulation of breast cancer growth

The complex interaction of hormones and growth factors regulate normal breast growth. However, this interaction between hormones, growth factors and cytokines is also involved in the growth of breast cancer cells, regulating biochemical signals that can induce, activate or repress genes. The tumor microenvironment will influence the breast cancer progression.[13]

1.2.1. Hormone regulation

The steroid hormones (estrogens and progestogens) are the main female sex hormones that regulate mammary gland and the presence of these receptors is important for breast cancer characterization and for treatment decision.[14]

Estrogens play an important role in maturation, proliferation, differentiation, and apoptosis and influence the growth and development of breast cancer.[15–17] Increased exposure to estrogen implicit in early menarche, late menopause and obesity is related with an increased risk of breast cancer.[18,19] The effects of this steroid hormone are mediated by two nuclear estrogen receptors: ERα and ERβ. ERα mediates the effects of estrogen on target genes including several protein regulation cell cycle and apoptosis, and certain growth factors and growth factor receptors, triggering directly or indirectly pathways that promote growth and proliferation both in normal and cancerous breast tissue. It has been reported that ERβ usually antagonizes ERα[20] and its expression is decreased during breast cancer progression.[21] In addition, previous studies have shown that ERβ expression in breast cancer cell lines promoted apoptosis and inhibited cell proliferation.[22] However, these data have recently been found conflicting because it has been noted that most used ERβ antibodies are not appropriately specific, which might have influenced previous results.[23]

ER induces and regulates the expression of progesterone receptor (PR) and thus the effect of progesterone in the development of the mammary gland and breast cancer.[24] Progesterone is a steroid hormone the effects of which are mediated by its nuclear receptor (PR) and which plays a major role in lobular alveolar development.[25] Progesterone is also implicated in the cyclical proliferation in the breast during the menstrual cycle.[26] PR is expressed in two isoforms: PR-A and PR-B.[27] It has been reported that increased expression of PR-B is associated with an increased risk of breast cancer.[28]

Tumors with ER and PR exhibit stronger clinical responses to hormonal treatment.[29] For tumors that express ER, endocrine therapies aim to block the ER pathway by depriving the receptor of ligand or directly inhibiting the receptor. [30] Adjuvant therapy with tamoxifen (an estrogen receptor modulator) decreases the rate of recurrence and reduces the death rate by one-third.[31] The absence of PR might be a mechanism for antiestrogen resistance and therefore a poor response to endocrine treatment.[32]

1.2.2. Growth factor regulation

Growth factor pathways mediate many of steroid hormone actions and strongly interact with steroid hormone receptors or their downstream target gene products to control breast cancer growth and progression.[33]

The self-sufficiency in growth signals is one of the six capabilities that define cancer. Growth factors and their receptors are overexpressed in many cancers and they act by autocrine, paracrine or autocrine stimulation. [34]

Insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF) and human epidermal growth factor (HER) pathways, for example, have been reported to have an important role in the development of breast cancer.

Increased concentrations of circulating IGF-I is correlated with the risk of breast cancer.[35] This growth factor stimulates proliferation of breast cancer cells and inhibits cell death.[36] EGF is overexpressed in breast cancer and it has been correlated with poor prognosis,[37,38] and its receptor EGFR is activated by gene amplification or point mutations in a subgroup of breast cancer.[39,40] It has been showed that the EGFR pathway is involved in proliferation and migration in breast cancer cells.[41,42] TGFβ expression has been shown to enhance invasion and metastasis of breast cancer. It is also correlated with poor prognosis.[43–45] VEGF has a crucial role in tumor angiogenesis and it is essential for vascular homeostasis.[46] HER belongs to EGFR family and it is involved in cell proliferation, cell survival and cell adhesion.[47,48]

In addition to these pathways, fibroblast growth factor signaling also plays an important role in the growth of breast cancer cells.

ii. Fibroblast Growth Factor pathways

2.1. Fibroblast Growth Factor family

Fibroblast Growth Factors (FGFs) are 17 to 34 kDa glycoproteins and they share 13-71% amino acid homology.[49] FGFs carry functions in development, tissue homeostasis and metabolism.[50]

The first members were found in an extract of bovine pituitary and brain tissue, from which basic FGF (bFGF) and acidic FGF (aFGF) were isolated and later renamed as FGF2 and FGF1, respectively.[51,52] Several FGF gene clusters suggest that FGF family was generated by chromosomal and gene translocation and duplication during evolution.[49] FGF exists only in multicellular organisms.[50]

The human FGF family consists of 22 members, FGF1 to FGF23; however, mouse FGF15 and human FGF19 are orthologues. 18 of these 22 members are ligands of FGFs receptors. FGFs are classified in seven subfamilies according phylogenetic analysis and according to their mechanism of action – paracrine, intracrine and endocrine FGFs (Figure 1). [50]

Intracrine FGFs are not secreted extracellularly: they lack the signal peptide for secretion function as intracellular molecules. They do not bind to fibroblast growth factor receptors (FGFR), but they have been suggested to interact with intracellular domains of voltage gated sodium channels. It is known that intracrine FGFs regulate the electrical excitability of neurons.[53] It is also been shown that high expression of FGF13 after surgery in prostate cancer is associated with a shortened time to biochemical recurrence.[54]

Paracrine FGFs (also called canonical FGFs), consist of 15 members that bind to tyrosine kinase FGFRs on the surface of neighboring cells, in the presence of heparin sulphate protein glycans (HSPGs).[55,56] The binding to heparin or heparan sulphate cofactor is essential for the formation of stable interaction with FGFR.[55] Paracrine FGFs promote differentiation and growth in embryonic development.[50]

Endocrine FGFs (or hormone-like FGFs) have a low affinity to heparin/heparin sulfate and they have to form complexes with the transmembrane proteins α/β-Klotho (co-receptors) to bind to the extracellular domain of FGFR (Figure 2). [57,58] Endocrine FGFs regulate vitamin D, lipid, phosphate and bile acid metabolism.[59,60]

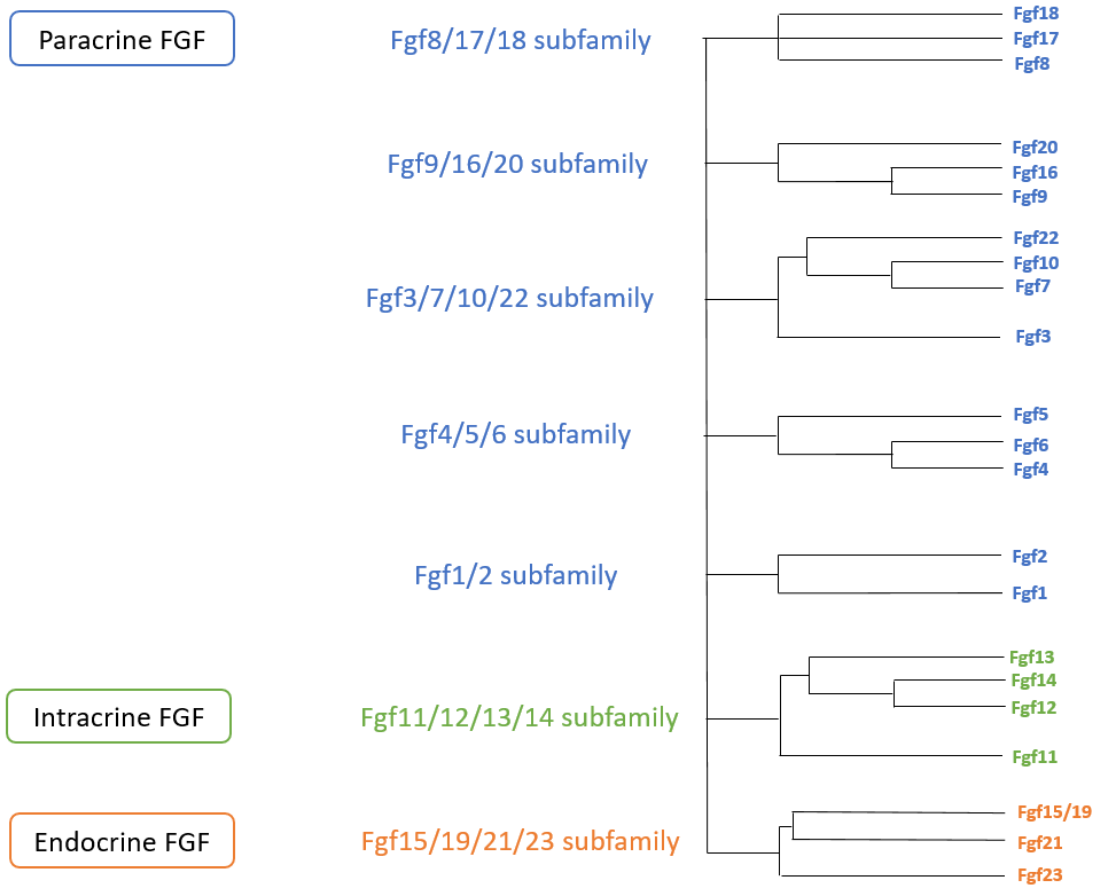

Figure 1 – Classification of Fibroblast Growth Factor family according the phylogenetic analysis and the mechanism of action.

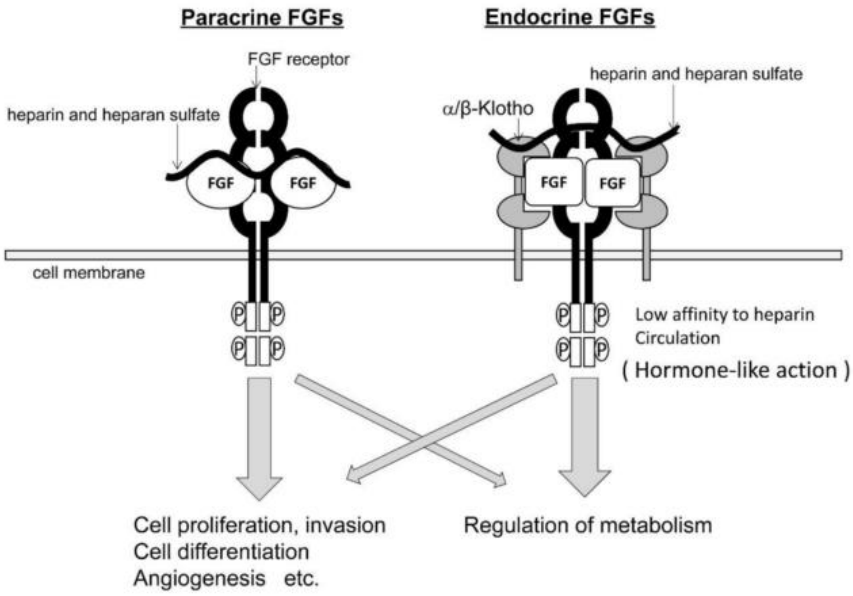

Figure 2 – Mechanisms in signal transduction in paracrine FGFs and endocrine FGFs. (Teishima et al., 2019)

Despite acting mainly as secreted factors, there is evidence of nuclear localization and function of some FGFs.[61]

During development, FGFs have important roles early in gastrulation and in organogenesis such as in the development of brain[62], limbs, liver and pancreas[63] and kidney and urinary tract.[64] In the adult, FGFs are important for tissue repair[65], wound healing[66] and metabolic homeostasis.[60] The FGF pathways critically control epithelial–mesenchymal crosstalk and through these mechanisms regulate differentiation, angiogenesis, survival and invasiveness.[59,67]

Genetic changes including amplifications, translocations and mutations in FGFs and FGFRs leading to aberrant expression have been observed in many human cancers.[68–70] For example, FGF1 is overexpressed in ovarian tumors and is related with poor prognosis[71]; FGF2, FGF6 and FGF8 are upregulated in prostate cancer[72–74]; FGFR1 is amplified and FGFR2 mutated and FGF8 elevated in subgroups breast cancer[75] and FGF18 is overexpressed in colorectal cancer[76].

2.2. Fibroblast Growth Factor Receptors

FGFRs are a family of transmembrane receptor tyrosine kinases that serve as receptors for FGFs and consist of three extracellular immunoglobulin(Ig)-like domains (I-III) linked to an intracellular tyrosine kinase via a transmembrane α-helix.[77,78] There are four classical FGFRs (FGFR1 – FGFR4), however, the formation of heterodimers and alternative splicing enhances the complexity of this system. Due to

alternative splicing, FGFR 1-3 have two versions of Ig-like III domains (IIIb and IIIc), determining ligand binding specificity.[79–81] The IIIb splice form is restricted to epithelial lineages and IIIc is expressed in mesenchymal lineages. [82–84] Distinct FGFs can activate either IIIb or IIIc.[58] Corresponding alternative splicing does not exist in FGFR4, therefore there are seven FGFRs with ligand binding specificities.[79]

Between the IgI and IgII domains, there is a stretch of seven to eight acidic residues called acid box, with an important role in the auto inhibition of the receptor.[85]

There is a lot of redundancy in FGF/FGFR system, considering that several FGF ligands can bind and activate the same FGFRs.[79]

FGF binding to FGFRs induce their dimerization and the phosphorylation that trigger the activation of four key downstream signaling pathways: RAS-RAF-MAPK (proliferation and differentiation), PI3K-AKT (cell survival), STAT (cell division and apoptosis) and PLC$_\gamma$ (cell morphology and migration) (figure 3). [86–88] Dysregulated FGFR signaling is involved in different types of cancer.[77]

There are some paradoxical responses in the FGF pathways. For example, FGF-ERK pathway activation is responsible for promoting proliferation in oligodendrocytes and endothelial cells but it also causes cell cycle arrest in chondrocytes.[89–91] The cellular responses to FGF/FGFR signaling vary depending on the cell type and the state of differentiation.[77,92]

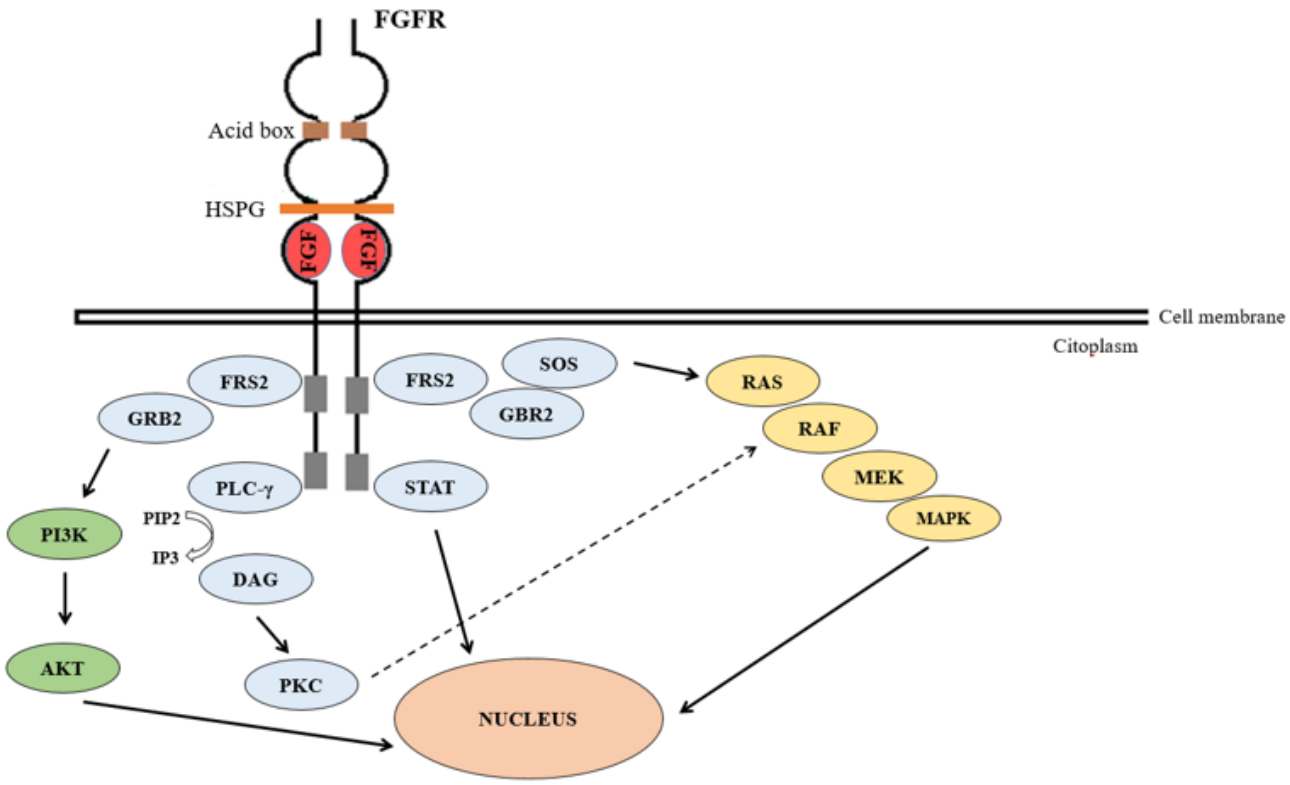

Figure 3 – Simplified representation of FGF signaling

There is also a fifth related receptor, known as FGF receptor-like protein 1 (FGFRL1) or FGFR5, which has no tyrosine kinase domain and it might negatively regulate FGF signaling.[93,94] FGF2, FGF3, FGF4, FGF8, FGF10, and FGF22 are able to bind to this receptor with high affinity.[94]

Concerning the mammary gland development, the FGF/FGFR signaling is involved in the ductal morphogenesis.[95] For example, it has been shown that during embryonic development, the deletion of FGFR2IIIb or FGF10 causes complete absence of mammary glands.[96]

In breast cancer, incorrect activation of FGFR1 has been shown in mammary hyperplasia and invasive lesions.[97] Furthermore, FGFR1 gene amplification is the most common aberration found in breast cancer.[98] Amplification of FGFR2 has also been identified in triple-negative breast cancers.[99]

iii. Fibroblast Growth Factor 8

Fibroblast Growth Factor 8 (FGF8) is a 28-35 kDa protein that functions as a mitogenic embryonic epithelial factor. It belongs to the fibroblast growth factor family.[88] It was first identified as a androgen-induced growth factor (AIGF) from the conditioned medium of mouse mammary carcinoma cell line SC3 after an androgen treatment.[100]

FGF8 belongs to the FGF8/17/18 subfamily and it shares a 70-80% homology of the amino acid sequence with FGF17 and FGF18. These FGFs not only have similar gene structure, but they also overlap in expression patterns and receptor-binding specificities.[49] The binding of FGF8 to FGFRs 1-4 and FGFRL1 triggers its action.[88]

FGF8 has been shown to play an important role in cell proliferation in both embryogenesis and tumorigenesis.[75] Mouse embryos homozygous for FGF8-null mutation die at embryonic day 9.5, due to the main role of FGF8 in early embryogenesis.[101] The expression of FGF8 also controls gastrulation and somatogenesis, a segmental patterning process during early embryogenesis.[102]

FGF8 is widely expressed during embryonic development, and it specifically regulates migration of cells that undergo epithelial-mesenchymal transition in gastrulation[101]. It is also involved in brain, central nervous system, face, limb, ear and eye development and differentiation.[103,104] Nevertheless, in adult normal tissues, the expression is restricted. Low levels have been detected in hormone-regulated tissues such as kidney, prostate, testis and breast.[105–107] FGF8 has also been shown to induce osteoblast proliferation and differentiation in vitro.[108,109]

FGF8 upregulated expression in the lactating breast suggests that FGF8 might be involved in the regulation of lactating cells.[110]

Mutations in human FGF8 gene are associated with congenital cleft lip and/or palate[111] and with deficiency in gonadotropin-releasing hormone.[112]

3.1. FGF8 splicing and isoforms

Alternative splicing is a key factor that increases cellular and functional complexity without increasing genome size.[113]

FGF8 gene is located on chromosome 10q24[114] and it consists of six exons. The first exon is divided into four subexons (1A to 1D) that can be alternatively spliced and generate four different human isoforms (a, b, e and) and eight mouse isoforms (a-h)[115–117] (figure 4).

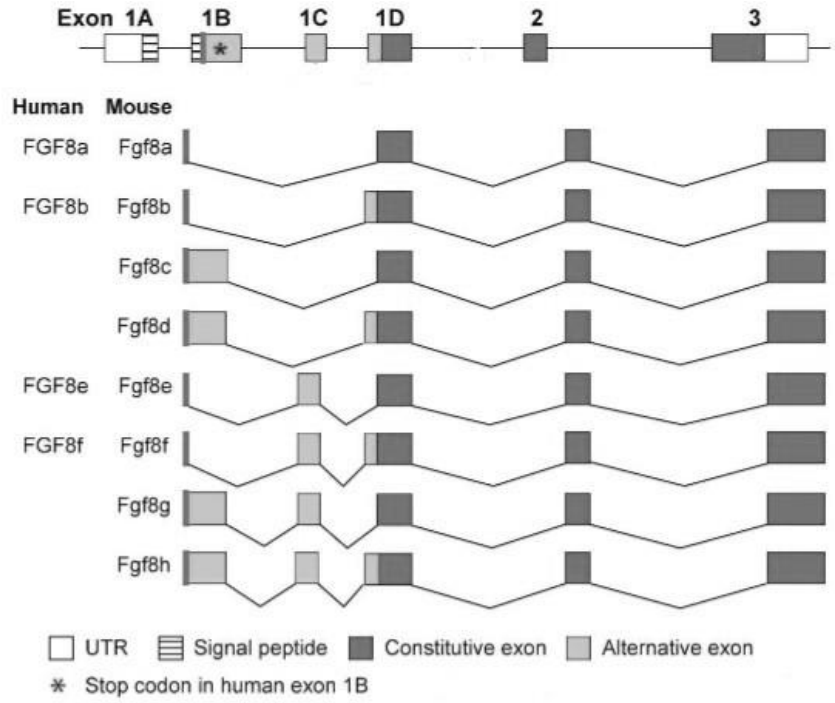

Figure 4 – Structure of FGF8 gene and FGF8 isoforms in human and mouse. (adapted from Sunmonu et al., 2011)

The FGF8 isoforms are similar in their C-terminal domains but they differ in their N-termini. FGF8a and FGF8b are highly conserved[105] while e, f, g and h isoforms are only present in placental mammals.[117]

FGF8a and FGF8b have different roles in brain development and they regulate proteins essential for cerebellar differentiation.[118] It has been demonstrated that FGF8a downregulates osteoclastogenesis.[109]

FGF8b has the ability to activate the receptors, specifically FGFR2IIIC, FGFR3IIIc, FGFR4 and FGFRL1. FGF8a, however, appears to have weaker binding to FGFRs than FGF8b.[119,120]

3.2. Expression of FGF8 in cancer

FGF8 is expressed in cancer cell lines derived from human ovarian, prostate and breast tumors.[75] Prostate and breast cancer cell lines transfected with FGF8 exhibited changes in morphology and motility and increased growth properties.[121,122] FGF8 expression is also associated with rich vasculature of growing tumors.[123] It has been shown that the expression of FGF8 is correlated with the expression of androgen receptor.[124]

FGF8 was detected in benign breast and in breast cancer, however the expression is increased in breast cancer, when compared to non-malignant tissue. [106] It has been shown that more than 50% of human breast cancers express FGF8.[125] It has also been suggested that the expression of FGFR1 is regulated by FGF8 and FGF signaling, which might be important in breast tumors that overexpress FGFs.[126]

FGF8b is the isoform expressed in hormone-dependent tumors and it has mitogenic and angiogenic potential. [121,127] It has been reported that FGF8b may be involved in the progression of hormonal cancers through stimulation of proliferation and angiogenesis. [122,123,128] This growth factor also induces morphological changes and promotes invasiveness of breast cancer cell lines.[121] FGF8b promotes breast cancer growth, increasing CyclinD1 and Ki67 levels and therefore stimulating cell cycle progression and protecting cells from apoptosis.[129]

In addition to FGF8b, FGF8a and FGF8e have also been detected in prostate cancer. Isoforms a and e are more expressed in prostate cancer than in benign tissue prostate samples. FGF8b, however, has similar expression in both cancer and benign prostate tissue.[106,130] The expression of this growth factor in prostate cancer is correlated with poor prognosis[72] and it is also involved in bone metastasis.[131]

Human hematological tumor cell lines were analyzed and it was demonstrated that 64% of them expressed FGF8, frequently co-expressed with high affinity to the receptors.[132]

It has also been shown that FGF8 downregulates the expression of thrombospondin-1, an antiangiogenic factor, which might be involved in the induction of the angiogenic phenotype of cancers that express FGF8.[133]

The paracrine effects of FGF8 promote angiogenesis and osteoblastic differentiation and therefore tumor progression.[75]

Chapter II – Objectives

This study has the following aims:

1. To study the proliferation of the MDA-MB-231 breast cancer cells transfected to express the FGF8 isoforms FGF8a, FGF8b, or FGF8e in comparison to the empty vector -transfected control MDA-MB-231 Mock.

2. To study the migration of MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e compared to MDA-MB-231 Mock.

3. To evaluate the expression of the FGF8 isoforms FGF8a, FGF8 b and FGF8e in malignant and non-malignant human breast tissue samples.

Chapter III – Material and Methods

i. Cell culture

Triple-negative breast cancer human cell line MDA-MB-231 (HTB-26™, ATCC) had been obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in a humidified environment at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Walkersville, MDA, USA), supplemented with 10% heat inactivated fetal bovine serum (iFBS) (Gibco, Life Technologies, Paisley, UK), 1% penicillin/streptomycin (Gibco, Life Technologies, Paisley, UK) and 1% glutaMAX™ (Gibco, Life Technologies, Paisley, UK).

MDA-MB-231 cells were transfected previously as referred in Mattila, 2006 with coding regions for FGF8a, FGF8b and FGF8e. Control transfection was performed using only an empty vector (MDA MB-231 Mock).

Transfected cells (MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e, MDA-MB-231 Mock) were cultured as above and 300-400 μg/mL of G418 (Geneticin) (Gibco, Life Technologies, Paisley, UK) was also added to the medium.

1.1. Cell pellet for RNA extraction

Cells were cultured until optical confluence of 70%-90%, washed with phosphate buffered saline (PBS) (Gibco, Life Technologies, Paisley, UK) and they were detached with trypsin (Life Technologies, Paisley, UK). Medium was added to inactivated trypsin and the cells were centrifuged at 200 g for 5 minutes (Thermo Electron, Osterode am Harz, Germany). Cell pellet was collected, and cell number was determined using an automatic cell counter (Bio-Rad, Hercules, CA, USA).

1.2. Cell proliferation assay

Cells were cultured until optical confluence of 60%-70%, washed with phosphate buffered saline (PBS) (Gibco, Life Technologies, Paisley, UK) and they were detached with trypsin (Gibco, Life Technologies, Paisley, UK). Medium was added to inactivated trypsin and the cells were centrifuged at 200 g for 5 minutes (Thermo Electron, Osterode am Harz, Germany). Cells were resuspended in medium and cell number was determined using an automatic cell counter (Bio-Rad, Hercules, CA, USA). Cells were plated in a 96-well plate ($4x10^3$ cells per well) and maintained in the Incucyte® S3 live-cell microscope (Essen Bioscience, USA) at 37°C and 5% CO_2. Experiment was performed with 9 technical replicates. Cells were monitored, and data was obtained at each 2 hours for 3 days. Data analysis was performed with IncuCyte® S3 Software (v2018B, Essen Bioscience, USA).

1.3. Scratch wound and cell migration assay

Scratch wound assay is a method used to measure directional cell migration in vitro, mimicking cell migration during wound healing in vivo.[135] Cells were cultured until optical confluence of 70%-90%, washed with phosphate buffered saline (PBS) (Gibco, Life Technologies, Paisley, UK) and they were detached with trypsin (Life Technologies, Paisley, UK). Medium was added to inactivate trypsin and the cells were centrifuged at 200 g for 5 minutes (Thermo Electron, Osterode am Harz, Germany). Cells were resuspended in medium and cell number was determined using an automatic cell counter (Bio-Rad, Hercules, CA, USA). Cells were plated in a 96-well plate ($1,5x10^4$ cells per well) and maintained in the incubator for 8 hours to allow them to adhere. Scratch wound was performed with WoundMaker™ (Essen Bioscience, USA). The plate was transferred to Incucyte® S3 live-cell microscope (Essen Bioscience, USA) at 37°C and 5% CO_2. Experiment was performed with 9 technical replicates. Cells were monitored, and data was obtained at each 2 hours for 3 days. Data analysis was performed with IncuCyte® S3 Software (v2018B, Essen Bioscience, USA).

ii. RNA extraction

RNA was extracted from the cell pellet using Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturers' protocol. RNA was eluted in 30 μL of RNase-free water. NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) was used to measure the RNA concentration.

iii. Patient samples

Human breast tissue samples were obtained from 23 patients: 8 non-malignant breast tissue samples and 15 malignant breast tissue samples, as indicated in table 1. The patient samples, based on informed consent from each tissue donor, were obtained from the Auria Biobank in 2017. The collection and use of breast specimens was approved by the local Ethical Committee. Non-malignant breast tissue refers to healthy patient's breast sample and for tissue adjacent to a tumor. For this study, previously extracted RNAs from patient samples were used.

Table 1 – Human breast samples

Sample ID	Tissue
B0100	Malignant tissue
B0200	Non-malignant tissue
B0300	Non-malignant tissue
B0400	Non-malignant tissue
B0500	Non-malignant tissue
B0600	Non-malignant tissue
B0700	Malignant tissue
B0800	Malignant tissue
B0900	Malignant tissue
B1000	Malignant tissue
B1100	Non-malignant tissue
B1200	Malignant tissue
B1300	Malignant tissue
B1400	Non-malignant tissue
B1500	Malignant tissue
B1600	Malignant tissue
B1700	Malignant tissue
B1800	Non-malignant tissue
B1900	Malignant tissue
B2000	Malignant tissue
B2100	Malignant tissue
B2200	Malignant tissue
B2400	Malignant tissue
B2500	Malignant tissue

iv. cDNA synthesis

cDNA from cell lines and patient samples was synthesized from 1 μg of the total RNA. Water was added to each sample in a final volume of 13μL. 1μL of oligonucleotides (New England BioLabs, Inc, NEB) and 0,5μL of dNTPS (Thermo Fisher Scientific, Vilnius, Lithuania) were added. The first reaction was performed in the thermocycler SuperCycler™ Trinity (Kyratec) (Fisher Biotec, Wembley, Australia) at 65° for 5 minutes. The master mix was made using 4,5μL of Reverse Transcriptase buffer (Thermo Fisher Scientific, Vilnius,

Lithuania), 0,5µL of RNA inhibitor and 0,5µL of Maxima Reverse Transcriptase (Thermo Fisher Scientific, Vilnius, Lithuania) per sample. The reaction was performed using the thermocycler SuperCycler™ Trinity (Kyratec) (Fisher Biotec, Wembley, Australia) at 50°C for 30 minutes and 85°C for 5 minutes. cDNA was stored at -20°C.

v. Primer design, qPCR and agarose gel electrophoresis of qPCR products

Two different primers (forward and reverse) were tested for FGF8a, FGF8b and FGF8e. Some primers were designed using the website https://www.ncbi.nlm.nih.gov/ and confirmed in https://www.ensembl.org/index.html (hereinafter referred as first set of primers). The second set of primers was chosen according to a previous publication.[105]

The expression of FGF8 isoforms was evaluated by qPCR. The analysis was normalized for a housekeeping gene - TBP - which is known to be expressed at approximately equal levels in the cells. Both sets of primers are identified in table 2 and 3.

Table 2 – First set of primers used in qPCR reactions

Oligo name	Sequence 5' – 3'	Tm (°C)
FGF8a	FW: GCT GAG CTG CCT GCT GTT	58
	RV: CCG TCT CCA CGA TGA GCT TT	60
FGF8b	FW: CCA AGC CCA GGT AAC TGT TCA	61
	RV: CCG TCT CCA CGA TGA GCT TT	60
FGF8e	FW: TGT CTC CCA ACA GCA TGT GAG	61
	RV: GTG CGG CTG TAG AGT TGG TA	60
TBP	FW: ACT TCA CAT CAC AGC TCC CC	60
	RV: GAA TAT AAT CCC AAG CGG TTT G	58

Table 3 – Second set of primers used in qPCR reactions

Oligo name	Sequence 5' – 3'	Tm (°C)
FGF8a	FW: CCA AGC CCA GCA TGT GAG GGA	65
	RV: TCG GAC TCG AAC TCT GCT TCC AAA	65
FGF8b	FW: CTC CAA GCC CAG GTA ACT GTT	61
	RV: TCG GAC TCG AAC TCT GCT TCC AAA	65

FGF8e	FW: CTC GCT TCC CTG TTC CGG GCT	67
	RV: TCG GAC TCG AAC TCT GCT TCC AAA	65
TBP	FW: ACT TCA CAT CAC AGC TCC CC	60
	RV: GAA TAT AAT CCC AAG CGG TTT G	58

The master mix used per reaction was the following: 0.9µL of H_2O, 0.3 µL of forward primer, 0.3 µL of reverse primer and 7.5 µL of SybrGreen (Thermo Fisher Scientific, Vilnius, Lithuania).

The quantitative endpoint for qPCR is the threshold cycle (C_t), which means that qPCR is in the exponential phase. C_t is inversely related to the amount of amplicon.[136] A primer efficiency test was also performed. Several fold cDNA dilutions were compared to the obtained C_t values. A standard curve was calculated and efficiency was determined by the following formula:

$$E = -1 + 10^{-1/slope}$$

The reactions were performed using in the thermocycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) according to the program in the figure 5. The data was collected and analyzed using the BioRad CFX manager™ software (Bio-Rad, Hercules, CA, USA).

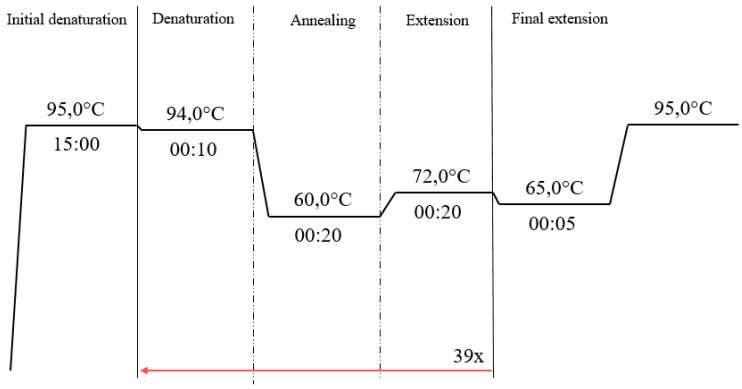

Figure 5 - qPCR program

To confirm the transfection, an agarose gel electrophoresis of the qPCR products of MDA-MB-231 FGF8a cells was performed. 1% of agarose (Bioline, MA, USA) was dissolved in 1x TBE (electrophoresis buffer, prepared by the laboratory) and warmed. One drop of ethidium bromide (Invitrogen ™, CA, USA) was added. Gel set for 30 minutes. Electrophoresis tank was filled with 1x TBE. 1µL of DNA loading dye (Thermo Scientific, Vilnius, Lithuania) per 15 µL of sample was added. Samples and DNA ladder (1 kb, New England Biolabs, USA) were loaded and gel run with 100V for 1hour. Gel was observed and pictures were taken in ChemiDoc™(Bio-Rad, Hercules, CA, USA).

vi. Data analysis

The qPCR data obtained was analyzed through the Ct comparative method[137], using the following formulas:

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (target\ gene) - \Delta Ct\ (reference\ gene)$$

$$Relative\ gene\ expression = 2^{-\Delta\Delta Ct}$$

6.1. Statistical analysis

The statistical analysis was performed using GraphPad Prism 5.0 software. The expression of FGF8 isoforms in patient samples obtained from qPCR was analyzed by a Mann–Whitney test. The data obtained in Incucyte® was analyzed by Kruskal–Wallis test. Differences between experimental conditions were statistically significant at $p<0.05$.

Chapter IV – Results

i. Expression of FGF8 isoforms in cell lines

MDA-MB-231 Mock, MDA-MB-231 FGF8a, MDA-MB-231 FGF8b and MDA-MB-231 FGF8e were maintained in culture and pictures were taken (figure 6).

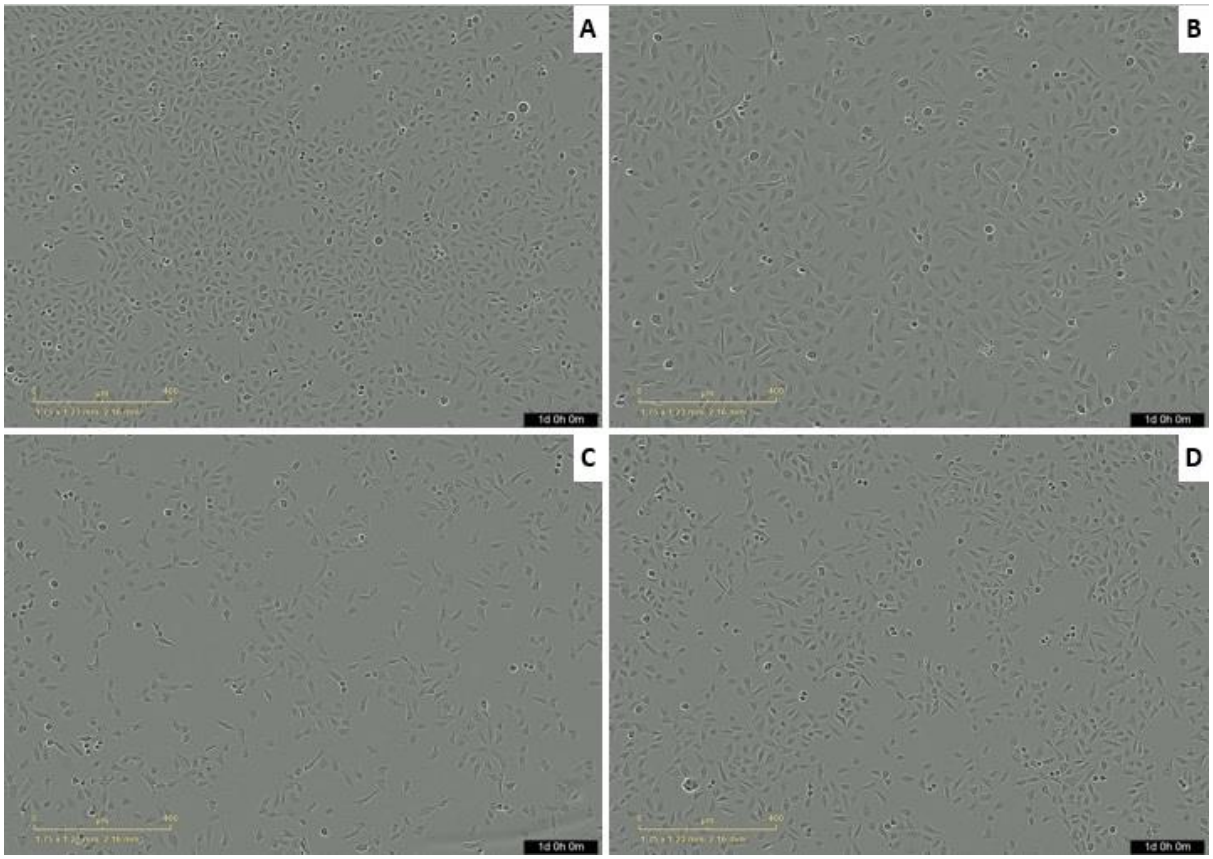

Figure 6 – Pictures of the cells. A) MDA-MB-231 Mock. B) MDA-MB-231 FGF8a. C) MDA-MB-231 FGF8b. D) MDA-MB-231 FGF8e

The relative expression of FGF8 isoforms was obtained from C_t comparative method and the results are presented as power. The figures 7 to 12 show the expression of FGF8 isoforms in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231, which expression was defined as 1, as a control.

Using the first set of primers (figure 7) or the second set (figure 8), FGF8a was detected in MDA-MB-231 FGF8b cells, however higher expression was detected using the first set. No expression of FGF8a was detected in MDA-MB-231 FGF8a cells, contrary to what might have been expected.

Figure 7 – Expression of FGF8a in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the first set of primers.

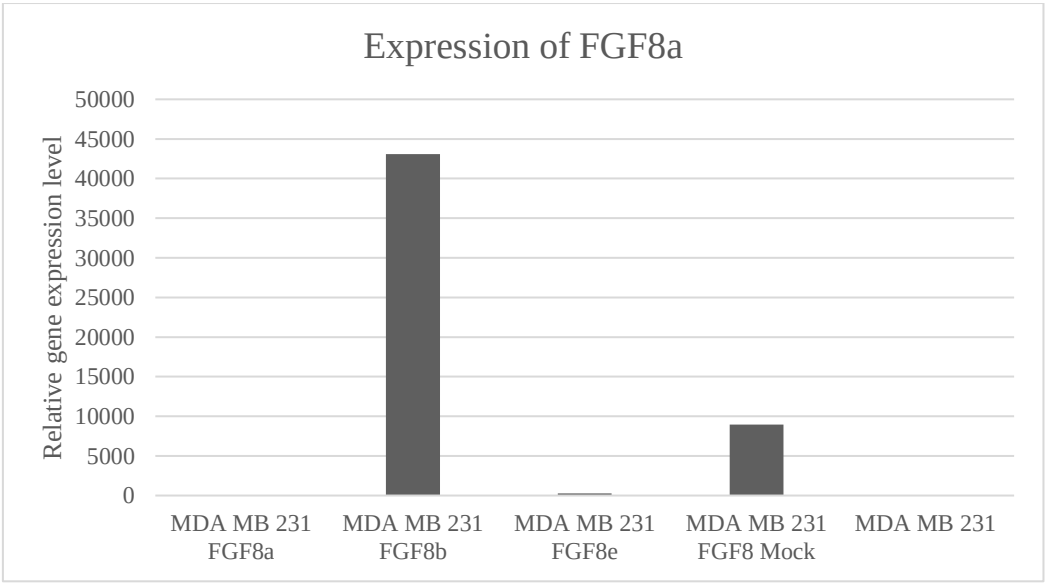

Figure 8– Expression of FGF8a in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the second set of primers.

An agarose gel electrophoresis of MDA-MB-231 FGF8a using primers for FGF8a qPCR products was performed (figure 9). No product was detected when second set of primers was used. A band was detected in MDA-MB-231 FGF8a cells, with the first set of primers. However, the size of FGF8a is 2kb, which does not correspond to the band visible on the gel.

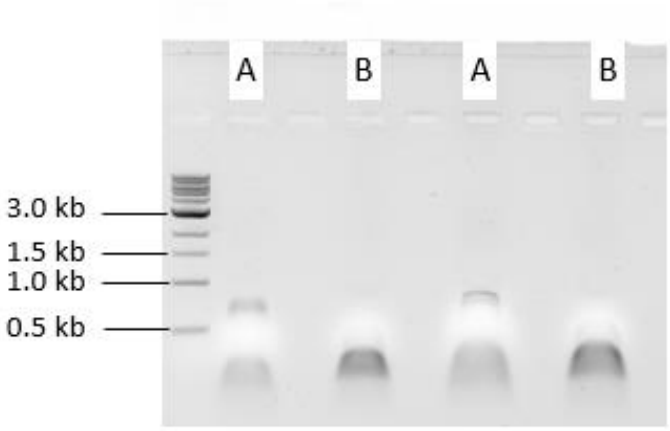

Figure 9 – Agarose gel electrophoresis of qPCR products. A) MDA-MB-231 FGF8a cells with first set of primers. B) MDA-MB-231 FGF8a cells with second set of primers.

FGF8b was detected with both set of primers in MDA-MB-231 cells transfected with FGF8b (figures 10 and 11). Expression was relatively higher using the first set of primers. There was no detected expression of FGF8b in any other cell line, comparing to MDA-MB-231 cells.

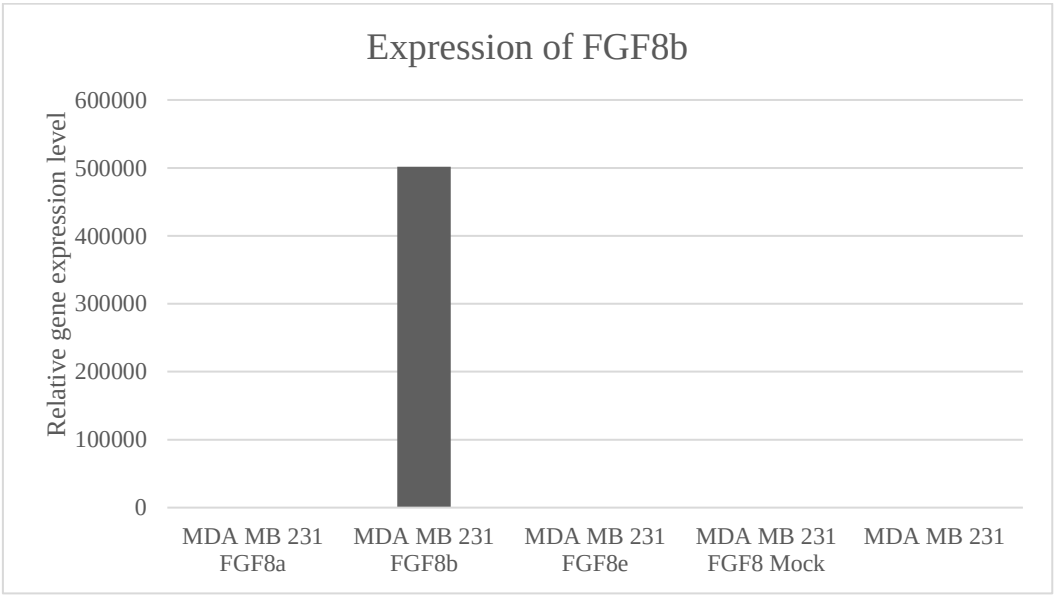

Figure 10 – Expression of FGF8b in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the first set of primers.

Figure 11 – Expression of FGF8b in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the second set of primers.

Using the first set of primers, FGF8e expression is higher in MDA-MB-231 FGF8e compared to MDA-MB-231 cells. Some expression was also detected in MDA-MB-231 FGF8b and MDA-MB-231 Mock, but relatively lower than MDA-MB-231 FGF8e (figure 12).

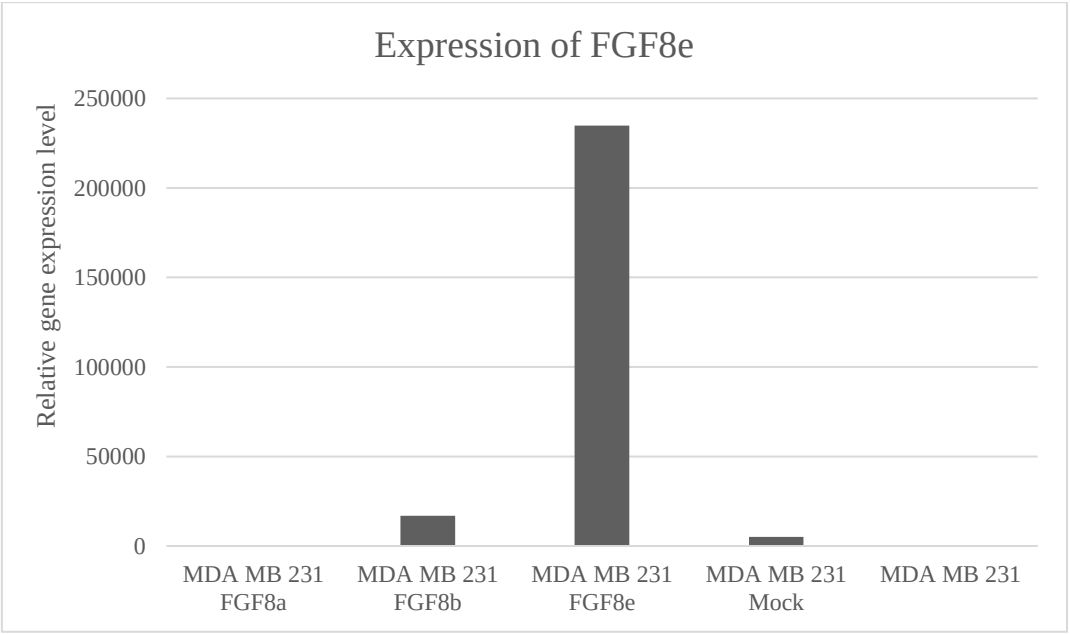

Figure 12 – Expression of FGF8e in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the first set of primers.

The analysis of the qPCR results with the second set of primers revealed some expression of FGF8e in MDA-MB-231 FGF8e cells, however lower than previous qPCR with the first set of primers. It was also detected FGF8e expression in MDA-MB-231 Mock cells (figure 13).

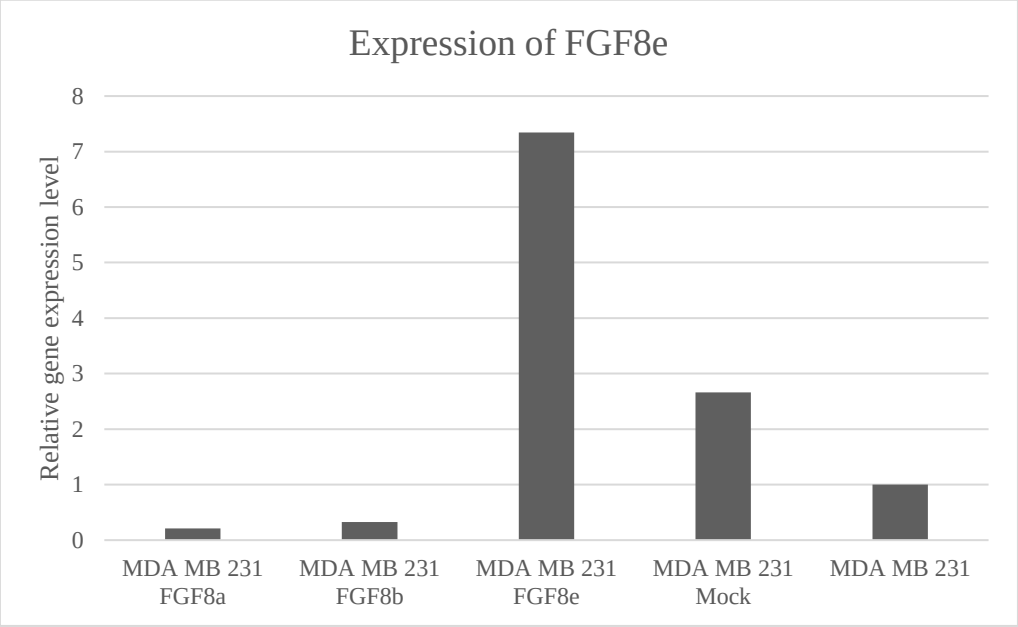

Figure 13 – Expression of FGF8e in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock compared to MDA-MB-231 using the second set of primers.

1.1. Primer efficiency test

The primer efficiency test was made in order to choose the primers for the following experiments. The test was performed using several cDNA dilutions compared to C_t value, for the both set of primers. The slope obtained by the qPCR standard curve allows calculating the efficiency of the primers, using the formula.

The primer efficiency test for FGF8a was not performed, due to high C_q values obtained with both sets of primers.

For the first set of primers, the calculated efficiency for FGF8b primer was 99,86% (figure 14) and 89,17% for FGF8e primer (figure 15).

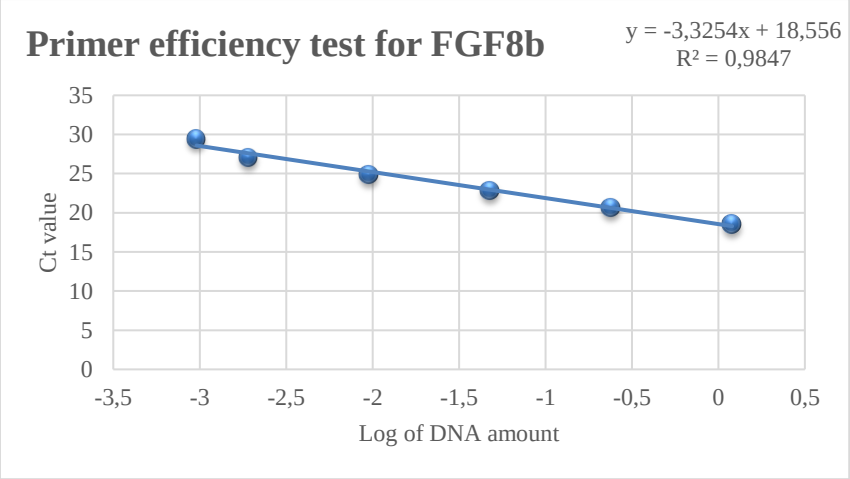

Figure 14 –Standard curve for primer efficiency test for FGF8b, with the first set of primers.

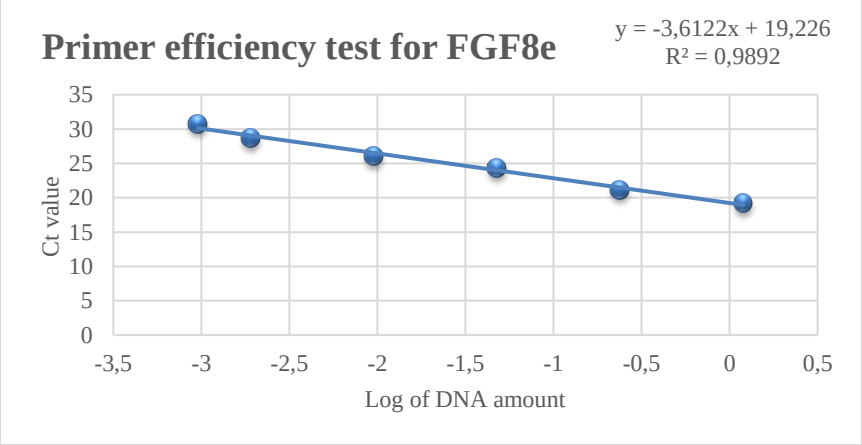

Figure 15 – Standard curve for primer efficiency test for FGF8e, with the first set of primers.po

Using the second set of primers, the calculated efficiency for FGF8b primer was 106% (figure 16) and 206% for FGF8e primer (figure 17).

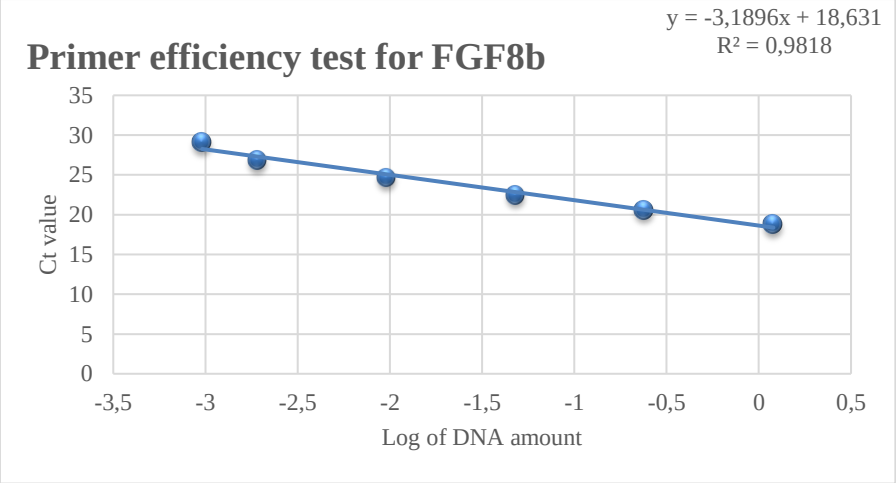

Figure 16 – Standard curve for primer efficiency test for FGF8b, with the second set of primers.

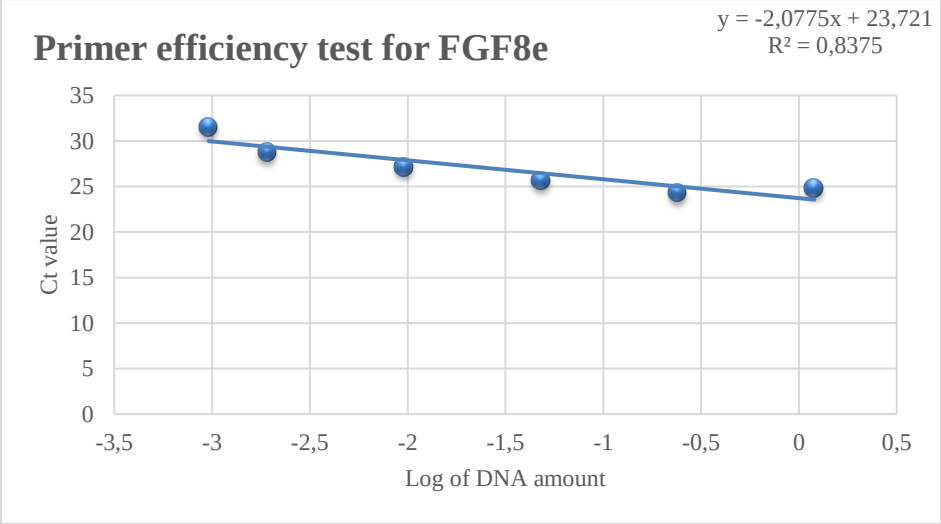

Figure 17 – Standard curve for primer efficiency test for FGF8e, with the second set of primers

Efficiency over 100% might indicate formation of primers dimers or no-specific amplicons. Therefore, the first set was chosen for the following experiments.

ii. Proliferation assay

For proliferation assay, cells were cultured in the incubator with the Incucyte live cell imaging system. The assay was performed measuring the well confluence over three days (cells reached 100% confluence) and data was collected every two hours. The average of the replicas for each cell line was used.

MDA-MB-231 FGF8e cells showed a significantly higher proliferation (*p<0,05*) compared to MDA-MB-231 FGF8a, MDA-MB-231 FGF8b and MDA-MB-231 Mock (figure 18). There was no significant difference in the proliferation rate between MDA-MB-231 Mock, MDA-MB-231 FGF8a and MDA-MB-231 FGF8b.

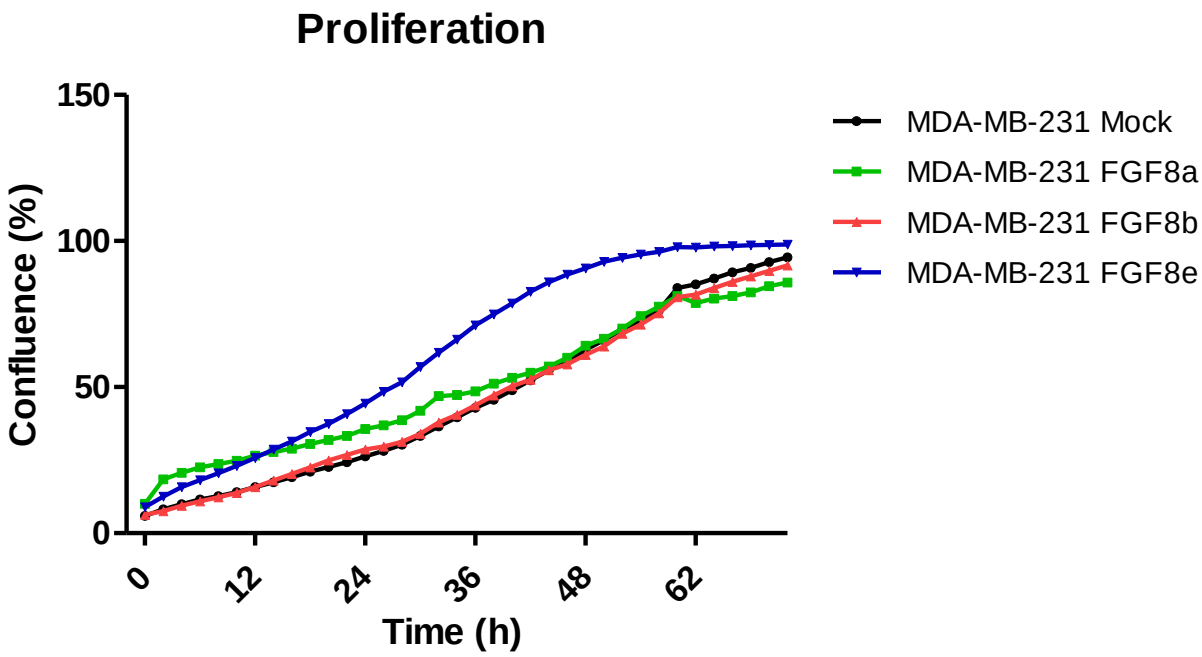

Figure 18 – Incucyte assay for proliferation. Confluence of MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock was measured over time. Experiment was performed three times with eight parallel wells.

iii. Migration assay

The scratch wound assay allows evaluating cell migration. The scratch wound was made when the wells were 100% confluent. The 96-well plate was kept in kept in the incubator with live cell imaging.

The assay was performed measuring the wound confluence over three days (wound reached 100% confluence) and data was collected every two hours. The average of the replicas for each cell line was used and data was normalized.

The assay revealed that MDA-MB-231 FGF8a had the highest migration rate (*p<0,05*) compared to MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock. No differences were found between MDA-MB-231 FGF8e and MDA-MB-231 Mock. MDA-MB-231 FGF8b presented significant differences (*p<0,05*) comparing to the other cell lines (figure 17).

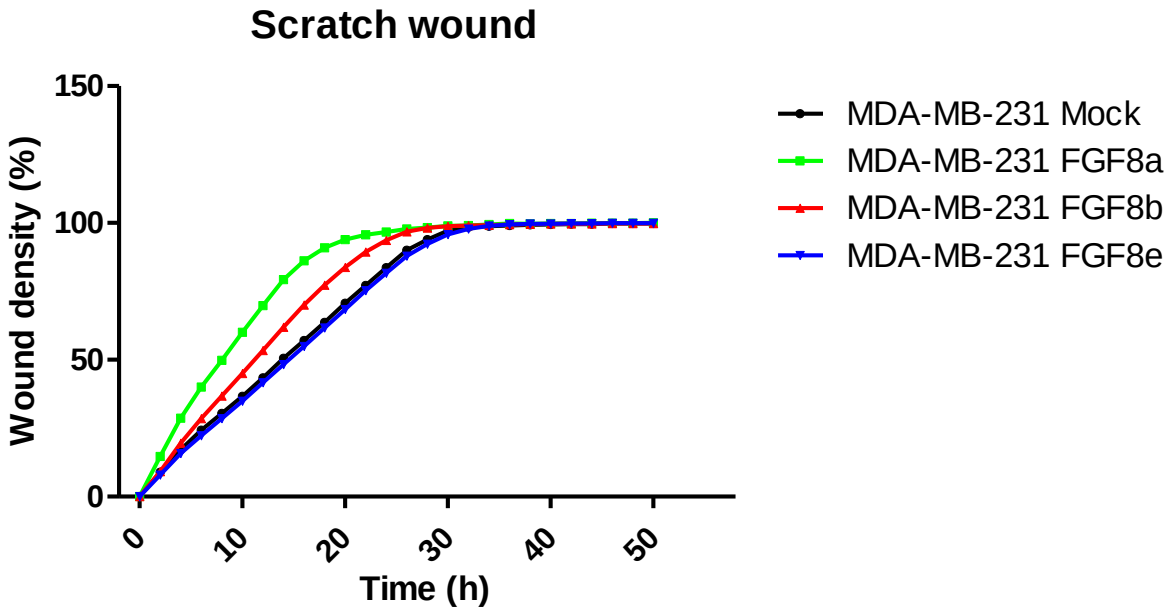

Figure 19 – Incucyte assay for migration. Wound density in MDA-MB-231 FGF8a, MDA-MB-231 FGF8b, MDA-MB-231 FGF8e and MDA-MB-231 Mock was measured over time. Experiment was performed three times with eight parallel wells.

iv. Expression of FGF8 isoforms in malignant and non-malignant breast tissue

Due to the lack of primer efficiency test for FGF8a and to the results obtained in the qPCR and electrophoresis to test the expression in the cell lines, the expression of FGF8a in breast tissue was not evaluated.

The expression of FGF8b and FGF8e in both malignant and non-malignant breast tissue samples was evaluated by qPCR and analyzed by comparative method and statistical analysis.

With comparative method, the mean of the expression of FGF8b and FGF8e in non-malignant breast tissue was calculated and defined as 1 and the expression in malignant breast tissue was compared with it.

The expression of FGF8b and FGF8e was also compared between non-malignant tissue. B1400 showed the lowest expression of both isoforms and it was defined as 1 and the expression in the other non-malignant tissue was compared with this one.

In this method, and concerning FGF8, three malignant breast samples (B2000, B2200 and B2300) showed relatively higher expression compared to non-malignant breast tissue B1400. Some other samples seemed to express FGF8b, but at a lower level. The expression in non-malignant tissue was uniform (figure 20).

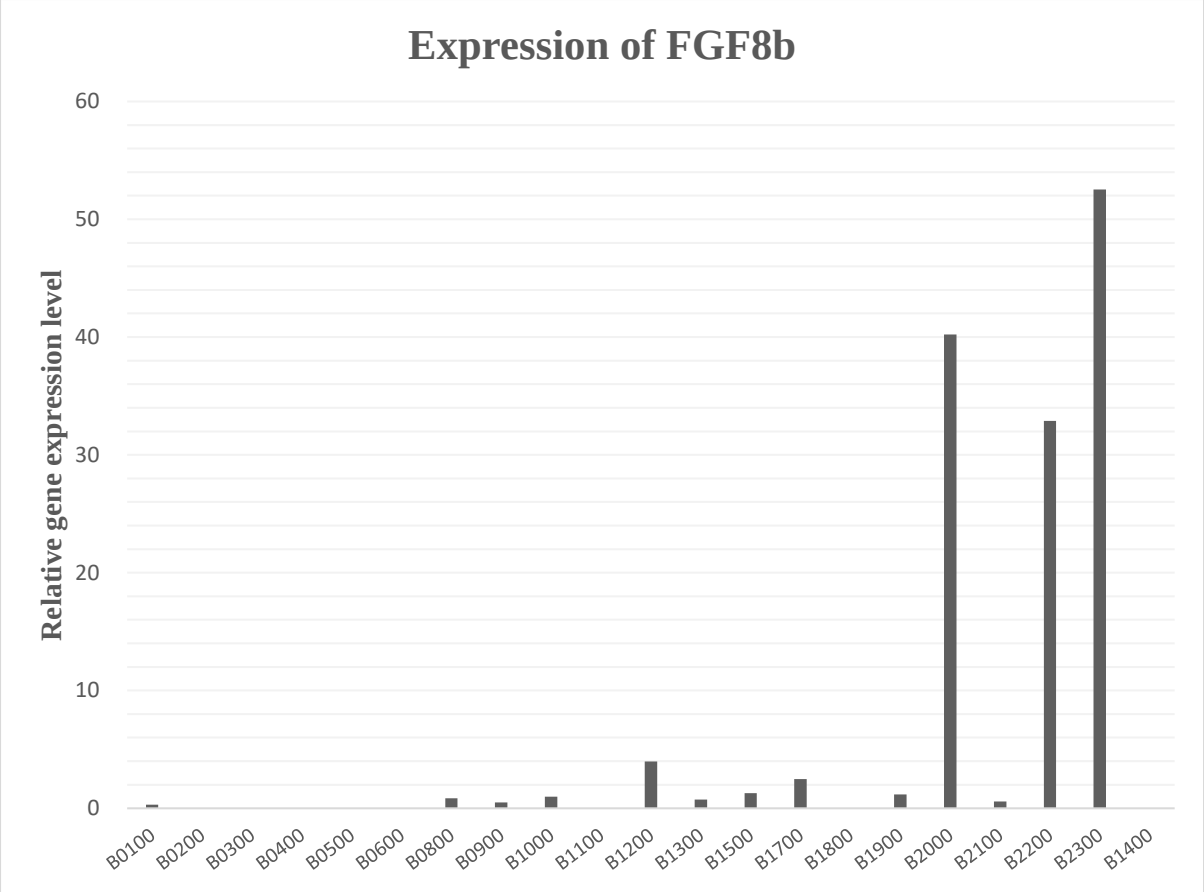

Figure 20 – Relative expression of FGF8b in malignant and non-malignant breast tissue, analyzed by the comparative method. The experiment was performed 4 times, with similar results and the mean was calculated.

The expression of FGF8e analyzed by comparative method showed relative higher expression of FGF8e in eight malignant breast samples. B2000 presented the highest relative expression level, followed by B2300, B2200 and 1900. Some other samples showed some expression compared to non-malignant tissue but at a lower level. The expression in non-malignant tissue was uniform (figure 21).

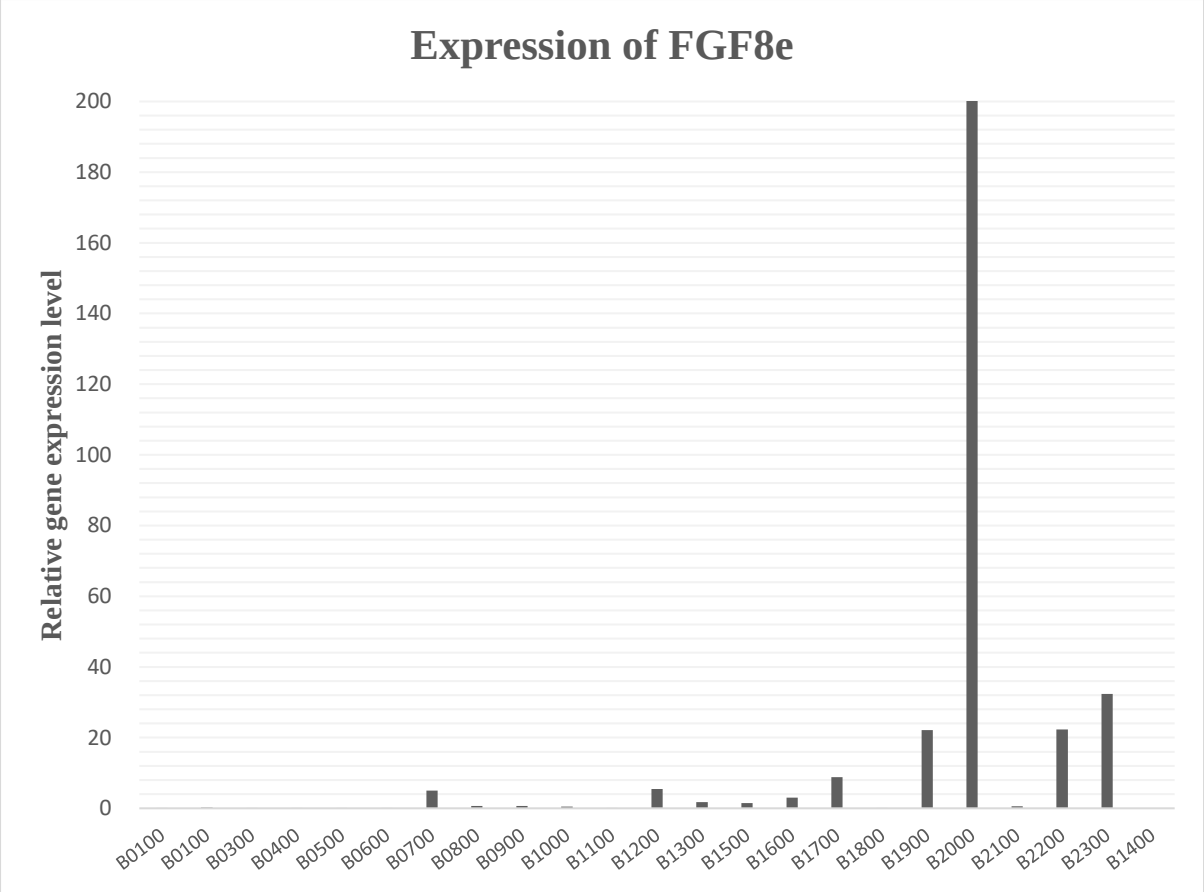

Figure 21 – Relative expression of FGF8b in malignant and non-malignant breast tissue, analyzed by the comparative method. The experiment was performed 4 times, with similar results and the mean was calculated.

The statistical analysis was made, regarding differences in the expression of FGF8b and FGF8e between malignant and non-malignant breast tissue. The comparison was made using the $\triangle C_t$ of each sample.

Although the previous results showed higher expression of FGF8b in some malignant breast samples, there was no difference detected between malignant and non-malignant breast tissue (*p>0,05*) (figure 24). Values under zero indicate that the expression level of the gene FGF8b is higher than that of the housekeeping gene (TBP).

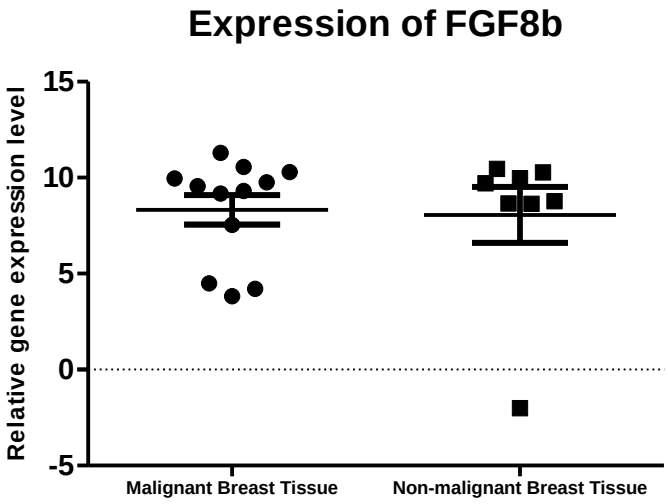

Figure 22 – Comparison of the relative gene expression level of FGF8b between malignant and non-malignant breast tissue.

The evaluation of FGF8e expression presented similar results, showing no difference between the two groups (*p>0,05*) (figure 25). Values under zero indicate that the expression level of FGF8e is higher than that of housekeeping gene (TBP).

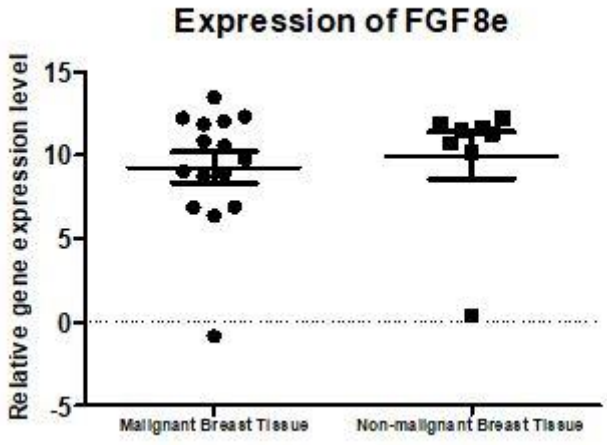

Figure 213 – Comparison of the relative gene expression level of FGF8e between malignant and non-malignant breast tissue.

Chapter V – Discussion

Primers were used to confirm the transfection in MDA-MB-231 cells and to confirm their efficacy, in order to use them in the evaluation of FGF8 isoforms in breast tissue. The primers used in previous publications proved to be less efficient as the ones that were designed for this study. Values over 100% in primer efficiency test might reveal the formation of primer dimers or non-specific products. It has been considered that primers with efficiency between 90% and 110% might be used.[138]

Despite of the electrophoresis performed with qPCR products of MDA-MB-231 FGF8a showed a band, it did not have the expected size. The results suggest that either the primers were not the indicated ones or that the cells were not expressing FGF8a as expected.

FGF8b was detected in MDA-MB-231 FGF8b cells and FGF8e was detected in MDA-MB-231 FGF8e cells, which indicate that the transfection was efficient.

Previous studies reported that prostate cells transfected with FGF8b increased their proliferation.[127,139] The experiments of this study suggested that MDA-MB-231 cells transfected with FGF8b proliferate at the same level as MDA-MB-231 Mock and MDA-MB-231 FGF8a. This might suggest that FGF8a and FGF8b do not influence proliferation in this particular breast cancer cell line, as they show the same proliferation rate as the control. MDA-MB-231 FGF8e showed the highest proliferation rate, compared to any other cell lines. FGF8e has been detected in prostate cancer[105], however the characteristics and mechanisms of this isoform have not yet been understood.

It has been demonstrated that FGF8b enhance growth and migration in both transfected prostate and breast cancer cell lines,[122,140] which is contradictory to the results of the present study. Migration assay showed that MDA-MB-231 cells transfected with FGF8a were the ones with higher migration rate, with a significant difference compared to Mock cells. FGF8b transfected cells showed also increased migration compared to MDA-MB-231 Mock cells.

The results also showed that MDA-MB-231 FGF8e and MDA-MB-231 mock migrate at the same level. Interestingly, MDA-MB-231 FGF8a exhibited the highest migration rate. There are no published results regarding FGF8a migration and proliferation, therefore, this finding might be interesting for future studies. Unfortunately, however, it was not possible to show that convincingly that these results are due to the expression of FGF8a.

Previous results regarding expression of FGF8 in malignant and non-malignant breast tissue were contradictory.[106,107,110] It has been reported that FGF8 expression is increased in human breast cancer compared to non-malignant tissue[75]. However, it has also been reported that FGF8 is expressed not only in cancer cells but also in normal epithelial cells.[75,106]

In this study, FGF8b and FGF8e isoforms were detected in both malignant and non-malignant breast tissue. Relative expression showed high expression levels of FGF8b and FGF8e in some malignant breast samples when compared to others. Relative expression method only shows a relative comparison and it does not indicate significant differences, which is insufficient to analyze discrepancies in the expression of FGF8 isoforms. Using these relative values, the statistical analysis between malignant and non-malignant breast tissue did not show significant differences, in either FGF8b or FGF8e.

In our study, stored RNA from patient samples was used. It has been shown that RNA can be affected by degradation events, even in low temperatures.[141] Therefore, future studies might consider evaluate the FGF8 expression in fresh breast samples or samples collected for a shorth time only. Regarding FGF8a, new primers should be redesigned and tested and the identity of the transfected gene in the clone used confirmed. The migration assay showed interesting data that might be considered, particularly if FGF8a expression is detected in malignant breast tissue samples.

The qPCR showed that some breast cancer tissue samples expressed FGF8b and e at an elevated level. In addition, compared to normal breast tissue (HBT), the expression levels of the FGF8 isoforms b and e showed great variability , which might be expected regarding differences between cancers of different patients and heterogeneity within the individual tumors.

Chapter VI – Conclusions

The data presented and discussed herein allows forming the following conclusions:

1. MDA-MB-231 cells transfected with FGF8e isoform revealed higher proliferation than MDA-MB-231 FGF8a, MDA-MB-231 FGF8b and MDA-MB-231 Mock.
2. MDA-MB-231 FGF8a cells show increased migration compared to MDA-MB-231 FGF8e and MDA-MB-231 Mock cells. MDA-MB-231 FGF8a show the highest migration rate.
3. Expression of FGF8b and FGF8e was elevated in some samples of breast cancer patients compared to the mean level of nonmalignant breast samples.
4. The breast cancer samples showed marked heterogeneity regarding the level of FGF8b and FGF8e expression.
5. Due to a low number of individual numbers analyzed, the differences of FGF8b expression between malignant and non-malignant breast tissue were not statistically significant.

Chapter VII – References

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