Establishment of the Ba/F3 method to test the oncogenic potential of cancer gene mutations

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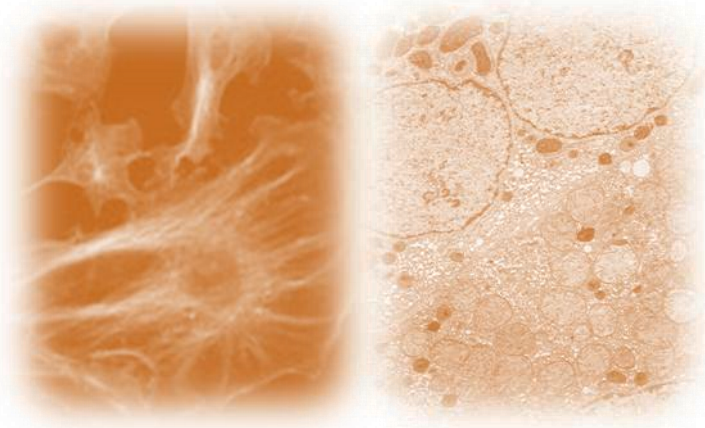

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Establishment of the Ba/F3 method to test the oncogenic potential of cancer gene mutations

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Aknowledgements

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The Norwegian Radiumhospital, Oslo, 21th Mai 2013

Eldri Undlien Due

Abstract

Breast cancer is the most common cancer type among women in Norway and 2839 patients were diagnosed in 2010. Breast cancer is a genetic disease where both germline and somatic mutations lead to cancer development and progression. Each breast cancer patient has a unique genomic and phenotypic background. Alterations and dysregulation of signaling pathways like PI3K/AKT among others, have been reported to have an important impact on treatment response and disease progression. More knowledge is needed about the deregulated genes and their mechanisms in breast cancer. The interleukin-3 (IL-3)-dependent pro lymphoid B-cell line Ba/F3 can be used to study the oncogenic potential of selected cancer genes. Here, our aim was to establish this method to be used in our laboratory. Expression of an oncogenic allele decides if the Ba/F3 cells grow in absence of IL-3 or not. Three missense mutations, *TP53* K120E, *TP53* H179R and *PIK3CA* P110 S405F, were tested. Plasmids carrying the mutated genes were constructed by *in vitro* site-directed mutagenesis. The validation of the mutagenesis was performed using Sanger sequencing. Plasmids carrying the mutated genes and control plasmids were transfected by electroporation, where the permeability of the cells was increased by an electric field and the plasmids were transferred into the Ba/F3 cells. After transfection, the Ba/F3 cells were cultured in the presence of IL-3 and Blasticidin for 10 days, to select for transfected cells. The parental cells died as they were not resistant against Blasticidin, whereas the Ba/F3 cells with plasmids survived. The transfected cells were further cultured in the absence of IL-3 and the cultures were microscopically evaluated and photographed every week. Microscopic evaluation of transfected Ba/F3 cell lines showed oncogenic mutations for *TP53* H179R, *PIK3CA* P110 S405F and the positive control *PIK3CA* P110 E545K. However, *TP53* K120E was non-oncogenic. The cell viability was determined with Trypan blue staining and cell viability assay. According the viability assay, the cells were not viable indicating that we were unsuccessful in creating stable cell lines. In summary, we have successfully established the Ba/F3 method in our laboratory and tested the oncogenic potential of known mutations in the *TP53* and *PIK3CA* genes.

Sammendrag

Brystkreft er den mest vanlige krefttypen blant kvinner i Norge og 2839 pasienter ble diagnostisert i 2010. Dette er en genetisk sykdom hvor både arvelige og somatiske mutasjoner kan føre til kreftutvikling. Hver brystkreft pasient har en unik genetisk og fenotypisk bakgrunn. Endringer og dysregulering av signalveier som PI3K/AKT blant flere, har vært rapportert å ha en viktig innvirkning på behandlingsrespons og sykdomsutvikling. Mere kunnskap er nødvendig om deregulerte gener og deres mekanismer i brystkreft. En interleukin-3 (IL-3) avhengig pro lymfoid B-cellelinje Ba/F3 kan brukes for å studere onkogenisk potensial av utvalgte gener. Målet var å etablere denne metoden i vårt laboratorium. Ekspresjonen av et onkogenisk allel bestemmer om Ba/F3 cellene vokser i fravær av IL-3 eller ikke. Tre missens mutasjoner *TP53* K120E, *TP53* H179R og *PIK3CA* P110 S405F ble testet. Plasmider som bærer mutante gener ble konstruert med *in vitro* punkt mutagenese. Sanger sekvensering ble utført for å validere mutagenesen. Plasmider med mutante gener og kontroll plasmider ble transfektert med elektroporering, hvor cellepermeabiliteten på Ba/F3 cellene økte under påvirkning av høy spenning og plasmidene ble overført inn i cellene. Etter transfeksjonen ble Ba/F3 cellene dyrket med IL-3 og Blasticidin i 10 dager for å få selektert for transfekterte celler. De parentale cellene døde fordi de ikke var resistente mot Blasticidin, mens Ba/F3 celler med plasmider overlevde. De transfekterte cellene ble dyrket videre uten IL-3 i mediet og cellekulturene ble mikroskopisk evaluert og fotografert hver uke. Mikroskopisk evaluering av de transfekterte Ba/F3 cellelinjene viste onkogeniske mutasjoner for *TP53* H179R, *PIK3CA* P110 S405F og positiv kontroll *PIK3CA* P110 E545K. *TP53* K120E var ikke onkogenisk. Celleveksten ble bestemt ved Trypan blå farging av cellene og celleviabilitetsanalyse. I følge denne analysen var cellene døde og dette indikerte at vi ikke lyktes med å etablere stabile cellelinjer. Som en oppsummering så var etableringen av Ba/F3 metoden i vårt laboratorium vellykket, og vi fikk testet det onkogeniske potensialet til kjente mutasjoner i *TP53* og *PIK3CA* genene.

Aim of the study

The aim of this master project was to establish a Ba/F3 method in our laboratory. The Ba/F3 cell line is a murine IL-3 dependent pro lymphoid B-cell line and can be used to study the oncogenic potential of selected cancer gene mutations. Furthermore, we aimed to study the oncogenic potential of three mutations; two mutants in the *TP53* and one mutant in the *PIK3CA* gene. After transfection of parental Ba/F3 cells, the expression of oncogenic alleles leads to cell growth in the absence of IL-3, and cell viability can be measured. By using this functional assay we can characterize whether gene mutations are oncogenic or non-oncogenic. This model system can be useful as a tool in assessing the oncogenic potential of cancer gene mutations, the downstream signaling of these, as well as in testing whether the mutations are druggable.

Abbreviations

3,4,5 PIP_3	Phosphatidylinositol 3,4,5 triphosphate
4,5-PIP_2	Phosphatidylinositol 4,5 biphosphate
AB	Applied Biosystems
ABD, RBD, C2	Adaptor binding domain, Ras-binding domain , C2 domain, domains in the *PIK3CA* gene
ADH	Atypical ductal hyperplasia
ADP	Adenosine diphosphate
AG	Adenine Guanine
AJCC	The American joint committee on cancer
ALH	Atypical lobular hyperplasia
AML	Acute myeloid leukemia
Amp	Ampicillin
ATP	Adenosine triphosphate
AUG	Adenine Uracil Guanine
Ba/F3	Murine IL-3 dependent pro-B-cell line
BALB/C mice	Albino laboratory-bred strain of a house mouse
BRCA1/BRCA2	Breast Cancer 1, early onset/ Breast cancer 2, early onset
BSD	Blasticidin
cDNA	Complementary DNA
COSMIC	Database
CTG	Cell titer Glo®
CYP3A4/5, CYP2D6	Cytochrome p450 enzymes
DBD	DNA binding domain
DCIS	Ductal carcinoma *in situ*
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
E	Glutamic acid
EGFR	Epidermal growth factor receptor
ENU	*N*-ethyl-*N*-nitrosourea
ER	Estrogen receptor

F	Phenylalanine
FBS	Fetal bovine serum
G1-G3	Histological grade1 - grade3
Gab2, IRS	Adapter proteins in intracellular downstream signaling
GFP	Green fluorescence protein
GFR	Growth factor receptor
GT	Guanine Thymine
H	Histidine
HER 1, 2, 3, 4	Human epidermal growth factor receptor 1, 2, 3, 4
HTR	Hormone replacement therapy
IDC	Invasive ductal carcinoma
IDC NOS	Invasive ductal carcinomas not otherwise specified
IL-2	Interleukin -2
IL-3	Interleukin-3
IL-7	Interleukin -7
ILC	Invasive lobular carcinoma
IPTG	Isopropyl-1-thio-β-D- galactopyranoside
JAK2, JAK3	Janus kinases
JH1, JH2	Domains in Janus kinases
K	Lysine
kb	kilo base
Ki67	A protein used as cellular marker for proliferation
LB	Luria broth
LCIS	Lobular carcinoma *in situ*
LFH	Laminar flow hood
MDM2	Gene encoding E3 ubiquitin-protein ligase Mdm2
miR	MicroRNA
MOHITO	Mouse hematopoietic Interleukin dependent cell line of T-cell origin
NBCG	Norwegian breast cancer group
NGS	Next generation sequencing
NMD	Nonsense –mediated decay

P210 $^{bcr/abl}$	Protein, derived from a hybrid gene created from chromosomal translocation that generates the Philadelphia chromosome.
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PIK3CA	Phosphatidylinositol-4,5,bisphosphate 3-kinase, catalytic subunit alpha
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
R	Arginine
rcf	Relative centrifugal force
RTK	Receptor Tyrosine kinase
S	Serine
TAM	Tamoxifen
TCGA	The cancer genome atlas network
Tm	Melting temperature
TNM	Tumor Node Metastasis
TP53	Tumor protein p53
TX	Texas
v.	Version
wt	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. Introduction

1.1. Cancer

Cancer is a leading cause of death worldwide with an estimate of 12,7 million new cases and 7,6 million cancer deaths in 2008. Aging and growth of the world population are increasing. Changes in lifestyle added to pre-existing risk factors will increase the number of cancer incidence in many developing countries (1).

Cancer is the most frequent genetic disease (2) and studies on cancer patient materials show clonal accumulations of somatic mutations over time (3). Over the past few decades, development of new sequencing- and molecular profiling methods has revealed the complexity of alterations in the cancer genomes.

Hanahan and Weinberg have characterized human cancer and its development (4;5). The six hallmarks of cancer are resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, evading growth suppressors, and sustaining proliferative signaling. These characteristics enable tumors to growth and metastasize. Underlying these hallmarks are inflammation and genomic instability.

In 2011, two emerging hallmarks, reprogramming energy metabolism and evading immune destruction, were also included in the characterization of cancers (Figure 1) (5). Tumor tissue also comprises of normal cells such as fibroblast and endothelial cells which interact with each other. These cells among others are part of the tumor microenvironment and active participants in tumorigenesis by providing growth signals, which affect the proliferation. Another contribution from stroma is the supply of signals to resist apoptosis to increase the survival of tumor cells.

Cancer cells can be affected by altered stress level resulting in phenotypic stress such as metabolic, proteotoxic, mitotic, oxidative and DNA damage stress (6). These phenotypes are not initiating tumorigenesis but characterize many tumor types. They are not unique to cancer cells only, but can also be observed in other conditions, such as chronic inflammation.

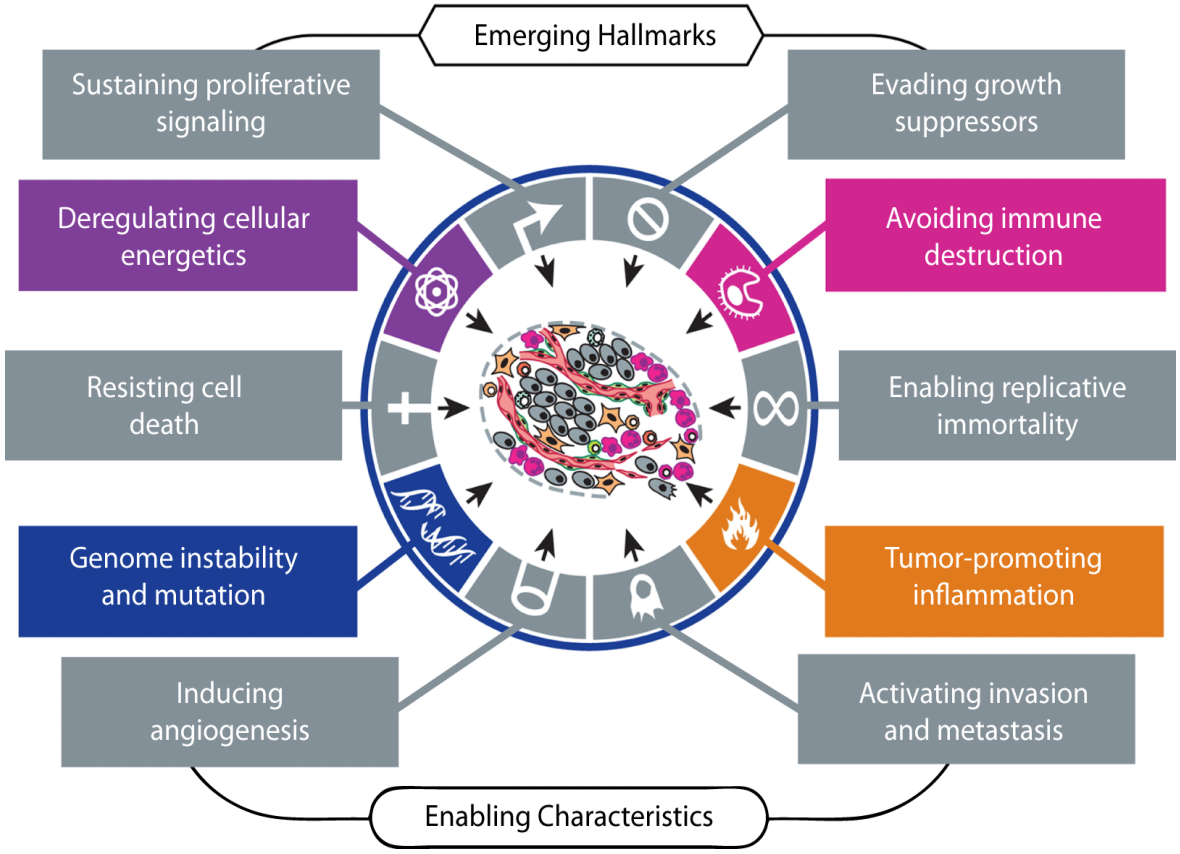


Figure 1. Enabling characteristics and emerging hallmarks of cancer.

The six hallmarks of cancer are resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, evading growth suppressors and sustaining proliferative signaling (grey text boxes). Underlying these hallmarks are tumor-promoting inflammation and genomic instability and mutation (blue and orange text boxes). The two emerging hallmarks are deregulating cellular energetics and avoiding immune destruction (purple and violet test boxes). The figure is modified from Hanahan and Weinberg (4;5).

1.2. Breast Cancer

Breast cancer is the most frequently diagnosed cancer among women worldwide. In 2008, 1,38 million new cancer cases were diagnosed (1). The disease caused over 400 000 deaths yearly (7). Breast cancer is a heterogenic disease (8). The subchapters below address the aspects of heterogeneity on the epidemiological, clinical, and the molecular level.

1.2.1. Survival, incidence and mortality of breast cancer in Norway

The survival of breast cancer has increased as a result of early diagnoses, improved treatment strategies and better surgery. Almost 90 % of the breast cancer patients are alive five years after diagnosis without any sign of relapse of the disease, (Figure 2) (9). In 2010, the number of cases was 2839 and the relative mortality was 673 (9).

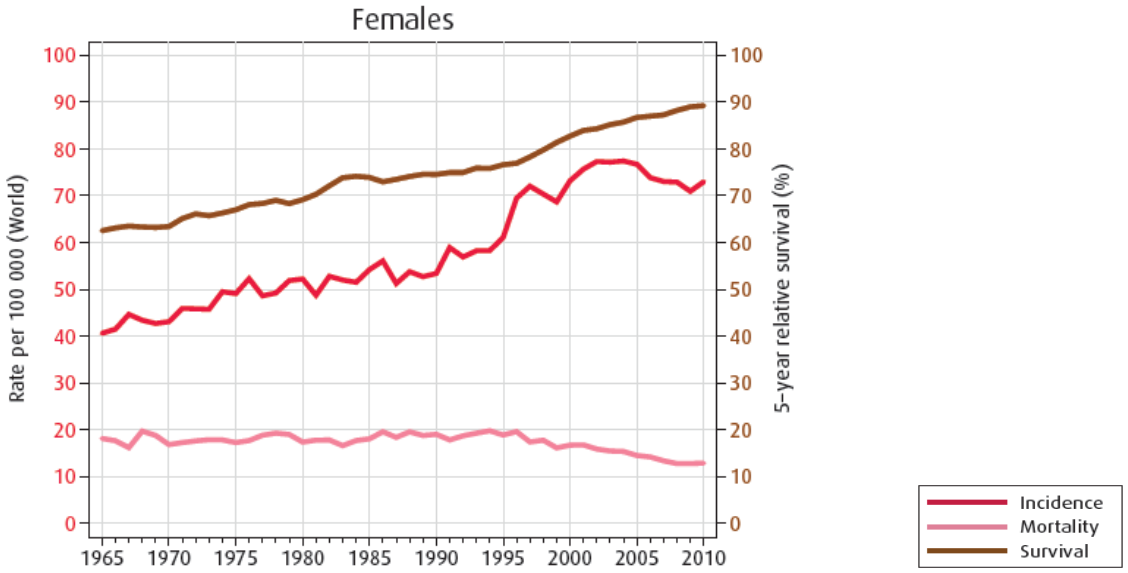


Figure 2. Trends in the incidence, mortality and 5-years survival proportions of breast cancer in Norway. The survival has increased in the last two decades (9).

1.2.2. Genetic risk factors

Genetic alterations associated with developing breast cancer can be either inherited or acquired. Out of the total number of breast cancers only 5-10% represent the familial type (10), as illustrated in Figure 3. These patients have a mutation of the *BRCA1* or *BRCA2* tumor suppressor genes. Patients with these mutations are hereditarily predisposed for breast cancer and have a high risk of developing the disease (11). Only 20 % of the familial types have mutations in *BRCA1* and *BRCA2* genes. A large proportion of the familial breast cancers have unknown familial predisposing genes and may be due to combinations of genetic variants of different loci (10).

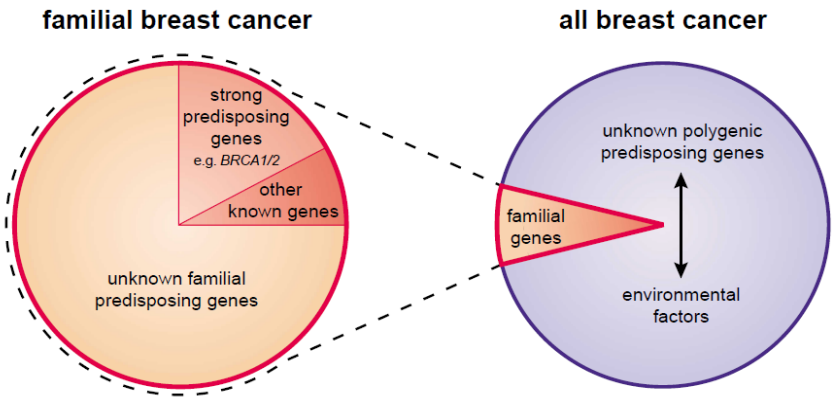


Figure 3. Diagram showing the proportion of familial breast cancer among all breast cancer. 5-10% of total number of all breast cancers represent the familial type (right) and the genes involved are *BRCA1/2* **.**These two genes are mutated in only 20 % of the familial cancers. A large proportion of the familial breast cancers have unknown familial predisposing genes (left) (10).

1.2.3. Environment and lifestyle factors

The high incidence rate of breast cancer is attributed to lifestyle factors. These include physical inactivity, hormone replacement therapy (HTR), overweight or obesity, alcohol consumption and dietary habits (12). All these factors may affect not only the incidence, but also mortality and treatment outcomes. For example, breast cancer patients with obesity have been found to have a poorer prognosis (13).

Dense breast tissue is also a risk factor for breast cancer. Mammographic density is characterized by increased number of breast epithelial cells and stromal tissue (14). Reproductive parameters such as low parity, late age at first pregnancy (>35 years), late menopause (>55 vs <45 years) and early menarche (<12 vs 16 years) are also risk factors (12).

More than 80 % of breast cancer patients are over 50 years old. A possible reason is the exposure to endogenous and environmental carcinogens, which lead to accumulation of gene mutations over time (12). The environmental influence may also lead to hyper methylation which can lead to silencing of tumor suppressor genes such as *BRCA1/2* and *TP53* (12;15).

1.2.4. Normal breast anatomy and breast cancer development

Location of an adult female breast is between the second and sixth ribs, and it consists of skin, subcutaneous tissue and breast tissue. Each breast has 15-20 lobules surrounded by adipose tissue (Figure 4). The lobules produce milk, and the milk ducts carry milk from the lobules to the nipple. Blood supply is transported from internal mammary and lateral thoracic arteries. More than 95% of lymphatic drainage goes through the axillary lymph nodes (16).

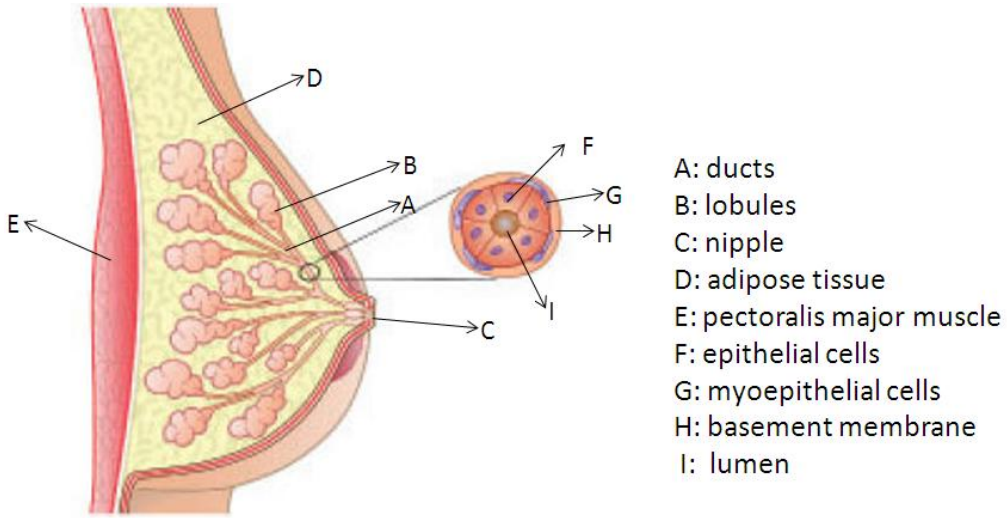


Figure 4. Anatomy of the breast. The figure shows the organization of the ducts and lobules with surrounding adipose tissue in the human breast. The cross section shows the lumen surrounded with luminal epithelial cells which are aligned in a polar manner. The myoepithelial cells surround the luminal epithelial cells in a non-continuous layer. The basement membrane is surrounded by these cells and aligned by fibroblasts. The entire structure is surrounded by stroma (17).

Atypical hyperplasia can either be atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH). ADH is characterized by abnormal cells within the duct (Figure 5). ALH occurs in the epithelial cells lining of the milk lobules, which become irregular in size and shape. In *in situ* carcinomas, the tumor is still encapsulated by the basement membrane. The most common type is ductal carcinoma *in situ* (DCIS) and this cancer type are situated inside the milk duct (18). In the lobular carcinoma *in situ* (LCIS) abnormal cells are located in the lobules (19). In the invasive stage the degradation of the basement membrane has taken place, and the tumor cells invade the surrounding tissue (Figure 5). Invasive ductal carcinoma (IDC) means that the cancer is situated in the milk ducts. The cancer cells have broken through the

duct wall and have started to invade the surrounded tissue in the breast (20). Invasive lobular carcinoma (ILC) are located in in the milk-producing lobules and invades through the lobule wall and the breast tissue (21). Typical for ILC`s are small, round cells with less cytoplasm, which infiltrate the stroma without destroying it. ILC can have increased propensity for both bilateral disease, multifocal and multicentric distribution (22). Some of the tumor cells metastasize and migrate to different organs through blood and lymph vessels.

Accumulation of acquired genetic alterations leads to tumor initiation and progression. Epigenetic and microenvironmental changes also play a role in this development (2;23). Epigenetic changes, such as DNA methylation, may lead to changes in the microenvironment. The microenvironment comprises of fibroblasts, myofibroblast, leukocytes, myoepithelial and epithelial cells among others. Studies have also shown that macrophages play a role in promoting angiogenesis, invasion and metastatic spread (23).

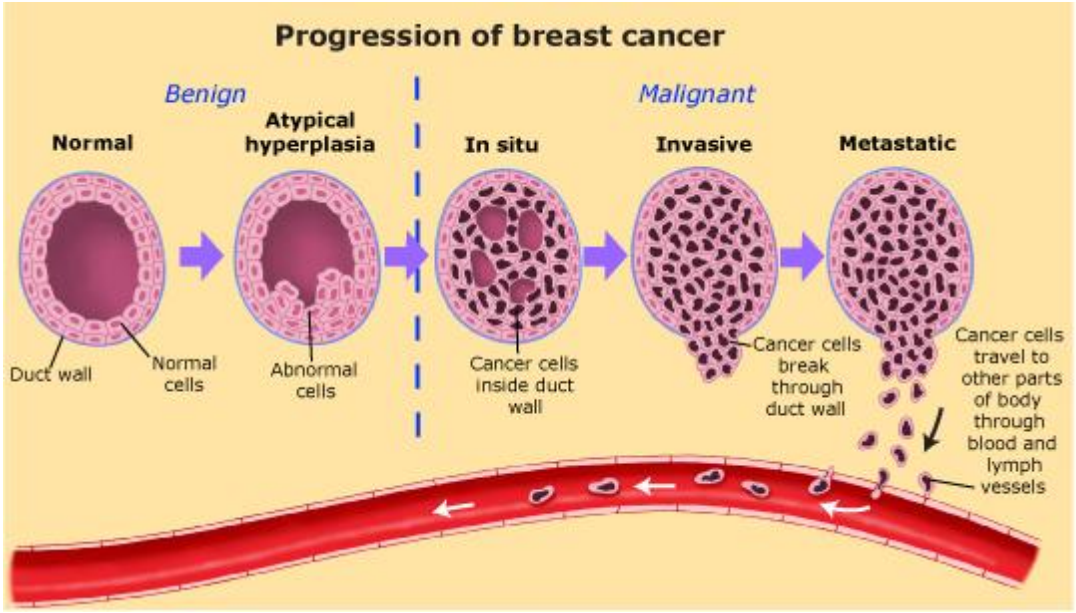


Figure 5. A hypothetic model of the breast cancer progression through the different stages in a duct of the breast. The figure of the normal breast duct illustrates an organized lining of epithelial cells surrounded by a basement membrane. The cancer development is illustrated through the hyperplasitic, *in situ*, invasive, and metastatic stage (24).

The majority of the breast cancer cases (50-80%) are classified as invasive ductal carcinomas, Not Otherwise Specified (IDC NOS). The remaining ~25 % of cancer cases are defined as histologically "special types", Weigelt and colleagues analyzed 11 of these by using

immunohistochemistry and genome-wide expression profiling (25). These special types are ILC, tubular, mucinous A, mucinous B, neuroendocrine, apocrine, IDC with osteoclastic giant cells, micropapillary, adenoid cystic, metaplastic and medullary carcinoma. Correct classifications of these special types can facilitate more optimal treatment and accurate the prognostic information for breast cancer patients.

1.2.5. Breast cancer classifications

Classification systems are useful tools for the clinicians when planning the treatment and estimating the prognosis of the patient. Different subtypes of breast cancer display differential aggressiveness and responses to the treatment. Some tumors are more malignant than others and require more aggressive treatment (26).

1.2.5.1. Histological grades

The grade refers as to how much the tumor cells resemble normal cells of the same tissue type (27). For breast cancer a modification of the Bloom-Richardson system is the most commonly used for the histological grading. The system is based on a description which combines architectural and nuclear characteristics of the tumor cells, and is based on a sum of the scores from of the following three parameters: tubule formation, nuclear pleomorphism and mitotic activity (Table 1) (16). The histological grades G1-G3 characterize the level of differentiation. Grade 1 is well differentiated and has a good prognosis, whereas Grade 3 is poorly differentiated with poor prognosis (28).

Table 1. The histological grades for breast cancer. The sum of scores is based on tubule formation, nuclear pleomorphism and mitotic activity (16).

Histological grade	Sum of scores	Characterization
G1	3-5	Well differentiated
G2	6-7	Moderately differentiated
G3	8-9	Poor differentiated

1.2.5.2. Breast cancer stages

The Tumor, Node, Metastasis (TNM) staging system was developed by The American Joint Committee on Cancer (AJCC). It is based on the tumor size (T), regional lymph node involvement (N) and whether the breast cancer has metastasized (M) or not (16;26;29). A combination of the different categories of T, N and M determines the stage of the breast cancer. Stage 0 describes non-invasive breast cancer and stage IV invasive metastatic breast cancer (Table 2).

Table 2. A simplified table of the Tumor, Node, Metastasis (TNM) staging system.

TNM system	Category	Short description
T (Describes the primary tumor)	Tx	No classification due to missing information
	T0	No primary tumor
	Tis (DCIS or LCIS)	Ductal or lobular carcinoma in situ
	Tumor size: T1 =<2 cm T2 >2.0=<5.0 cm T3 >5 cm T4 Independent on size	There are also subclasses within these categories.
N (Describes whether or not the cancer has reached nearby lymph nodes)	Nx	No cancer detected or measured in nearby lymph nodes.
	N0	Nearby lymph nodes do not contain any cancer.
	N1, N2, N3	Number based on lymph node involvement and how much cancer is found in them. The greater the number the greater extent of the lymph node involvement. Also subclasses in these categories.
M (Describes whether or not the cancer has spread to other parts of the body)	Mx	Metastasis cannot be measured or found.
	M0	There is no distant metastasis.
	M1	Distant metastasis is present.

1.2.5.3. Hormone receptor status

The level of the endocrine receptors is also important in describing the breast tumor. The endocrine receptors are estrogen receptor (ER) and progesterone receptor (PR). If the breast cancer cells are ER+, the growth will be promoted in by the presence of estrogen. PR+ tumors will grow in response to progesterone. The hormone receptor status is used to guide the treatment of the patient. Approximately 75% of the breast cancers are ER+ and the ER status can change over time. Of the ER positive cases 65% are positive for PR (30).

1.2.5.4. The proliferation marker Ki67

Ki67 is a protein marker which gives information about the proliferation rate of the cancer cells. The proliferation index is defined as the percentage of Ki67 positive cells. The proliferation rate describes how fast the cancer cells are growing and can be used as a measurement of aggressiveness.

1.2.6. Molecular subtypes

Breast cancer is a heterogenous disease. It is composed of a spectrum of different tumor subtypes with distinct cellular origins, etiologies and somatic changes (31). Gene expression profiling has contributed to a taxonomy called "intrinsic subtypes of breast cancer". These subtypes are named basal-like, Her2-enriched, luminal B, luminal A and a normal breast like. These subtypes are correlated with patient outcome (32). A sixth subtype named "Claudin-low" group was suggested as a subtype in 2007 and was further characterized in 2010 (33;34). The claudin-low subtype is similar to the mammary epithelial stem cell profile (34), and is ER, PR and human epidermal growth factor receptor 2 (HER2) negative. HER2 will be described more detailed in the subchapter 1.2.9.

Curtis *et al.* (35) suggested ten groups based on an integrated genomic/transcriptomic study. This study describes how copy number alterations affect the gene expression and which novel subgroups should be the focus of further investigations (35).

1.2.7. Treatment of breast cancer

The classifications above are useful in determining which treatments the patients should receive. There are national guidelines for recommendations for surgery, radiotherapy and

systemic treatment. The treatment is multimodal (36) and may consist of surgery and different treatments such as chemotherapy, adjuvant hormonal therapy, immunotherapy, radiotherapy and targeted therapy. Some examples of targeted therapy of breast cancer are briefly described in the sub chapters below.

1.2.8. Targeting estrogen receptor

Tamoxifen (TAM) treatment is the usual endocrine therapy for (ER) positive breast cancer in pre-menopausal women. The pro-drug TAM is a non-steroid compound and has a competitive ER antagonist effect. Most of the metabolism of TAM occurs in the liver. The cytochrome p450 enzymes CYP3A4/5 and CYP2D6 metabolize TAM to the active metabolite Endoxifen (37). Norwegian Breast Cancer Group (NBCG) recommends that medicines which could inhibit CYP2DG activity should not been used in combination with TAM. Endocrine therapy can also give positive "side effect" on the skeletal on postmenopausal women (38). The drug has agonist activities in bone and liver. It mimics the effect of estrogen and prevents osteoporosis.

Five years adjuvant treatment with TAM has increased the survival by 33 % during the first 15 years after the start of the treatment (39). Recommendation for the treatment of postmenopausal women in Norway after surgery is two years with an aromatase inhibitor and a subsequent three years with TAM. Alternatively, aromatase inhibitor can be given for five years. An aromatase inhibitor stops the estrogen production in postmenopausal women resulting in less estrogen available for the stimulation of the ER receptor. The reason why premenopausal women are not treated with aromatase inhibitor is that the drug cannot stop the ovaries from producing estrogen. For premenopausal women the treatment is five years with adjuvant TAM treatment (40).

1.2.9. Targeting human epidermal growth factor receptor 2 (HER2)

Approximately 20-25% of the breast cancers have amplification and / or overexpression of the *HER2/neu/ERBB-2* proto oncogene. This tumor type is related to worse outcome and higher recurrence rate compared to the *HER2* negative breast cancer (41). Genetic alterations lead to overexpression and constitutive activation of the HER2 protein. In addition to HER2 also HER1, HER3 and HER4 exist and they are transmembrane tyrosine kinase receptors (42). Their oncogenic functions are among others stimulation of PI3K/AKT/mTOR signaling

pathway (43) followed by cellular proliferation and survival of cancer cells. PI3K/AKT signaling pathway and the *PIK3CA* gene are described more in detailed in the sub-chapters 1.3.2 and 1.4.5.

Trastuzumab (Herceptin) is a humanized murine monoclonal antibody. The antibody works as an antagonist and targets and blocks the HER2 receptor by binding to its extracellular domain (Figure 6). Inhibition of both growth and survival of cancer cells occur (42). Approximately 30% of HER2 positive breast cancers respond to Trastuzumab treatment (44). Studies have been focusing on the mechanisms of resistance against this drug and the research is still ongoing in this field. A suggested biomarker for prognoses for this therapy is *PTEN* expression combined with *PIK3CA* mutation status (45). Loss of *PTEN* combined with mutations in *PIK3CA* in HER2 positive breast cancer predicts Trastuzumab resistance (43;44).

Juntilla *et al.* (46) focused on activators in the PI3K pathway and Trastuzumab resistance. They found that combination of agents was more effective in preventing tumor growth than single agent. Breast cancer cells with loss of PTEN or activating mutations in the PI3K pathway show resistance to Trastuzumab but are sensitive to Lapatinib (44). Lapatinib is a tyrosine kinase inhibitor that inhibits the receptor signaling by binding to the ATP-binding pocket of the EGFR and HER2 protein kinase domains. Trastuzumab and Lapatinib act at different sites of the receptor. A combination of these two drugs may be beneficial. Trial are ongoing to evaluate Lapatinib in combination with endocrine or chemotherapeutic drugs and its role in an adjuvant setting (47). Esteva *et al.* (48) found more Trastuzumab resistance and worse survival in HER2 positive breast cancer patients with loss of *PTEN* in combinations with multiple components altered in PI3K pathway. These selected studies illustrate the complexity of targeted therapy of HER2+ breast cancer tumors. Martini *et al.* (41) noted that integration studies on expression profile, copy-number alterations, and methylations together with the mutational status will refine the cancer picture. Then the predicted response to targeted therapies can be more precise.

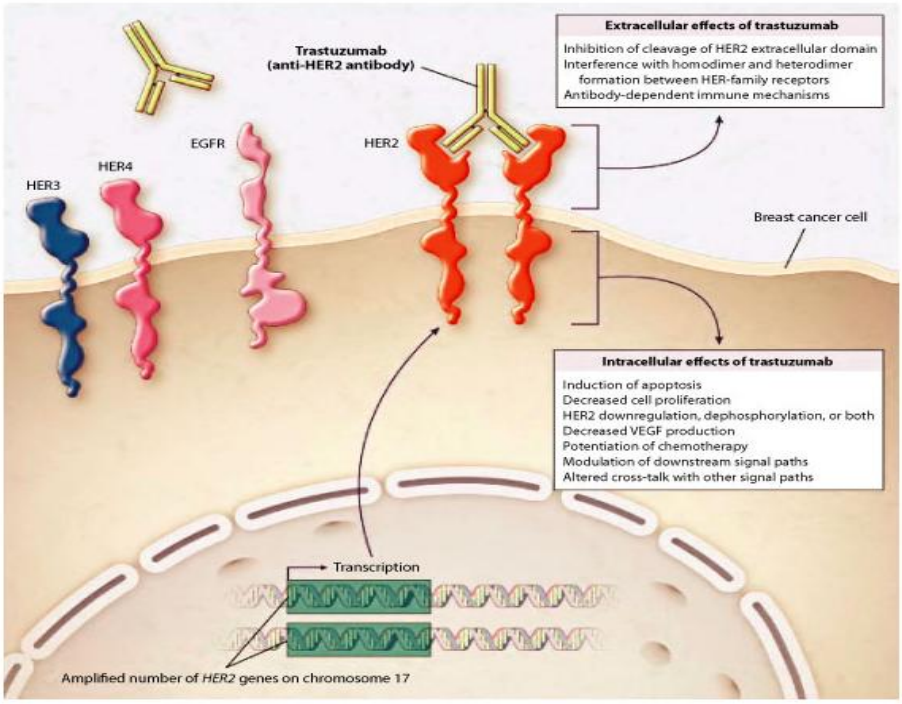


Figure 6. Mechanism of Trastuzumab. The drug is a humanized murine monoclonal antibody and it acts as an antagonist by targeting and blocking the HER2 receptor. It binds to the extracellular juxtamembrane domain of the receptor (49).

1.2.10. Integrating genomic levels in breast cancer

In the last years the researchers have increased the focus on integrating at different –omics levels. An article from The Cancer Genome Atlas Network (TCGA) described analysis of 825 primary breast cancer samples by combining data from five platforms, DNA methylation, exome sequencing, DNA methylation, genomic DNA copy number arrays and exome sequencing (50). One of the findings was that the somatic mutation frequencies were more than 10% in only three genes across all breast cancers: *TP53*, *PIK3CA* and *GATA3*. Ellis *et al.* summarized six recent integration studies including this article from TCGA. One of their conclusion marks was to identify driving genetic events together with the subtype of breast cancer. This is important for diagnosis and treatment purposes of this patient group (51).

1.3. Signaling pathways

Biological and physiological processes in a multicellular organism are coordinated and regulated by cell signaling. The cells cooperate by receiving and sending signals to each other. The environmental factor, such as temperature shock, nutrient molecules or differences in extracellular ion concentrations, can also influence the cells (52) .

The cell signaling regulates many processes such as differentiation, metabolism, proliferation and movement. Cell signaling is divided in three different types which are paracrine (between neighboring cells), synaptic (across the synaptic cleft) and juxtacrine (the transmitting cell and the responding cell are in directly contact) (52). The next subchapters focus on the p53 protein signaling pathway and the MDM2-p53 loop as a regulator for interactions within this pathway (53). The phosphatidylinositol 3-kinase (PI3K) signaling pathway is also presented. This pathway is the second most affected pathway with genetic aberrations after the p53 signaling pathway in cancer (44). More than 70 % of all breast cancers have mutations in genes involved in the PI3K pathway (54). PI3K and its downstream components show extensive cross-talking with several other signaling pathways. This leads to a complex network of signals (55). One of the main focuses is to understand the mechanisms in this network when developing combination therapies. The influence of activation of alternative cascades and feedback loops are also important to study (44).

1.3.1. The p53 signaling pathway

P53 protein is encoded by the *TP53* tumor suppressor gene which has the highest mutation frequency with 31 % of all human tumors (7). Under normal conditions the p53 levels in the cells are low because p53 is destabilized mainly by the action of MDM2. When the cells are affected by various types of stresses, p53 is released from MDM2 (Figure 7). Increased p53 levels lead to various phenotypic changes and are partially dependent on the strength, duration and nature of the activating signal (53). P53 senses stress signals from oncogenes, DNA damage, telomere erosion or metabolic deprivation (7). The stress response can result in cell cycle arrest by inhibiting cell cycle progression, induction of apoptosis, DNA repair and metabolic homeostasis maintenance (56).

MDM2 protein is an E3 ubiquitin ligase and also a Caspase 3 substrate. It regulates the p53 expression and function (57). MDM2 can bind to the transactivating domain of p53 and thus block the function of the protein. In addition, MDM2 can transport p53 from the nucleus into the proteasome for degradation (51). P53 is a tetrameric transcriptional factor. One of its targets is the *MDM2* gene leading to increased *MDM2* expression. The MDM2-p53 autoregulatory feedback loop is the centrum of the p53 pathway. Another member of the MDM2 family is MDMX which also contributes to p53 degradation (53).

The crystal structure of the DNA binding domain (DBD) of the wild type p53 together with the target DNA is important for the understanding how the *TP53* mutations affect the p53 activity (7). Mutant p53 can also affect the micro-RNA expression (58;59), and after micro-RNA processing the level in the cells have changed. In response to DNA damage, p53 enhances the post transcriptional maturation of several micro RNA (miR-16-1, miR-143, miR-145, miR-34) with growth-suppressive function (58). Several studies have also implicated miR-34 family of miRNAs in the p53 network (60). P53 is a part of various physiological processes as fertility, cell metabolism and mitochondrial respiration, autophagy, cell adhesion, stem cell maintenance and development (3).

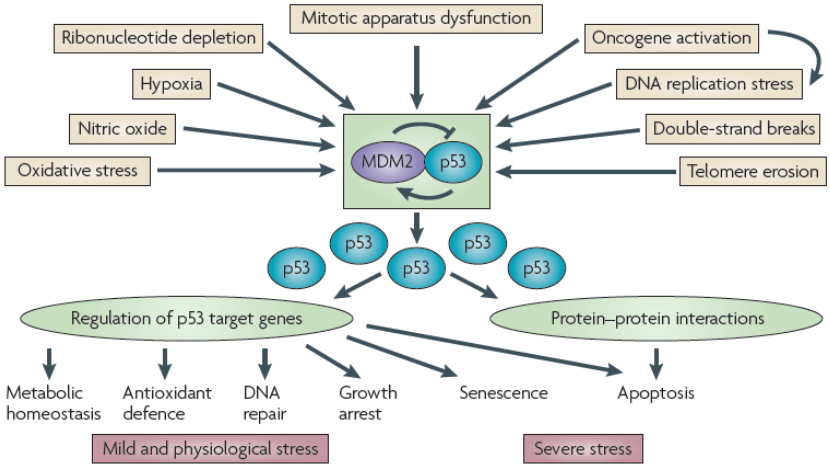


Figure 7. A simplified scheme of the p53 pathway. Different stress signals lead to p53 release from MDM2, resulting in increased p53 levels. p53 effects transactivation of target genes, usually by repression. The stress response can be cell cycle arrest by inhibiting cell cycle progression, induction of apoptosis, DNA repair and metabolic homeostasis maintenance (53;56).

1.3.2. The PI3K/AKT signaling pathway

Normal tissues control the production of growth promoting signals and regulate the cell growth through regulation of the cell cycle. In cancer, this homeostasis is deregulated. In cancer cells the cell signaling is deregulated leading to abnormal activation of the pathway (5). The PI3K pathway is a part of this growth factor receptor (GFR) signaling and a key mediator for cell growth, proliferation and metabolism. Genomic alterations at different levels in the pathway lead to its abnormal activation and cancer development (44).

The *PIK3CA* proto-oncogene is encoding the catalytic subunit P110 α of PI3K and is the most frequently mutated gene (26% in COSMIC database) in breast cancer (7). Phosphatidylinositol 3-kinase (PI3K) superfamily has received considerable attention after the discovery that PI3K activity is associated with viral onco-proteins (44). Its role in prevention of apoptosis and growth regulation contribute to this focus. PI3Ks are grouped into three classes I, II, III. Class Ia PI3Ks comprises of PIK3Cα, PIK3Cβ, PIK3Cδ. They are proteins with regulatory subunit p85 and catalytic subunit p110 (44). The *PIK3CA* gene is encoding p110α subunit (61).

The PI3K/AKT signaling pathway is triggered after growth factor activation of a receptor tyrosine kinase (RTK). Phosphatidylinositol is a phospholipid with an inositol head and it is a component of eukaryote cell membranes. The inositol head can be phosphorylated at multiple sites by phosphoinositide kinases (PIKs) (44). Adaptors, such as Gab2 or IRS family protein are then recruited. Gab2 or IRS family proteins bind to the regulatory p85 subunit of PI3K. Then the catalytic subunits p110α, β and δ are activated and the complex transformed to phosphorylate 4, 5-phosphoinositide (4, 5-PIP_2) into second messenger 3,4,5-phospoinosite triphosphate (PIP_3). PIP_3 recruits proteins such as AKT and PDK1 to the plasma membrane. AKT is phosphorylated by PDK1 and PDK2. Activation of AKT leads to modulation of different substrates (Figure 8) (62). AKT kinase is a nodal point in a complex network of signaling pathways. These substrates are important for survival, cell cycles and cell proliferation.

PTEN and SHIP-1 are phosphatases and negative regulators of PI3K signaling by limiting the production of the second messenger PIP3. Up to 33% of all breast cancers have *PTEN* loss or decreased expression and this leads to tumor progression by activating of AKT /mTOR-dependent cell proliferation (44).

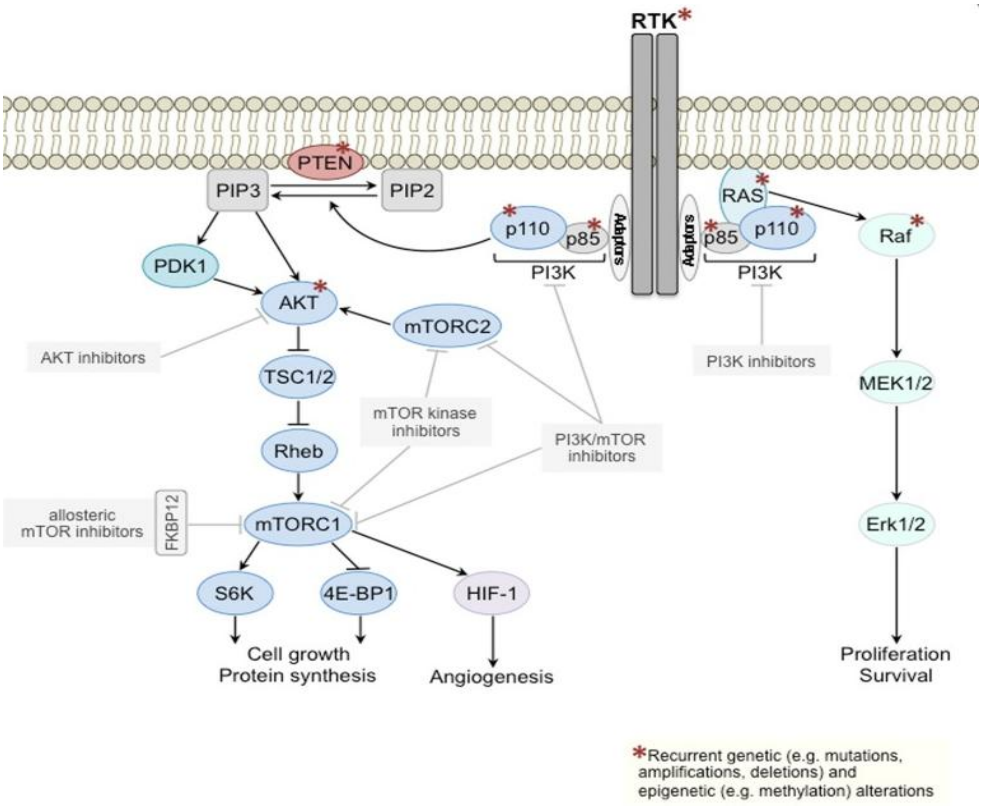


Figure 8. A simplified scheme of the PI3K/AKT signaling pathway. The PI3K/AKT pathway is frequently activated in human cancer. After binding of a growth factor to the RTK, PI3Ks are recruited to the plasma membrane receptor where they phosphorylate PIP_2 to generate PIP_3. AKT kinase binds to PIP_3 and becomes activated by phosphorylation. A cascade of signals occurs and leads to regulation of cell growth, protein synthesis, proliferation, survival and angiogenesis (63) .

1.4. Cancer gene mutations

1.4.1. Different types of mutations

There is a lot of variation in DNA sequences in healthy human genomes. The sequences can be altered in different ways. Pathogenic DNA changes can lead to either disease or a susceptibility to diseases. These DNA changes occur in the regulatory region or in the coding part of a gene (52). There are also unknown numbers of functional elements in intronic and intergenic DNA (2). The somatic changes in cancer cells consist of different classes of DNA changes, like substitution of one base to another, small and large insertions and deletions of the DNA sequence, copy number increases and reductions, DNA rearrangements and epigenetic changes. Also somatic mutations in the mitochondria genome have been reported in different cancer cells (2).

1.4.2. Pathogenic DNA changes affecting one or few nucleotides

A deletion is a removal of one or more nucleotides in the DNA whereas an insertion is addition of one or more nucleotides. Point mutation is an exchange of one nucleotide to another. A missense mutation which results in a codon change, leads to amino acid change in the protein. There are different types of substitutions. A conservative substitution means amino acid change within the same class (uncharged polar, uncharged non-polar, acidic, basic). This has less effect on the protein structure. The opposite situation is a non-conservative substitution with amino acid changes between different classes and it has more effect on the protein structure. Different codons can encode the same amino acid. A silent mutation is a nucleotide substitution in the codon without any amino acid changes in the protein.

The genetic code comprises of 64 codons, of which three are stop codons. A nonsense mutation has occurred when a nucleotide substitution has led to a stop codon. A truncated protein can be more harmful than the absence of a protein because it can interfere with the function of the normal product (52). Nonsense –mediated decay (NMD) is a cellular mechanism which detects and degrades mRNAs containing premature terminations codons. It is a possible protection mechanism. The spliceosome recognizes the Guanine Thymine (GT) sequence as the beginning of all exons and Adenine Guanine (AG) sequence in the end of all exons in mRNAs. A splice site mutation in theses sequences can be an insertion, a deletion or a point mutation. The spliceosome will not recognize these sequences when they are changed and splicing will be disrupted. Silence and missense mutations can have a pathogenic effect on splicing (52).

Variants such as small deletions and insertions within an exon, whole exon deletions, abnormal splicing and duplications can cause a frame shift. The reading frame starts with initiator codon Adenine Uracil Guanine (AUG). Downstream changes that add or remove a number of nucleotides not divided in three, will cause changes in the reading frame. When 3 of 64 codons are stop codons and the reading is out of the frame, a stop codon will soon be reached (52).

1.4.3. Pathogenic DNA changes affecting many nucleotides in chromosomal structure

The term amplification (copy number increase) is used when the genome has multiple copies of a chromosomal region. The genes dosage is increased and the consequence is larger amount of the gene product. The opposite term is deletion (copy number reduction). Large chromosomal regions are deleted and loss of genes within these regions occurs. A somatic genetic change such as deletion may cause a loss of heterozygosity. It means loss of one allele.

1.4.4. Driver and passenger mutations

Somatic mutations in a cancer cell genome are suggested to be classified according to its consequences for the cancer development. The first class called driver mutation consists of mutations which are able to favor and foster cells with proliferation and survival properties. The second class named passenger mutation is an individual genetic variation. One single cell needs a set of mutations to get the properties to proliferate autonomously and invade tissue and metastasize (2). Figure 9 describes the development of a cancer cell through the cell divisions from the fertilization to chemotherapy resistance at the recurrence phase.

Driver mutations are in cancer genes and the number of driver mutations varies between the cancer types. Driver mutations will cause a clonal expansion. They are positively selected in the tissue microenvironment from which the cancer developed and they contribute to the oncogenesis (2).

Cells with passenger mutations do not have growth advantage, thus they will not contribute to the tumor development. These mutations are more or less randomly distributed (2). McFarland and colleagues investigated the impact of deleterious passenger mutations on cancer progression (64) . Many passengers fall within protein coding genes and can have deleterious effect on cancer cells. They have used a stochastic model to analyze cancer genomics data (from COSMIC and TCGA database) and two strategies were followed. First, they increased the overall mutation rate and thus increased the rate of passenger accumulation. In addition they magnified the deleterious effect of passengers. Both strategies reduced the cancer size. They also focused on passenger-mediated therapies (64).

A meta study of Stephens *et al.* (65) focused on driver mutations and mutational processes in 100 breast cancer tumors. They studied mutations in exons of protein coding genes and somatic copy number changes. The number of somatic mutations varies markedly between the tumors. The number of driver mutations in the individual cancer varied from maximum six to a single driver. Somatic driver substitutions and small insertion/deletions were found in the following genes: *AKT1*, *BRCA1*, *CDH1*, *GATA3*, *PIK3CA*, *PTEN*, *RB1* and *TP53*. These genes have earlier been described to be involved in breast cancer development. Stephens *et al.* conclude that driver mutations are operative in many cancer genes. In some cases, genes with driver mutations are associated with sub-clonal evolution, but in others in the root cancer clone (65).

Stratton *et al.* (2) emphasized the importance to identify rare sub clones with drug resistance mutations before the cancer therapy is chosen. It is a challenge to distinguish clusters of driver mutations from random passenger mutations. This is dependent on driver mutation frequency and the prevalence of passenger mutations (2;66). Targeted therapy on the most common driver which is dominant and has expanded will not succeed if the cells have become resistant. Combined treatment are then feasible.

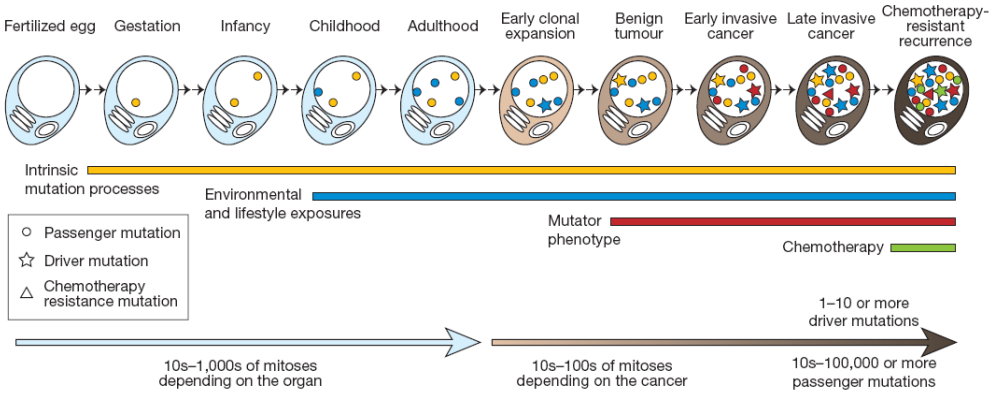


Figure 9. Development from the fertilized egg to a single cell within a cancer by mitotic cell divisions. The illustration also shows the timing of somatic mutations acquired by the cancer cell and contribution of other processes (2).

Stephens *et al.* (65) found that seven out of 40 genes (*TP53, PIK3CA, ERBB2, MYC, FGFR1/ZNF703, GATA3, CCND1)* were mutated in more than 10% of the 100 breast cancers. The *PIK3CA* and the *TP53* genes are the two most frequently altered genes in human cancer.

Reasonable statistic indicates that they are likely to be driver genes (67). The *PIK3CA* and *TP53* mutations will be discussed in more detail in the two following chapters.

1.4.5. Mutations in the *PIK3CA* gene

PIK3CA gene has hotspots (mutated codon in the gene) in exon 9 (helical domain) and exon 20 (kinase domain) (Figure 10) (68). There is also a third hotspot in exon 7 (C2 domain). Saal *et al.* (69) found mutations in seven primary breast tumor of a total number of 292 (2,4 %) in hotspot 7. Samuels *et al.* (70) reported that mutations generally arise late in *PIK3CA* in the tumor development.

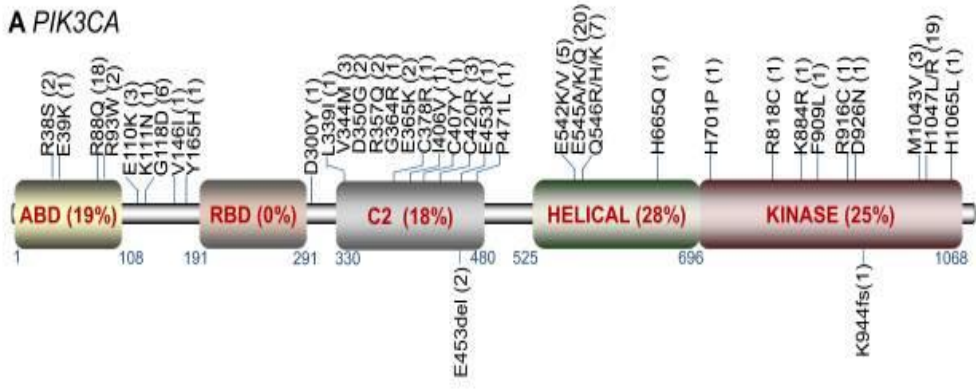


Figure 10. Distribution of nonsynonymous (alter the amino acid sequence of a protein) mutations in the PIK3CA gene (61). P110α gene product of *PIK3CA* is a catalytic subunit of phosphatidylinositol 3-kinase (PI3K). It consists of 5 domains: ABD, Ras-binding domain (RBD), C2 domain, helical domain and kinase catalytic domain (modified from Cheung *et al.* (61).

1.4.6. Mutations in the *TP53* gene

The *TP53* gene consists of different domains where all have been shown to contain missense mutations at different frequencies (Figure 11) (7). The Li- fraumeni syndrome is associated with germline mutations in the *TP53* gene (16). Occurrence of different hotspot mutations in *TP53* varies between the different tumor types, grade and survival of breast cancer (7). Mutation rates between the breast cancer subtypes vary between 5-95 % (71). The basal-like and HER2-enriched subtypes have the highest frequency of *TP53* mutations and they are associated with poor prognosis. Also *TP53* mutation types may differ between breast cancer subtypes. For example basal-like and *BRCA1*-mutated breast cancer has a high incidence of

protein-truncating mutations. This supports the fact that *TP53* mutations have diverse effects in different cells of origin and conditions of cancer development (71).

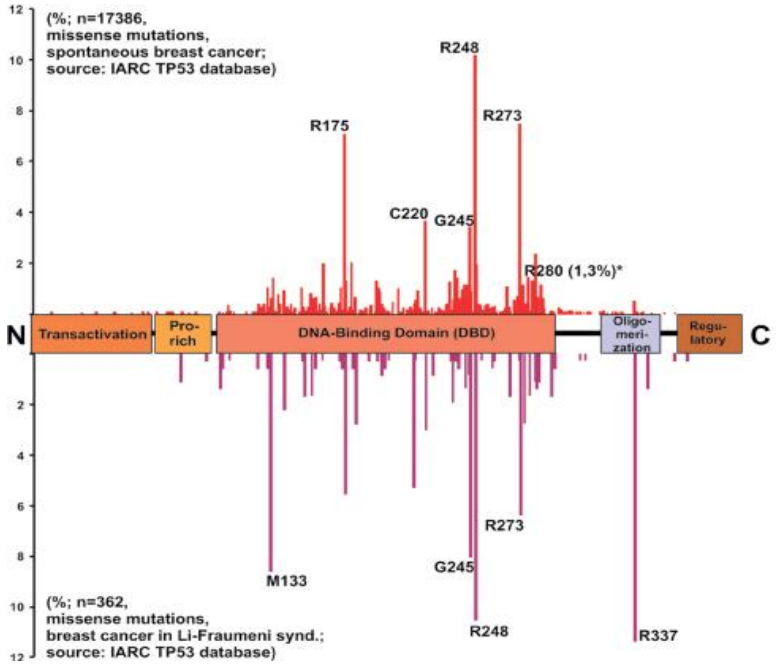


Figure 11. Frequency of p53 missense alterations in breast cancer. "Human p53 domain structure with indicated frequency (percent bars) of missense changes in *TP53* found in spontaneous (above) or Li-Fraumeni-associated (below) breast cancer. The five most frequently changed codons are indicated by numbers and residue names". (modified from Walerych *et al.* (7).

1.4.7. Experimental targeting of p53

Tumor cells do often overexpress mutant p53 which leads to reduced sensitivity to conventional chemotherapy and radiotherapy (72). Activation of mutant p53 by small drug molecules appears to be a greater challenge than for the wild type p53. One reason is that a large range of different mutant proteins expressed in the tumor might lead to different structural alterations. P53 is normally expressed at low levels but accumulate upon stress such as DNA damage. Targeting p53 leads to tumor suppression. A major concern of activating p53 are toxic side effects on normal tissues and cells (72).

P53 is a transcriptional factor and not an enzyme and it has complicated protein-protein interactions (53). These challenges have to be overcome in developing targeted drugs. P53 therapeutics has been tested in xenograft models (immunosuppressed mouse with a primary tumor implant) by interrupting the p53 and MDM2 interaction. P53 can then be activated or

stabilized and tumor regression occurs. One class of p53 targeted drugs is the imidazoline derivatives called Nutlins. They interact with p53-binding pocket of the MDM2 molecule and dissociate p53 from MDM2 (53). An activation of p53 will then leads to induction of apoptosis. MDM2 inhibition can be a promising treatment strategy for Luminal B disease with wild-type *TP53* (51). There is still a lack of knowledge on whether such small molecules affect healthy cells and tissue. PRIMA-1 and MIRA-1 are two small molecules which target cells that express mutant p53 and thus initiate apoptosis. More studies have to be done before testing these drugs in clinical trials (72).

1.5. Ba/F3 as a model system

It is important to study the effect of different mutations in order to understand therapy resistance and to develop better treatment strategies. For that purpose, researchers have developed cell line model systems, such as the Ba/F3 model. The Ba/F3 cells were first characterized by Palacios and Steinmetz who isolated IL-3 dependent pro-B cells from bone marrow of normal and autoimmune-prone BALB/c mice (73). These clones carried B cell lineage surface antigen B-220. They died within 24-36 hours in the absence of IL-3 and the mean doubling time was about 14-18 hours in a logarithmic growth phase. Daley and Baltimore (74) did the first landmark experiment and launched the use of Ba/F3 cells by infection of the Ba/F3 cells with retroviral constructs encoding the protein derived from the hybrid gene created by the Philadelphia chromosome ($P210^{bcr/abl}$). Cell lines expressing $P210^{bcr/abl}$ were IL-3 independent and tumorigenic in nude mice. Since then the Ba/F3 cells have been used as a tool in cancer research (75).

The Ba/F3 cells have many properties which can be exploited in a model system. These cells are fast growing suspension cells with a rapid experimental turnaround. Both virus transduction and electroporation have been used for transfection of the Ba/F3 cells. The Ba/F3 cell line with active tyrosine kinase or other oncogene has the possibility to survive in the absence of IL-3 (75). Protein kinases are able to function as dominant oncogenes. Following are some examples of how Ba/F3 cells have been applied in kinase drug discovery. Transfected Ba/F3 cells can be used when characterizing whether they express oncogenic mutations *i.e.* have testing the transforming potential or not. It is also possible to study downstream signaling mechanisms of mutant kinases. Warmuth *et al.* (75) summarizes the use of Ba/F3 model system to investigate mutants in the Janus kinases Jak 2 and Jak3.

Research groups have found that mutants in genes encoding these kinases have potential to function as oncogenes in hematopoietic malignancies (76;77). Walters *et al.* found different regulations of JH1 domain through JH2 domain in these two kinases. The structural basis of this phenomenon has to be investigated by crystallographic studies. They also presented a study by using Ba/F3 cells to identify gain of function mutations in three types of Jak 3 kinases. These findings are clinically important information in numerous malignancies such as acute myeloid leukemia (AML) (76).

Ba/F3 model system is also used for testing the response of various drugs for oncogenic mutants. The effect of inhibition of cell growth by targeted drugs varies, and the consequences are growth arrest and apoptosis. The Ba/F3 cells will be rescued by adding IL-3 to the growth medium when testing the drug. Then the number of viable cells will not decrease. This is used as a control to be compared with drug testing in growth media without IL-3. Because of the growth property of the Ba/F3 cells, this model system is suitable to be adapted to high throughput drug screening systems. Melnick *et al.* (78) describes a robotic system for testing a panel of 35 activated tyrosine-kinase dependent cellular assays in dose – response format against a set of 1400 kinase inhibitors in a single experiment.

It is important to predict mutations that cause resistance to a drug. The Ba/F3 model system gives the possibility to screen for mutations inducing treatment resistance. The system has also limitations and one example is that other several distinct resistance mechanisms can be involved. It is difficult to separate between these mechanisms. Bradeen *et al.* (79) have developed a rapid mutagenesis assay to avoid these limitations. Their protocol is based on a chemical mutagenization of Bcr-Abl cDNA with *N*-ethyl-*N*-nitrosourea (ENU), which is a potent inducer of random point mutations. Then the mutant gene is introduced into Ba/F3 cells. This assay was used to test for resistance profile of four drugs alone and in combinations.

2. Material and methods

The names of the reagents and equipment together with catalog numbers, manufactures and suppliers used are listed in the Appendix A.

2.1. Cell line and plasmids

The detailed information about the Ba/F3 cells and plasmids used in this study are listed in Table 3. Plasmid *TP53* wild type (wt) and *PIK3CA* wt contained both Blasticidin- and Ampicillin resistance genes and were used as DNA templates in the mutagenesis and also as controls for normal transfection and viability. Figure 12 shows the map of the plasmid vectors for the human *TP53* wt coding sequence and the vector pLenti 6.3 for the human *PIK3CA* wt coding sequence.

Table 3. Overview of cell line and plasmids.

Cell lines and plasmids	**Description**	**Supplier**
Ba/F3 cell line	Ba/F3 is a murine, interleukin-3 (IL-3) –dependent pro lymphoid cell line (75).	Gordon Mills laboratory, Houston, Texas, USA
Plasmid *PIK3CA* WT	Plasmid background was Plenti 6.3 (10,8 kilobase (kb). *PIK3CA* coding sequence was 3,3 kb and total size was 14,1 kb. The plasmid contained both Blasticidin- and Ampicillin resistant genes.	Gordon Mills laboratory, Houston, Texas, USA
Plasmid *TP53* WT	Human cDNA SC119832 (80) *TP53* coding sequence with accession no: NM_000546.2 (1,2 kb) was sub cloned into the vector pCMV6-A-BSD (PS100022) (81) (5.5 kb). The total size was 6,7 kb. The plasmid contained both Blasticidin- and Ampicillin resistant genes.	OriGene Technologies Inc., USA
Plasmid *PIK3CA*, E545K	Plasmid background was Plenti 6.3 (82) (10,8 kb) and contained both Blasticidin and Ampicillin resistant genes.	Gordon Mills laboratory, Houston, Texas, USA
Plasmid *LacZ*	Plasmid background was Plenti 6.3.	Gordon Mills laboratory, Houston, Texas, USA

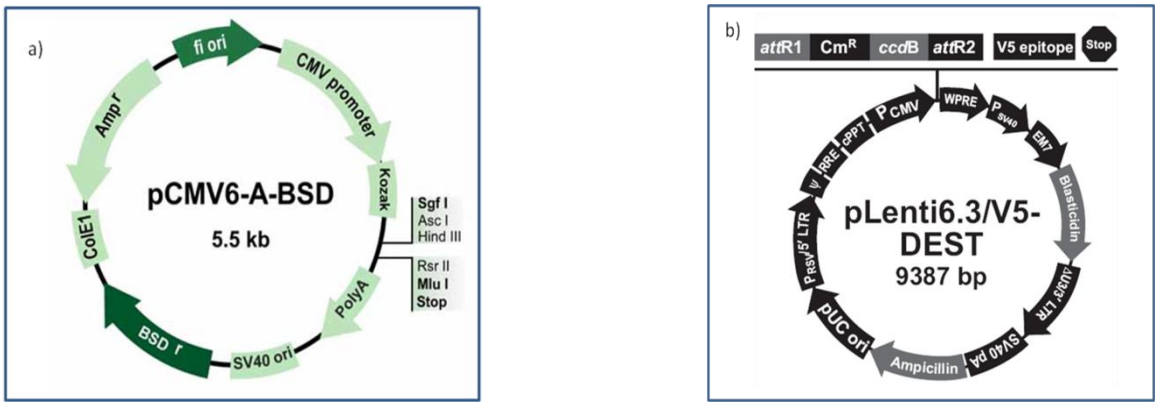


Figure 12. The map of the plasmid vectors (83;84). a) Human *TP53* wt coding sequence was sub-cloned into pCMV6-A-BSD vector by Origene. b) Human *PIK3CA* wt coding sequence was sub-cloned into pLenti6.3/V5-DEST vector by M.D. Andersson. The plasmids were both Blasticidin (BSD) - and Ampicillin (Amp^r) resistant.

2.2. Cell culturing

Cell culturing was performed in sterile condition in laminar flow hood (LFH). 75% ethanol (Antibac AS, Norway) was used for disinfection. The cells were cultured in a Corning petri dish, 100 x20 mm (Corning Incorporated, NY USA) in a CO_2 incubator (Nerliens Meszansky, Norway) at 37 °C with 5 % CO_2. RPMI 1640 with L-Glutamine (Gibco®, USA), 5 % fetal bovine serum (FBS) (Gibco®) and Recombinant Mouse Interleukin-3 (IL-3) 5 ng/ml (Gibco®) was used for the parental Ba/F3 cells. Blasticidin S HCL (Invitrogen, USA) was used as a selection antibiotics for the transfected Ba/F3 cells.

The Ba/F3 cells were stored in -170 °C in liquid N_2 tank (Taylor-Warton, Germany) with 10 % Dimethyl Sulfoxide (DMSO) (Thermo Scientific,USA) and 90 % FBS (Gibco®). The cells were cultured for 14 days and the confluence level was approximately 80 % before starting the experiments. The Ba/F3 cells are suspension cells and were split by transferring cell suspension directly to a new dish containing fresh media. The split ratio for the parental Ba/F3 cell line was 1/10. When performing new experiments the cell suspension was centrifuged (Hettich Rotina 420, Germany) at 1000 rotation per minutes (rpm) for 5 minutes, the supernatant was removed and the cell pellet was resuspended and diluted in fresh Ba/F3 medium.

2.3. Mycoplasma detection

Mycoplasma is a small prokaryote organism < 1 µm. It competes with the cultured cells by using nutrients in the medium. Supernatant of cultured Ba/F3 cells was tested for mycoplasma contamination. The mycoplasma test was performed by using MycoAlert^{R} Mycoplasma Detection kit (Lonza Nottingham, UK) which contained both MycoAlert^{R} Reagent and Substrate. It is based on a bioluminescent reaction as described in Figure 13.

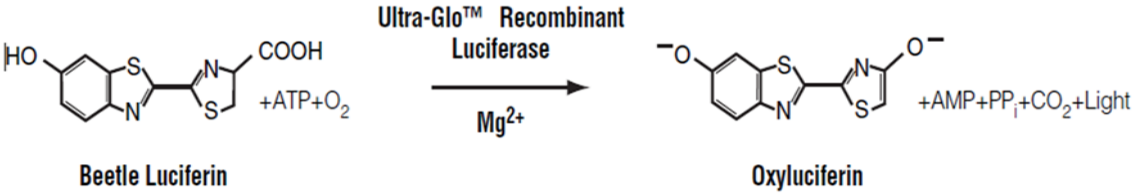


Figure 13. The Luciferase reaction. Beetle Luciferin changes to Oxyluciferin and the reaction is catalyzed by Luciferase, Mg2+, ATP and O_2. The luminescence signal is proportional to the presence of ATP which reflects viable cells in the culture (85).

The MycoAlert^{R} Reagent was added to the cell culture supernatant at room temperature to lyse the mycoplasma. The background luminescence was measured before adding the MycoAlert^{R} substrate. If the cell culture contains mycoplasma, released enzymes from lysed mycoplasma react with the substrate catalyzing the conversion from ADP to ATP. The luciferase reaction is catalyzed by ATP, O_2, Mg^{2+} and Luciferase. The intensity of emitted light is proportional to the ATP concentrations and was measured using Promega Glomax 96 microplate Luminometer (Promega, USA). Duplicates from cell supernatant from early passage were measured (86).

2.4. Cell counting

The cell number was determined by counting using a Bürker chamber (Assistant, Germany). The cell suspension was diluted 1/2 with Trypan blue (Invitrogen, USA) and 10 µl of the colored cell suspension was transferred to the chamber. Separation between blue dead cells and bright living cells was easier by using this color method. Cells were counted in five of 16 sub chambers as shown in Figure 14. The average number of living cells was calculated by the following formula:

Cell number average x dilution factor x 10000 = number of cells/milliliter (ml).

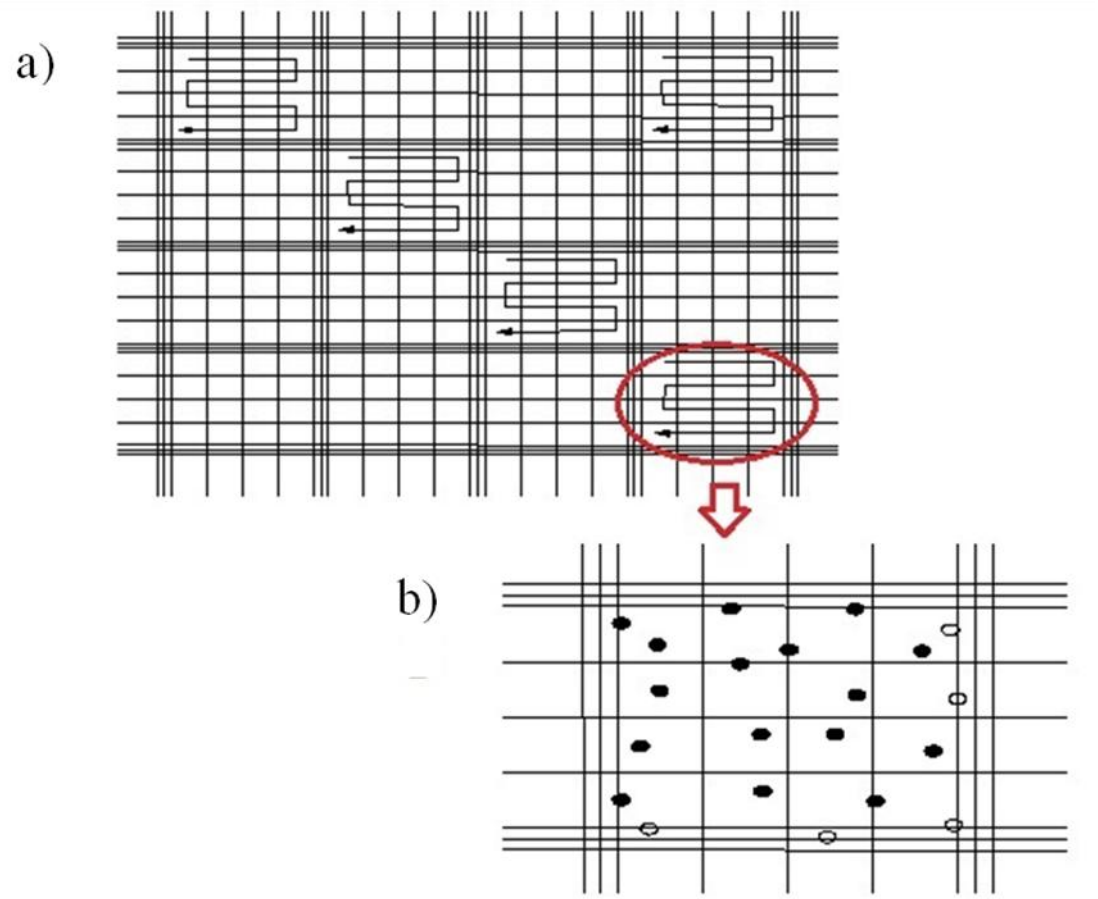


Figure 14. Bürker cell counting chamber. a) Five of 16 sub chambers were counted, marked by arrows. b) The black spots represent cells which were counted. The white spots represents cells which were touching two out of the four edges and were not counted (87).

2.5. Cell viability assay

The detection method of viable cells in the culture was the Cell titer Glo^{R} (CTG) luminescent cell viability assay (Promega). It is based on the Luciferase reaction as described in Figure 13. 20 µl CTG reagent was added to the wells and thus lysed the cells. After incubation with CTG reagent for 35 minutes in dark at room temperature, the luminescence signals were measured on a Wallac 1450 Micro Beta TriLux luminescence Counter (PerkinElmer, USA) with the MicroBeta Windows Workstation (PerkinElmer).

2.6. *In vitro* site directed mutagenesis

2.6.1. Primer design for mutagenesis

The Ensemble Genome Browser (88) was used to get the sequence information for the genes of interest. The Clustal Omega software (89) was used for alignment of *TP53* reference sequences to the reference sequence in the Seqscape v.2.5 software. This was done to decide which transcript variant to choose for ordering of the custom made *TP53* wt vector. Snipper.chip.org (90) was used to find where the mutation is located in the *PIK3CA* gene. The length of the primers was limited to 45 bp, as a longer primer could affect the mutagenesis efficiency due to likelihood of secondary structure formation (91). The primer design program which was used is from Stratagene, USA. It is designed for the QuickChange II XL site-directed mutagenesis kit (Stratagene) (92). The sequences of the mutagenesis primers with the introduced mutations are shown in Table 4.

Table 4. Mutagenesis primers.

Primer name	Length (nucleotide)	Melting temperature (Tm)	Primer Sequence (5`to 3`)
PIK3CA S405F sense	37	78.28 °C	5`-ctgctcgactttgccttttcatttgctctgttaaagg-3`
PIK3CA S405F antisense	37	78.28 °C	5`-cctttaacagagcaaatgaaaaggcaaagtcgagcag-3`
TP53 K120E sense	33	80.38 °C	5`-gcattctgggacagccgagtctgtgacttgcac-3`
TP53 K120E antisense	33	80.38 °C	5`-gtgcaagtcacagactcggctgtcccagaatgc-3`
TP53 H179R sense	25	80.02 °C	5`-gctgcccccaccgtgagcgctgctc-3`
TP53 H179R antisense	25	80.02 °C	5`-gagcagcgctcacggtgggggcagc-3`

2.6.2. Site directed mutagenesis

In vitro Site-directed mutagenesis is a method used to create plasmids containing a mutant gene. In this study we created plasmids with mutated *PIK3CA* and *TP53* genes (Figure 15).

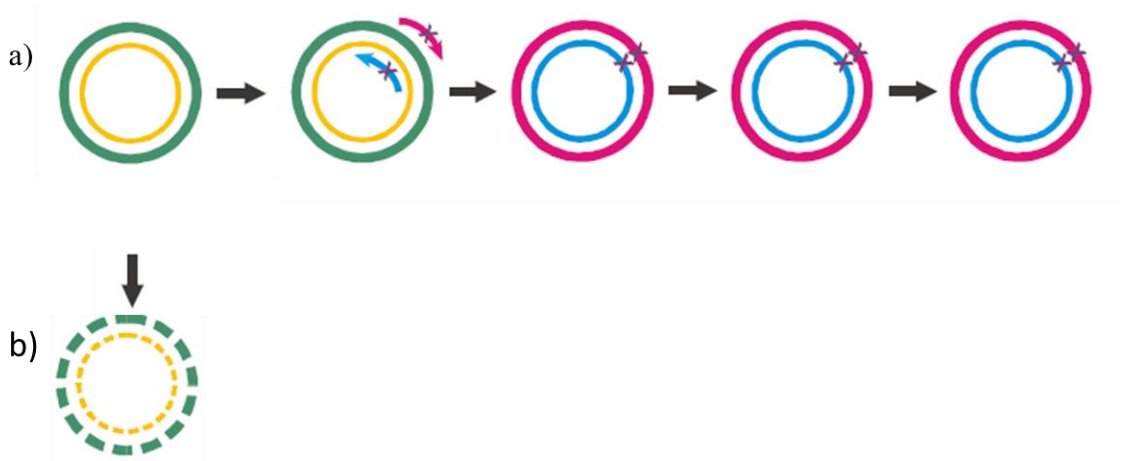


Figure 15. *In vitro* site-directed mutagenesis. a) Mutant strand synthesis after denaturation by thermal cycling. b) digestion of parental methylated and hemi methylated DNA with Dpn1. The mutant molecules were then transformed into competent cells for nick repair (93).

The mutagenesis was performed by using Quickchange ^{R}II XL site-directed mutagenesis kit. Control and the sample reactions were pipetted and run on the PCR machine as described in Table 5, 6 and 7 (93). The water used in the PCR reactions was Distilled Water, DNase/RNase Free (Gibco®).

Table 5. Control reaction. Pipetting scheme.

Reagent	**Volume for each tube (µl)**
10 X reaction buffer	5
pWhitescript 4.5-kb control plasmid (5 ng/µl)	2
Oligonucleotide control primer # 1 (100 ng/µl)	1.25
Oligonucleotide control primer# 2 (100 ng/µl)	1.25
dNTP mix	1
Quick solution reagents	3
Distilled Water, DNase/RNase Free	36.5
PfuUltra HF DNA polymerase (2.5 U/µl)	1
Total volume of control reaction	51

Table 6. Sample reaction. Pipetting scheme.

Reagent	Volume for each tube (µl)
10 X reaction buffer	5
dsDNA template (5 ng/µl)	2
Oligonucleotide sense primer (100 ng/µl)	1.25
Oligonucleotide antisense primer(100 ng/µl)	1.25
dNTP mix	1
Quick solution reagents	3
Nuclease free water	36.5
PfuUltra HF DNA polymerase (2.5 U/µl)	1
Total volume of sample reaction	51

Table 7. The PCR program for the mutagenesis.

Cycles	Temperature	Time
1	95 °C	1 minute (min)
12[a] or 18[b]	95 °C 60 °C 68 °C	50 seconds(sec) 50 sec 1 min/kb of plasmid length
1	68 °C	5min[c]/ 7min[d]/12min[e]
	4 °C	∞

a) for the pWhitescript control b) for the *PIK3CA*- and the *TP53* wt plasmid c) for the pWhitescript control d) for the *TP53* wt plasmid e) for the *PIK3CA* wt plasmid

The PCR products were digested with Dpn I endonuclease at 37 °C for 1 hour to degrade the wt plasmid which is susceptible to degradation because it is methylated. The mutant plasmids were transformed into ultra-competent XL10 Gold E.coli cells for nick repair. The Hte phenotype which was representative in these bacteria increased the transformation efficiency of ligated DNA, and their content of *lacI*^{Q}*ZΔM15* gene permitted blue-white screening. These competent cells also contained *end A1*- and *recA* mutations which improved the quality of the plasmid mini prep DNA and ensured insert stability (93). 45 µl of the competent cells were aliquoted into pre cooled tubes together with β-Mercaptoethanol, carefully mixed and incubated on ice for 10 min. 2 µl of sample plasmids, pWhitescript control and TP53 wt plasmid and 1 µl of the other controls were added in each tube of competent cells and incubated on ice for 30 minutes. Transformations were performed by a heat shock at 42 °C for 30-40 seconds at a heating block before the tubes were transferred to ice for 2 minutes to stop

the reactions. Pre-warmed Luria broth (LB) medium pH 7,0 was added to the reactions before incubation in 37 °C for 1 hour at 220 rpm. LB agar plates contained 0,05 mg/ml Ampicillin (Invitrogen, USA). The LB agar plates were added 100 µl of 10 mM Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma-Aldrich, USA) and 110 µl of 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma-Aldrich). After the transformation, colonies can be screened for β-galactosidase production (β-gal^{+}) by virtue of a blue colony phenotype (93). Whitescript control plasmid, pUC18 control plasmid (diluted 1/40 with LB medium) and sample plasmid were spread out on the LB agar surface and air dried. The plates were incubated at 37°C over night.

2.7. Culturing of transformed bacteria, mini- and midi preparation of plasmid DNA

2.7.1. Mini preparations

Mini preparations were performed using with QIAprep^{R} spin mini prep kit (Qiagen,USA) (94) to isolate plasmid DNA in small scale in order to identify the clones that carried mutated plasmids. Ampicillin (0,1 mg/ml) was added to 5 ml pre warmed LB medium in falcon tubes (BD Biosciences, USA). Single bacterial clones from the LB plates were transferred to the falcon tubes and then spread out in separate sectors on a new LB plate for storage. The cultures were incubated by shaking at 200 rpm, 37 °C ≥ 16 hours. The LB-plate with seeded bacteria in sectors was placed in an incubator at 37 °C ≥ 16 hours before storing at 4 °C. After incubation the falcon tubes were centrifuged (Hettich Rotina 420, Germany) at 3000 rpm for 10 minutes. The supernatants were removed and the mini prep protocol (94) was followed.

2.7.2. Midi preparations

Single transformated bacterial clones were picked and cultured for midi preparation to isolate a larger amount of plasmid DNAs. Each of the transfected bacterial clones picked from the LB plate, were transferred to separate Falcon tubes containing 5 ml LB medium and final Ampicillin concentration 0,1mg/ml. The tubes were incubated by shaking (New Brunswick Scientific E24, USA) at 200 rpm and 37 °C for approximate six hours. 1000 µl of each of the bacterial suspensions were added to sterile Falcon Erlenmeyer Culture flasks (BD

Biosciences, USA) containing 100 ml of LB medium and Ampicillin (0,1 mg/ml). The flasks were incubated over night at 37 °C 200 rpm.

Next day, 100 µl of minimum 99 % sterile Glycerol (Sigma Aldrich, USA) and 500 µl of each bacterial suspension were mixed in 2 ml Cryo tubes (Greiner, Germany) to a final Glycerol concentration of ~17 % and frozen at -80°C for long time storage. The six different glycerol stocks have *TP53* wt, *PIK3CA* wt, *Lac*Z, *TP53* H179R, *TP53* K120E and *PIK3CA* S405F genes represented in the plasmids. The bacteria were pelleted by centrifugation at 4754 relative centrifugal force (rcf) for 13 minutes. Thereafter the supernatants were removed and Cell Resuspension Solution from the midi prep kit PureYield™ Plasmid Midi prep System (Promega) was added to the pellets and the kit protocol (95) was followed.

2.8. DNA Sequencing

2.8.1. Primer design for sequencing

The sequencing primers were design with Primer3 program (91).

Table 8. Sequencing primers.

primer name	Length (nucleotide)	Tm	Primer Sequence (5`to 3`)
PIK3CA ex6FM13BAF sense	20	58.84 °C	5`-tgtaaaacgacggccagtaccttgttccaatcccaggt-3`
PIK3CA ex8RM13BAF antisense	20	58.29 °C	5`-caggaaacagctatgacctggccaaagattcaaagcca-3`
TP53 ex4FM13BAF sense	20	58.47 °C	5`-tgtaaaacgacggccagttgtcccttcccagaaaacct-3`
TP53 ex6RM13BAF antisense	20	58.94 °C	5`-caggaaacagctatgaccataagatgctgaggaggggc -3`

2.8.2. Sequencing and data analysis

The validation of the site-directed mutagenesis was performed by the BigDye^R^Direct Sequencing Kit (Applied Biosystem, USA) on the Applied Biosystems 3730 DNA analyzer (Applied Biosystems) (96). Sequencing Analysis software v. 5.2 (Applied Biosystems) was used for data analyzes. The workflow of the sequencing method is shown in Figure 16.

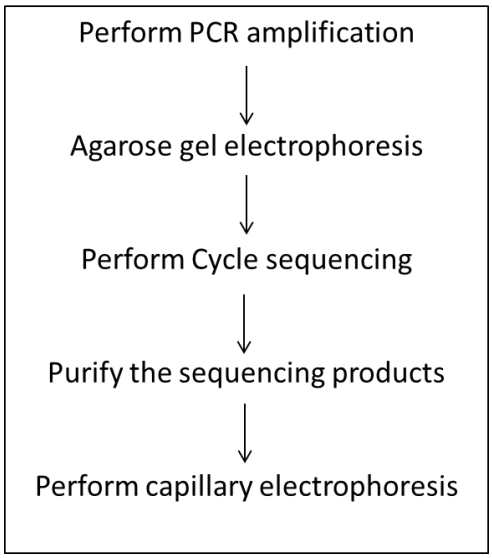


Figure 16. Workflow of the sequencing method.

The sequences of the PCR primers are described in Table 8 and the PCR reactions were prepared as described in Table 9. A negative control without any DNA template was included in the analyses.

Table 9. Pipetting scheme for the PCR reaction in one well.

Reagent	**Volume for each well (µl)**
Mutant Plasmid DNA (5 ng/ul)	1
M13 universal sense primer (0,8 µM)	0.75
M13 universal antisense primer (0,8 µM)	0.75
BigDye^{R} direct PCR Mastermix	5
Nuclease free water	2.5
Total volume of each reaction	10

The PCR amplifications were run with DNA Engine tetrad 2 Peltier Thermal Cycler (Bio-Rad laboratories, USA). The PCR amplification conditions are listed in Table 10.

Table 10. Time and temperature conditions of PCR amplification.

Cycles	Temperature	Time
1	96 C°	5 min
35	94 C° 59 C° 68 C°	30 sec 45 sec 45 sec
1	72 C°	2 min
1	4 C°	∞

2 µl of each of the PCR products were mixed with 2 µl gel loading buffer and applied on an 1.5 % agarose gel contained GelRed™ nucleic Acid Gel Stain (Biotium, USA). 2µl DNA ladder øx 174-Hae III digest (TaKaRa, Japan) was used in a separate well. The gel was run for 30 minutes at 200 Voltage (V) and the GeneGenius Bio Imaging System (Syngene, UK) was used for visualization. The sequencing reactions for each sample were prepared as described in Table 11 and 3 µl sequencing reaction mix was added to each samples.

Table 11. Pipetting scheme for the sequence reaction in one well.

Reagent	Volume for each well (ul)
BigDye Direct Sequencing Master Mix	2
One sequencing primer: BigDye® Direct M13 sense primer **or** BigDye® Direct M13 antisense primer	1
Total volume of sequencing reaction mix	3

The sequence reactions were run on the DNA Engine tetrad 2 Peltier Thermal Cycler and the sequence reaction conditions are displayed in Table 12.

Table 12. Time and temperature conditions during cycle sequencing.

Cycles	Temperature	Time
1	37 C°	15 min
1	80 C°	2 min
1	96 C°	1 min
25	96 C° 50 C° 60 C°	10 sec 5 sec 4 min
1	4 C°	∞

Purification of the sequencing product was performed by using BigDye^{R}XTerminator^{TM} Purification Kit (Applied Biosystems) (97). 45 µl SAM^{TM} and 10 µl Xterminator^{TM} solution were added to each sample. The sample mix was added on the 96 well plate, sealed, vortexed and centrifuged. All empty wells were filled with nuclease free water before starting of the capillary electrophoreses. The 96 well plate was sealed with septa (Applied Biosystems) before sequencing on the 3730 DNA analyser. The principle of the Applied Biosystems 3730 DNA analyzer is: fluorescent labelled DNA fragments are separated according to molecular weight in a denaturing flowable polymer (98). Negatively charged DNA molecules from the buffered cycle sequencing reaction enter the capillary by an electro kinetic injection. Before reaching the positive electrode the fluorescence labelled DNA fragments move across the path of a laser beam and start to fluoresce. An optical detection in the instrument detects the fluorescence and converts it to digital data by data collection software. In one capillary injection all four bases can be detected and distinguished. Each dye emits light with different wavelength and all four colours represent all four bases. The sequencing results were analysed and reviewed by sequencing Analysis software v. 5.2 (Applied Biosystems).

2.9. Transfection of Ba/F3 cells

Before the transfection of Ba/F3 cells, the plasmid DNA was concentrated by a speedvac procedure (DNA120 SpeedVac® ThermoSavant (Houm, Norway) to get a final concentration of 1 µg/µl. One million parental Ba/F3 cells were used for each transfection. The cells were washed with Phosphate buffered saline (PBS) (GIBCO®) and resuspended in 80 µl Resuspendation buffer (R). Then 30 µl of plasmid DNAs with concentration 1 µg/µl were transferred to the sample tubes. The pulse conditions during the transfections on the Neon Device (Figure 17) were one pulse with 1635 Voltage for 20 milliseconds (protocol from MD Andersson). The Electrolytic buffer (E2) was added to the Neon^{TM} Tube before the sample was pipetted by the Neon^{TM} pipette and inserted in to the pipetting station. After the electroporation the transfected Ba/F3 cells were transferred to six-well plate containing Ba/F3 medium with IL-3. *PIK3CA* S405F, *TP53* K120E and *TP53* H179R were the sample plasmids that were transfected. The control samples were Mock, parental Ba/F3, *PIK3CA* P110 wt, *PIK3CA* P110 E545K, *Lac*Z and *TP53* wt. These controls were used in order to be able to compare growth rate of the cell cultures and the transfections.

Three days after the transfections the selection with Blasticidin (Invitrogen) was started. The final concentration of Blasticidin in the cell cultures were 20 µg/ml. After maintenance and Blasticidin selection for a ten days period, the cell cultures were transferred to a 48 well plate and cultured with Ba/F3 medium without IL-3. The transfected cell cultures were evaluated microscopically and photographed. Ampoules of transfected samples *PIK3CA* P110 S405F, *TP53* H179R and *PIK3CA* P110 E545K were mixed with DMSO and stored in the liquid Nitrogen tank to have a backup stock. The cell viability was confirmed by Trypan blue staining and CTG assay.

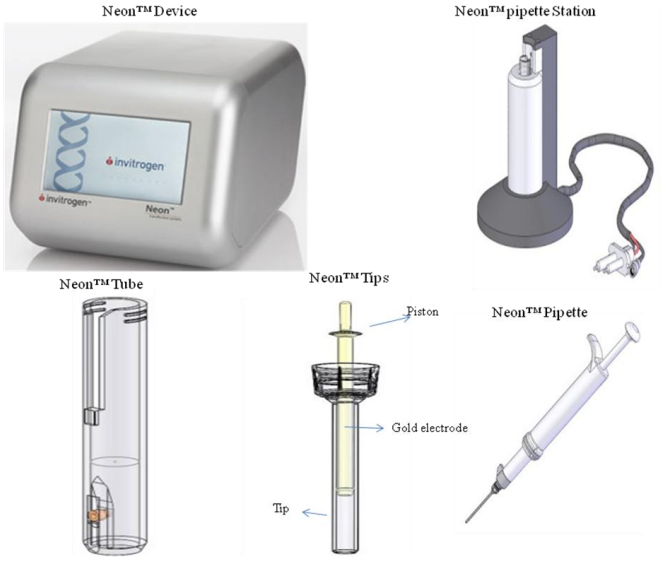


Figure 17. Neon™ transfection System. The bench top device was used together with a pipette station, a tube, pipette tips, pipette and 100 µl Neon™ kit (99).

3. Results

3.1. Mycoplasma testing of the parental Ba/F3 cell line

The parental Ba/F3 cell culture was examined for Mycoplasma infection. A Mycoplasma contamination may lead to reduction in proliferation rate of the cells. The reason for this is a competition between the cells and the Mycoplasma for using nutrients in the culturing media. MycoAlert^{R} reagent was added to two duplicates of supernatant of parental Ba/F3 cells. The luminescence values were read before and after the addition of the substrate. Possible mycoplasma enzymes react with the substrate and catalyze the reaction of ADP to ATP. The luminescence signal is proportional to the presence of ATP that reflects the activity of any mycoplasma enzymes. If the ratio of the second luminescence value against the first luminescence value is below one there is no mycoplasma in the culture. The result from the mycoplasma test (Table 13) show that the Ba/F3 cells were free from mycoplasma contamination as the luminescence ratio was below one.

Table 13. Results of the mycoplasma test on the parental Ba/F3 cell line. The column with the luminescence ratio shows the results of two duplicates of supernatant of the cell culture. The ratios were below one and the cell culture was not infected by mycoplasma.

Run number	Luminescence ratio
1	0.30
2	0.31

3.2. Optimization of the cell number of Ba/F3 cell line on 384-well plate

The purpose of this experiment was to find the optimal number of parental Ba/F3 cells for 384 well plate after three days culturing. The total volume of cell suspension was 50 µl in each well in a 384 well plate. The optimal number of parental Ba/F3 cells in a 384 well plate was going to be used for optimization of the Blasticidin concentration, and also for testing of parental Ba/F3 cell growth in media with- and without IL-3 in the same plate format.

Six different dilutions of cell suspensions were made: 500, 800, 1100, 1400, 1700 and 2000 cells/50 µl. Eight replicas of each dilution were added in one row in the 384 well plate. After

72 hours incubation the cell viability was assayed with CTG. The results are shown in Figure 18. The optimal parental cell number selected to use for further analyzes is 700 / well in a 50 µl total volume. The growth curve shows uniformly increased in the exponential phase, and the middle part of that phase was selected for the optimal cell number. In addition, the cells were evaluated visually in the microscope.

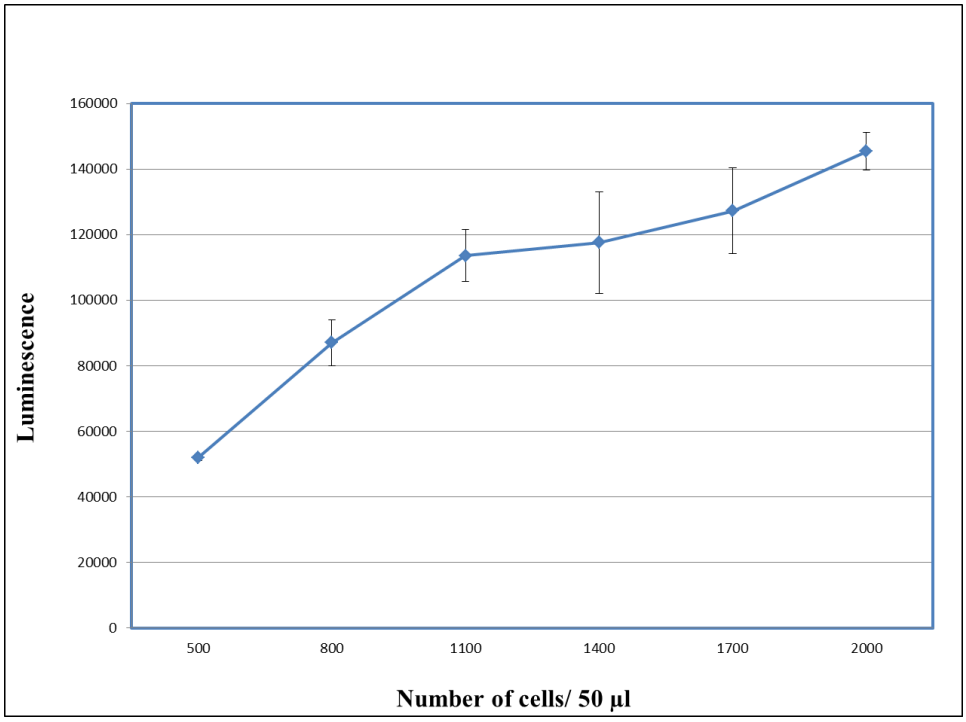


Figure 18. Growth curve of parental Ba/F3 cells. The X-axis represents the number of cells/50 µl total volume and the Y-axis illustrates the luminescence values. Each data point represents the average value of eight replicates.

3.3. Optimization of Blasticidin concentration

Blasticidin was used as a selection marker for transfected Ba/F3 cells. The goal for this optimization was to find the lowest concentration of Blasticidin needed for the parental cells to die. The parental Ba/F3 cells are not Blasticidin resistant. 700 parental cells were added in 384 well plate and Blasticidin was added to a final concentrations: 10, 15, 20, 25 µg/ml. Five replicates of each cell suspension with different Blasticidin concentration was pipetted on to the 384 well plate. One row with 5 replicas of parental cells without Blasticidin, and one row with five replicas with only media were used as controls in the experiment. After three days culturing the cell viability was measured with CTG. Optimal Blasticidin concentration used

for selection of transfected Ba/F3 cells is 20 µg/ml (Figure 19). The experiment was performed twice.

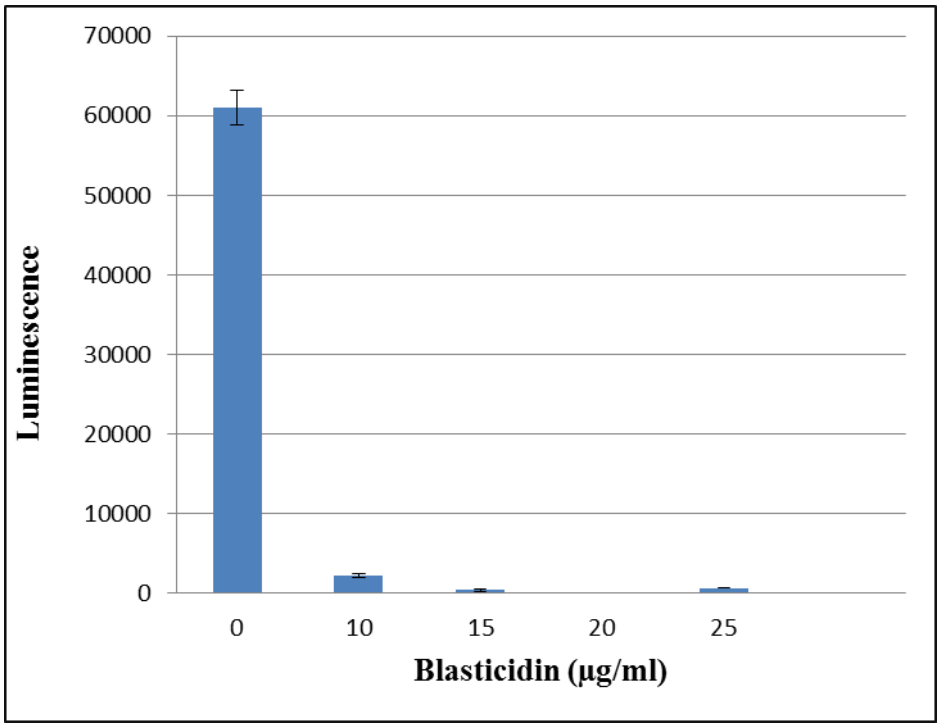


Figure 19. Optimization of Blasticidin concentration for Ba/F3 cells. The X-axis represents different Blasticidin concentrations and the Y-axis illustrates the luminescence values. Each data point represents the average value of five replicates.

3.4. Testing of cell growth of parental Ba/F3 with and without IL3 in the culture medium

Ba/F3 cells are IL-3 dependent (75). To confirm this we made dilutions of 700 cells / 50 µl total volume with five replicates, with IL-3 and without IL-3 in a 384 well plate. After three days culturing the cell suspensions were stained with Trypan blue and cell viability measured with CTG. The results in Figure 20 image a) show no living cells in media without IL-3, but only precipitations from the staining solution. Image b) shows bright spots with viable Ba/F3 cells in media with IL-3. Image c) and d) are from the 384 plate wells before the CTG reagent was added. Image number c) is cell suspension without IL-3 and shows no living cells. Image number d) is cell suspension with IL-3 and there are many viable cells growing in clusters.

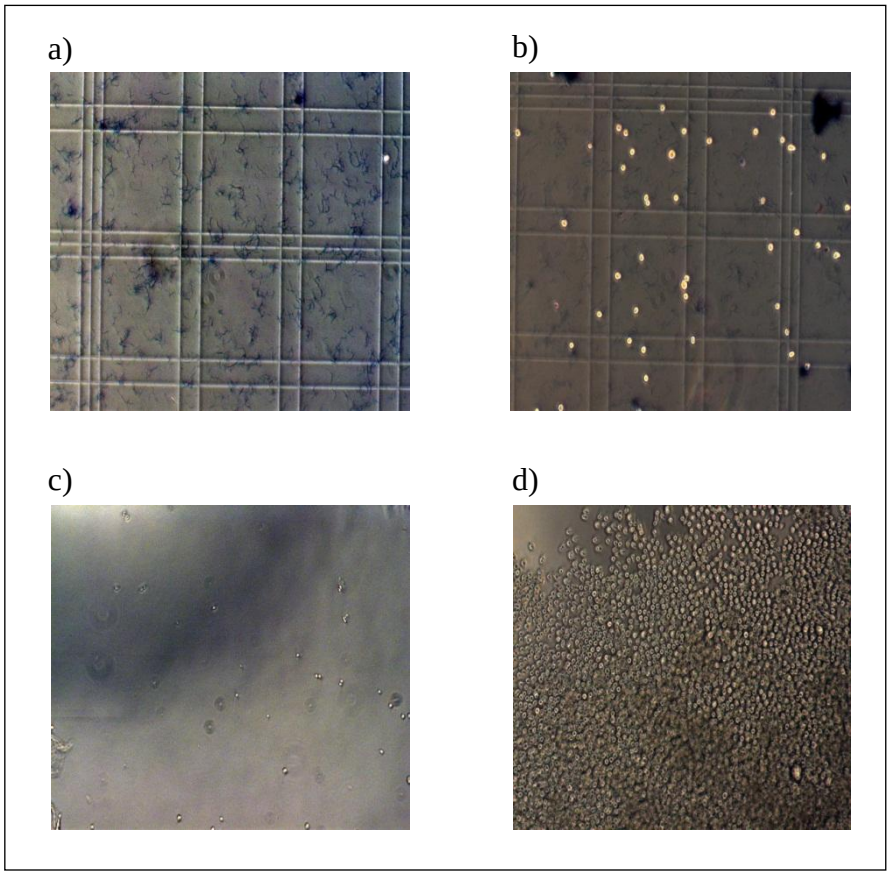


Figure 20. Images of Bürker chambers and wells in a 384 well plate. a) Bürker chamber with no living cells in medium without IL-3, only precipitations from the staining solution. b) Bürker chamber with bright spots represented viable Ba/F3 cells in medium with IL-3. c) cell suspension in a 384 well with medium without IL-3 with no living cells. d) cell suspension in a 384 well with medium with IL-3 with many living cells growing in clusters. (10x) magnification.

The cell viability assay was performed and the results are shown in Figure 21. The average and standard deviation of the luminescence values of five replicates with and without IL-3 in the media are represented by the columns. There is higher luminescence value with IL-3 and no signal without IL-3. The differences in the pictures 20 a-d and the luminescence numbers confirm that the parental Ba/F3 cells are IL-3 dependent.

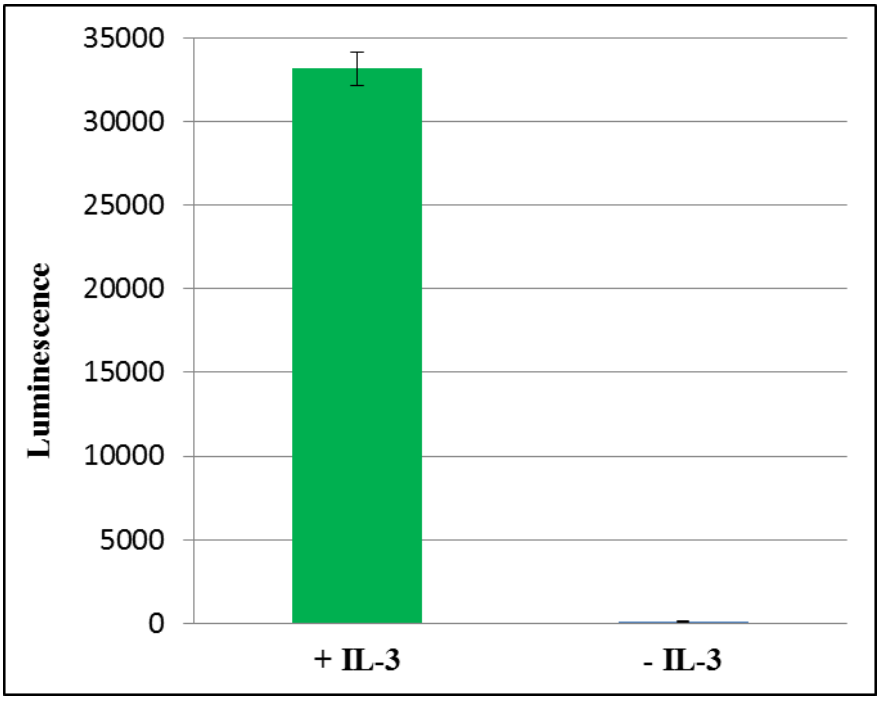


Figure 21. Cell viability assay to confirm the IL-3 dependency of Ba/F3. These average luminescence numbers of five replicates confirmed parental Ba/F3 cells dependency of IL-3 in the culturing media.

3.5. *In vitro* site directed mutagenesis

In order to get plasmids containing the mutants we performed site directed mutagenesis. After mutagenesis, three - six clones of transformed bacteria with plasmids *PIK3CA* P110 S405F, *TP53* K120E and *TP53* H179R were picked for mini preparations. DNAs were isolated and Tables 14 and 15 show high concentrations, amounts and good quality of DNA. Only 10 ng DNA is needed for confirmation of the results by DNA sequencing.

Table 14. Nanodrop results of *PIK3CA* P110 S405F plasmid mini preparations.

Sample parallel	DNA concentration (C) µg/ml	260/280 ratio	Total amount of DNA (µg)
1.1 (*PIK3CA* P110 S405F)	169,04	1.86	8,45
1.2 (*PIK3CA* P110 S405F)	88,28	1.85	4,41
1.3 (*PIK3CA* P110 S405F)	114,27	1.85	5,71
1.4 (*PIK3CA* P110 S405F)	169,40	1.86	8,47
1.5 (*PIK3CA* P110 S405F)	166,41	1.86	8,32
1.6 (*PIK3CA* P110 S405F)	109,01	1.87	5,45

Table 15. Nanodrop results of plasmid mini preparations of *TP53* K120E and *TP53* H179R.

Sample parallel	DNA concentration (C) µg/ml	260/280 ratio	Total amount of DNA (µg)
1.1 (*TP53* K120E)	266.24	1.86	12.91
1.2 (*TP53* K120E)	94.02	1.86	4.56
1.3 (*TP53* K120E)	136.01	1.88	6.59
2.1(*TP53* H179R)	134.60	1.87	6.53
2.2 (*TP53* H179R)	148.13	1.86	7.18
2.3 (*TP53* H179R)	107.36	1.88	5.21

The correct mutations were confirmed by sequencing and the Tables 16 and 17 confirmed that all bacterial clones contained the mutant plasmids.

Table 16. Sequencing results of *PIK3CA* P110 S405F plasmid mini preparations Table 14.

Sample parallel	Sequence result, forward	Sequence result, reverse
1.1	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote
1.2	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote
1.3	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote
1.4	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote
1.5	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote
1.6	*PIK3CA* S405F, homozygote	*PIK3CA* S405F, homozygote

Table 17. Sequencing results of plasmid mini preparations of *TP53* K120E and *TP53* H179R Table 15.

Sample parallel	Sequence results, forward	Sequence results, reverse
1.1	*TP53* K120E, homozygote	*TP53* K120E, homozygote
1.2	*TP53* K120E, homozygote	*TP53* K120E, homozygote
1.3	*TP53* K120E, homozygote	*TP53* K120E, homozygote
2.1	*TP53* H179R, homozygote	*TP53* H179R, homozygote
2.2	*TP53* H179R, homozygote	*TP53* H179R, homozygote
2.3	*TP53* H179R, homozygote	*TP53* H179R, homozygote

Electropherograms of the forward- and the reverse sequences shows that the plasmid DNA containing the homozygote mutant *PIK3CA* P110 S405F (Figure 22).

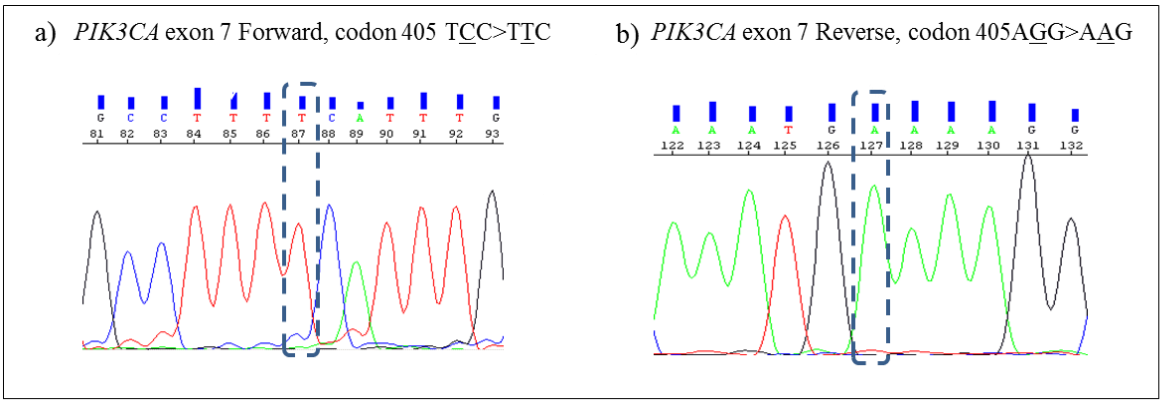


Figure 22. Electropherograms of the forward and reverse sequence of the mutant *PIK3CA* P110 S405F. The forward sequence is read in 5'→3' direction and the reverse sequence in 3'→5' direction. a) *PIK3CA* P110 S405F, exon7 forward. b) *PIK3CA* P110 S405F, exon7 reverse.

Similary is seen for the homozygote mutants *TP53* K120E and *TP53* H179R, electropherograms of the forward and the reverse sequence of plasmid DNA containing the mutants (Figure 23). The sequence results of the mutant plasmids generated from the mutagenesis were thus confirmed.

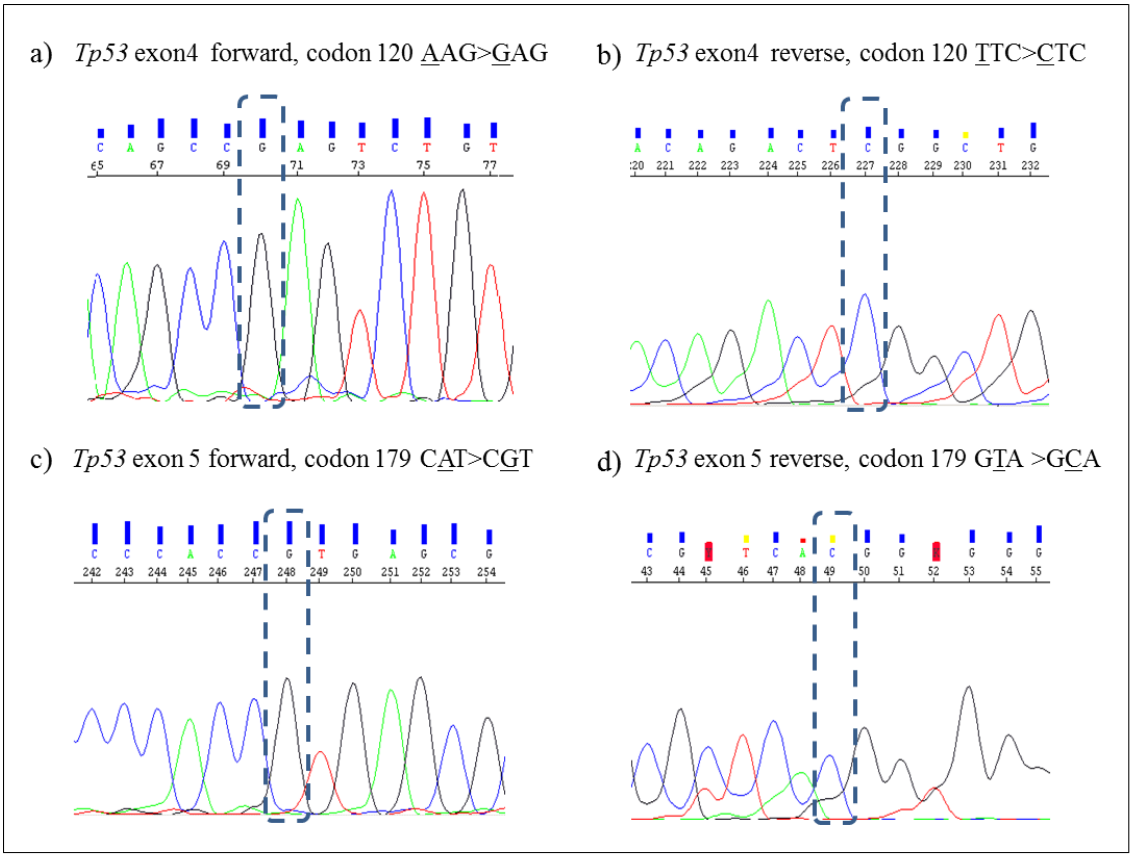


Figure 23. Electropherograms of the forward and the reverse sequence of the mutant *TP53* exon4 K120E and the mutant *TP53* exon5 H179R. The forward sequence is read in 5`→3` direction and the reverse sequence in 3`→5` direction. a) *TP53* K120E, exon 4 forward. b) *TP53* K120E, exon 4 reverse. c) *TP53* H179R, exon 5 forward. d) *TP53* H179R , exon 5 reverse.

3.6. Plasmid midi preparation

Clones which contained the right mutant gene were picked for large scale culturing in order to isolate DNA midi preparations. The concentrations and total DNA amounts are shown in Table 18. The table shows high concentrations, and good quality of DNA. To obtain the recommend plasmid concentration for the transfections which is 30 µg of 1 µg/µl, the plasmid DNA samples were speedvacced.

Table 18. Plasmid DNA concentrations after plasmid midi preparation.

Sample	DNAconcentration (C) µg/ml	260/280 ratio	Total amount of DNA (µg)
PIK3CA P110 S405F	216.74	1.86	~71.5
PIK3CA P110 S405F	166.40	1.90	~54.9
TP53 K120E	360.12	1.86	72
TP53 H179R	275.78	1.88	124.1
TP53 wt	220.39	1.88	50.7
LacZ	134.01	1.85	45.6
PIK3CA P110 wt	195.31	1.86	72.3
PIK3CA P110 E545K	302.99	1.87	75.7
PIK3CA P110 S405F	145.43	1.86	64

3.7. Transfection of Ba/F3 cells to obtain stable cell lines

After constructions of plasmids containing mutant genes, the plasmids were transfected into the parental Ba/F3 cells by electroporation. As we have confirmed that parental Ba/F3 are dependent on IL-3 in the culturing media, they are not Blasticidin resistant. After Blasticidin selection the cells were grown in medium without IL3 in order to find out whether the mutations were oncogenic. After the transfections, Blasticidin selections and maintenance of the cell cultures were done every third day with medium without IL-3. The cell suspensions were examined microscopically to visualize changes and cell growth. The cell cultures were also photographed.

Figure 24 show pictures of the transfected Ba/F3 cell line *TP53* H179R. Seven days after the first Blasticidin addition the microscopic examination of the cell culture shows two different cell types, viable transfected Ba/F3 cells and dead parental cells. 14 days after the first Blasticidin addition, the cells grow fast before they change their morphology on the 29th day. The transfection and the Blasticidin selection have worked. It indicates that the mutation *TP53* H179R is oncogenic because of the presence of living cells after maintenance with medium without IL-3. According to the IL-3 experiment, subchapter 3.4, all parental cells died after three days without IL-3.

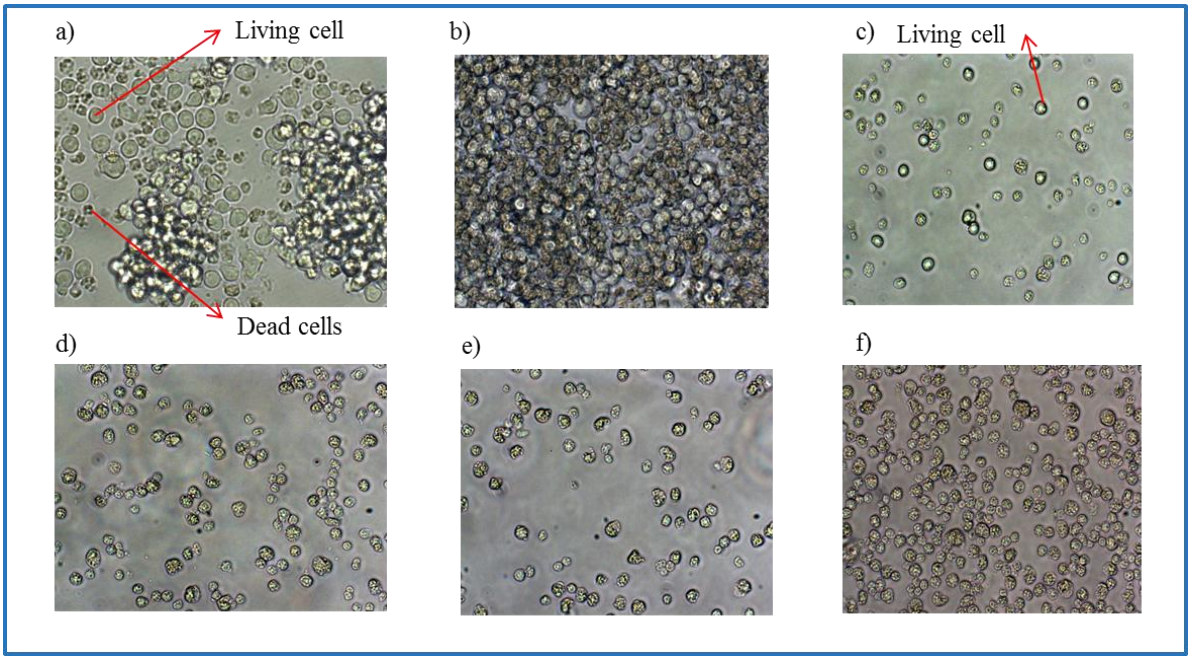


Figure 24. Images of the wells with transfected Ba/F3 cell line *TP53* H179R. a) Seven days after the first Blasticidin addition. The image shows two different cell types, viable transfected Ba/F3 cells and dead parental cells. b) 14 days after the first Blasticidin addition. The image shows fast growing living cells and dead cells. c) 23 days after the first Blasticidin addition. The image shows a few living cells among dead cells. d) 29 days after the first Blasticidin addition. The image shows that the cells have changed their morphology. e) 35 days after he first Blasticidin addition. It was split 1/2, 29 days after the first Blasticidin addition. The image shows that the cells have same appearance as in d). f) 43 days after the first Blasticidin addition. It was split 1/2 38 days after the first Blasticidin addition. The image shows that the cells have same appearance as in d). (40 x) magnification is used.

Seven days after the first Blasticidin addition of the transfected Ba/F3 cell line *PIK3CA* P110 S405F the microscopic examination of the cell culture shows cell suspension with dead cells and few living cells (Figure 25). After 14 days after the first Blasticidin addition the viable cell number has increased before they have changed their morphology on the 29th day of the first Blasticidin addition. The transfection and the Blasticidin selection are sucessfull. It is likely that the mutation *PIK3CA* P110 S405F is oncogenic because of presence of living cells after maintance with medium without IL-3.

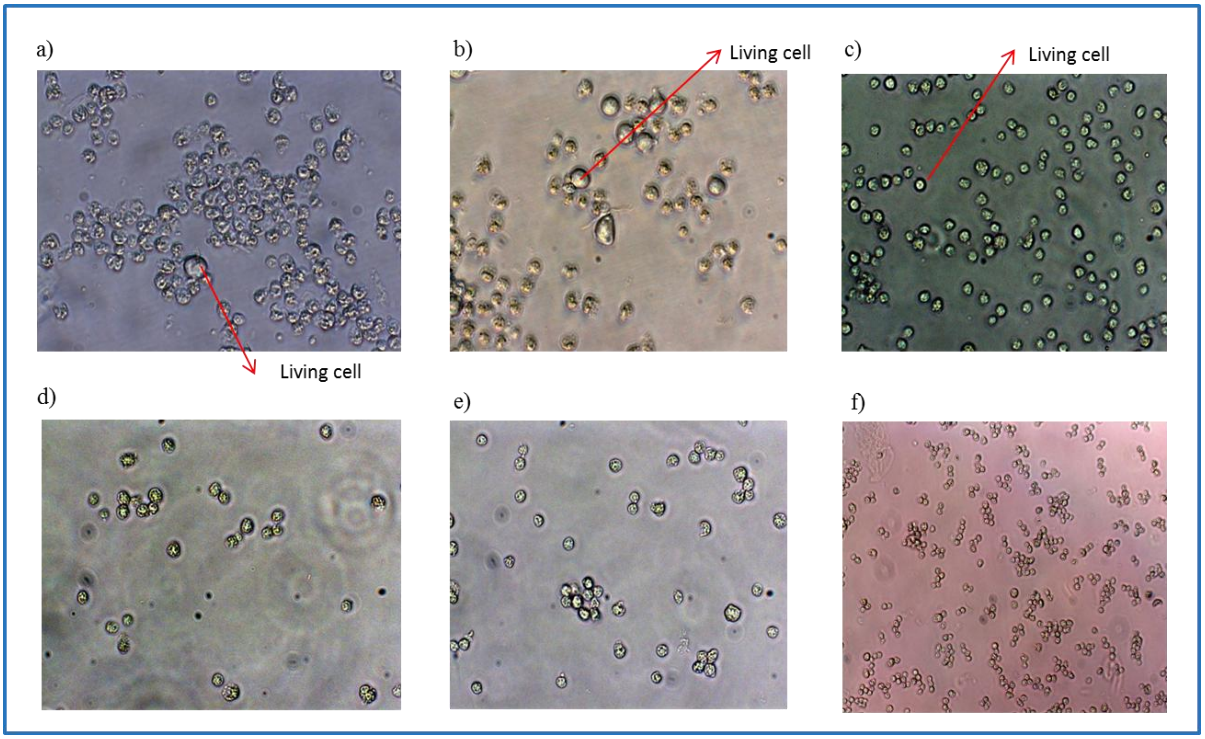


Figure 25. Images of the wells with cultured transfected Ba/F3 cell line *PIK3CA* P110 S405F a) Seven days after the first Blasticidin addition. The image shows cell suspension with dead cells and few living cells. b) 14 days after the first Blasticidin addition. The number of living cells has increased. c) 23 days after the first Blasticidin addition. The image shows both living cells and dead cells. d) 29 days after the first Blasticidin addition. The cells have changed their morphology. e) 35 days after the first Blasticidin addition. It was split 1/2 29 days after the first Blasticidin addition. The cells look the same as the cells in the image d). f) 43 days after the first Blasticidin addition. The cell suspension was split 1/2 38 days after the first Blasticidin addition. The cells look the same as the cells in the image d). a)-e): (40 x) magnification, f): (20x) magnification.

By microscopic evaluation of the transfected Ba/F3 cell line *TP53* K120E we saw only dead cells without any changes in the appearance (Figure 26). This indicates that the cell line is not oncogenic or that the transfection has not succeeded.

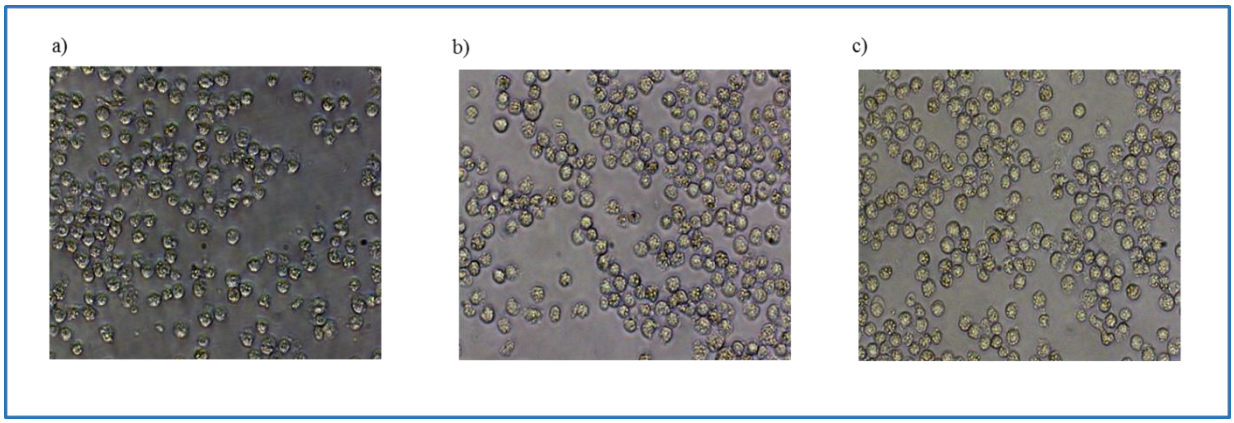


Figure 26. Images of the wells with cultured transfected Ba/F3 cell line TP53 K120E. All images show a well containing cell suspension with dead cells 21 days, 29 days and 35 days after the first Blasticidin addition. (40x) magnification is used.

The *PIK3CA* P110 E545K is a known oncogenic mutation (personal communication: Gordon Mills, M.D. Andersson, Houston, Tx, USA) and was used as a positive transfection and viability control. Seven days after the first Blasticidin addition the microscopic examination of the cell culture shows cell suspension of dead cells, but on the 21 days after the first Blasticidin addition many viable cells were observed (Figure 27). On the 29 days after the first Blasticidin addition the cells have changed their morphology. Also here transfection and Blasticidin selection have worked. The cell line worked as a positive control.

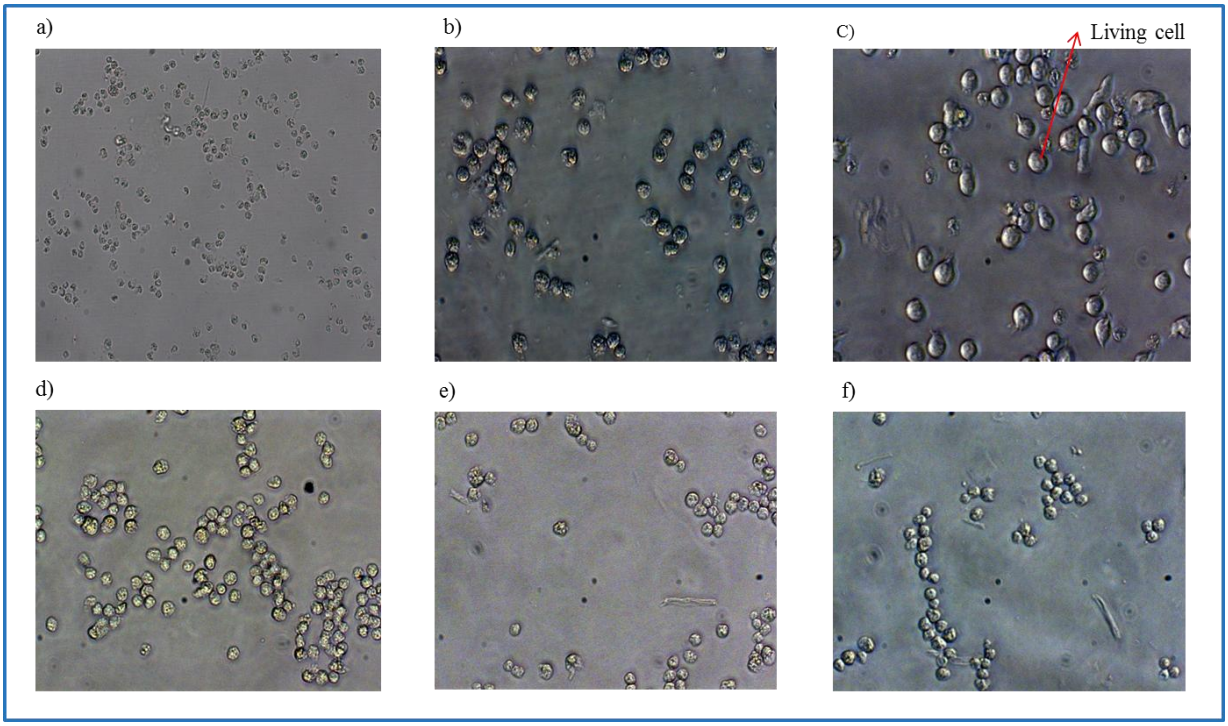
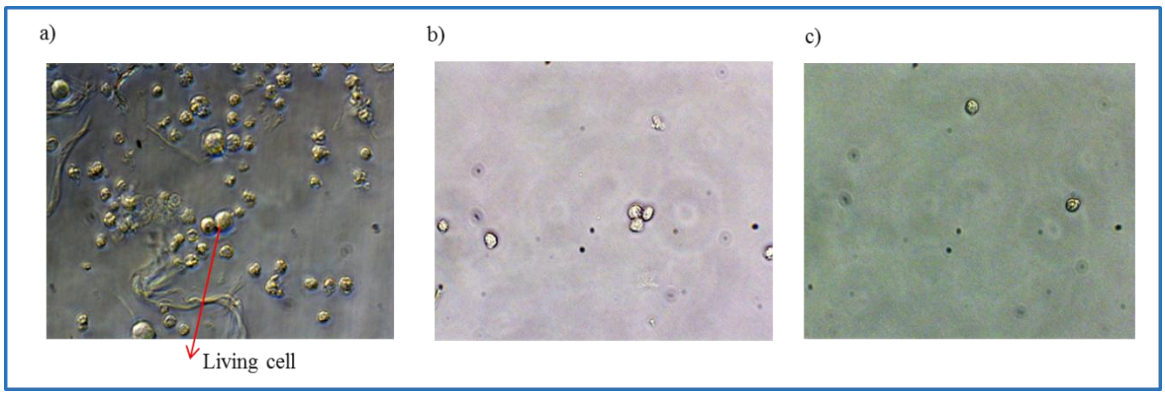


Figure 27. **Images of the wells with cultured transfected Ba/F3 cell line *PIK3CA* P110 E545K** a) Seven days after the first Blasticidin addition. No visible living cells. b) 14 days after the first Blasticidin addition. The image shows no visible cells. c) 21 days after the first Blasticidin addition. The image show many viable cells. d) 29 days after the first Blasticidin addition. The image shows that the cells have changed their morphology. e) 35 days after the first Blasticidin addition. The cells were split 1/2 29 days after the first Blasticidin addition. The cells look like cells in image d). f) 43 days after the first Blasticidin addition. The cells look like cells in image d). a): (20 x) magnification. b)-f): (40x) magnification.

The *TP53* wt plasmid was used as a double stranded DNA template in the mutagenesis. Figure 28 shows images of the transfected cell line *TP53* wt plasmid. On the 21 day after the first Blasticidin addition we saw dead cells and few living cells. On the 29 days after the first Blasticidin addition we saw only few dead cells and the same observation was done on the 35 days. The transfection has worked but the cells did not grow without IL-3.



Figur 28. **Images of the wells with cultured transfected Ba/F3 cell line *TP53* wt.** a) 21 days after the first Blasticidin addition. The image shows dead cells and few viable cells. b) 29 days after the first Blasticidin addition. Only few dead cells, no living cells were observed. c) 35 days after the first Blasticidin addition. Only few dead cells, no living cells were observed. All images show wells with cell suspension, (40x) magnification.

The *PIK3CA* P110 wt plasmid was used as a double stranded DNA template in the mutagenesis. Figure 29 shows image of the transfected Ba/F3 cell line *PIK3CA* P110 wt. The images shows cell culture with only dead cells without any changes in the morphology.

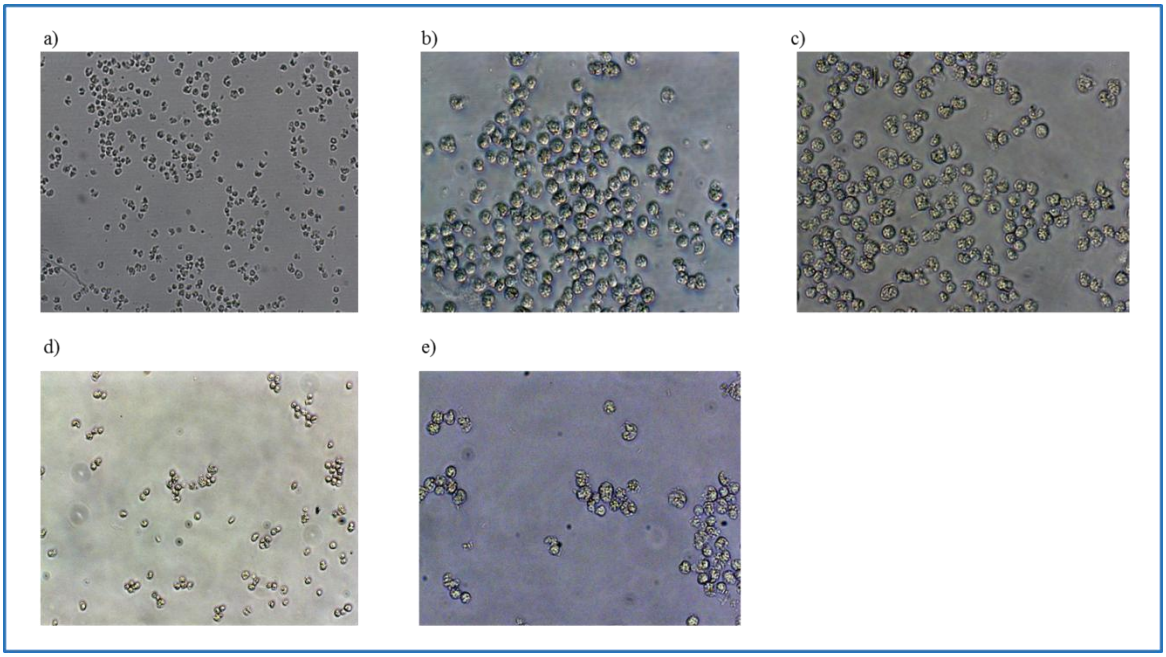


Figure 29. Images of the wells with cultured transfected Ba/F3 cell line *PIK3CA* P110 wt. All images show wells with cell suspension with dead cells seven days, 14 days. 21 days, 29 days and 35 days after the first Blasticidin addition, a) and d) (20x) magnification. b), c) and e) (40x) magnification.

The *Lac*Z plasmid was used as a normal control for monitoring the transfection efficiency. Cells with the *Lac*Z plasmid were dead (Figure 30) . It worked as a normal control.

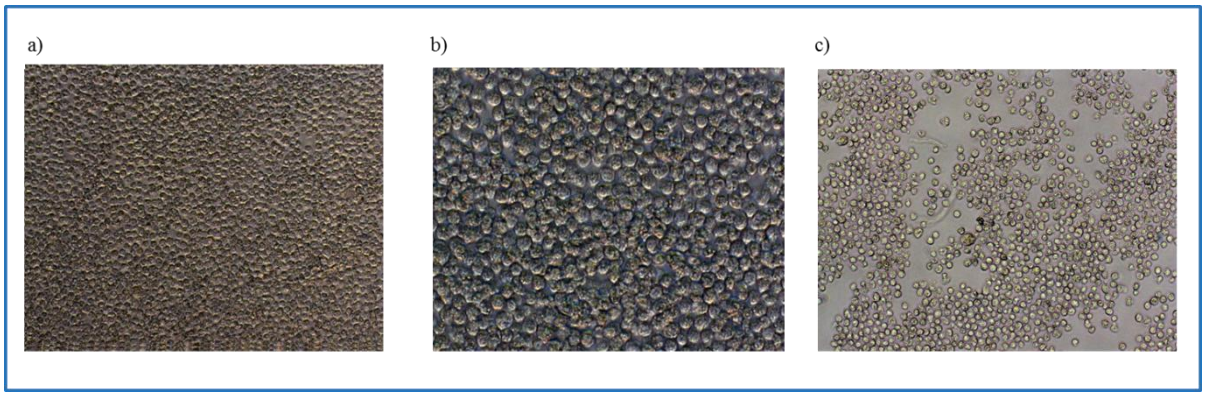


Figure 30. Images of the wells with cultured transfected Ba/F3 *Lac*Z. All images show cell suspension with dead cells 21days, 29 days and 35 days after the first Blasticidin addition, a) and c) (20x) magnification, b) (40x) magnification.

Parental Mock+/- control cells were subjected to electroporation. Mock + is treated with Blasticidin and Mock- is not treated with this selection marker. Figure 31a-e) show only dead cells as these are not resistant to Blasticidin. Mock – in 31f) is more confluent since it grew fast as it was not treated with Blasticidin, but the cells died due to lack of IL-3.

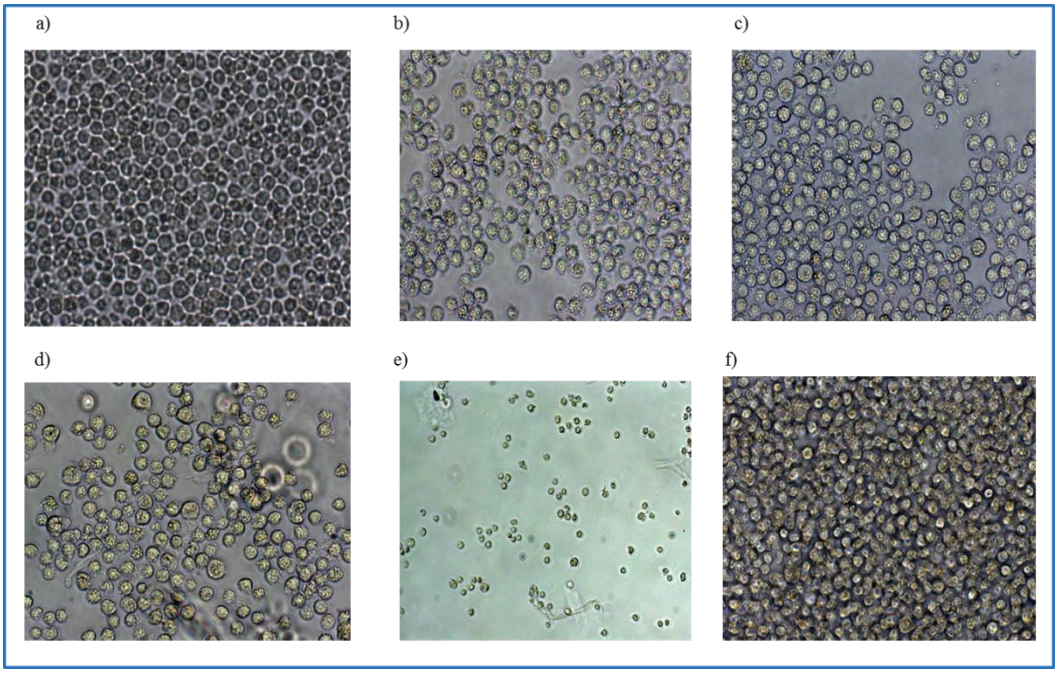


Figure 31. Images of the wells in a 48 well plate containing Mock treated Ba/F3 cells with and without Blasticidin in the medium. a)-e): The cells were subjected to electroporation and treated with Blasticidin (Mock+). a)-e): Cell suspension with dead cells seven days, 14 days, 21 days, 35 days and 43 days after the first Blasticidin addition. f): The cells were subjected to electroporation and not treated with Blasticidin (Mock-). Cell suspension with many dead cells. The parental cells divided fast before they died because of lack of IL3 in the medium. a) - d) and f) (40x) magnification. e) (20x) magnification.

Figure 32 shows the parental Ba/F3 cell cultures which are not subjected to electroporation. Images a) and b) show few dead cells due to the Blasticidin treatment. Images c) and d) show many dead Ba/F3 cells. The cell culture was not treated with Blasticidin. The cells grew fast before they died due to maintenance with medium without IL-3.

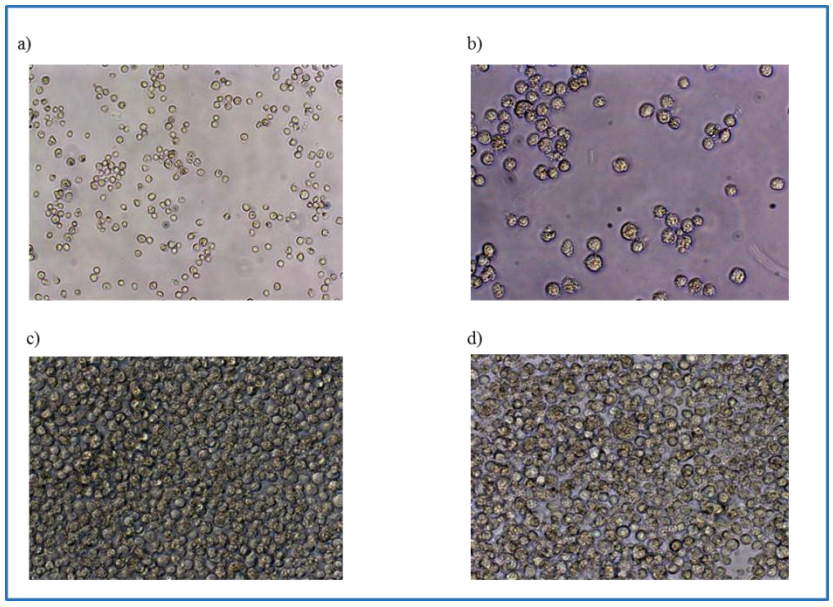


Figure 32. Images of the wells with cultured parental Ba/F3 cells. a) and b) show treatment with Blasticidin, c) and d) show no treatment with Blasticidin. a): The image is taken 21 days after Blasticidin addition and shows few dead cells. b): The image is taken 35 days after Blasticidin addition and show few dead cells. c) and d) show many dead cells. The parental Ba/F3cells divided fast before they died because of lack of IL-3 in the medium. a) (20x) and b), c), and d) (40x) magnification.

We wanted to confirm whether the transfected cells were viable and did Trypan blue staining and a CTG assay after one week culturing in a 384 plate. Figure 33 show images of cell suspensions stained with Trypan blue and confirms that the cells were dead. The CTG results showed no luminescence signal in all replicates for all samples and controls (data not shown), so the microscopic observations of Trypan blue stained cells were confirmed. This indicates that we were not able to create stable cell lines after ~ six week culturing for the transfected Ba/F3 cell lines *TP53* H179R, *PIK3CA* P110 S405F and the positive control *PIK3CA* P110 E545K. We also cultured a parallel transfected Ba/F3 cell line *PIK3CA* P110 S405F from a separate transfection for ~three months, but this also contained only dead cells.

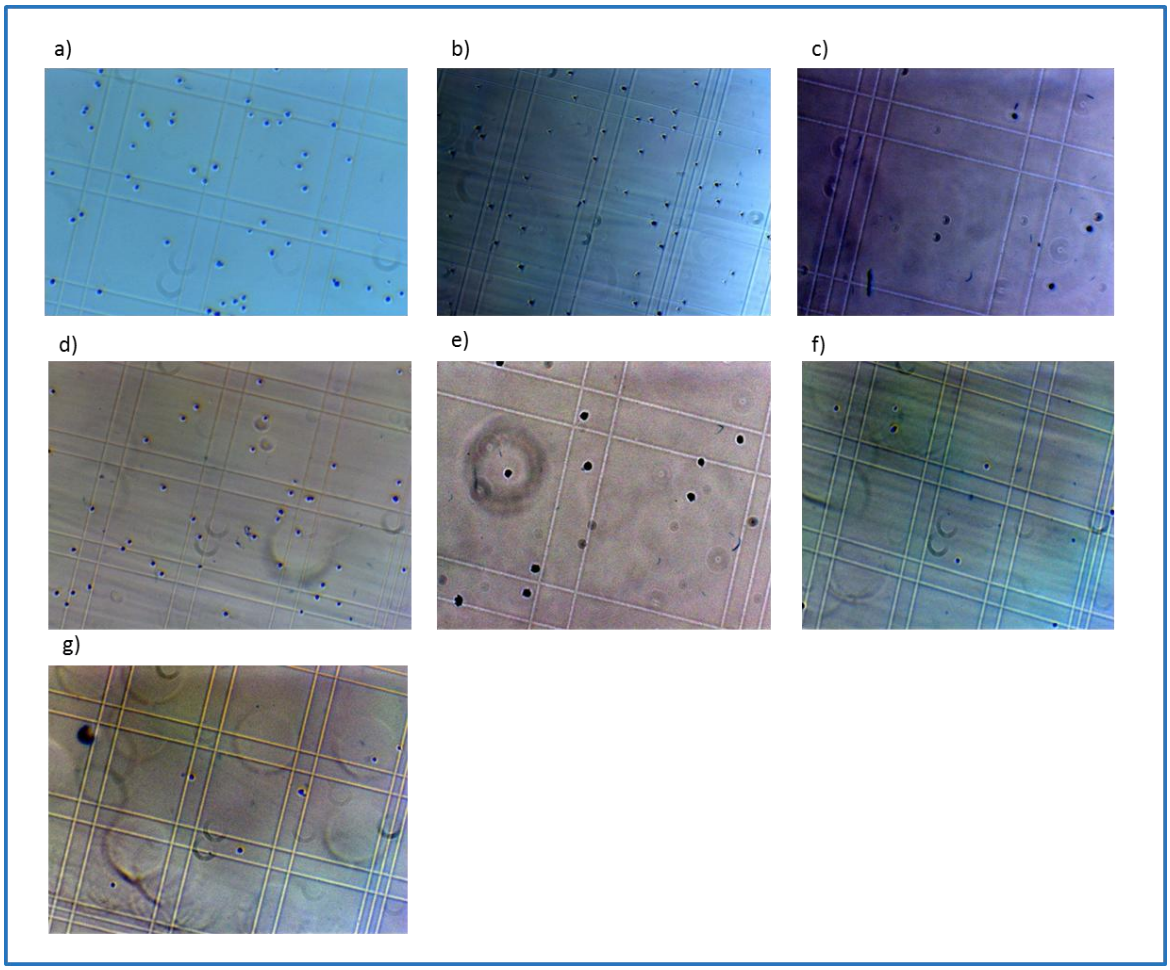


Figure 33. Microscopic observations of cells viability by using Trypan blue staining (1/2 dilution) of 10 µl cell suspension. The images show dead blue stained cells. a) Transfected Ba/F3 cell line *TP53* K120E. b) Transfected Ba/F3 cell line *TP53* H179R. c) Transfected cell line *PIK3CA* P110 S405F (1. parallel). d) Transfected cell line *PIK3CA* P110 S405F (2. parallel). e) Mock +, negative control. f) Transfected Ba/F3 cell line *PIK3CA* P110 wt, normal control. g) Transfected Ba/F3 cell line *PIK3CA* P110 E545K, positive oncogenic control. a), b), d), f) and g) have (10x) magnification. c) and e) have (20x) magnification. The microscopic filter 2-0,4 was used in a), d), f) and g) and filter 1-0,4 was used in e).

4. Discussion

4.1. Experimental considerations

4.1.1. *In vitro* cell culture of Ba/F3 cells

The Ba/F3 cell line is used as a model system in cancer research. Ba/F3 cells are fast growing suspension cells and therefore the experimental turnaround is both easy and rapid (75). The cell line is IL-3 dependent and this property can be exploited in the detection of oncogenic mutations. Mutant genes can be expressed in transfected Ba/F3 cells and they may lead to IL-3 independence (74). The Ba/F3 cell can be used to study the potency of cancer gene mutations, and to test for drugs to predict response in the clinic (75).

The purpose of this study was to establish the Ba/F3 model system for testing the oncogenic potential of cancer gene mutants in Ba/F3 cell cultures. In this study Blasticidin and IL-3 was used as selection markers for the transfected Ba/F3 cells. Blasticidin treatment and maintenance of the cell cultures was done for a 10 days period after the transfection to select the transfected Ba/F3 cells containing plasmids with Blasticidin resistance. Parental cells without plasmid died in this period because they were not resistant to Blasticidin. After the selection period the cells were maintained without IL-3. If the transfected Ba/F3 cells started to grow after the maintenance without IL-3 in the medium, then they have an oncogenic mutation in its genomes. If all the cells in one sample died without IL-3, it indicated that the mutation was not oncogenic.

The transfected Ba/F3 cell lines were observed regularly in a microscope. First, we observed that the cell line *TP53* H179R started to grow fast seven days after the first Blasticidin addition. Also the transfected cell line *PIK3CA* S405F was observed with few living cells at the same time, but the living cell number had increased one week later. The transfected positive control cell line *PIK3CA* P110 E545K was observed with growth 21 days after the first Blasticidin addition. The transfected Ba/F3 cells where then grown further in an attempt to generate stable transfected cell lines. The maintenance started without IL-3 for the first experiment about 6 weeks after the last Blasticidin addition, and eight days after the second experiment. The maintenance by changing medium without IL-3 should have started three days after the last Blasticidin treatment. The delay in changing of medium of viable cell cultures may lead to pH changes and lack of nutrients in the cell cultures. The transfection,

the Blasticidin selection period and removal of IL-3 from the medium are all stressful for the Ba/F3 cells. The change of medium three days after the Blasticidin treatment did not take place due to misunderstanding of the protocol. This is the most likably explanation why we were not able to create stable cell lines. Other possible reasons could be that there were few living cells in the culture, which were washed away while maintaining the cells thus, no cells survive. The viable cell cultures were expanded from 48-well plate to six-well plate. It is possible that the cells became stressed by changing to this plate format. The Ba/F3 cells grow in clusters and by diluting them in six-well format they might be unable to signal to each other. However, the positive control was cultured in the 48-well format throughout the period and also these cells died. Another possible reason why the Ba/F3 cells died could be that the transfected viable cells have lost the plasmids because we cultured without Blasticidin after the ten days selection period. We did this according to the protocol obtained from our collaborator at M.D. Andersson. Generating stable cell lines can be challenging. For example, our collaborator did five experiments before they achieved to get stable clones for the positive control *PIK3CA* E545K and the *PIK3CA* P110 wt. They did ten experiments without establishment of a stable cell line for the negative control *Lac*Z with Plenti 6.3 background. An alternative method for the Blasticidin selection was reported by Walters and his colleagues (76). Ba/F3 cells were transduced (100) with supernatant containing viral constructs. After incubation the cells were selected for Green fluorescence protein marker (GFP) expression by cell sorting. GFP positive cells were cultured for five-ten days in medium without IL-3. If the Ba/F3 cells with plasmids containing the mutant *TP53* K120E, *TP53* H179R and *PIK3CA* P110 S405F have expressed GFP marker, we could have selected for expression of GFP by cell sorting instead of selection with Blasticidin. The cell viability test could have been done right after we observed the cell growth. The cell cultures could have been split in two, one part to assess cell viability and one part for further culturing. Then we would have had both luminescence values and images of the possible oncogenic mutations. Instead, Trypan blue staining and cell viability assay were done on the cell cultures after 3 month for one sample and ~ six weeks for two other cell lines and control cell lines. At this point the cells had died. This might explain the changes in the cell morphology between 21-29 days after the first Blasticidin addition. We found that the *TP53* H179R and *PIK3CA* P110 S405F were possible oncogenic mutants. The mutant in the transfected cell line *TP53* K120E was non-oncogenic assuming that the transfection worked. This cell line was observed with only dead cells after culturing with medium without IL-3. These results have to be confirmed by further CTG viability testing.

4.1.2. Controls used in the experiments

It is important to have controls at all stages of the experiments as sources of error can be identified that can affect the test results. Transfected Ba/F3 cell lines *Lac*Z, *TP53* wt, *PIK3CA* P110 wt, Mock treated parental Ba/F3 cells and parental Ba/F3 cells with and without (+/-) Blasticidin were used as negative controls, whereas *PIK3CA* E545K where used as a positive control in the experiments. Mock treated Ba/F3 cells (+/-) Blasticidin did not contain any plasmids, but were subjected to electroporation. Comparison of these negative controls together with parental Ba/F3 cells (+/-) Blasticidin without electroporation could tell us if the electric shock have affected the parental cells. The *Lac*Z control was confluent with living cells after the last Blasticidin addition. After removing IL3 from the medium the cells died. The results from the cell growth experiment of parental cells with and without IL-3 showed that after three days without IL-3, there were no viable cells left in the culture. The image in Figure 30 a) shows only dead cells from the 21 day after the first Blasticidin addition. This negative control gave us information about how the selection markers have worked in our experiment. It also confirmed the fact that cells cannot survive without IL-3 unless they carry an oncogenic mutant. The luminescence value from the cell viability test of the negative control is useful for comparison for transfected mutant Ba/F3 cells. The Ba/F3 cell line *PIK3CA* P110 wt was observed without any living cells throughout the culturing. This was as expected since the transfected Ba/F3 cells contained wild type plasmids.

The transfected Ba/F3 cell lines *TP53* wt grew more slowly compared to the other cell lines after the transfection. A possible explanation was that these cells did not tolerate the transfection as good as the others. Few living cells were observed 21 days after the first Blasticidin addition (Figure 28a). This observation was unexpected since the transfected Ba/F3 cells contained wild type plasmids and should not have been able to grow without IL-3. The most probable explanation could be that there was some IL-3 left in the medium because of lower consumption of IL-3. That might explain why the *TP53* wt transfected cells started to grow after a while, but died when we changed medium without IL-3 every third day. On the 29th day after the first Blasticidin addition we observed only few dead cells left in the cell suspension. Many living transfected cells were observed in the positive oncogenic control *PIK3CA* E545K 21 days after the first Blasticidin addition. Some transfected cell lines with

mutants with oncogenic potential use longer time before they start growing after the last maintenance with IL-3.

4.1.3. Utilization of the Ba/F3 model system

Warmuth and his colleagues (75) point out that the Ba/F3 cells properties as fast growing suspension cells makes this cell line ideal for automatic culturing systems. Generation of cellular potency and selective drug profile can be achieved within one - two weeks (78). They further suggested that the Ba/F3 method might be an alternative to the more lab intensive and less sensitive fibroblast or epithelial transformation assays (75). It was easy to maintain the cultured Ba/F3 cells because they were suspension cells. The cell culture was easy to grow and it took only two weeks to get optimal confluence level before the experiments could starts. In the present study, the Ba/F3 cells were transfected by electroporation. An alternative transfection method is to use a viral system with high titer lentivirus generated by cotransfection of HEK293FT cells with viral constructs. Ba/F3 cells would then be transduced by viral supernatant, incubated without IL-3 before viability testing (61). Walters *et al.* used virus transfection of Ba/F3 cells combined with cell sorting of GFP-positive cells before further analyses (76). Lentivirus vectors have the ability to mediate stable expression and mediate potent transduction (101) into diving and non-diving cells both *in vivo* and *in vitro* (100). The transfection efficiency might be better with lentivirus. The laboratory facility must have a higher security level by using a viral system for the transfections. The equipment and the optimized conditions for the electroporation procedure were the same as our collaborators used at the M.D. Andersson. The Ba/F3 cells were easy to transfect by electroporation and the success rate is in general ~ 40 % by using the Neon ^{TM} transfection system according to the collaborators at M.D. Andersson. The first landmark experiment on Ba/F3 cells was done by Daley *et al.* in 1988 (74). They managed to render Ba/F3 cells IL-3 independent by the oncogenic fusion kinase Bcr-Abl. Since then the Ba/F3 model system has been used of several laboratories and many studies have been done with the Ba/F3 cells (61;76;78;102;103). Most of the works have been focused on studies of oncogenic potential of kinase mutations (such as mutations in *JAK 1,2,3, ERBB 2,3,4*). Many studies have also been done to on determination the efficacy of kinase drug inhibitors and to predict clinical response to drugs. The model system is also a valuable tool in studying signaling mechanisms downstream of mutant kinases (75).

In our study we managed to establish the model system and we did found two possible oncogenic mutations. In addition, we found one passenger mutation. We did not succeed to establish stable cell lines of the two cell lines with oncogenic potential. If we do drug testing on them in the future, we have to establish stable cell lines as the transfection efficiency on Ba/F3 cells on our electroporation system is not high enough. We get most reliable results from drug screening by testing drugs on stable oncogenic clones. A stable cell line is created when the "gene of interest" has been integrated in the host genome and multiplied. Together with the host genetic material it can be divided into daughter cells and remain intact for a long period. An unstable cell line can lose "the gene of interest" and the experiments become unsuccessful.

This model system gives a possibility to test for oncogenic potential for cancer gene mutations found in patients. For example, mutants may be identified by deep sequencing of the tumor, and the Ba/F3 model system can be used to test the oncogenic potential of the identified mutation. The oncogenic mutations may then be further tested with drugs to find targeted therapy against the particular mutants. It may also be possible to introduce up to three mutations in one gene in the mutagenesis. Another possibility could be to assays with two different mutant genes. Then the Ba/F3 cells have to be transfected with two different mutant vectors that have different selection markers.

4.1.4. Other methods to study oncogenic mutations

There are other methods to test the oncogenic potential of mutations. For example, traditional *in vitro* kinase assay measures the kinase activity. If the gene encoding the kinase has a mutation, the control mechanism of the kinase can be disturbed. The kinase becomes active and it phosphorylates the substrate continuously. The phosphorylated substrate is labeled with radioactive ATP before it can be visualized by autoradiography (104). Another method is the soft agar formation assay. Transfected cells expressing mutants or wild type genes are seeded on a top layer of 0,4 % agarose. The bottom layer of the well contains higher concentration of agarose. After two-three weeks maintenance, the colonies are stained with crystal violet and counted. The colony numbers describe the growth rate of the transfected cells (105). This is an assay for anchorage-independent growth which means that for example cancer cells can grow and form colonies independent of each other.

A novel system to study and verify oncogenic capacity of mutations in the kinome and phosphatome was published by Kleppe and colleagues (106). They established a model system based on a mouse CD4+ and CD8+ T-cell line (named MOHITO for MOuse Hematopoietic Interleukin dependent cell line of T-cell Origin) to study oncogenic signaling of T-cell malignancies. MOHITO cells are dependent on both Interleukin 2 (IL-2) and Interleukin 7 (IL-7) but the latter cytokine is the major survival and proliferation stimulus. MOHITO cells are transduced with a bicistronic BCR-ABL1-IRES-GFP construct which allows translation of both BCR-ABL1 and GFP proteins. The initial fraction of GTP positive cells was 30%. After IL-7 withdrawal a constant increase of GTP positive clones were observed and the transduced cells take over. Protein analyses showed high level of autophosphorylated BCR-ABL1 and phosphorylation of its downstream target Stat5. Transformed cells became sensitive to Imatinib, which induced cell death and blocked proliferation (106). In this way Kleppe and colleagues could compare the signaling downstream of oncogenic kinases in the B- and the T-cell context. This model system can be used in the same manner as the Ba/F3 method. It is a similar system, MOHITO is for the T-cell signaling and Ba/F3 cells are for the B-cells signaling.

Shah *et al.* worked with infusion of Ba/F3 cells with stable luciferase expression into mouse in a kinase inhibitor resistance study (103). They first characterized the Imatinib-resistant Ba/F3 cells with BCR-ABL mutants *in vitro*. Then they moved to the *in vivo* experiments by testing of growth inhibition of Imatinib-resistant mutant BCR-ABL-expressing Ba/F3 by the drug BMS-354825.

4.2. Biological considerations

Stratton and colleagues described a driver mutation as a mutation that conferred growth advantage on the cancer cell. The passenger mutation has no clonal growth advantage and do not contribute to the tumor development (2). In the literature the genes that harbor few or no driver gene mutations have been labeled as driver genes (67). These are genes which are epigenetically altered, underexpressed or overexpressed in the tumor and may play an important role in the neoplastic process. Vogelstein and colleagues suggested categorizing driver genes as either “Mut-driver genes” or “Epi-driver genes”. Mut-driver genes contain sufficient number or type of driver gene mutations to distinguish them from other genes. Epi-driver genes are altered through chromatin modification or in DNA methylation which persist when the tumor cells divides (67).

The mutations tested in our study were from breast tumor samples used in two mice xenograft models established in our institute. These xenograft models have also been studied for example on metabolic, genomic and transcriptomic levels (107;108). The mutations that we tested are in the *PIK3CA* and *TP53* genes. These are two of the most frequently altered genes in breast cancer (65;109). Mutation of *PIK3CA* can lead to constitutive activation. The majority of the genes in the PI3K pathway are often mutated or amplified in many types of cancer. This signaling cascade regulates proliferation, growth and survival (109). About 23% of the breast cancers have mutations in the tumor suppressor gene *TP53*. The wild type p53 has an important role as a transcriptional factor induced by stress. It can promote cell cycle arrest, apoptosis and senescence and is a potent tumor suppressor. An oncogenic mutation in the *TP53* gene can lead to increased cell proliferation. Levin and Oren have found that *TP53* mutation is found a late event and presumably play a part in the progression to invasive, advanced and metastatic disease (53).

The somatic mutations in tumor samples are mostly missense and heterozygous. Mutations generally result in loss of function. The mutated allele loses its function whereas the wild type allele retains the function. The mutant *TP53* may interact with the wild type allele and mask the function of wild type allele in a dominant negative fashion. In some cases the mutated protein might show gain of function by acquiring novel functions. This leads to increased proliferation of abnormal cells. Codon 179 in the *TP53* gene is reported as a hotspot region in breast cancer (110). The mutation *TP53* H179R, Histidine to Arginine found in one of the xenograft breast tumor is reported in 0,63% of the total mutated cases (18 of a total of 2878 mutations in breast cancer) (111). This mutation is reported to have both dominant-negative activity and gain of function in various functional assays. The *TP53* K120E is a rare mutation and only 2 breast cancer cases are reported to have this mutation (of a total of 2878 mutations reported) (111). Few functional studies on this mutation associate it with loss of function. These data support our findings that *TP53* H179R is an oncogenic mutation and that *TP53* K120E is non-oncogenic. Brachmann and colleagues predicted correlation between the *TP53* mutations in cancer and dominant-negative activity on *TP53* mutations in a yeast assay (112). They studied the same codon, but with different amino acid change. The oncogenic mutation *PIK3CA* P110 S405F was found in the same xenograft tumor as the non-oncogenic *TP53* K120E mutation and *PIK3CA* seems to be a driver gene in this xenograft tumor. The mutation *PIK3CA* P110 S405F is in the C2 domain in exon 7. In addition to hotspots in exon 9 and 20,

a third hotspot region in exon 7 was reported in the *PIK3CA* gene. One study reported a mutant frequency of seven of a total number of 292 breast cancer tumor samples (2,4%) (69). Most of the mutation screening on *PIK3CA* have been done in the hotspots in exon 9 (Helical domain) and exon 20 (Kinase domain) and few has screened exon 7. The *PIK3CA* mutation screening should also include exon 7 since "important" mutations maybe missed. Based on our findings, the *PIK3CA* S405F seems to be an oncogenic mutation. In a metastudy of 100 breast cancer tumors done by Stephens and his colleagues, they found that the maximum numbers of oncogenic mutations in an individual breast cancer was six, and 28 cases had only a single driver. They found driver mutations in 40 genes and 73 combinations of mutated genes. This illustrates the genetic diversity in breast cancer (65). In some cases one mutation with oncogenic potential can be enough in the development of cancer. It is possible that the oncogenic mutations we found in this study, may lead to breast cancer progression, however more testing have to be done.

Vogelstein and colleagues state that all known driver genes can be classified into 1 - 12 pathways and that they can be organized into 3 core cellular processes: the cell survival, the genome maintenance and the cell fate (Figure 34) (67).

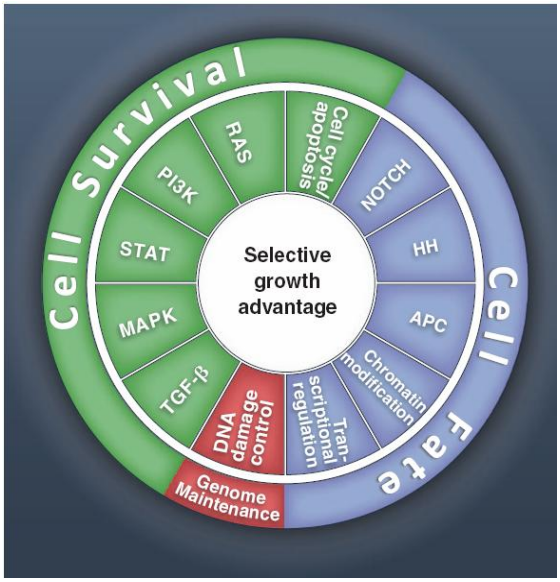


Figure 34. Cancer cell signaling pathways and the cellular processes they regulate (67). All driver genes can be classified into 1-12 pathways (middle circle) that confer a selective growth advantage (inner circle). These pathways can be further organized into three core cellular processes (outer circle).

The *PIK3CA* gene belongs to the cell survival and the *TP53* to the genome maintenance pathways (67). The cell signaling pathways are a complex network with many parallel and regulation systems at different levels. Most of the signaling pathways incorporate feedback loops to turn on and off the signaling cascades. By having a positive feedback loop, the output of a process stimulates its own production, and for a negative feedback loop the output inhibits its own production (113). One example is the p53-MDM2 autoregulatory feedback loop (53). A positive regulation is when p53 as a transcriptional factor target *MDM-2* and the *MDM-2* expression increases. MDM2 can inhibit p53 through binding to its transactivating domain.

Another regulation of signaling cascades is cross-talking and the most common is the one between the substrates for different kinases in several pathways. The substrates can also be shared between different pathways and interact with other signaling cascades. There are multiple cross-talk mechanisms from the PI3K pathway to other signaling pathways like the Ras-MAPK pathway. PI3K pathway can also modulate Raf which is a Ras target. AKT phosphorylates C-Raf and B-Raf and this inhibition reduces the activity of the MAPK pathway. It is important for proper regulation of differentiation in some tissues and for cell survival (55).

Cancer cells can have oncogenic pathway redundancy. Multiple pathways are able to assist tumor growth and development. For example PI3K/AKT/mTOR and MEK/ERK signaling pathways cooperate in many tumors to drive growth and survival of the tumor (114). Due to activation of alternative cascades, feedback loops and cross-talks in the signaling network a single targeted therapy approach can fail. Use of drug combinations can inhibit the whole pathway and prevent feedback or cross-talks mechanism (55). Combination therapy was found to be more efficient in the inhibition of tumors in mice in a study done by Junttila *et al.* (46). By targeting two independent signaling pathways with two or more drugs there is smaller chance to develop resistance against the treatment (67). The small molecule kinase inhibitors are binding the kinase as a small ligand or interfering with the enzymatic activity of the protein (67). PI3K signaling pathway is the most frequently targeted pathway (51). Boyault *et al.* showed that the majority of the mutations in breast cancer tumors occurred in the *TP53* and *PI3K* pathway. Targeted therapies of these two pathways can provide benefit for a substantial proportion of breast cancer patients (115).

The mutation frequency we found in the IAC database for the Tp53 H179R was 0,63%. This might be too low to do further *in vivo* experiments from mice to human clinical trials. It could be a possibility to use the Ba/F3 model system to test for life-pro-longing drugs for patients with metastasis where no other treatments work. First, deep sequencing can be done on the tumor sample from the patient to identify candidate mutations for functional testing. The mutant gene can be tested for oncogenic potential in the Ba/F3 assay. If the mutant gene is oncogenic we can do further drug testing in this system. The most effectively drug inhibiting the proliferation of the transfected Ba/F3 cells with the oncogenic mutant, can then be used to treat the patient. The Ba/F3 assay has many sub-procedures before it come to the transfection and the cell culturing part; therefore it is not a quick method. It will take too long time to do drug experiments of the oncogenic mutation in mice experiments for the metastatic patient group.

The two established breast cancer xenograft models are described as basal-like and luminal-like phenotype based on histopathological characteristics, ER status and profound diversity in gene expression pattern (107). Moestue and colleague found effect of long term treatment of PI3K inhibitors MK2206 or BEZ235 in the tumor-bearing mice which have the basal-like but not in the luminal-like phenotype (116). In our study we found the oncogenic mutations *PIK3CA* S405F and the non-oncogenic mutation *TP53* K120E in the tumor from the basal-like and the oncogenic mutation *TP53* H179R in the tumor from the luminal-like xenograft mice. The study from Moestue showed treatment response in the xenograft which has an oncogenic mutation in the *PIK3CA* gene. This strengthens our findings that the *PIK3CA* mutation was oncogenic in the BaF3 model system. Other treatment regimens can be used for testing in these two xenograft models. When the results from our study are confirmed, they can be used for further planning and result evaluations of the experiments on these two xenograft models.

Some issues were raised by Yi *et al.* about clinical use of genomic data for personalized cancer therapy. Both non-functional passenger mutations and detection of artifacts variants of no significance can be identified from the sequence results (105). If therapeutic decisions were taken from these analyses it might results in therapeutic failure if the functional importance of the mutants is not tested upfront. Another issue is that novel gene variants of unknown significance are being discovered in large-scale studies (117-119). Low frequency of non-hotspot mutations in *PIK3CA, KRAS, NRAS* and *ERBB2* have been shown to have

transforming activity in functional analyses (105). “Methods based on mutation frequency can only prioritize genes for further analysis but cannot unambiguously identify driver genes that are mutated at low frequencies” (67).

Breast cancer is heterogeneous and consists of many different diseases. It is unique to each woman in a complex biological system. A correct diagnosis and etiology-matched treatment is based on identification of driving genetic events within the context of the tumor cell of origin (51). Experimental model systems are therefore important in studying mechanisms of breast cancer and to develop better treatment strategies.

5. Conclusions

In this master thesis, we successfully established the Ba/F3 method in our laboratory. The parental Ba/F3 cell line is an IL-3 dependent murine pro lymphoid B-cell line. This cell line can be used as a model system to assess the oncogenic potential of cancer gene mutations, as well as their downstream signaling. Parental BaF3 cells are transfected with cancer gene mutations by electroporation, and oncogenic mutants will continue to grow without IL-3. Three mutations and controls were tested for oncogenic potential by using this method. The transfected Ba/F3 cell line with mutations *TP53* H179R, *PIK3CA*P110 S405F and *PIK3CA*P110 E545K were oncogenic, as evaluated microscopically. *TP53* K120E was non-oncogenic because no cell growth was observed in the absence of IL-3. The negative controls *TP53* wt, *PIK3CA* wt, *LacZ*, Mocks +/- Blasticidin and parental Ba/F3 cells +/- Blasticidin did not grow without IL-3. According to the cell viability tests, the cells were not viable after 1.5-3 months incubation, indicating that we were unsuccessful in creating stable cell lines. All microscopic observations have to be confirmed with a new cell viability assay.

6. Future aspects.

We successfully established the Ba/F3 model system in our laboratory. The microscopic evaluations of the two oncogenic mutations *TP53* H179R and *PIK3CA*P110 S405F and the non-oncogenic *TP53* K120E in this study have to be confirmed by a new cell viability assay. We would then be able to quantify the viable cells using a luminescence reader. This would be more accurate than visual microscopic evaluations. It is possible to create stable cell lines of the transfected Ba/F3 cells with oncogenic mutations and the controls. We would then be able to do further experiments on the mutants tested, such as drug experiments. By using the Ba/F3 model system, targeted therapy against the oncogenic mutations can be tested. Deep sequencing of patients' cancer genome is getting more common. The BaF3 system can be used for testing the oncogenic potential of mutated genes identified by sequencing. It is now easier to get access to next generation sequencing results from breast cancer tumor materials at our department. By using this functional method for example on new mutations found in several patients and also on tumor samples showing aggressiveness, we can assess if the mutations are oncogenic or not. The BaF3 method can be further developed for testing two different genes with mutations at the same time by using two different vectors with different selection markers. Most of the breast cancer patients have tumor genomes which are complex and deregulated. To test for mutations in multiple genes at the same time would mimic the *in vivo* situation. It is also possible to test up to three mutations for one gene at the same time by using this method.

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Appendix

Appendix A

Table A1. Reagents and equipments

Name	Producer	Supplier	Cat.nr.
Corning Petri dish	Corning Incorporated, NY USA	Fisher Scientific	430167
RPMI media + L-Glutamine	GIBCO®	Life Technologies™	21875-091
FBS	GIBCO® Invitrogen™, USA	Life Technologies™	26140
IL-3	GIBCO®, USA	Life Technologies™	PMC0034 and PMC0035
Blasticidin S HCl	Invitrogen	Life Technologies™	R210-01
DMSO	Thermo Scientific, USA	Fisher Scientific	20688
Waterbath, sub aqua 12 puls	Grant	VWR International AS	-
Saarstedt tube 15 ml	Saarstedt	Saarstedt	62.554.502
Saarstedt tube 50 ml	Saarstedt	Saarstedt	62.547.254
Cryo tube 2 ml	Greiner bio-one , Germany	BD Biosciences	126277
Centrifuge, Rotina 420, Hettich Zentrifugen	Andreas Hettich GmbH & Co.KG, Germany	DJB Labcare	-
Light Microscop, Axiovert 40 CFL	Zeiss	-	-
Ampicillin sodium salt	GIBCO®, USA	Life Technologies™	11593-027
TP53 wt Plasmid	Origene, USA	Bionordika	CW102028
MycoAlert® Mycoplasma Detection kit	Lonza, USA	Medprobe	LT07-218
Nuclon 96 microtiterplate	Nunc, Denmark	Thermo Scientific	136101
Bürker Chamber	Assistent	VWR	-
Trypan Blue stain 0,4%	Gibco	Invitrogen	15250-061
CTG	Promega , USA	Nerliens Meszansky	G7571/2/3
QIAprep® spin miniprep kit	Qiagen	Qiagen, Norway	27104
Nanodrop	Saween Werner	Saween Werner	-
Falcon tube	BD Biosciences	BD Biosciences	352059
Wallac 1450 Micro Beta TriLux luminescence Counter	Perkin Elmer, USA	-	-

Name	Producer	Supplier	Cat.nr.
MicroBeta Windows Workstation	Perkin Elmer, USA	-	-
DPBS, Dulbecco`s Phospate buffered Saline – $CaCl_{2,}$ - $MgCl_2$	GIBCO®, USA	Life Technologies™	14190-169
Distilled Water, DNase/RNase Free	GIBCO®, USA	Life Technologies™	10977-049
Applied Biosystems 3730 DNA analyzer	Applied Biosystems, USA	Life Technologies™	-
Sequencing Analysis software v5.2	Applied Biosystems, USA	Life Technologies™	-
BigDye®Direct Sequencing Kit	Applied Biosystems, USA	Life Technologies™	-
DNA Engine tetrad 2 Peltier Thermal Cycler)	Bio-Rad laboratories, USA	Bio-Rad	-
øx 174-Hae III digest	TaKaRa	Clontech	3405 A
GeneGenius Bio Imaging System	Syngene	VWR	-
BigDye®XTerminator™ Purifiaction Kit	Applied Biosystems	4376484	4276484
96-well plate septa	Applied Biosystems	Life Technologies™	4315933
3730 DNA analyzer	Applied Biosystems	Life Technologies™	-
POP-7™ Performance Optimized Polymer	Applied Biosystems	Life Technologies™	4363929
Sequencing analyzes software v5.2	Applied Biosystems	Life Technologies™	-
Sequencing primers	Eurogentec	Bionordica	-
Neon™ transfection System	Invitrogen	Life Technologies™	-
DNA120 SpeedVac® ThermoSavant	-	Dip.Ing.Houm	-
BD Falcon 250 ml Erlenmeyer Culture Flask	BD Biosciences, USA	BD Biosciences	355119
Glyserol, minimum 99%	SigmaAldrich	VWR	G5516
Eppendorf tube ,1,5 ml	Eppendorf, Germany	VWR	003 0123.328
100 µl Neon™ Kit	Invitrogen	Life Technologies™	-
6 well plate	Thermo Scientific, Denmark	Thermo Scientific, Denmark	140675

Name	**Producer**	**Supplier**	**Cat.nr.**
48 well plate	Thermo Scientific, Denmark	Thermo Scientific, Denmark	150687
Steril filter, 0,2 µm	Millipore, Ireland	Millipore	-
GelRed ™ nucleic Acid Gel Stain	Biotium, USA	-	41003-1

Appendix B

Table B1. Ba/F3 culture medium. Calculated volumes for different total amount of the medium.

500 ml:		**250 ml:**	
RPMI (13 X 35ml +20 ml)	475 ml	RPMI 6 x35 ml+27,5 ml)	237,5 ml
FBS (Final concentration 5%)	25 ml	FBS (Final concentration 5%)	12,5 ml
IL-3(100 ng/ml)	25 ul	IL-3 (100 ng/ml)	12,5 ul

125 ml:		**62,5 ml:**	
RPMI (3x35 ml +13,7 ml)	118,7 ml	RPMI (1x35 ml + 27,5 ml)	59,3 ml
FBS (Final concentration 5%)	6,25 ml	FBS (Final concentration 5%)	3,1 ml
IL-3 (100 ng/ml)	6,25 ul	IL-3 (100 ng/ml)	3,1 ul

31,2 ml	
RPMI	29.7 ml
FBS (Final concentration 5%)	1,6 ml
IL-3 (100 ng/ml)	1,6 ul

Appendix C

DNA sequencing on the ABI 3730 DNA Analyzer

Kits: BigDye® Direct Cycle Sequencing Kit and BigDye® XTerminator™ Purification Kit

Use: DNA sequencing

Sample: Genomic DNA (5 ng/µl)

Principle: Chapter 1 "Introduction to DNA Sequencing" in the DNA Sequencing by Capillary Electrophoresis, Applied Biosystems Chemistry Guide[1]

Chemical/reagent	Supplier	Ordering information	Lab location	User location
10x 3730 Buffer with EDTA	Applied Biosystems	4335613	K02-107 PCR lab I (fridge)	K02-078 Instrument lab
φX 174-Hae III digest	TaKaRa	3405 A	K02-107 PCR lab I (fridge)	K02-078 Instrument lab
Big Dye® Direct Cycle Sequencing Kit	Applied Biosystems	4458688	K02-088 DNA lab (freezer 4)	K02-088 DNA lab
BigDye® XTerminator™ Purification Kit	Applied Biosystems	4376484	K02-085 Cooling room	K02-088 DNA lab
Bromophenol blue	Bio-Rad Laboratories	161-0404	K02-097 Chemical room	K02-097 Chemical room
Certified™ Molecular Biology Agarose	Bio-Rad Laboratories	161-3102	K02-097 Chemical room	K02-097 Chemical room
DNase/RNase free H_2O	GIBCO	10977-35	K02-088 DNA lab	K02-088 DNA lab
EDTA disodium salt	BDH®	100935V	K02-097 Chemical room	K02-097 Chemical room
Ficoll® PM 400	Sigma-Aldrich® Norway AS	F4375	K02-097 Chemical room	K02-097 Chemical room
Glacial Acetic Acid	MERCK	100063	K02-097 Chemical room	K02-097 Chemical room

[1] The *Applied Biosystems Chemistry Guide* may be downloaded from http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041003.pdf

Chemical/reagent	Supplier	Ordering information	Lab location	User location
GelRed™ Nucleic Acid Gel Stain	Biotium	41003-1	K02-107 PCR lab I (fridge)	K02-107 PCR lab I
POP-7™ Performance Optimized Polymer	Applied Biosystems	4363929	K02-107 PCR lab I (fridge)	K02-078 Instrument lab
Trizma® base	Sigma-Aldrich® Norway AS	T1503	K02-097 Chemical room	K02-097 Chemical room
Laboratory equipment				
96-well plate septa	Applied Biosystems	4315933	K02-107 PCR lab I	
Bijou bottle 129B, 7ml, Sterilin	Heger	10036	K02-088 DNA-lab	
Disposable Scalpels	Swann-Morton	0503	K02-088 DNA lab	
Domed Cap Strip	Thermo Scientific	AB-0602	K02-088 DNA lab	
Eppendorf PCR Clean Safe-Lock Tubes 1,5 ml	Eppendorf	0030 123.328	K02-088 DNA lab	
Gloves (nitrile)	OUS storageroom	RH20203 (Small)/ RH20201(Medium)	K02-088 DNA lab	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Applied Biosystems	4306737	K02-088 DNA lab	
Reagent Reservoir 50 ml	Costar	4870	K02-088 DNA lab	
Therma-Fast®96, Non-Skirted	Thermo Scientific	AB-0600/G	K02-088 DNA lab	
Instruments				
3730 DNA Analyzer	Applied Biosystems	-	K02-078 Instrument lab	
DNA Engine Tetrad 2 Peltier Thermal Cycler	Bio-Rad Laboratories	-	K02-107 PCR lab I	
Eppendorf Centrifuge 5804	Eppendorf	-	K02-107 PCR lab I	
Eppendorf Centrifuge 5810R	Eppendorf	-	K02-088 DNA lab	

Instruments **(continue)**			
Eppendorf Mini Spin®	Eppendorf	-	K02-088 DNA lab
GeneGenius Bio Imaging System	Syngene	-	K02-107 PCR lab I
Illumina High-Speed Microplate Shaker	Illumina	-	K02-078 Instrument lab
Powerpac 300 Electrophoresis Power Supply	Bio-Rad Laboratories	-	K02-107 PCR lab I
SeqScape v.2.7	Applied Biosystems	-	Personal computer
Sub-Cell® Model 192	Bio-Rad Laboratories	-	K02-107 PCR lab I

Safety precautions

Chemical/reagent	Laboratory classification request	User location	R-phrases	S/P-phrases	Classification according to danger
10x 3730 Buffer with EDTA	SN1	K02-078 Instrument lab			**1**
Agarose CAS: 9012-36-6	SN1	K02-097 Chemical room			**1**
BigDye® Direct M13 Forward primer	SN1	K02-088 DNA lab			**1**
BigDye® Direct M13 Reverse primer	SN1	K02-088 DNA lab			**1**
BigDye® Direct PCR Master Mix **<u>Hazardous substances:</u>** Hydroxylated Organoamine (1-10%) CAS: Proprietary Glycerol (10-30%) CAS: 56-81-5	SN1	K02-088 DNA lab	Xi; R36/37/38 Xi; R36/37/38	S24/26/37/60 S24/26/37/60	**2** Wear personal protective equipment
BigDye® Direct Sequencing Master Mix **<u>Hazardous substances:</u>** Hydroxylated Organoamine (1-10%) CAS: Proprietary Glycerol (10-30%) CAS: 56-81-5	SN1	K02-088 DNA lab	Xi; R36/37/38 Xi; R36/37/38	S24/26/37/60 S24/26/37/60	**2** Wear personal protective equipment
Control DNA CEPH	SN1	K02-088 DNA lab			**1**

Chemical/reagent	Laboratory classification request	User location	R-phrases	S/P-phrases	Classification according to danger
DNase/RNase free H_2O	SN1	K02-088 DNA lab			**1** Wear gloves!
Gel Loading buffer (0,1% bromophenol blue)	SN1	K02-107 PCR lab I			**1**
GelRed™ Nucleic Acid Stain, 10,000 x in DMSO **Hazardous substances:** DMSO CAS: 67-68-5	SN1	K02-107 PCR lab I	Xi; R36/37/38	S23/26/36	**2** Handle with care. The substance has not been fully tested.
SAM™ Solution **Hazardous substances:** Trade Secret (1-10%) CAS: Proprietary Trade Secret (10-30%) CAS: Proprietary	SN1	K02-088 DNA lab	Xi; R36/37/38 Xi; R36/38	S24/26/37/60 S24/26/37/60	**2** Wear personal protective equipment
TAE buffer (50x) **Hazardous substances:** Trizma® base CAS: 77-86-1 Glacial Acetic Acid CAS: 64-19-7 EDTA dinatriumsalt dihydrat CAS: 6381-92-6	SN2	K02-107 PCR lab I	Xi; R36/38/43/52/53	S24/26/37/61 + P338	**2**
TAE buffer (1x)	SN1	K02-107 PCR lab I			**1**

Chemical/reagent	Laboratory classification request	User location	R-phrases	S/P-phrases	Classification according to danger
XTerminator™ Solution **Hazardous substances:** Trade Secret (10-30%) CAS: Proprietary Trade Secret (10-30%) CAS: Proprietary	SN1	K02-088 DNA lab	Xi; R36 Xi; R36/37/38	S24/26/37/60 S24/26/37/60	**2** Wear personal protective equipment

Xi = irritant. **R10:** Flammable. **R35:** Causes severe burns. **R36:** Irritating to eyes. **R37:** Irritating respiratory system. **R38:** Irritating to skin. **R43:** May cause sensitization by skin contact. **R52:** Harmful to aquatic organisms. **R53:** May cause long-term adverse effects in the aquatic environment. **S23:** Do not breathe gas/fumes/vapor/spray. **S24:** Avoid contact with skin. **S26:** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. **S36:** Wear suitable protective clothing. **S37:** Wear suitable gloves. **S45:** In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). **R51:** Toxic to aquatic organisms. **R52:** Harmful to aquatic organisms. **S60:** This material and its container must be disposed of as hazardous waste. **S61:** Avoid release to the environment. Refer to special instructions/safety data sheet. ***P338:*** Remove contact lenses if present and easy to do and continue rinsing.

Disposal

Chemical/reagent	Disposal number
10x 3730 Buffer with EDTA diluted 1:10	Not dangerous waste
Agarose gel (1,5%)	Yellow risk container
BigDye® Direct Cycle Sequencing Kit	These materials and its containers must be disposed of as hazardous waste. Yellow risk container.
BigDye® XTerminator™ Purification Kit	These materials and its containers must be disposed of as hazardous waste. Yellow risk container.
DNase/RNase free water	Not dangerous waste
TAE buffer (50x)	**7152** Organic disposal without halogens
TAE buffer (1x)	Not dangerous waste

Protocol: BigDye® Direct Cycle Sequencing Kit[2]

Preparations and considerations

- Remember that M13 PCR primers are a requirement using this method
- Make sure that the Tetrad 2 Thermal Cycler (Bio-Rad) is available for use
- For optimal performance, do not freeze and thaw the BigDye® Direct Sequencing Master Mix more than five times
- Make sure that the level of POP-7™ polymer is sufficient for your sample analyses
- **PROTECT** the capillaries from **drying** (if they dry, they die)

STEP 1: PCR amplification

1.1) For each reaction, mix the components as displayed in table 1 in a MicroAmp™ Optical 96-Well Reaction Plate with Barcode. Use 1,0 µl DNase/RNase free water instead of genomic DNA to include negative controls.

Table 1: *Component concentrations and volumes required to PCR amplify one sample*

Components	Volume
Genomic DNA (5 ng/µl)	1,0 µl
M13-tailed Fwd PCR primer (0,8 µM)	0,75µl
M13-tailed Rev PCR primer (0,8 µM)	0,75 µl
BigDye® Direct PCR Master Mix	5,0 µl
DNase/RNase free water	2,5 µl
Total volume for each reaction	**10,0 µl**

1.2) Mix the components well by pipetting up and down, seal the plate with caps (Domed Cap Strip, Thermo Scientific) and centrifuge the reaction plate briefly

1.3) Run the PCR amplification in a thermal cycler as displayed in table 2

[2] The *BigDye® Direct Cycle Sequencing Kit protocol* may be downloaded from http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_091370.pdf

Table 2: *Time and temperature conditions during PCR amplification*

Stage	Tetrad 2 Thermal Cycler (Bio-Rad)	
	Temp	**Time**
Hold	96°C	5 min
Cycle (35 cycles)	94°C	30 sec
	63°C*	45 sec
	68°C	45 sec
Hold	72°C	2 min
Hold	4°C	∞

* Annealing temperatures depend on the PCR primer T_m values

STEP 2: Agarose gel electrophoresis (optional)

2.1) Prepare an 1,5% agarose gel (table 8)

2.2) Mix 2,0 µl PCR-product and 2,0 µl gel loading buffer and load the entire volume into a gel well

2.3) Load 2,0 µl DNA ladder into the necessary number of gel wells

2.4) Electrophoresis: 200 V for 30 min

2.5) Use the GeneGenius Bio Imaging System for visualization

Stopping point 1: PCR products can be stored at 4°C over night or at –15°C or –25°C for long-term storage.

STEP 3: Cycle sequencing

3.1) For each reaction, mix the components as displayed in table 3. Mix well and centrifuge the tubes briefly. Keep the premix on ice and in the dark using aluminum foil to avoid fluorescence bleaching.

Table 3: *Sequencing reaction mix components and volumes required to cycle sequence one sample*

Components	Volume for each reaction
BigDye® Direct Sequencing Master Mix	2,0 µl
Sequencing primer: • BigDye® Direct M13 Fwd Primer **or** • BigDye® Direct M13 Rev Primer	1,0 µl
Total volume for each reaction	**3,0 µl**

3.2) Add 3,0 µl sequencing reaction mix to each sample

3.3) Seal the plate with caps and centrifuge the plate briefly

3.4) Run the sequencing reactions in a thermal cycler as displayed in table 4

Table 4: *Time and temperature conditions during cycle sequencing*

Stage	Tetrad 2 Thermal Cycler (Bio-Rad)	
	Temp	**Time**
Hold	37°C	15 min
Hold	80°C	2 min
Hold	96°C	1 min
Cycle (25 cycles)	96°C	10 sec
	50°C	5 sec
	60°C	4 min
Hold	4°C	∞

Stopping point 2: The reaction plate can be stored at 4°C over night, or at –15°C or –25°C for long-term storage.

STEP 4: Purify the sequencing products

Remember to keep the reaction plates in the dark to avoid fluorescence bleaching

4.1) Centrifuge the reaction plate at 100 x G for 1 min

4.2) Premix the SAM™ Solution and XTerminator™ Solution as displayed in table 5

Before using the BigDye® XTerminator™ Purification Kit[3]:

- Make sure there are no particles in the SAM™ Solution. Heat the solution to 37°C and resuspend the solution if particles are present.
- Homogenize the XTerminator™ Solution using a high-speed vortexer for 10 sec. Avoid pipetting from the top of the liquid due to rapid sedimentation
- Use a sterile scalpel and cut the pipette tips to create wide-bore tips (orifice > 1,0 mm) to aspirate the XTerminator™ Solution
- Calculate to use 15% more of the SAM™ and XTerminator™ Solution than needed by the number of samples to be purified through dead volume. The ratio of SAM™ and XTerminator™ Solution should be 4,5:1 (v/v).

[3] The *BigDye® XTerminator™ Purification Kit protocol* may be downloaded from http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042772.pdf

Table 5: *BigDye® XTerminator™ Purification Kit components and volumes to purify 96 samples*

Components	Volume for each well	Volume for 96 wells
SAM™ Solution	45 µl	4968 µl
XTerminator™ Solution	10 µl	1104 µl
Total volume	**55 µl**	**6072 µl**

4.3) Add 55 µl of the SAM™ and XTerminator™ Solution premix to each sample.
Mix the solution in between to prevent bead sedimentation. Cap seal the plate.

4.4) Vortex the reaction plate for 30 min at 2000 rpm

4.5) Centrifuge the reaction plate at 1000 x G for 2 min

Stopping point 3: Sealed reaction plates can be stored up to 48 hours in room temperature or up to 10 days at 4°C or –20°C before proceeding with capillary electrophoresis.

Short protocol: BigDye® Direct Cycle Sequencing Kit

Plate ID Forward:

Plate ID Reverse:

Fragment sequenced:

PCR mix

Component	Volume pr reaction (µL)	Volume for 96 wells (µL)
Genomic DNA (5 ng/µl)	1.0	
M13 Fwd PCR Primer (0,8 µM)	0.75	78.0
M13 Rev PCR Primer (0,8 µM)	0.75	78.0
BigDye® Direct PCR Master Mix	5.0	520
DNase/RNase free water	2.5	260
Total volume pr reaction	10.0	936 µL/104 = 9 µL

Sequencing mix		**FORWARD**	**REVERSE**
Component	**Volume (µL)**	**Volume for 96 wells (µL)**	**Volume for 96 wells (µL)**
BigDye® Direct Sequencing Master Mix	2.0	216	216
Sequencing primer: • BigDye® Direct M13 Fwd Primer **or** • BigDye® Direct M13 Rev Primer	 1.0 1.0	108	108
Total volume pr reaction	**3.0**	324 µl/108 = 3 µl	324 µl/108 = 3 µl

BigDye® XTerminator™ Purification Kit

Component	**Volume (µL)**	**Volume for 96 wells (µL)**
SAM™ Solution	45	4968
XTerminator®Solution	10	1104
Total volume pr reaction	**55**	6072

Agarose gel (1,5%)

Component	**Amount**
Agarose (BioRad, Cat.nr: 161-3102)	5,25 g
TAE buffer (1x)	to 350 ml
GelRed Nucleotid Acid Stain (Biotinum, Cat.nr: 41003-1)	35,0 µl

Additional reagents

50x TAE buffer

Chemicals

Trizma® base, Sigma-Aldrich® Norway AS (Prod. No. T1503)

EDTA disodium salt, BDH® (Prod. 100935V)

Glacial Acetic Acid, MERCK (Cat. No. 100063)

Procedure

Table 6: *Component volumes and amounts to make 1 litre of TAE buffer (50x)*

Components	**Amount**	**R/H and S/P sentences**
Trizma® base	242 g	H315/319/335, P261/305+351+338
EDTA disodium salt	100 ml 0,5 M EDTA (pH = 8,0)	R36/52/53, S61
Glacial Acetic Acid	57,1 ml	H226/314, P280/305+351+338/310
MQ-water	up to 1000 ml	-

1) Scale in 242 g Trizma® base

2) Add 500 ml MQ-water

3) Add 100 ml 0,5 M EDTA (pH = 8,0) and 57,1 ml Glacial Acetic Acid

4) Add MQ-water to adjust to 1000 ml

Store the buffer in room temperature

1x TAE buffer

Procedure

Table 7: *Component volumes to make 1 litre of TAE buffer (1x)*

Components	**Amount**	**R/H and S/P sentences**
TAE buffer (50x)	100 ml	Xi; R36/38/43/52/53, S24/26/37/61 + P338
MQ-water	up to 1000 ml	-

Store the buffer in room temperature

Agarose gel (1,5% agarose)

Chemicals

Agarose, Bio-Rad Laboratories (Cat. No. 161-3102)

GelRed™ Nucleotid Acid Stain, Biotium (Cat. No. 41003-1)

1x TAE buffer

Procedure

Table 8: *Component volumes and amounts to make an 1,5% agarose gel*

Components	Amount	R/H and S/P sentences
Agarose	5,25 g	-
TAE buffer (1x)	350 ml	-
GelRed™ NucleicAcid Stain	35,0 µl	Xi; R36/37/38, S23/26/36

1) Put together the gel chamber and place the gel well combs

2) Mix 5,25 g Agarose and 350 ml 1x TAE buffer in an Erlenmeyer flask

3) Heat to boiling in a microwave

4) Swirl the flask gently until all the agarose is completely dissolved

5) Cool the gel solution to about 65°C

6) Add 35,0 µl GelRed™ Nucleotid Acid Stain (Biotium)

7) Pour the gel in the gel chamber and let it harden for about 30 min

8) Remove the combs and clean the combs and chamber using tap water

Store the gel by 4°C. Wrap the gel in plastic foil to avoid drying.

Gel loading buffer (0,1% Bromophenol blue)

Chemicals

Bromophenol blue, Bio-Rad Laboratories (Cat. No. 161-0404)

Ficoll® PM 400, Sigma-Aldrich® Norway AS (Prod. No. F4375)

1x TAE buffer

Procedure

Table 9: *Component volumes and amounts to make 0,1% Bromophenol blue gel loading buffer*

Components	Amount	R/H and S/P sentences
Bromophenol blue	0,025 g	S22/24/25
Ficoll® PM 400	5,0 g	-
1x TAE buffer	25 ml	-

1) Scale in 0,025 g Bromophenol blue and 5,0 g Ficoll in a 50 ml tube

2) Add 25 ml 1x TAE buffer

3) Ficoll needs time to dissolve properly. Vortex the tube for about 24 hours to get a homogenous solution

Keep the buffer in room temperature for short-term storage, and by 4°C for long-term storage

1x Sequencing buffer

Chemicals

10x 3730 Buffer with EDTA, Applied Biosystems (Part. No. 4335613)

Procedure

Table 10: *Component volumes to make 1 litre of Sequencing buffer (1x)*

Components	**Amount**	**R/H and S/P sentences**
10x 3730 Buffer with EDTA	100 ml	-
MQ-water	up to 1000 ml	-

Store the buffer at 4°C

Xi = irritant. **R36:** Irritating to eyes. **R37:** Irritating respiratory system. **R38:** Irritating to skin. **R43:** May cause sensitization by skin contact. **R52:** Harmful to aquatic organisms. **R53:** May cause long-term adverse effects in the aquatic environment. **H226:** Flammable liquid and vapor. **H314:** Causes severe skin burns and eye damage. **H315:** Causes skin irritation. **H319:** Causes serious eye irritation. **H335:** May cause respiratory irritation.

S22: Do not breathe dust. **S23:** Do not breathe gas/fumes/vapor/spray. **S24:** Avoid contact with skin. **S25:** Avoid contact with eyes. **S26:** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. **S36:** Wear suitable protective clothing. **S37:** Wear suitable gloves. **S61:** Avoid release to the environment. Refer to special instructions/safety data sheet. **P261:** Avoid breathing dust/fume/gas/mist/vapor/spray. **P280:** Wear protective gloves/protective clothing/eye protection/face protection. **P310:** Immediately call a POISON CENTER or doctor/physician. **P338:** Remove contact lenses if present and easy to do and continue rinsing. **P305+351+338:** IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do and continue rinsing.

Appendix D

Principle of the Neon Transfection system (Invitrogen, USA)

The Neon^{TM} tips contain a gold-plated electrode and works as an electroporation chamber by delivering a high electrical field to a biological sample. The advantage of this equipment compared to standard cuvette based electroporation, is that the gap size between the electrodes becomes larger, while the surface area of each electrode becomes smaller. The consequences are uniform electrical field, less ion formation, minimal PH changes and negligible heat changes. Different types of samples such as nucleic acids, proteins and siRNA can be transfected into all mammalian cell types. Together with this system Neon^{TM} Kits (2 formats: 10 µl or 100 µl sample (Invitrogen) with reagents and pipette tips are used for the transfections. The cell counts pr. reaction are between $1x10^4$ -$5x10^6$ and the sample volume varies between 10 µl -100 µl. The system can be combined with various culture plate formats (60mm, 6-well, 48-well and 24 well).

Appendix E

Table E1. Observations and numbers of bacterial colonies containing sample- and control plasmids after the first performed mutagenesis on the LB agar plates.

Plasmid	Description	+: With X-Gal and IPTG -: Without X-Gal and IPTG	Results
pWhitescript	Mutagenesis control	+	Negative (only one colony)
PIK3CA P110 S405F	Sample	+	Fewer number of white colonies
PIK3CA P110 S405F	Sample	-	Many white colonies
PUC18	Transformation control	+	Average of total number of colonies: 38. Average of number of blue colonies: 17.

Table E2. Observations and numbers of bacterial colonies containing sample- and control plasmids after the second performed mutagenesis of the LB agar plates.

Plasmid	Description	+: With X-Gal and IPTG -: Without X-Gal and IPTG	Results
pWhitescript	Mutagenesis control	+	Many blue colonies
TP53 A120G	Sample	+	White, separate colonies, fewer than the LB agar plate without X-gal and IPTG
TP53 A120G	Sample	-	White, separate colonies
TP53 A179G	Sample	+	White, separate colonies, fewer than the LB agar plate without X-gal and IPTG
TP53 A179G	Sample	-	White, separate colonies
TP53 WT	WT control	-	Many white colonies
PIK3CA P110 WT (plenti 6.3)	WT control	-	Many white colonies
PIK3CA P110 E545K (Plenti 6.3)	Mutant control	-	Many white colonies
LacZ (Plenti 6.3)	Normal control	-	Many white colonies
PUC18	Transformation control	+	-Total number of colonies: ~86 -Number of blue colonies: ~24

On both mutagenesis the X-gal concentration was 0,005 % instead of 2% which is the protocol recommends. This is a possible reason why it was < 90% blue colonies on the PUC18 transformation control.