Title of thesis

Mechanism of metastasis of triple negative breast cancer

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Abstract

Triple Negative Breast Cancer (TNBC) is increasingly recognised as a serious, worldwide public health concern. TNBC has the ability to recur after treatment and appears to be greatest in the first few years. The phenotypical changes of the TNBC metastasis represent a unique that heterogeneous tumour cell population with special biological features that permit travel to distant sites and the establishment of a clinically disseminated disease. Triple-negative tumours do not express the oestrogen-ERα receptor, progesterone PgR receptor and the epidermal growth factor receptor (Her2). However, the three biomarkers are used clinically to guide treatment. The main aim of this research is to elucidate recently identified molecular pathways that contribute to TNBC metastasis. It also provides a useful approach to understand the heterogeneity of TNBC at its different stages. Several protein candidates show differential expression between metastatic and non-metastatic tumours. This research had hypothesised that overexpression by the group of proteins increases the metastatic propensity of TNBC. In this research, I studied selected protein candidates extracted from human breast cancer tumour tissue and breast cancer cell lines (MDA-MB-468 and MCF-7) using quantitative detection by in-gel digestion mass spectrometry and proteomics, western blotting (WB), immunocytochemistry (ICC), CRISPR/Cas9 and Wound Healing. Here, I used the CRISPR/Cas9 techniques to induce EGFR and STAT1 overexpression and then performed wound-healing experiments, which revealed that STAT1 overexpression from its genomic locus significantly, decreased the potential of the cells to close the wound. Also, the present study demonstrates the overexpression of

Abstract

EGFR in 26 tumour samples of TNBC patients with a p-value < 0.05. The data obtained provides valuable information in order to understand the mechanisms of metastasis.

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Abbreviations

ABI - Aminothiazole-based Inhibitor
APS - Ammonium Persulfate
BAD- Bcl-2-associated death promoter
BL1- Basal-Like 1
BL2- Basal-Like 2
BLBC- Basal Like breast cancer
BMI- Body Mass Index
Breast Cancer - breast cancer
BSA- Bovine Serum Albumin
CAN- acetonitrile
CRISPR- clustered regularly interspaced short palindromic repeat
DAB - 3, 3- diaminobenzidine
DCIS - ductal carcinoma in situ
DFS - Disease Free Survival
DTT- Dithiothreitol
EGFR - Epidermal Growth Factor Receptor
ERK family- Extracellular-signal-Regulated Kinases
ERα - Estrogen Receptor alpha
ES - European Society
GE- Gene Expression
GOF- Gain-of-function
GRB2- Growth factor receptor-bound protein2
gRNA- guide Ribonucleic acid
Her2 - Human Epidermal Growth Factor Receptor 2
HRT- Hormone Replacement therapy
IM - Immunomodulatory
IRS1- Insulin receptor substrate 1
LAR- Luminal Androgen Receptor
LOF- Loss-of-function
LREC-Local Research Ethics Committee
M- Mesenchymal
MAPK- Mitogen-activated protein kinase
MBC- Metastasis breast cancer
MDM2- Murine double minute 2
MMPS- Matrix Metalloproteinase
MSL- Mesenchymal Stem-Like
mTOR - mammalian Target Of Rapamycin
NHS- National Health Service
OS - Overall Survival
PAGE- Polyacrylamide Gel Electrophoresis
PCNA- Proliferating Cell Nuclear Antigen

PDK1- 3-phosphoinositide-dependent protein kinase 1
PgR - Progesterone Receptor
PI3K- Phosphoinositide-3 kinase
PIP2- Phosphatidylinositol bisphosphate 2
PIP3- Phosphatidylinositol triphosphate 3
PTEN- Phosphatase and tensin homologue deleted on chromosome ten
PVDF- Polyvinylidene fluoride
RAPTOR- Regulatory associated protein of TOR
SDS - Sodium dodecyl sulfate
sgRNA- Single Guide Ribonucleic Acid
Src - Sarcome genes
STAT1- Signal Transducer and Activator of Transcription 1
TBS- Tris buffer saline
TEMED- N, N, N, N-tetramethylethylenediamine
TNBC - Triple Negative breast cancer
TRAIL - tumour necrosis factor-related apoptosis ligand
uPA - rokinase-type Plasminogen Activator
V- Voltage
VEGF - Vascular Endothelial Growth Factor
vPA - vampire bat Plasminogen Activator.

Chapter 1 Introduction of Breast Cancer and Triple Negative Breast Cancer

1.1 Structure and Function of the Normal Breast Organ

An understanding of the anatomy of the breast is essential in order to enable an assessment of any abnormality of the structure in breast. Although, the breasts have been studied since early anatomy research, our knowledge about breast structure is not complete. Recent studies focusing on the anatomy of the breast have discovered that the duct system is comprised of a smaller number of main ducts. This recent information add new knowledge to previous assumptions (Moore et al. 2013, Johnson and Cutler 2016) . In the past, the mammary gland was classified as a branch of tubuloalveolar structure with hormone responsive lobules surrounded by a loose connective tissue stroma. However, recent research has shown that the glands making up the breast are in fact made up of adipose tissue separated by bands of connective tissue.

In the literature on the fundamental knowledge of the anatomy of the breast (Cooper 1840), the milk ducts (thickened ectodermal ridge) of the breast are closely intertwined, small, compressible and superficial. This clearly explains why they do not display typical dilated “sinuses” and do not typically store large amounts of milk (Geddes 2007) . Additionally, the amount of adipose tissue between the glandular tissues in the breast is highly variable during lactation (Figure 1-1).

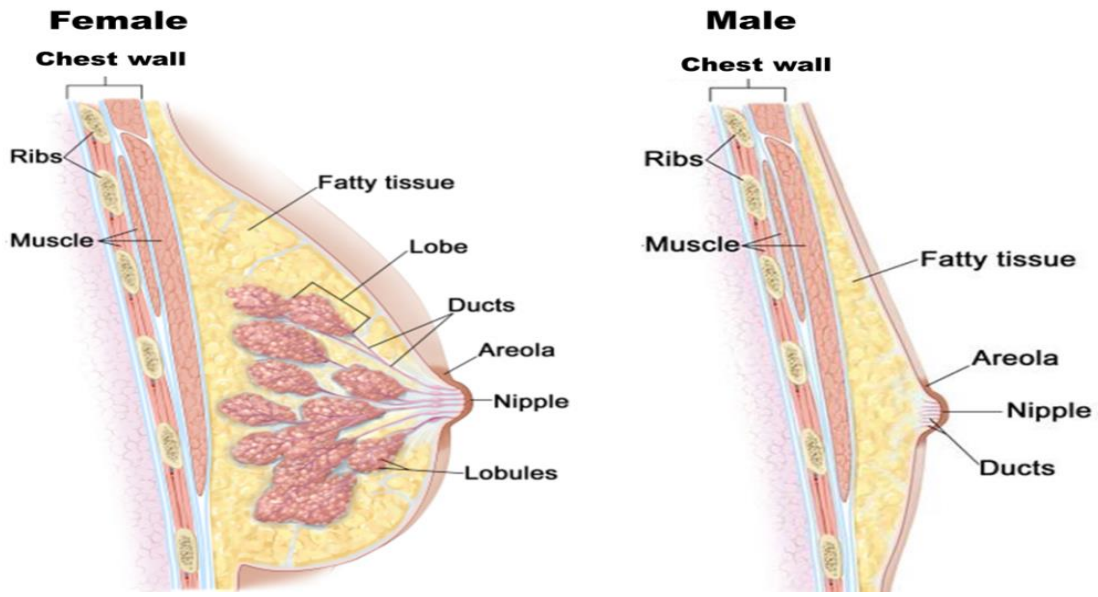


Figure 1-1 Anatomy of the male and female normal breast tissue.

The fatty tissue, nipple, ducts, lobe and lobules are shown on the inside of the breast (Guilford et al. 2017).

After lactation, the breast regresses to a differentiated state through the process of involution, which occurs following the cycle of pregnancy, parturition, and lactation (Johnson and Cutler 2016). With the reduction of oestrogen and progesterone in menopause, the breast involutes reverting to a near prepubertal structure. Many factors control these complex developmental processes, such as a combination of hormonal stimulation, growth factors and other physical elements constituting the microenvironment of the mammary gland (Johnson and Cutler 2016).

Knowledge of the mechanical properties of the breast tissue is essential for early cancer detection, for example, the young modulus of breast tissue is highly dependent on the tissue preload compression level. Recent research clearly indicates that a wide variation in

moduli exists, not only between different types of tissue, but also within each type of tissue; these differentiations were most evident in normal fat and fibrous glandular tissue (Ramião et al. 2016).

There is a large volume of published studies describing how the function of human tissues and cells depends on a programme of gene expression, which occurs in a highly regulated manner during development. This regulatory programme utilises signals, such as hormones, environmental insults and molecules from the microenvironment (Kehat et al. 2001). Numerous early studies have attempted to explain that the growth factors and hormones in the Extracellular Matrix (ECM), which surrounds tissue contains signalling molecules that are responsible for the maintenance of the tissue structure and its morphology. However, both mechanical and biochemical connections between the ECM and the nuclear skeleton are involved in the mechanisms leading to changes in chromatin structure and gene expression (Emerman et al. 1979).

1.2 Breast Cancer Molecular Biology Subtype

Human breast cancer is caused by a genetic alteration of somatic cells in the breast tissue leading to the transformation of breast ductal epithelial cells and to malignant growth (Guilford et al. 2017) (Figure 1-2).

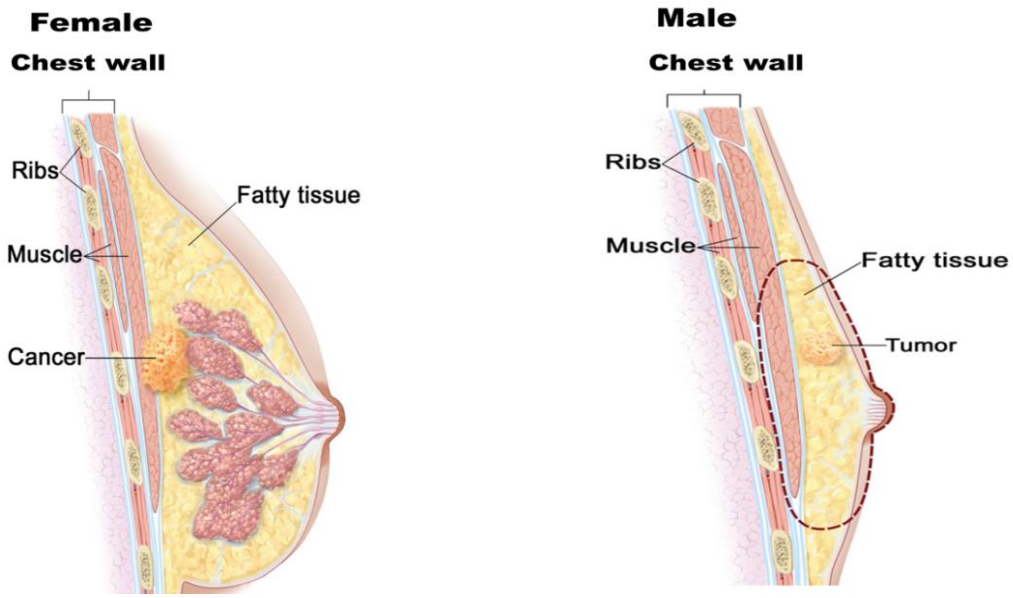


Figure 1-2 Anatomy of the female and male (right) tumour breast tissue

The fatty tissue, nipple, ducts, lobe and lobules are shown on the inside of the breast

These alterations in DNA may be either inherited or somatic, but the susceptibility to the disease is always inherited. The chromosome 17q21 is shown (Hall et al. 1990) to be the location of the gene that gives a predisposition by inherited susceptibility to breast cancer, especially in families with a history of early onset of the disease.

Genetic research has found that the region of chromosome 17q, which includes several plausible candidate genes that could carry the inherited susceptibility, such as a gene for a truncated form of the human epidermal growth factor receptor [her2; MIM 164870 (Mendelian inheritance in man)], which is identical to [erbb2 (MIM 190150)] (Hall et al. 1990). The second gene in chromosome 17q is Her2, this acts as an oncogene in NIH 3T3

cells and plays a role in the amplification process in many primary breast tumours, which is associated with poor prognosis, at least for node positive tumours (Slamon et al. 1989).

Genetic data analysis studies (Albertazzi et al. 1998) have showed other candidate genes in this region, such as nm23, whose expression is associated with lymph node metastasis in primary breast carcinomas (Wen et al. 1992). Family history of breast cancer is a significant risk factor in all populations. Epidemiological evidence has consistently found that the risk of breast cancer in women is increased by whether or not their mother had breast cancer, or whether other female members in their family have had breast cancer (Hall et al. 1990, Gluz et al. 2009).

Several molecular and gene expression analyses have documented the establishment of breast cancer subtypes and defined five distinct subtypes of morphologically similar breast cancer (luminal A and luminal B are estrogen receptor-positive tumours, while normal breast -like and HER2 over-expression are oestrogen- negative tumours (Prat et al. 2010)). There is a growing body of literature that recognises the importance of breast cancer subtypes and one of these five subtypes is the basal-like subtype and TNBC, which include basal-type characteristics, basal-epithelial phenotype, basal breast cancer and basaloid breast cancer (Foulkes et al. 2010).

These molecular subtypes of breast cancer are characterised by a gene expression profile that is similar to that of the basal myoepithelial layer of normal breast cancer. Additionally, express receptors are essential in breast tumour diagnosis, of which the most important

ones are Oestrogen Receptor (ERα), Progesterone Receptor (PR) and Human Epidermal growth factor Receptor2 (HER2). Based on the Immunohistochemical (IHC) analyses of the ERα, PR and HER2, breast cancer can be classified into different categories, such as Luminal A-like (ER-positive (ER+)) PR-positive (PR+), HER2-negative (HER2-), Luminal B-like (ER+ and PR+,HER2-positive (HER2+)), and triple negative breast cancer (Table 1-1).

Table 1-1 Breast cancer subtypes according to receptors expression.

Breast Cancer Subtype(s)	**Features**
Luminal A	**ER^+,PR^+ and $HER2^-$**
Luminal B	**ER^+,PR^+ and $HER2^+$**
HER2	**ER^-,PR^- and $HER2^+$**
TNBC	**ER^-,PR^- and $HER2^-$**
Normal breast cancer	**Expression genes characteristic of adipose tissue**

The most frequent subtype of breast cancer is Luminal A-like, the second most common subtype is TNBC (Ma et al. 2006). These biological subtypes are considered when a new treatment strategies are developed, alongside tumour grade, lymph node status, lymph vascular invasion, tumour size and patient ages (Dawson et al. 2013).

The decline in survival rate is generally the result of delay of and a poor diagnosis of breast cancer (Møller et al. 2016). This particularly impact women from minority communities, for example, in United State (US), Hispanic and black women with breast cancer showed a higher risk of death compared with non-Hispanic Caucasian women (Li et al. 2003).

Specifically, black women (black Caribbean and black African) with breast cancer had a later stage distribution and decreased survival rate than white patients (DeSantis et al. 2016).

The variations in survival rate of women who have had breast cancer are due to socioeconomic status, treatment, Body Mass Index (BMI) and comorbid conditions, which contribute partially to the excess mortality in black and Hispanic women with breast cancer (Warner et al. 2015).The incidence rate of breast cancer is increased slightly in African and African American women and decreased among Hispanic women while it is stable among non-Hispanic Caucasian women, Asian American /Pacific Islanders, and Native Americans (including, Alaska Native Americans from 2006 to 2010 (DeSantis et al. 2014).

In the United Kingdom (UK), breast cancer accounted for 15% of all reported cancer cases in 2015, with 370 cases of male breast cancer and 54,800 new cases of breast cancer in women in 2015 (Rabie et al. 2019). The death rate for breast cancer patients was 15% according to cancer statistics in 2015 (Maddams et al. 2009) (Figure 1-3).

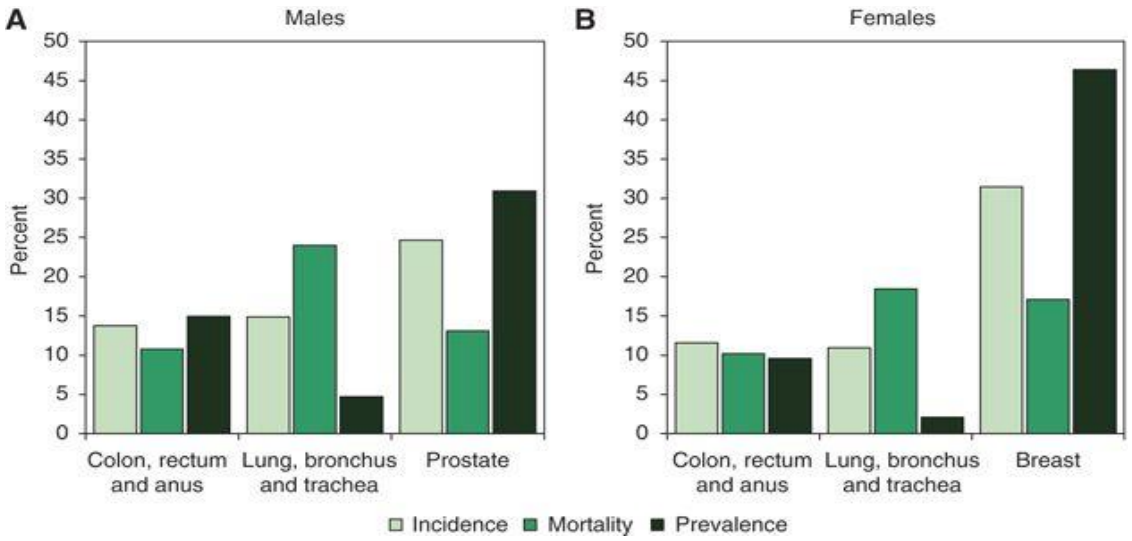


Figure 1-3 Cancer incidence, mortality and Prevalence in the UK for 2008 (Maddams et al. 2009).NB: breast cancer in males is rare and statistics had to be independently sought.

Mapping the genes responsible for causing breast cancer is critical to diagnose the early symptoms for the development of breast cancer in the general population. Excluding skin cancers, breast cancer is the most common cancer diagnosed in women in the US, approximately one in three women develop cancer and it is the second leading cause of cancer death among women after lung cancer (DeSantis et al. 2016).

DeSantis et al. (2014) found that the highest incidence rate of breast cancer had occurred in Caucasian women at age < 40; however, this rate is growing among Caucasian and African American women, particularly in women aged 50 to 59 years. In terms of the risk factors for breast cancer, data in relation to breast cancer cases in developed countries suggest that the most important factors in getting a diagnosis are those that influence exposure to oestrogen, including the reproductive and hormonal factors such as early age

menarche, having children at a later age, not having children and late menopause hormone replacement therapy.

Additionally, women in developed countries are at an increased risk of breast cancer compared with those women from less developed countries. The reason for this fact can be explained by the differences in lifestyle, such as having fewer children and a limited duration of breastfeeding (Stuebe 2009). Many factors that influence the risk of developing breast cancer are hormone replacement therapy, exposure to ionizing radiation, being overweight. In particular obesity in post-menopausal women has been connected to breast cancer recurrence. Poor survival in pre-and post–menopausal breast cancer cases increases the risk of postmenopausal breast cancer; however, it is one of the few modifiable risk factors for breast cancer (Porter et al. 2017, Porter et al. 2016).

The biological mechanism behind this association between breast cancer survival and obesity is not fully understood, but one possible explanation involves the relationship between mediators and adipocytokines on the one hand; and hormones and inflammatory cytokines on the other. This relationship influence cell survival or apoptosis, migrations and proliferation, for example, the higher level of insulin in obese women is linked to a poorer prognosis in breast cancer, as the BMI is linked to breast cancer mortality (Chan et al. 2014, Wiseman 2008).

However, molecular biology, pathology and clinical evidence show that breast cancer is heterogeneous (Tavassoli and Devilee 2003). While most epidemiologic research has

treated breast cancer as a single disease that is linked with a common set of risk factors. This heterogeneity is apparent at the molecular level and can be characterised by biomarkers.

The most widely studied and classified markers in breast cancer are the Estrogen receptor and the Progesterone receptor. Based on their expression breast cancer can be classified into either hormone receptor-positive or negative phenotypes. However, when compared hormone receptor–positive tumours exhibit a stronger clinical response to hormonal treatment and a better differentiated morphological appearance, and incidence rates increase with aging, rather than decreasing after menopause (Yasui and Potter 1999, Althuis et al. 2004).

1.3 Breast Cancer Screening and Risk Factors

Genomics studies have provided a molecular portrait of breast cancer. This molecular portrait has provided clear information about the relationship between phenotype diversity and gene expression pattern (Zhang et al. 2016, Perou et al. 2000). Prognosis is now determined for breast cancer patients using gene expression signatures and analytic approaches such as meta-analysis, functional enrichment analysis, transcriptional network analysis and next generation analysis. A number of breast cancer studies disagree and argue on the relative importance of risk factors and it is the subject to considerable debate.

As discussed previously, breast cancer was shown to have five distinct stable subtypes of morphologically similar breast cancer, e.g. Luminal A, Luminal B, Her2 over-expressing, TNBC (close to Basal-Like) and breast cancer. These subtypes may be associated with different traditional risk factors, for example, the incidence of luminal B in breast cancer may be increased in women who gain substantial weight after age eighteen (Tamimi et al. 2012).

Furthermore, menopausal status may also interact with the development of breast cancer for example when a women has more than five years of Hormone Replacement Therapy (HRT) there is a higher incidence rate of Her2 overexpressing cancer occurring. Luminal cancer is also more significant in obese or overweight women (Turkoz et al. 2013).

There is a growing body of literature on Quality Of Life (QOL) has received considerable attention, specifically in relation to lifestyle studies and in the breast cancer research in order to early diagnosis (Rahou et al. 2016). Additionally, the clinical trials have discovered that changes in QOL are linked to changes in clinical variables, including survival, which contribute to improved treatment (Montazeri et al. 2001, Wilsoff and Hjorth 1997).

Cancer cells originate from a malignant transformation of normal tissue progenitor and stem cells (Mimeault et al. 2007) , which differs from earlier beliefs that cancer cells rise from mature tissue cells; however the exact origin of cancer cell remains unclear (Wu et al. 2016, Sell 2004). Much of the current literature explains the correlation between tissue - specific cancer risk and the lifetime population size in tissue - specific stem cells during

cell-division. According to the role of the accumulative number of errors occurring through cell-division, the pathways of malignant transformation can be influenced by both intrinsic mechanism and extrinsic factors (Tomasetti and Vogelstein 2015).

Existing studies recognise the essential role acted by the first intrinsic mechanism to cancer development, which includes the mutation that results from random errors in the DNA replication process. The power of these factors can cause DNA damage after cell division through the accumulation of mutations (specifically driver mutations) and genetic alterations, which increases the risk of developing cancer. Therefore, cancer risk would originate from those uncontrollable intrinsic mechanisms and also from those highly modifiable extrinsic factors (Wu et al. 2016). Second, the route of extrinsic factors of different mutagenesis rates, such as environmental factors (ultraviolet UV radiation) and carcinogenesis (Tomasetti and Vogelstein 2015).

In terms of tumour microenvironment, a tumour has been defined as organs that have the ability to exceed normal healthy tissue. Thus, the biology of cancer can be understood through studying the cell type, specifically those within the tissue and the tumour microenvironment which develops construct during the tumorigenesis (Albini and Sporn 2007). Extensive studies during the last three decades has shown a large number of activity oncogenic mutations in tumour cells and inactivity mutations in tumour suppressor genes that are responsible for the acquisition of the malignant phenotype. In terms of metastasis, epithelial cells acquire unlimited replicative potential independent power by acquiring the mesenchymal, the fibroblast-like properties and decreased intracellular

adhesion and increased motility, which creates a cancer cell with invasive and metastatic properties (Larue and Bellacosa 2005).

Moreover, it has become clear that these mutations alone or in themselves are not powerful and/or sufficient to give a malignant phenotype this is only manifested with a permissive microenvironment, also known malignant teratocarcinoma cells (Dean et al. 2005). The breast cancer microenvironment is populated by many cells such as the adipocytes of the mammary fat, fibroblast, hematopoietic cells (leukocytes and lymphocytes) and blood vessels. It has become apparent that tumours require the establishment of a vascularise to progress above a certain size and to become malignant (Qian and Pollard 2010).

1.4 The Molecular Pathways Involved Breast Cancer

Questions have been raised about different genetic methodologies that have been used in the last 20 years to identify multiple susceptibility loci that vary in population frequency.

There is increasing concern that some breast cancer cases are associated with somatic mutations in breast cells, which are acquired throughout a women's lifetime. In hereditary breast cancer, specific genetic factors are involved in the inherited cancer risk and inherited mutations in the *BRCA1* or *BRCA2* genes are clearly described. However, mutations in *ATM, CDH1, CHEK2, PALB2, PTEN, STK11*, and *TP53* also confer breast cancer risk (Godet and Gilkes 2017).

Understanding the functional scenario of hereditary mutations has opened new paths for breast cancer prevention and is uncovering promising treatment strategies (Menashe et al. 2010, Miki et al. 1994, Nelen et al. 1996). Homeostat is a monitor of physiological parameters and functions in human body stability such as blood pressure, temperature, water-salt balance and acid-base balance, which help cells to continue to live and work normally and regularly in a suitable environment as required (Marieb and Hoehn 2007). The homeostat imbalance can influence fluid composition and environmental alteration activities; also, homeostatic mechanism converts such an imbalance and starts a new establishment to all physiological values in a specific range. For thus, the new homeostat develops inside the tumour due to the cancer's ability to adapt to the environment through evaluating and monitoring changes in disease, metabolic, neuroendocrine immune and physiological parameters (Banfalvi 2014).

There is a correlation between these parameters and tumour progression and they can act as prognostic disease members. Lactate, enzymatic activities and cholesterol are the typical parameters of new metabolic alterations (Nogueira and Hay 2013, Paesano et al. 2005). Numerous studies have attempted to explain the energy of cancer cells, it has been clearly seen that tumour cells rely mainly on glycolysis for energy production even in the presences of sufficient oxygen, which known Warburg effect to sustain a high proliferation rate and up-regulated in human cancer (Diaz-Ruiz et al. 2011). This is associated with aggressive tumour outcome by catalysing the inter- conversion of pyruvate and lactate, which is the main enzyme responsible for catalysis (Fiume et al. 2014). These genes are thought to contribute to breast cancer susceptibility through different cellular mechanisms.

Three pathways and one signalling cascade are highlighted in the following analysis which is based on genome-wide association studies of breast cancer.

1.4.1 The First Pathway (Syndecan-1 Signalling)

The first pathway identified through the genome-wide studies of breast cancer is syndecan-1 signalling which contains 13 genes involved in different cellular processes that are mediated by syndecan-1 (SDC1). This gene encodes a trans-membrane heparin sulphate proteoglycan that mediates a signal transduction cascade leading to cell proliferation, cell migration and cell adhesion following interaction with extracellular matrix proteins. The evidence for the potential role of syndecan-1 in the development of breast cancer has been linked with unfavorable breast cancer prognoses (Leivonen et al. 2004). Maeda et al. (2006) suggested that the expression of syndecan-1 by stromal fibroblasts can promote breast carcinoma growth in-vivo tumour angiogenesis.

1.4.2 The Second Pathway (c-Met)

The second pathway linked to breast cancer is c-Met signalling which consists of 33 genes participating in signal transduction mechanisms that are induced by the tyrosine kinase proto-oncogene c-Met (MET). Induction and stimulation of this pathway can lead to enhanced cell motility, proliferation, invasion and metastasis. The ligand hepatocyte growth factor and c-Met have been shown to be dysregulated and correlated with poor prognosis in a number of human malignancies (Christensen et al. 2005).

1.4.3 The Third Pathway (Hormone Signalling)

The third pathways is known as hormone pathway, Wagner et al. (2007) identified 22 genes involved in the cellular mechanisms induced by either growth hormone or insulin receptors in growth hormones signalling. These two receptors as well as the Insulin like Growth Factor (IGF) receptor are all trans-membrane tyrosine kinase receptors, which induce cell growth and proliferations. Changes in the activity of IGF receptor could lead to hyperplasia and the development of tumours.

1.4.4 Intrinsic Subtypes in Breast Cancer

One interesting finding is the canonical signalling cascade RAS/ RAF/ MAPK as the common denominator of pathways that associated with breast cancer risk (Menashe et al. 2010). It plays an essential role in transmitting extracellular signals from growth factors to promote growth, proliferation and differentiation. The activity of this pathway has been linked to multiple human malignancies (Downward 2003).

Another pathway activated by constitutive activation of Wnt signalling enhances the self-renewal of mammary progenitor cells. Continuous stimulation of this pathway leads to the formation of breast tumours (Jones and Kemp 2008). Upregulation of the Wnt/Ca2b pathway genes FZD7 and PRKCB1 raises the possibility that the non-canonical Wnt pathway might be involved in tumour formation in TNBC. Other findings argue are that the FZD7 only initiate Wnt/b-catenin signalling, whereas PRKCB1 upregulation in TNBC might be induced via other signalling pathways.

Yang et al. (2011) explained that canonical Wnt signalling regulates cell fate decisions throughout embryonic development and is involved in breast cancer. Activation of this pathway is transduced through the FZD family receptor, and the LRP5 Lipoprotein receptor related protein five co-receptor to initiate the β-catenin signalling cascade. Moreover, by Casein Kinase 1(CK1) and Glycogen Synthesis Kinase 3 (GSK3), sequential phosphorylation results in β-catenin ubiquitination and as mentioned in the literature review, the Notch Extracellular Domain (NECD) of the Notch receptor is pulled away from the Notch Intracellular Domain (NICD) and is endocytosed by the ligand bearing cell (Nichols et al. 2007).

Notch singling pathway was found in mammalian and could be explained by involving the interaction of Delta/Serrate/Log-2 (DSL) ligands, such as Delta like 1, -3,-4 and Jagged 1 and 2 with one of the four Notch receptors (Notch 1-4). Additionally, the abnormal regulation of the mammary epithelial tissue many further gives rise to different breast cancer molecular subtypes, this explains the heterogeneous nature of human breast cancer tumour (Skibinski and Kuperwasser 2015).

Maria (2016) found that the master regulators of embryonic development Cripto-1, Notch, CSL, and Wnt/β-Catenin act as key role in modulating mammary gland morphogenesis and cell fate in the embryo through fatal and adult mammary stem cells. Cripto-1 Notch/CSL and Wnt/β-Catenin signalling pathways are enhanced in breast cancer (Edeling et al. 2016).

Cripto-1 is revealed in a number of different types of human cancer, such as breast cancer. Measurement of the level of the soluble Cripto-1 in the plasma or serum of breast cancer patient could provide a potential clinical diagnostic and prognostic significance (Klauzinska et al. 2014). Additionally, Cripto-1 is involved in the reprogramming of differentiation of the breast cancer cells into tumour initiating cells by induction of an Epithelial-mesenchymal transition, for example, the Cripto-1 /GRP78/BIP complex promotes the tumour growth by involving the expression of GRP78/BIP on the surface of tumour cells, which binds to GPI-anchored proteins (Shani et al. 2008). Additionally, Cripto-1 has a strong indication to act as an angiogenic activity of Wnt/β-Catenin and Criptio-1 modulates Notch /CSL singlling pathways, which is involved in the aetiology of breast cancer. The reason for this is that Cripto-1 takes a role of a chaperone protein for the Notch receptor in the Golgi/Endoplasmic Reticulum. By enhancing the Notch receptors, Notch signalling is essential and critical for maintaining ER^+ /PR^+/ HER-2 breast tumours and also cell cycle regulation in ER^+ /PR^+/ HER-2 (Dai et al. 2015).

Additionally, Notch signalling is present in 50% of breast cancers. It is implicated in regulating tumour stroma interactions, metastasis and chemotherapeutic resistance in breast cancer , which directly induces the Vascular Endothelial Growth Factor Receptor 3 (VEGFR-3) in blood vessel endothelial cells and regulated vascular development and subsequently lymphomagenesis (Shawber et al. 2007).

A growing of body literature argues that Canonical Wnt/β-Catenin signalling can be activated in different types of cancer through mutations in the Wnt/β-Catenin, Ape and

Axin. Several normal molecular mechanisms that might contribute to aberrant Wnt/β-Catenin activations and progression in breast cancer have been described in proteasome-mediated degradation (Smith et al. 2004).

1.4.5 Oestrogen Receptor (ER α) Pathway

Oestrogen Receptor α (ERα) is a transcription factor that regulates gene expression events that culminate in cell division, and this is an important property that contributes to its critical role in mammary gland development (Doisneau-Sixou et al. 2003). Additionally, ERα is a member of the nuclear receptor superfamily, which comprises of 48 proteins that have a diversity of roles and are major contributors to the functioning of the endocrine system (Germain et al. 2006). ERα has a DNA-Binding Domain (DBD) and is able to directly regulate gene expression events and a Ligand-Binding Domain (LBD) that renders it responds to an activating ligand (Arao et al. 2013).

The role of ERα in initiating timely and controlled cell division during mammary gland development and during post-pubertal physiological functions, such as pregnancy, is a coordinated process that involves other hormones and their nuclear receptor transcription factors, including progesterone and prolactin (Brisken and Ataca 2015). The ability of ERα to associate with DNA and the initiate gene transcription is subverted in disease. ERα becomes a driving transcription factor that is no longer regulated by control mechanisms, and this explains in an oestrogen-induced tumour. ERα functions as a gene regulating

transcription factor, but the ERα-mediated cell division occurs in an uncontrolled manner and the result is tumour growth initiation and cancer progression.

One of the first targeted agents in the treatment of cancer was the Selective Oestrogen Receptor Modulator (SERM), such as tamoxifen. This is an effective treatment for ER^+ breast cancers (Fisher et al. 2005) because it can mimic oestrogen and bind to the LBD pocket of the ERα. However, unlike oestrogen, it alters the structure and function of ERα; for thus, this transcription factor is no longer capable of regulating gene expression (Shiau et al. 1998). Tamoxifen has remained the mainstay to treat ER^+ disease (Jordan 2003), but many women develop endocrine resistance and tamoxifen subsequently then fails. This led to the development of novel agents that block the ERα function, resulting in pure steroidal anti-oestrogens, such as Fulvestrant (Faslodex) and a class of compounds termed Aromatase Inhibitors (AIs). Fulvestrant binds to the LBD of ERα, but unlike tamoxifen, it induces degradation of the ER protein. This drug has been an effective treatment in tamoxifen-resistant contexts (Howell and Abram 2005). The research community has studied the ERα pathway for decades and findings have revealed a rather complex picture, where ERα associates with hundreds of proteins, interacts with thousands of regions in the genome and can regulate a multitude of target genes and non-coding RNAs many of which are only now being identified.

1.4.6 HER2 Receptor Pathway

In the literature on the molecular pathways of breast cancer, the relative importance of Her2 has been subject to considerable debate, because Her2 is highly expressed in breast

cancer, ovarian cancer, and gastric cancer. Her2 has a role in tumourigenesis and the accessibility of its extracellular domain make Her2 an ideal target for the targeted delivery of anti-tumour drugs (Tai et al. 2010).

The cancer research community has succeeded in the development of the humanised monoclonal anti-Her2 antibody (Trastuzumab) for the treatment of breast cancer, as well as, to develop various Her2 specific antibodies, dimerisation inhibitors and kinase inhibitors for cancer therapy (Tai et al. 2010). There is no natural ligand for Her2 and various artificial ligands targeting of Her2 have been developed and applied in various targeted drug delivery systems, for example, Her2 targeted cancer therapy, which includes numerous strategies including the blockage of receptor dimerization, inhibition of the tyrosine kinase activity, and the interruption of the downstream signal pathway; the tumourigenic action of Her2 is not limited to a potential proliferative effect (Nahta et al. 2006).

The key role for the Her family in enhancing metastatic potential rests in their ability to promote secretion of basement membrane degradative enzymes, such as the Matrix Metalloproteases (MMPs) (Tan et al. 1997), which determine modifications in the tissue architecture through the breakdown of the matrix and consequent perturbations of cell–cell and cell–matrix interactions. These alterations, together with changes in the integrin and cadherin function frequently observed in tumour cells with activated Her, facilitate the communication between tumour cells and their escape from control by the microenvironment (O-charoenrat and Rhys-Evans 1999).

1.5 Breast Cancer Biomarkers and New Clinical Treatment

According to the definition of the National Cancer Institute–USA, a bio-marker is a biological molecule found in blood or body fluid or tissue that is a sign of normal or abnormal process or condition or disease (Henry and Hayes 2012). It is referred to as molecular marker or bio signature; it can be any molecule such as DNA, RNA or proteins. The perfect biomarker should be easily detectable, highly sensitive and specific for its target phenotype, as well as economically feasible, because biomarkers may be used to monitor the body responses to a treatment for cancer.

Paul et.al (2013) and Raben et.al (2004) reported that defining the molecular mechanism that gives rise to the cancer phenotype is also believed to represent a critical step in developing an effective therapeutic system for cancer patients. The lack of sensitive technology to detect the cancer cells from minute quantities of available tissue is a major problem associated with early diagnoses. Furthermore, the lack of a technology platform has considerably slowed the identification of reliable biomarkers to accurately diagnose most types of cancer (Wardwell and Massion 2005).

There has been a growing interest in the systems biology approaches in order to discover new biomarkers that could help early diagnosis, prognosis, classification of disease subtypes, and prediction of treatment response, as well as identification of potential targets for drug therapy (Leth-Larsen et al. 2010). The effective system of treatment requires a

specific genotyping, measurement of genes expression, protein abundance or proteins in the cancerous tissue. So if the expression profile connected with the underlying pathogenesis of the cancer is determined one can presumably choose a treatment system that is best suited for a specific type of cancer (Sawyers et al. 2002, Meyerson and Carbone 2005, Hwang et al. 2006).

Several studies have documented that mammography is the only method of screening for breast cancer which is shown to have decreased mortality more than imaging modalities, such as thermography, breast specific gamma imaging, positron emission mammography and optical imaging for breast screening (Group 2006, Tabár et al. 2001). However, mammography alone does not perform as well as mammography with supplemental screening in high risk women. Supplemental screening with breast Magnetic Resonance Imaging (Finn et al. 2009, Lee et al. 2010) and ultrasound is recommended in selected intermediate and high risk populations and also for women with a family history of breast cancer and genetic predisposition (Mainiero et al. 2016).

A considerable amount of literature has been published on breast cancer, these studies endorse that patients who have a high risk of developing breast cancer should have the combination of mammography and MRI tests (Lee et al. 2010, Plevritis et al. 2006).
There is a growing body of literature that gene expression profiling allows for the study and investigations of the complexity of breast cancer by using microarray-based technologies (Li et al. 2016). This investigation includes the predicting of breast cancer recurrence, by

using new technologies such as the 70-gene Mamma Print microarray assay (Van't Veer et al. 2002) and the 21-gene Oncotype DX assay (Cronin et al. 2007).

Additionally, for identifying clinically relevant molecular subtypes of breast cancer, the 50-gene PAM50 assay (Parker et al. 2009) is used. Existing research recognises the critical role played by the mammographic abnormalities, proliferative breast cancer with or without atypia, family clustering and inherited germ-line abnormalities as molecular bio-markers for breast cancer. These bio-markers are utilised as endpoints in short-term chemoprevention trials (Janssens et al. 2004). Using gene assays is suggested to be useful in the treatment management (Prat et al. 2012). ERa and PgR expression alone are inaccurate to determine a patient's optimal treatment system, because, the amount of 'cross-talk' between the different pathways is considered significant (Fuqua and Cui 2004). Clinically, ERa predicts for response to endocrine therapy such as antiestrogen (tamoxifen) administration or ovarian suppression. However, the prognostic value of ERa expression and the value of biomarkers could change after five years of long-term follow-up (Fuqua and Cui 2004).

Equivalently, HER2 testing positive is useful for selecting targeted therapy with monoclonal antibodies Trastuzumab against HER2 protein overexpression, amplification of the *HER2* gene, or both. This treatement is as aggressive as the behavior of the tumour (Gajria and Chandarlapaty 2011). In addition, Trastuzumab (Herceptin) a humanised monoclonal antibody against the extracellular domain of HER2 (HER2 belongs to a family of four transmembrane receptor tyrosine kinases), has been observed to benefit patients

with HER2-positive cancer, when regulated weekly or every three weeks, alone , or in combination with chemotherapy (Piccart-Gebhart et al. 2005).

Preliminary evidence showed the significance of ERα and AR co-expression in ER+, HER2- MBC. ER+, AR+ patients, also showed evidence of significantly improved EFS, when treated with Palbociclib and endocrine therapy compared with AR-, ER+ patients (Giacinti and Giordano 2006). There is evidence that the AR expression is associated with pRB (the retino-blastoma (Rb)) expression that represents a mechanism by which cell cycle inhibition with Palbociclib is particularly efficacious in these patients (Sherr 1996, Giacinti and Giordano 2006). Generally, Palbociclib (PD 0332991) was the first oral CDK4/6 inhibitor successfully implemented in clinical practice as a single agent. It was investigated in a phase II trial that included 36 heavily pretreated patients with Rb-positive metastatic breast cancer and patients with HR+/Her-2 negative subtypes of breast cancer (DeMichele et al. 2013). One in vitro study showed that the HR+/HER2 negative subtype seems to be more sensitive to the drug compared to other subtypes. HER2+ cell lines that responded to Palbociclib have luminal features.

Preclinical data showed the synergistic effects of combining Palbociclib with tamoxifen in ER-positive cell lines, since Palbociclib increases the sensitivity of the ER-positive cells to endocrine therapy (Finn et al. 2009). During treatment, the inhibition of CDKs leads to the activation of autophagy. As a mechanism for treatment resistance, and the addition of an autophagy inhibitor to a CDK4/6 inhibitor can significantly reverse resistance and

decrease the dose of Palbociclib required to treat breast cancer patients, with a possible significant impact on toxicity and QOL (Vijayaraghavan et al. 2017).

Endocrinology and Her2 target therapies are not able to taken by TNBC patients and the treatment options are limited to chemotherapy, radiotherapy and surgery. There is an urgency to find intelligent drugs and the greatest obstacle is the vast heterogeneity of TNBC. Most studies have failed to clearly identify that unified alteration may serve as targets of a targeted therapy.

However, several genomic signature analyses have revealed potential molecular targets, which could basically be defined in four groups: agents that (**I**) cause damage to DNA (i.e. cisplatin, cyclophosphamide), (**II**) inhibit poly (ADP-ribose) polymerase (PARP inhibitors), (**III**) tyrosine-kinase inhibitors and (**IV**) inhibit downstream signalling pathways (mainly PI3K/AKT) (Fernández and Reigosa 2013, Brunello et al. 2013), for example, Bevacizumab, Olaparib/iniparib, Cetuximab, mTOR, inhibitors (mammalian target of rapamycin) and Bicalutamide /enzalutamide (“anti-androgen” targeted therapy) (D'Agostino Sr 2011, Arun et al. 2015, Carey et al. 2012)

Some preclinical studies showed that luminal TNBC which expresses the androgen receptor (AR), is sensitive to androgen deprivation (Rampurwala et al. 2016). Bicalutamide as a monotherapy demonstrated a clinical benefit of 19% of the AR positive (Lehmann and Pietenpol 2015). A phase II study is evaluating enzalutamide's safety and efficacy in TNBC

and AR positive patients, results are yet to be seen and targeted therapies against some identified biomarkers in TNBC have not proven a significant improvement; the problem has been the lack of dependable predictive biomarkers, which is essential before any of these treatments can be introduced in clinical practice (Gerratana et al. 2018).

1.6 Triple Negative Breast Cancer (TNBC)

Triple negative breast cancer (TNBC) is increasingly recognised as a serious, worldwide public health concern and more than 170,000 patients are diagnosed with TNBC each year (Ismail-Khan and Bui 2010). The risk appears to be greatest in the first few years after treatment since; breast cancer is a dynamic disease that evolves with time and as a function of therapy. Moreover, the phenotypical changes of the metastatic TNBC may represent a unique heterogeneous tumour cell population with special biological features that permit travel to distant sites and the establishment of a clinically disseminated disease. Triple-negative tumours does not express the nuclear hormone receptors such as (ERα), (PgR), nor the epidermal growth factor receptor Her2, however, in breast cancer, the three clinical bio-markers are used to guide treatment (Luo et al. 2010). Furthermore, 10% - 20% of breast cancer test negative for these three receptors, which means hormones are not supporting tumour growth.

Hormone-based therapy (Tamoxifen) to target ER- a positive cells, antibody-based therapy (trastuzmab) to target Her2/ Neu positive breast cancer are not effective when treating (TNBC), Additionally, TNBC is unlikely to respond to medications that target Her2 such as Herceptin (Liedtke and Rody 2015).

There are limited therapeutic options for treating (TNBC) when compared to receptor positive cases. Women with TNBC have a higher risk of death within five years of diagnosis, but not after this period of time. African American women are particularly at risk for developing TNBC and dying, they are three times more likely to die than other ethnic groups around the world (Dent et al. 2007).

Molecular researchers have reported that the origin of ER- and ER+ tumours include the existence of two independent pathways of carcinogenesis. Meaning, the development of all tumours through a single pathway resulting initially in ER+ neoplasms, which subsequently can be transformed into ER- tumours by epigenetic and /or genetic events (Zhu et al. 1997). The maintenance of the original receptor status of breast cancer over time reveals that the etiology of receptor negative and receptor positive cancer is distinct and can diverge early in the pathogenesis of these tumours.

1.6.1 Statistical Studies of TNBC

This literature review reveals that the earliest indication of TNBC was in 2005. Foulkes et.al (2010) stated that TNBC has a growing recognition by oncologists, geneticists, and pathologists. TNBC is a heterogeneous type of malignancy associated with poor prognosis and accounts for between 9% and 16% of all breast cancer cases (Montagna et al. 2013, Siegel et al. 2015). DeSantis et al. (2014) found that women with TNBC were dying more frequently than patients with other subtypes of breast cancer (42.2% versus 28%)

respectively, while the median time to death was 4.2 years for patients with TNBC compared with 6 years for those with other cancers.

Gluz (2009) explained that there were no universally accepted models explaining the factors which drive the TNBC development. Previous research has found the aggressiveness of TNBC is further indicated by the fact that the peak risk of recurrence is within the first three years after initial treatment of the disease with the majority of deaths occurring in the first five years (Dent et al. 2007). Many studies are increasingly recognizing that TNBC is an aggressive disease with outcomes inferior to those of other breast cancer subtypes based on the gene expression profile and the luminal androgen receptor (approximately LAR, 12%) (Hubalek et al. 2017).

The rate of survival is highest for the ER-positive, PR- positive and HER2 positive phenotypes. According to the American Cancer Society breast cancer statistics from 2013-2015 (Siegel et al. 2013) (which included data on incidence, mortality, survival, and screening) there were roughly 232,340 new cases of the invasive disease with 39,620 related deaths in the US. Yearly, there are 54,000 breast cancer cases diagnosed in the UK and the survival rate for this disease is lower than in comparable industrial countries. (Perou et al. 2000, Siegel et al. 2016).

Also, the heterogeneous nature of breast cancer has important implications for physicians and their patients (Onitilo et al. 2009). Moreover, many studies in gene expression analysis suggest that TNBC arises from basal cells of the mammary epithelium and is associated

with high mitotic activity and invasive tumours in younger patients and in premenopausal women (Greenwood et al. 2012, Sørlie et al. 2001). Approximately 75% of breast cancer is positive for ERα and/or PR protein expression. The remaining 20% - 25% of breast cancers are ERα and PR negative and are not amenable to selective oestrogen receptor modulators. However, few tumours in the triple negative group can express AR and the type of expression seems to be related to tumours undergoing apocrine differentiation; this observation has important biological and chemical significance (Niemeier et al. 2010).

1.6.2 Molecular Subtypes of TNBC

Several studies have revealed that TNBC is a heterogeneous disease at the molecular, clinical and histological levels (Lehmann and Pietenpol 2015, Pareja et al. 2016). Advanced biotechnologies such as next generation sequencing have led to the discovery of several molecular characteristics, including the inactivation of the BRCA pathway, MAP/ERK kinase (MEK) and phosphatidylinositol-3-kinase (PI3K) pathway activation, high level of tumour protein P53 mutations, loss of retinoblastoma protein (RB), high activation of MYC, enrichment for androgen receptor (AR) regulated gene expression signatures. Other potentially targetable molecules include the immune checkpoints Programmed Death (PD1) and Programmed Death-ligand 1(PDL1). Understanding of the molecular profiles of different types of tumours has allowed the development of promising therapeutic agents such as DNA-damaging agents, AR inhibitor and immune checkpoint inhibitors (Oualla et al. 2017).

Zheng et al. (2012) observed that several strategies have demonstrated that basal-like tumours are not necessarily TNBC. For instance, between 15% and - 45% of basal-like breast cancers (BLBC) have been shown to express ER and 14% of BLBC express Her-2 demonstrating that regardless of classification method, not all BLBC are TNBC. There were some studies suggesting that the molecular markers of TNBC, such as VEGF, EGFR, Src and mTOR, have been important for the design of clinical trials investigating targeted treatments (Lehmann et al. 2011). However, 16% - 44% of TNBC test negative for all these markers. Thus, it is difficult to develop targeted treatment strategies that would be universally applicable to all TNBC.

The rapidly growing area of literature on TNBC indicates that there are six stable subtypes (Uscanga-Perales et al. 2016) displaying unique Gene Expression (GE) signatures and ontologies, including; 2 basal-like subtypes BL1 and BL2. These have a higher expression of cell cycle related genes, DNA damage response genes and representative cell lines preferentially responding to cisplatin. Second, Mesenchymal (M) and a Mesenchymal Stem-Like (MSL) subtypes were enriched in GE for epithelial mesenchymal transition. Growth factor pathways and cell models responded to PI3K/m TOR inhibitors and Dasatinib (abl/src) inhibitor. Third, an immunomodulatory (IM) and a luminal androgen receptor (LAR) subtype, which includes patients with decreased relapse-free survival and was characterised by AR signalling (Table 1-2) (Uscanga-Perales et al. 2016)

Such TNBC patients have a poor outlook, independent of lymph-node status (Foulkes et al. 2004). An implication of this is the possibility that TNBC shares survival biology features

with basal-like breast cancer and is associated with poor clinical outcome and high rates of recurrence following chemotherapy. The negative prognosis of TNBC is in a large part due to the excessive risk of developing visceral metastases (Dent et al. 2007, Hwang et al. 2007).

Table 1-2 Molecular Subtypes and Characterises of TNBC

TNBC Subtypes	**Characterises of Triple Negative tumour**
-Basal Like -1 (BLI)	Cellular proliferation and response to cellular damage
-Basal Like -2 (BL2)	Growth factor signalling with myoepithelial markers
-Immunomodulatory Class	Signalling mediated by immune synapsis
-Mesenchymal Class	MET and differentiation
-Mesenchymal Stem Cell	MET, differentiation and stem cell potential, angiogenesis and growth factor signalling.
-Luminal Androgen Receptor	Hormone signalling mediated by androgen receptor

1.6.3 Risk Factors of TNBC

TNBC is associated with increased risk for visceral metastasis and lower risk for bone recurrences. In-vitro and in-vivo research, and epidemiological data have revealed that the reproductive hormones estrogen and progesterone play an essential role in breast cancer etiology (Persson 2000). Risk factors such as postmenopausal obesity, use of exogenous hormones and age at first menarche increase the risk of developing breast cancer by increasing systemic exposure to hormones, which leads directly to linking higher circulating levels of estradiol to postmenopausal breast cancer.

Additionally, the breast cancer progress can be affected by hormone related exposure (as one of the risk factors), including, hormone synthesis, metabolism, and protein expression. Oral contraceptive exogenous hormone use was strongly associated with ER- rather than ER+ tumours (Althuis et al. 2004).

Socioeconomic characteristics of population and older age, family history of breast cancer, earlier menarche age, induced abortion, nulliparity, and lack of breastfeeding all affectthe risk of developing breast cancerThese factors have been studied extensively and a large pool of data analysis has found that premenopausal women have approximately 20% - 30% higher risk of breast cancer among (Dolle et al. 2009). Additionally, previous studies have reported that the risk of oral contraceptive (OC), use is concentrated among younger premenopausal women (Rosenberg et al. 1996). These findings are consistent with he mechanism through which OC use impacts the risk of breast cancer in young women by studying the role of estrogen in promoting the growth and vascularization of cancer cells, in particular, the mechanism of transcription which affects estrogen binding to its receptor in ER+ in mammary and ovarian cancer cells. However, some publications have proposed a second mechanism whereby estrogen promotes the growth of ER- and ER+ cancer by enhancing angiogenesis and stromal cell recruitment (Gupta et al. 2007)One million cases of breast cancer are diagnosed annually around the world and it is estimated that over 170,000 harbor the triple-negative (estrogen receptor/progesterone receptor/Her2-negative) phenotype (Anders and Carey 2009). According to data from gene expression microarrays, most TNBC share characteristic with BL subtype. The BL molecular subtype

exhibits a unique molecular profile and a set of risk factors aggressive and early pattern of metastasis, limited treatment options, and poor prognosis.

Most population-based studies have found a higher proportion of TNBC tumours among premenopausal African American women, pregnancy at an early age, shorter duration of breast feeding, and elevated hip-to-waist ratio are might be particular risk factors. TNBC clinical diagnostics illustrate the preferential relapse in visceral organs which include the central nervous system. Although initial response to chemotherapy might be more profound, relapse is early and common among TNBC when compared with luminal breast cancers. The armamentarium of "targeted therapeutics" for TNBC is evolving and includes strategies to inhibit angiogenesis, epidermal growth factor receptors and other kinases (Anders and Carey 2009).

1.7 Biomarkers and Molecular Targets of TNBC

Breast cancer of the triple negative phenotype overlaps with BL breast cancer; however, TNBC has significant clinical implications, such as an association with poor survival rates, higher incidence of recurrence and distant metastasis. Furthermore, research has highlighted the need for a better understanding of TNBC and it has received considerable critical attention for the following reasons. First, the heterogeneity of this disease reflects the activation of different genetic pathways and various cellular targets in which these genetic changes occur. Second, chemotherapy, which is associated with high toxicity, is the main treatment option. TNBC patients are treated typically with a combination of radiation, chemotherapy and surgery. TNBC patients seem to achieve higher response

rates to chemotherapy, but this does not convert into Overall Survival (OS) or superior Progression Free Survival (PFS) (Rodríguez-Pinilla et al. 2006).

The classic diagnostic and therapeutic strategies for TNBC are discussed below. According to gene expression analysis, TNBC is a subtype of breast cancer with a heterogeneous behavior. This analysis is not only useful to understand the disease, but to identify the new molecular target for its treatment (Lehmann et al. 2011). Some of TNBC biomarkers as potential therapeutic targets are shown below (Table 1-3).

Table 1-3 TNBC biomarker and molecular therapeutic targets

TNBC Biomarkers	The Molecular Targets
Epidermal Growth Factor Receptor(EGFR)	marker of cellular proliferation, angiogenesis, metastasis and apoptosis inhibition
vascular endothelial growth factor (VEGF)	proliferation and maintains the integrity of the cell
C-kit and basal cytokeratin	stimulates survival and differentiation and induces invasiveness in the cancerous cell
P53	Cell cycle regulation and tumour apoptosis
hKi67	A cellular proliferation marker.
PARP	A family of cell signalling enzymes.
Hsp90	A cellular chaperone
COX2	inflammatory expression protein
TK	Cell grow and differentiate protein
Mtor	Association with a poor response to treatment

1.7.1 The Clinical Therapy of TNBC

The TNBC patients are classified into two types: some patients respond to treatment, while the majority has a poor outcome at the clinical level. Additionally, the nature of this disease is highly aggressive with poor viable therapeutic options and OS. It exhibits a higher incidence rate among African American and Hispanic women under 40 years of age. In recent years, there has been an increasing amount of literature on neoadjuvant therapeutic approaches. Clinical studies show neoadjuvant therapeutics such as anthracyclines and taxanes are improving survival rates in TNBC women with a clinical response rate up to 85%, while pathologic response (pCR) rate is 30%-40% (von Minckwitz et al. 2012). Clinical triple negative tumours display distinctive pattern of relapse with a high risk of developing distant metastasis and death through predilection for visceral lung and brain metastasis (Kennecke et al. 2010).

Patients with TNBC have a highest risk of recurrence and metastasis (Foulkes et al. 2010). Various targeted therapies have been investigated, but as yet none are currently approved, [for example, Bevacizumab, a VEGF- targeting anti-body was granted accelerated FDA approval with weekly paclitaxel for first line treatment of metastatic breast cancer, however, this approval was later revoked after follow up phase III clinical trials].

Preclinical studies have implicated AR as a potential tumour suppressor in ER-positive breast cancer with anti-proliferation effects owing to the cross talk between the steroid receptor signalling pathways. The reason is AR is activated by testosterone and

dihydrotestosterone, which is involved in the function of multiple female organs, such as the reproduction tract as well as, bones, kidney and muscles.

Moreover, testosterone and dihydrotestosterone act as prohormones of estradiol and bind directly to AR. The result of binding is the translocation of the receptor to the nucleus and a binding to the target genes and transcriptional activation (Zhu et al. 1997). Moreover, clinical data analysis has shown that the androgen signalling pathway plays an essential role in the development of normal and malignant breast tissue. Epidemiology studies have suggested that increased levels of androgen receptor are associated with an increased risk of breast cancer (Birrell et al. 2007, Wilson et al. 1981, Hickey et al. 2012).

A considerable amount of literature has been published on Antibody-Drug Conjugates (ADC). These studies have discovered that several ADC are encouraging the activity in TNBC such as, Sacituzumab govitecan (IMMU-132) is an antibody–SN-38 conjugate targeting TROP2, which is expressed in the majority of studied tumour specimens (approximately 88%). These were moderately to strongly positive for Trop-2 by immunohistochemistry research and clearly showed that Sacituzumab govitecan is well tolerated and induced early and durable responses in pretreated patients with metastatic TNBC (Bardia et al. 2017).

Biomarker research has found that isolating the glycoproteins on the surface of epithelial cancer cells can improve delivery of elevated concentrations of these molecules. This is because many of these targets are not necessarily cancer drivers or specific to breast

cancer (Garrido-Castro et al. 2019). Moreover, the Palbociclib ongoing trials in the management of BC studies in estrogen receptor (ER)+, HER2-negative Metastatic Breast Cancer (MBC) demonstrated a significant progression-free survival advantage for palbociclib/CDK4/6 inhibiter in combination with letrozole or fulvestrant in the first or second line setting compared to these therapies alone. These studies revealed preliminary efficacy signals for androgen receptor AR blockade in MBC, triple negative patients and predominately in AR+ and treated ER+ MBC patients with Palbociclib early in their metastatic treatment course (Ribnikar et al. 2019).

1.7.2 The Histologic Characterisation of TNBC

The immunohistochemical diagnosis of TNBC is a type of breast cancer that does not express ER, PR and HER2. However, the American College of Pathology, the American Society of Clinical Oncology and the St. Galen guidelines have changed the cutoff rate used to define the estrogen and progesterone receptor negativity. The accepted receptor cut off rate now is less than 1% whereas HER2 negativity is defined according to the IHC expression of score 0-1 or lack of gene amplification (Wolff et al. 2013).

Currently, endocrine therapy is used for patients with ERα expression of 1% and more in all stages of breast cancer (Iwamoto et al. 2012). The IHC of triple negative tumours shows that they are invasive ductal carcinomas characterised of high histologic grade, central necrosis, poor differentiation, high lymphatic infiltration and high proliferation rates. Additionally, the triple negative tumour phenotype also includes high grade histologic

subtypes of breast cancer including metaplastic carcinoma, adenoid cystic carcinoma apocrine, histocytoid carcinoma and medullary carcinoma (Bae et al. 2011).

1.7.3 TNBC Treatment Strategies

As a result, of the heterogeneity of TNBC, there is an ongoing efforts to develop new targeted therapies for the different subtypes. First, DNA- damaging chemotherapy and DNA repair targeting is discussed. The DNA repair system is essential to maintain the integrity and stability of the genome. BRCA1 and BRCA2 are tumour suppressor genes involved in homologous recombination- mediated repair of double stranded breaks. Mutation in BRCA1 and BRCA2 genes leads to impaired DNA repair function by homologous recombination and results in genomic instability (Hoeijmakers 2001). Advanced knowledge of the DNA repair mechanism has enabled the development of new therapeutic approaches in TNBC therapies exploiting the higher sensitivity of these tumours to DNA damaging agents, including platinum salts and poly ADP-Ribose Polymerase (PARP) inhibitors. However, the use of platinum compounds in the neoadjuvant have shown conflicting results, such as Carboplatiinum to Paclitaxel, and Liposomal doxorubicin (Sirohi et al. 2008). Some TNBC research explains that the combination of a platinum agent with PARP inhibitors might increase the effectiveness by increasing double- strand breaks to the point where even a competent repair system is overwhelmed (Comen and Robson 2010). PARP Inhibitor plays an essential role in the DNA repair system, by homologues recombination-mediated repair of double stranded

breaks and the lack of enzyme activity leads to persistent DNA lesions which induce apoptosis (O'shaughnessy et al. 2011).

Inhibitors of PARP have been developed as a new treatment for cancers with specific DNA repair deficiency such as TNBC, BRCA1and BRCA2 mutations,(e.g. Olaparib oral active PARP inhibiter). Several studies have documented that adjuvant endocrine therapies that stop the action of estrogen on breast tissue can block tumour growths thereby becoming essential treatment strategies, which reduce the risk of recurrence and death in women with estrogen receptor ER-positive disease (Group 2015). This is not an available as an option for TNBC. Depending on the data from medical records, the poorer prognosis of TNBC and their disproportionate burden on Hispanic and other medically disadvantaged groups makes it critical to identify factors that differentially influence the development of this subtype (Figure 1-41). Modifiable factors such as breast feeding have great public health significance, which may inform and influence prevention strategies to help close the gap in breast cancer survival across racial ethnic groups owing to differential occurrence of breast cancer subtype (Chen et al. 2016).

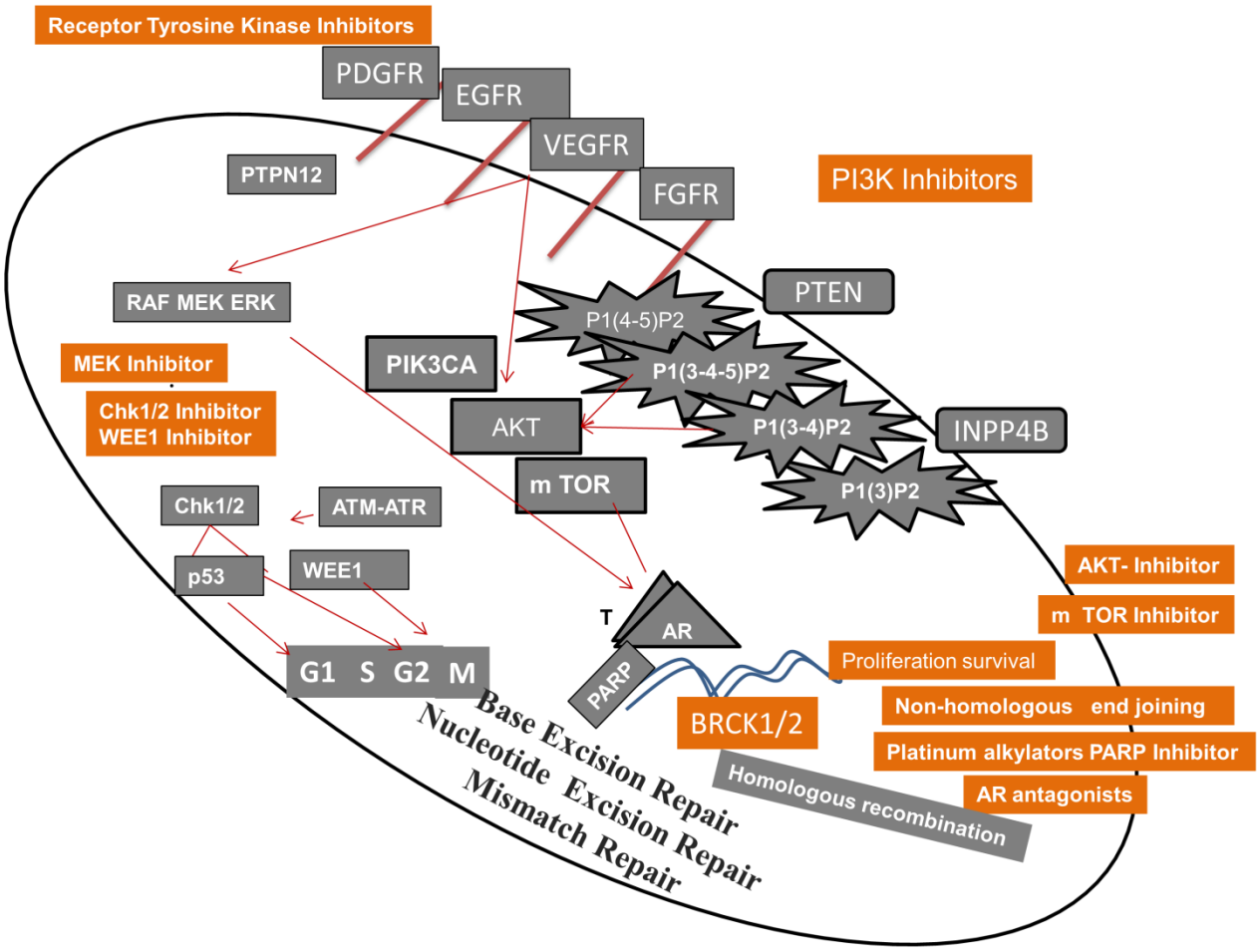


Figure 1-41 therapy pathways for metastatic breast cancer adapted from (Waks and Winer 2018).

1.8 Cancer Metastasis

Metastasis is a complex process of not only the invasion, but also the homing and proliferation of metastasis sites. It requires the activity of several genes. These include the

urokinase plasminogen activator receptor system such as urokinase-type plasminogen activator (Ménard et al. 2000) and the vampire bat plasminogen activator (vPA), cytokines (1L-1,1L-6,1L-8,1L-11 Tumour Necrosis Factor and Transforming Growth Factor-β1) chemokines and their receptor (CXCR) and matrix metalloproteinase (MMPS) (Sheridan et al. 2006).

In the traditional metastasis model, metastatic cells are rare and arise from the late stages of tumour progression. In recent, published studies of the expression profiling on human tumours such as breast carcinomas have challenged the long held view of these models refocused on the early formation of overt metastases. The findings of these current studies do not support the traditional metastasis models, and suggest that most cancer cells in a primary tumour may have a metastatic phenotype (Pantel and Brakenhoff 2004, Bernards and Weinberg 2002). In the biological process of metastasis, the presence or absence of lymph-node metastases can predict the likelihood of survival for most patients with cancer, because the relationship between lymph-node metastases and distant metastasis is strong. For instance, the lymph-node metastasis in the head and neck cancers is the strongest prognostic factor (Parkin et al. 1999).

Moreover, the number of lymph-node metastases and the extra-nodal spread are also prognostic factors, particularly, for the development of distant metastasis. In reviewing the literature, the metastatic pattern of other solid tumours, such as breast carcinoma is different from that of head –neck tumour. Even using sensitive analytic assays, 20%-30% of the patients with breast cancer were free of axillary lymph-node metastasis. Body et al.

(2006) highlighted that the ability of breast cancer cells to metastasise and proliferate in bone marrow and form colonies can be explained by their ability to destroy bone via osteoclast stimulation.

Current data suggests that the breast cancer cells seem to secrete factors such as parathyroid hormone related protein, which creates a fertile soil rich in cytokines and growth factors and leads to the development of metastasis in the skeleton as well as to increased growth of breast cancer. Multiple myeloma patients suffer significant skeletal morbidity during the natural course of their disease such as bone metastasis lesions; and this also affects an estimated 65%-75% of patients with breast carcinoma (Coleman 1997).

MBC is a fatal disease and is the main cause of almost all breast cancer deaths (Smith et al. 2004). The abnormal cancerous cells in the breast area grow and multiply without stopping thereby creating a tumour, which usually starts in the ducts or lobules. Advanced breast cancer or stage IV breast cancer is a relatively new name for metastatic breast cancer commonly referred to as the spread of cancer cells from where the cancer started to distant parts of the body. Away from a primary tumour, these cancer cells enter the blood stream or lymphatic system and spread to form a metastatic tumour, which means the new tumour came from the original tumour. In the last few years, there have been outstanding advances in breast cancer management that has led to improved prognosis, and early stage metastasis diagnosis by sensitive immunological, molecular procedures and cancer bio-markers (diagnostic markers) (Onitilo et al. 2009). It is thought that TNBC patients develop distant metastatic disease variables other than the number of organ sites

and that the location involved had no bearing on the outcome, however, patients with visceral disease generally have a poor outcome. Also, TNBC patients with brain and liver metastasis had poorer survival rates than those with bone and lung metastasis. In addition, pleural metastasis in patients were also associated with unfavorable survival outcomes, when compared to lung metastasis, that indicated that the route of the first metastasis correlated significantly with the survival of TNBC patients, with distant metastasis to the brain being the poorest survival indicator followed by liver, pleura, bone and lungs (Tseng et al. 2012).

1.9 Cancer Hallmarks

Existing research recognises the critical role played by six biological capabilities, which are acquired during the multistep development of human tumours. These are (I) evading growth suppressors; (II) sustaining proliferative signalling; (III) resisting cell death; (IV) enabling replicative immortality (V) angiogenesis and (VI) activating invasion and metastasis. Hanahan and Weinberg (2000) defined these as the six hallmarks of cancer. These hallmarks constitute an organising principle of rationalising the complexities of neoplastic disease. They have been studied over the last few decades because the notion of normal cells which evolve progressively from the neoplastic state, and the multistep process of human tumour development, could be rationalized by the need for incipient cancer cells to acquire the traits that permit them to become tumourigenic and then ultimately malignant. Furthermore, Hanahan and Weinberg (2000) provide a different point of view in that they argue that tumours are more than insular masses of proliferating cancer cells, instead they are compound tissues composed of multiple distinct cell types that

participate in heterotypic interactions with another cell. Evidence suggests that the hallmarks of cancer cell have a continuous power to provide the next generation of **cells** with these markers (Hanahan and Weinberg 2011). Enabling characteristics (genome instability and mutation, and tumour-promoting inflammation) and emerging hallmarks (evading immune destruction and immune inflammatory cells) are additional processes which need to be considered in order to understand the mechanism of the six hallmarks.

1.9.1 Genome instability and mutation

Acquisition of the hallmarks of cancer depends on a succession of the alterations of the genome materials of neoplastic cells. For example, in particular the mutant genotype allows and enable sub clone of cells to outgrowth and consequent dominance in a local tissue environment; and according to heritable phenotypes, the inactivation of tumour suppressor genes could be acquired by epigenetic mechanisms, such as in DNA methylation and histone modifications (Hanahan and Weinberg 2011, Berdasco and Esteller 2010)**.** Normally, cancer cells increase the rates of mutations (Salk et al. 2010), by these mechanisms. First, increasing the sensitivity of cell to mutagenic agents, through the breakdown of one or several components of the genomic maintenance machine or both. Second, accelerate the accumulation of mutations by compromising the surveillance system, which monitor the genomic integrity and force genetically damaged cells into senescence or apoptosis (Jackson and Bartek 2009, Hanahan and Weinberg 2000).

Dvorak (1986) explained that some tumours are densely infiltrated by immunity cells (both, innate and adaptive) and for this, mirror inflammatory condition arising in non-neoplastic tissues. This can be demonstrated by using the Immunohistochemical technique (Pages et al. 2010).

Tumour associated inflammatory response also had noticeable characteristics, such as unanticipated, paradoxical (effect of enhancing tumorigenesis) and progression, which helps incipient neoplasia to acquire hallmarks capability. A considerable amount of literature has been published on the intersection between inflammation and cancer pathogenesis. These studies explained that the immune cells are largely of the innate immune system (DeNardo et al. 2010, Qian and Pollard 2010).

Furthermore, inflammation can contribute to in multiple hallmark capability by suppling bioactive molecules to the tumour microenvironment, such as growth factors (sustain proliferation signals), proangiogenic factors, survival factors (limiting cell death) and extracellular matrix-modifying enzymes (supporting an invasion, metastasis and angiogenesis) (Karnoub and Weinberg 2007). Thus, Inflammation is present in the early stages of neoplastic progression and capable of fostering the development of incipient neoplasia to full-blown cancers (Qian and Pollard 2010, De Visser et al. 2006).

Also, inflammatory cells can release chemical molecules, particularly reactive oxygen species, which are actively mutagenic for accelerating the genetic evaluation of cancer cells toward heightened malignancy (Grivennikov et al. 2010).

Hanahan and Weinberg (2011) demonstrated that infiltrating cells of the immune **system** contribute to the development of tumours through conflicting ways: there are both tumour-antagonising and tuomur-promoting leukocytes. This could appear in various proportions in most neoplastic lesions, such as the presence of tumour antagonising NK (Natural Killer) cells and CTLs (Cytotoxic T Lymphocytes), which are tumour-antagonising but other immune cells are also present that are tumour-promoting by eventually enhancing the cancer hallmark capability.

Manavi (2007) discovered that STAT1 acts like a pro-immune and anti-tumour transcription factor, acting as a tumour suppressor but could also have and up-regulation role in the late stage of human cancers, such as breast cancer. STAT1 has been associated with an anti-tumour effect; however, accumulating evidence has linked increased STAT1 activation with increased tumour progression in multiple types of cancer, such as breast cancer (Hix et al. 2013). STAT1 regulates the DNA repair pathways and could be used as predictive biomarker for breast cancer chemotherapy and radiotherapy (Khodarev et al. 2004). Additionally, extensive studies were concerned with the action of single extracellular signalling polypeptides; however cells within tissues are exposed to multiple agents. An example is increased cAMP, which could blunt the activation of the STAT1 family, and prior treatment of the cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) can prevent 1L-6 induced activation of STAT1. It remains unclear how STAT1-dependent transcriptional initiation is regulated (Darnell 1997).

Metastasis is associated with specific molecular changes such as decreased cell-cell junction proteins and increased degradation of basement membrane proteins (Cheng et al. 2007, Fidler 2003). Meanwhile, accumulating evidence has suggested that the Circulating Tumour Cells (CTC) that are released from the primary tumour into the circulating blood are the main cause of tumour metastasis (Chaffer and Weinberg 2011, Zhang et al. 2012). However, CTC status has been shown to have prognostic relevance for overall survival but not for disease progression in TNBC (Hwang et al. 2012).

A cancer cell cannot be considered as an indistinct entity in an organism, but as a strongly linked entity within the whole body. To achieve the normal function of breast tissue and to assist in the production of milk, multiple cell types communicate to alter each other's behaviour and share a reciprocal relationship within the tissue microenvironment. Moreover, the double–layered ductal structures are encapsulated within a Basement Membrane (BM). The compartment outside the ductal structure contains the stromal type Extracellular Matrix (ECM) elements, such as collagen I and elastin, adipose cells, fibroblast, immune cells (mast, leukocytes) and blood vessels. All of these are essential for the organisation and architecture of the breast with in the surrounding BM. The exquisitely choreographed interactions among the epithelial cells, perturbation in stromal–epithelial and changes in the hormonal and cytokine milieu could encourage the cascades of events and responses to begin. This could result in loss of polarity and disruption to the epithelial compartment within the basement membrane. (Rizki and Bissell 2004).

The progression process from normal breast tissue to malignant is defined by pathological stages as determined by histological and cellular characteristics of the tissue, breast cancer progression stages and hyperplasia (usual or atypical), Ductal Carcinoma In Situ (DCIS) and invasive carcinoma. Moreover, acquisition of malignancy is accompanied by changes in cell morphology functions and genome integrity as well as, metaplastic changes in cell behaviour, for example, epithelial to mesenchymal transition and fibroblast to myofibroblast conversation in stroma (Nisticò et al. 2012, Rønnov-Jessen et al. 1995).

Tumour progressions are believed to result from the genetic instability within cancer cells that selects clonal expansion and results in a more aggressive tumour in the future. However, the genetic and phenotypic drift of breast cancer cells has not changed even after many years; similarly with the breast cancer cell line. It is possible to select clonogenic variants within a cell line that have different genetic and biological properties from the parental population (Feng Li et al. 2007, Fillmore and Kuperwasser 2008).

The complexity and diversity of the somatic mutational process in humans can be discovered by studying the mutational pattern within a cancer genome. However, the mechanistic basis of many mutational signatures remains speculative (Alexandrov et al. 2013). To elucidate the mutational signatures process, Alexandrov et al (2013) explains two major streams of investigation. First, the collection of a mutational signature from known mutagenesis or perturbation of DNA maintenance machinery in model systems compared with those found in human cancer. Second, by studying the diverse relationship between the mutational signatures and the characteristics of cancer biology, along with the

molecular profiling and epidemiology studies. Together, these studies provide an advance in our understanding of cancer aetiology and thereby prevention and treatment.

Information from a comprehensive electronic literature review (i.e Pup Med, Medline) between current meta-analysis and previous posted analysis provides clear evidence that CTC in peripheral blood is useful not only to TNBC metastasis but also to act as the prognostic indicator of non-metastasis TNBC (Kelley et al. 2015, Weaver and Yang 2013).

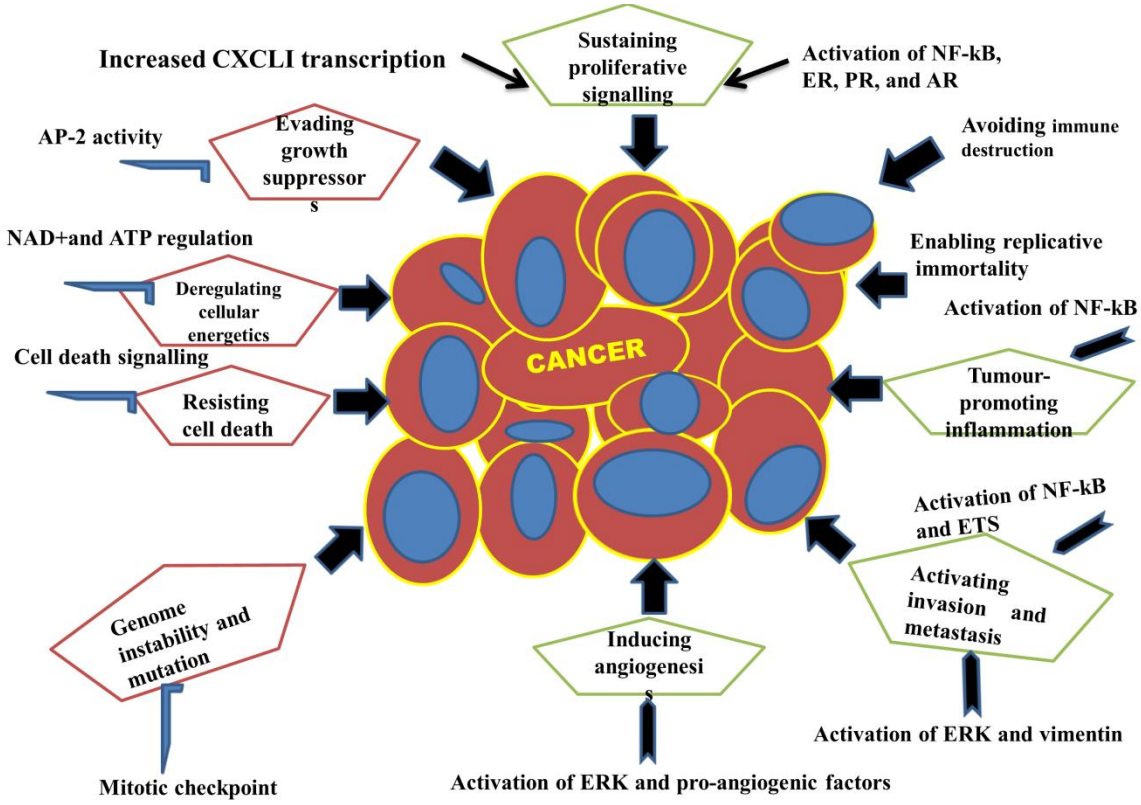


Figure 1-5 Multiple pathways

This schematic depicts multiple PARP-1-mediated processes which either stimulate or inhibit six of the eight "hallmarks of cancer"

1.10 Membrane Proteins, Quantitative Detection and Proteomics as an Approach to Targeted Therapy

There are many phenotypic changes associated with malignant transformation such as cell proliferation adhesion and migration. Most of these are mediated or initiated by

proteins linked with the plasma membrane which make these central in the biological process and a potentially effective drug target (Sheryl Harvey et al. 2001).

A number of researchers have reported that mutated cancer cells have often shown an alteration in the expression level of particular plasma membrane proteins or proteins associated with the membrane. This can be either a higher expression of a certain receptor such as Her-2 which contributes to tumour cell growth when activated by circulating or locally produced ligand or downregulation of certain adhesion molecules that prompt the cells to detach from the primary tumour and spread (Swanton et al. 2006).

More recent analysis has focused on the application of proteomics based on the assumption that many of the physiological changes in cancer cells are mediated by molecular alterations at the protein level, that are not expected to be revealed at the DNA/RNA level (Semmes et al. 2006). In addition, O.John (2006) suggested that the molecular changes arising in the tumour cell can be utilised to provide biomarkers that should guide the treatment course on the molecular level. Thus, there has been an impressive emergence of mass spectrometry based technology applied to the study of proteins; equally notable is the rapid adaptation of these technologies to the biomedical approach to the realm of clinical proteomics.

The applied proteomics technologies promise improved care to patients via biomarkers. Biomarkers can not only contribute to the earliest detection of disease but can also be used for (**I**) determining cancer risk, (**II**) stratifying disease stage and grade and (**III)** monitoring response to therapy.

1.10.1 The Role of Proteomics in Breast Cancer

A considerable amount of literature has been published on proteomics. These studies highlighted the role of proteomics in identifying new disease related biomarkers. Therefore, in the multiple reasons outlined, proteomics is already assisting for cancer diagnosis and the development of targeted treatment (Pei et al. 2007, Shi et al. 2006). Tumour cell lines are rich in cancer related proteins and it is for this reason that in-vitro tumour cell lines were first selected to study breast cancer and prostate cancer. It is estimated that approximately 30-35% of all open reading frames of the human genome encode polytypic transmembrane proteins (Whelan et al. 2009, Hirokawa et al. 1998).

However, membrane proteins remain under represented in proteomics studies due to poor water solubility, making the separation and mass spectrometry analysis difficult (Speers and Wu 2007). Recent trends in proteomics technique could help to overcome these limitations, for example, the development of the orbitrap mass analyser. By combining the new technology mass spectrometry could be applied to large-scale identification, characterisation and quantification of the proteome and the membrane proteome (Scigelova and Makarov 2006), for instance, the hybrid LTQ/Orbitrap system has the ability to detect as many as 2000 proteins in a single experiment. In proteomics experiments, a protein mixture is digested into peptides, separated and analysed by MS/MS (Aebersold and Mann 2003).

Peptide identification and mass spectrometry accuracy could constrain the sequences of peptide candidates (Zubarev et al. 1996) and increase the accuracy provided by the hybrid

system such as the LTQ/Orbitrap in reducing the identification of false positive peptide. However, the identification of peptide rarely covers the whole protein sequence which makes the detailed protein characterisation very difficult, in particular when it comes to the determination of post-translation modifications. All these limitations apply to the proteomics studies of breast cancer and TNBC. Despite the heterogeneity of TNBC, it should be noted that the data analysis generated from proteomics experiments has highlighted significant expression of candidate proteins. This finding suggests that combining proteomics data analysis screen with western blotting provides a strong approach for elucidation of the heterogeneity mechanism of TNBC.

1.11 The Target Proteins

Many prior studies have noted the importance of membranes in cell biology, providing a physical barrier between the cell, the environment and the various subcellular compartments (Leth-Larsen et al. 2010). The multi-display functions of cell membranes are mainly fulfilled by organised protein systems called membrane proteins. Approximately, 30% of the genome is encodes membrane proteins in eukaryotic cells, which control signalling and energy transduction e.g. control of communications between cell-cell intracellular ion concentration, excretion of cytotoxic substances and receptor signalling pathways (Stevens and Arkin 2000, Kabbani 2008).

Previous studies have reported that the cell surface membrane proteins or plasma membrane proteins are permanently bound to the lipid bilayer as integral or peripheral proteins, whereas others are associated to the membrane only briefly and under specific

conditions (Escribá et al. 2008). Kabbani et al. (2008) explained that plasma membrane proteins are trans-membrane proteins that bind hydrophilic signalling molecules that cannot cross the lipid bilayer and ultimately act as signal transducers to regulate cell processes.

1.11.1 The Role of Scribble Protein in Triple Negative Breast Cancer

Scribble protein plays an essential role in the maintenance and regulation of epithelial tissue polarity (Bilder and Perrimon 2000). By interacting with mitogen-activated protein kinase of the ERK family, it down-regulates their activation and ability to migrate to the nucleus. Nagasaka et.al (2010) revealed that Scribble protein is localised at the basolateral membrane and that it has the ability to inhibit the transition from growth G1 to synthesis S in the cell cycle. One of the hallmarks of cancer is a loss of cell polarity and subsequent tissue disorganisation as shown in Figure 1-6).

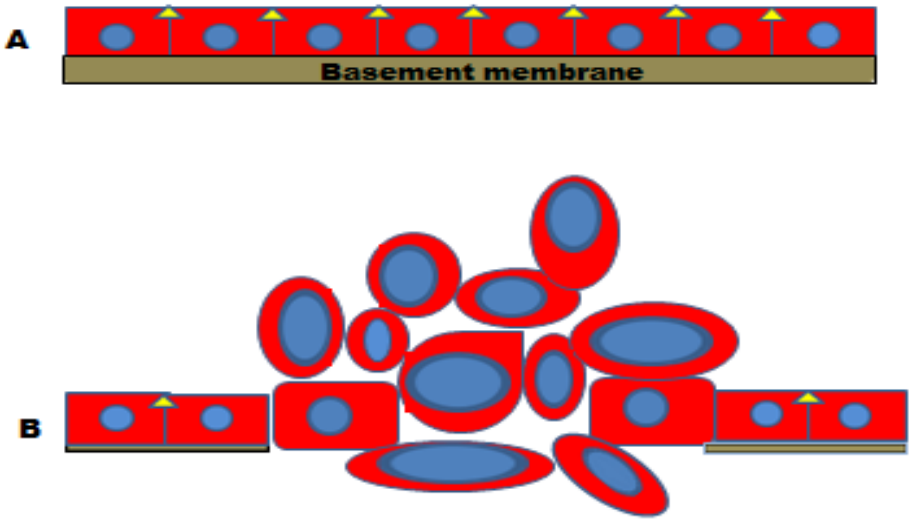


Figure 1-6 Disruption of cell polarity and tissue disorganization

This is a hallmark of advanced epithelial tumour. (A) Normal simple epithelium comprises a monolayer of individual cells that display distinct apical-basal polarity. Cells are tightly packed and connected to each other by the apical junctional complexes (blue), which separate apical (red) and basolateral (green) membrane domains. (B) Cells in high-grade epithelial tumours display loss of apical-basal polarity and overall tissue disorganization (Lee and Vasioukhin 2008).

Information available on cell polarity stems from a study on *Drosophila melanogaster* polarity proteins such as Lgl, Dig and Scribble (Boone 2008). These are potent tumour suppressors and the loss of these proteins results in neoplastic transformation of the epithelial tissues which are distributed, lining the internal and external surfaces of our bodies. Additionally, each biological function is achieved by the distinct structural organisation of epithelial cells within those tissues to create the integrity of their architecture. However, it remains unclear whether a loss of cell polarity is a consequence or a cause of cancer (Bilder and Perrimon 2000).

Royer and Lu (2011) revealed that the majority of human cancers are initiated from epithelial tissue and display loss of cell polarity and as consequence tissue disorganisation. A number of studies has found a decreased expression or loss of Scribble protein in primary tumours in human patients (Cavatorta et al. 2004). Michaelis et.al (2013) suggested that Scribble protein acts as a scaffold protein and is associated with the β catenin/cadherin complex. It is phosphorylated on canonical MAPK sites as well. Deficiency in Scribble protein results in the loss of epithelial apico–basal polarity manifested in the misdistribution of apical polarity and adherence junctions to the basolateral cell surface and abnormal cell growth. Moreover, Scribble regulates planar cell polarity of epithelial tissue and epithelial cell proliferation. Scribble is involved in the regulation of the microfilament networks and it is worth noting here that early proteomics studies using two-dimensional gel electrophoresis, carried out on breast cancer patients versus healthy controls, showed significant alterations, particularly stable for protein spots related to microfilament network proteins group.

These microfilament networks play essential roles in cellular functions, such as, adhesion, cell-cell interaction, proliferation, motility, migration and differentiation. In addition, the architecture of microfilament networks are functionally connected with each other and are known to be highly relevant for the tumourigenesis stages, such as, precancerous lesions which include enhanced metastasis potential malignancies, particularly when associated with loss of actin and poorly arranged actin-skeleton organisation (Cong-Rong Liu et al. 2004).

1.11.2 Epidermal Growth Factor Receptor (EGFR) in TNBC

A number of studies have identified Epidermal Growth Factor Receptor (EGFR) protein over-expression in 16%-36% of breast cancers. However, systematic studies evaluating gene amplification mRNA expression and protein expression in the same set of cases is lacking. EGFR is one of the four transmembrane growth factor receptor proteins that shares similarities in structure and function (Bhargava et al. 2005). The EGFR gene is located on the short arm of chromosome 7 and encodes a 170kDa trans-membrane protein. EGFR consists of an extracellular EGF-binding domain, a short trans-membrane region and an intracellular domain with ligand activated tyrosine kinase activity (Cohen et al. 1982).

In cancer biology, identification of the low abundance proteins is essential and many fractionation methods have been explored, including analyses by poly acrylamide gel electrophoresis which has become a standard tool in the molecular biology laboratory. Previous studies have identified that EGFR is known to be over-expressed in TNBC (Gluz et al. 2009, Liedtke et al. 2008). The question remains whether EGFR is a valid target; since many of the EGFR tyrosine-kinase inhibitors used on MBC showed only a 5% response rate. Furthermore, the European Society of Medical Oncology study (Pirker et al. 2010) on EGFR–targeted therapy where the related study examined results from 173 metastatic TNBC patients who were randomised to receive cetuximab, an anti-EGFR antibody, or cisplatin alone.

An overall, response rate of 20% was seen in patients who received the combination compared with a response rate of 10.3% in the cisplatin–alone arm. These promising clinical data suggested that EGFR is an important target in TNBC.

1.11.3 Signal Transducer and Activator of Transcription 1 (STAT1)

Previously, studies have discovered the importance of Signal Transducer and Activator of Transcription 1 (STAT1) in cancer. STAT1 belongs to a family of transcription factors which transduce signals generated by a wide variety of extracellular stimuli and are involved in many mechanisms, such as, the regulation of cell survival, proliferation and differentiation in nearly all tissue types (Ihle 2001). A search of the literature revealed some studies that have shown STAT1, as a pro-immune and anti-tumour transcription factor, and acts in the tumour suppressor role. However, other studies have shown that STAT1 has been found to be up-regulated in late stage human cancers including breast cancer (Manavi et al. 2007).

Additionally, It was discovered that STAT1 regulates DNA repair pathways as a predictive marker for breast cancer chemotherapy and radiotherapy resistance (Khodarev et al. 2004). Although, extensive research has been carried out on STAT1, a great many unanswered questions remain. Most studies were concerned with the action of single extracellular signalling polypeptides, but cells in tissues are often exposed to multiple agents simultaneously, for example, increased cAMP can blunt the activation of the STAT1 family, and prior treatment of cells with granulocyte-macrophage colony-stimulating factor

(GM-CSF) can prevent 1L-6 induced activation of STAT1. In addition, it still remains unknown how STAT1-dependent transcriptional initiation is regulated (Darnell 1997).

Existing research recognises the critical role played by STAT1 on the immune-system as a regulatory factor to the numerous genes involved in promoting chronic inflammatory disease, and inhibiting STAT1 signalling for the treatment of autoimmune disease is an active area of research. Both isoform (α and β) are highly expressed when the IFN-signalling pathway is activated and are associated with a tumour suppressor and pro-apoptotic function in some experimental system (Khodarev et al. 2004). STAT1 over-expression has been demonstrated in several human cancers, including head and neck cancer (Buettner et al. 2002) and breast cancer (Greenwood et al. 2012). In terms of cancer, STAT1 has been associated with an anti-tumour effect; however, accumulating evidence has linked increased STAT1 activation with increased tumour progression in multiple types of cancer, such as breast cancer (Hix et al. 2013).

1.11.4 The Role of STAT1 in breast cancer

On the basis of the evidence currently available, a large body of studies show correlated tumour –up-regulation of STAT1 with advanced breast cancer. Previous work of the Brown group (2007) had demonstrated increased tumour STAT1 immunostaining especially at the tumour stromal borders in human breast tumour specimens when compared with normal breast tissue. In addition, he found invasive carcinoma biopsy samples from human breast tumour sections show increased phospho-STAT1.

The immunostaining method observed significant staining and provides conclusive evidence that increased tumour STAT1 activation is proportional to an increase of disease progression in human breast cancer (Hix et al. 2013). Throughout this literature, new evidence reveals that STAT1 overexpression in breast cancer; might have a tumour promoting rather than tumour suppressor role (Kovacic et al. 2006, Perou et al. 1999).

Several studies in multiple cancer models have implicated constitutive STAT1 activation as tumour promoting, for example, STAT1 overexpression in human squamous carcinoma cells was found to induce pro-survival genes and resistance to genotoxic stress. The Ming Zhang group (2013) has used mouse carcinoma cell lines and mouse models to study the mechanism of STAT1 modulation on tumour progression. This group has been studying these models which were created with varying STAT1 protein expression levels and they showed that STAT1 activates anti-proliferative and pro-apoptotic genes, which has been classically associated with anti-viral and anti-tumour immunity. In addition, loss of STAT1 is associated with breast cancer development based on data using STAT1-/- models. However, it is essential to mention that the immune system in wild type mice does not function exactly the same as that in STAT-/-model. Based on these studies, STAT1 over-expression in breast cancer may have a tumour- suppression role (Hix et al. 2013).

In addition, this author demonstrated in human breast cancer biopsies that tumour cell expression of constitutively active STAT1 correlates with increasing disease progression from DCIS to invasive carcinoma. The question is what is the mechanism by which STAT1 promotes tumour progression in an immunocompetent model? Various methods have been

developed and introduced to measure STAT1 and to study its potential role in the regulation of DNA repair pathway genes and regulation of numerous pro-inflammatory cytokines and chemokines. Acute inflammation is highly beneficial for antitumour immunity. On the other hand, chronic inflammation is an established driver of tumourigenesis and is highly correlated with metastatic tumour progression (Hix et al. 2013). The more surprising correlation is with STAT1 signalling, which is implicated in several inflammation-driven diseases such as rheumatoid arthritis (de Prati et al. 2005).

1.12 CD74

While a variety of definitions of the term CD74 have been suggested, this literature review uses a trans-membrane glycoprotein that is associated with Major Histocompatibility Complex (MHC) class II and is an important chaperone that regulates the presentation of peptide antigen to T-cell receptors for the immune response. CD74 is present on the immature major histocompatibility class II complex where it blocks the peptide-binding groove. In addition, CD74 is expressed at a high level by antigen-presenting cells including B-cell monocytes, macrophages, and dendritic cells in normal tissues (Tian et al. 2012, Leng et al. 2003).

Although, cell surface expression of CD74 is low in many cell types, rapid internalisation with concomitant re-expression at the cell surface provides a steady-state level of CD74 at the cell surface that is sufficient for biological function (Tian et al. 2012). There is a growing body of literature showing that CD74 is proteolytically cleaved and removed at an intracellular site, which allows antigenic peptides to bind and the resulting mature MHC II is

then is transported to the cell surface. Recently, researchers have shown an increased interest in CD74 after investigating the relationship and molecular features in gastric cancer; Zheng et.al (2012) found that upregulation of CD74 is associated with increasing clinical stage and it provides an opportunity as a novel gastric cancer chemoprevention and treatment strategy. However, the clinical implications of CD74 as a marker for breast cancer are still unclear (Zheng et al. 2012).

1.12.1 CD74 and the function of Scribble

Metodieva et al. (2013) reported that over-expression of CD74 is linked to increased invasion and metastasis of breast tumours, particularly the tumours of TNBC. Furthermore, the over-expression of CD74 is a frequently observed phenomenon in breast cancer and is a direct cause for deregulation of Scribble. Interestingly, over-expression of CD74 leads to functional interaction with Scribble which affects not only the total abundance of Scribble, but also the pattern of its post-translational modifications in the C-terminal part of the protein. In a short review about post- transcriptional level, ribonucleic acid (RNAs) are linked with RNA-binding proteins (RBPs) to form ribonucleoprotein complexes in the cell (Tymoszuk et al. 2014). The RNA-binding proteins play essential roles in the biogenesis, stability, function, transport and cellular localisation, by influencing the structure and interactions of the RNAs; for example, a large number of RBPs in Eukaryotic cells are encoded and each has a unique RNA-binding activity and protein-protein interaction characteristic.

1.13 Mx1

A considerable amount of literature has been published on the Mx1 protein. These studies recognise the capacity of MX1 protein to inhibit the multiplication of many RNA viruses (Haller and Kochs 2002). Furthermore, Mx1 is an Interferon-induced GTPase, Interferons (IFNs) induce the synthesis of numerous proteins; some of which have antiviral activity and different subcellular localization and antiviral specificities (Der et al. 1998).

This type of activity is linked to the GTPase binding domain of the Mx1 protein. Additionally, the ability of the Mx1 protein of dot-forming is required in order to exert their antiviral activity against the influenza virus and other orthomyxoviruses (Engelhardt et al. 2004). Sequence analysis indicated that the Mx1 protein consists of 631 amino acids 30% of which are charged and contain at the a nuclear localisation signal at the carboxyl terminus that is essential for translocation of the Mx1 protein into nuclei (Noteborn et al. 1987). The Mx family proteins appear to have an important cellular function because Mx1 homology proteins have been identified in a wide variety of animal species including monkeys, pigs, cattle and rabbits (Horisberger and Gunst 1991).

Furthermore, the murine Mx1 protein is homologous, not only in the Mx family proteins, but also to two other cellular proteins (Nakayama et al. 1991). One is the product of yeast vesicular sorting protein VPS1 (Clary et al. 1990), whilst the other is the rat microtubule associated mechanochemical enzyme dynamin (Obar et al. 1990). Along this line, the Mx1 protein may therefore be involved in either protein sorting or intracellular motility. It was

clearly shown that MX1 is over-expressed in metastatic TNBC (Greenwood et al. 2012) as well as, in metastatic colorectal tumours and it is shown to be required for wound healing by cultured colorectal cells (Croner et al. 2014).

1.14 Combined Role of EGFR, STAT1 and Scribble as a Functional Group of Proteins in TNBC

As discussed above, several protein candidates show different expression between metastatic and non-metastatic breast tumours and cell lines. Although extensive research has been carried out on these proteins as single roles, recent studies found that over-expression of CD74 interferes with Scribble that is known to causes the tumour suppressor protein to shift its localisation from the original site in basolateral membrane and the sites of cell to cell contacts to the cytoplasm and the apical membrane. In addition, the major effect of this mechanism is to lead to down-regulation of Scribble in the long run, as the protein abundance decreased, when CD74 was over-expressed for a prolonged period. Thus, it appears to cause an overall decrease of Scribble abundance (Metodieva et al. 2013, Metodieva et al. 2016). Moreover, Scribble regulates planar cell polarity of epithelial tissue and epithelial cell proliferation. However, as pointed out previously, it remains unclear whether a loss of cell polarity is a consequence or a cause of human cancer.

Royer and Lu (2011) highlighted that the majority of human cancers are initiated from epithelial tissue and display loss of cell polarity and consequent tissue disorganisation. A numbers of researchers have found decreased expression or loss of Scribble protein in

primary tumours in human patients. Thus, a correlative study of CD74 overexpression and Scribble expression and/ or localisation would provide additional mechanistic information in this direction. Furthermore, as our knowledge regarding molecular biomarkers for breast cancer increases, prognostic indices are developed and combine the predictive power of individual molecular biomarkers with specific clinical and pathologic factors; in breast cancer, STAT1 expression was a predictive marker for poor survival as well as chemotherapy and radiotherapy resistance (Khodarev et al. 2004, Weichselbaum et al. 2008).

The expression syngeneic transcription mouse model of breast cancer showed a significant up– regulation of STAT1 and other IFN-y-activated genes in the highly metastatic TM-40D-MB tumour cells. Moreover, this experiment showed that constitutive overexpression of STAT1 in low metastatic TM-40D-MB cancer cells promotes aggressive tumour growth, where by knockdown of STAT1 in highly metastatic TM-40D-MB cell line significantly delays tumour growth. Thus, STAT1 could be combined with Scribble and CD74 in our proposed panel. Potential targets for treatment of TNBC include surface receptors such EGFR or c-KIT protein kinase components of MAP-kinase cascade and PEK B (AKT) pathway.

This type of cancer might initiate a more sensitive induction of DNA damage by specific chemotherapy agents that cause inters strand and double strand breaks and the inhibition of already defective DNA repair, such as Poly ADP- Ribose Polymerase1 (PARP1). Rakha et al. (2007) suggested that for TNBC, nodal status, tumour size and AR expression are

the most useful prognostic markers. This is because when they stratified the cases into lymph node-positive and lymph node negative subgroups, they found that in the lymph node-positive tumours, both size and AR expression retained their prognostic value. However, in the lymph node-negative group, basal phenotype was the sole marker that showed the prognostic value, whereas other markers, including the patient's age, tumour size and AR expression were not significant. In my study, I propose to add EGFR to the group of the other marker/target proteins discussed above, as EGFR is frequently overexpressed in TNBC and a target of approved rational based therapies. It is be interesting to see how EGFR expression relates to Scribble, CD74, Mx1 and STAT1 expression.

1.15 Immunocytochemistry (ICC)

Immunostaining is a fundamental technique used in many biological, medical and veterinary laboratories for the diagnosis and research of infectious and neoplastic diseases. Over the past century, there has been a dramatic increase in the ability to detect Ag in tissue sections, mainly by countering the deleterious effects of formaldehyde with Ag retrieval and increasing sensitivity of the detection system (Ramos-Vara 2005). However, the first paper on this topic had been published previously (Coons et al. 1941).

There is a very large body of literature on immunocytochemistry. It is in essence a microscopic imaging technique used to assess the presence of a specific antigen in cells by using a specific antibody for visualising and quantification purposes (Hirohashi et al.

2010, Ramin et al. 2012). Immunocytochemistry (ICC) is a variant of immunohistochemistry (IHC), which uses cultured cells instead of tissue sections. ICC is increasingly recognised as a prominent laboratory technique for evaluating the presence of a specific protein Ag in cells by detecting specific Ab-Ag interactions, where the antibody has been tagged with a visible label (Normanno et al. 2009).

The binding process of antigen (protein) with antibody is very similar to a key and lock which make them useful for demonstration and examination of both the presence and the sub-cellular localisation of an antigen and as a therapeutic agent (Oliver 2008). Although the sensitive immunological, molecular procedure and cancer bio markers (diagnostic markers) have been developed to detect the single tumour cells in lymph nodes during the early stage of metastases, it has certain limitations in the sensitive technology to detect the cancer cells in the early diagnosis from minute quantities of available tumour tissue from patients. Moreover, the lack of sensitive technology platforms has considerably slowed the identification of reliable biomarkers to accurately diagnose most types of cancer (Wardwell and Massion 2005).

In research, test detecting the anti-body epitopes in total cell homogenates by western blotting has been the preferred application. The protein blotting procedure has been expanded upon other applications for physical chemical analysis of protein-protein interactions including the detection of receptors after binding of their respective ligands (Burnette 1981, Karey and Sirbasku 1989).

1.15.1 Immunochemical Studies of TNBC:

TNBCs are heterogeneous diseases and are classified as estrogen receptor alpha (ERα)-negative, progesterone receptor-negative, and HER2/ERbB2/NEU-negative lesions (Prat and Perou 2011, Wang et al. 2019). Emerging data clearly indicate that TNBC is a heterogeneous class with a variable prognosis according to clinical, pathological and genetic factors (Montagna et al. 2013). Patients with TNBC experienced high rates of recurrence only in the period from one to four years after diagnosis and the risk of recurrence declined rapidly thereafter. No recurrence occurred after eight years of follow-up while in other breast cancer patients, the risk of recurrence and death was steady and continued for 17 years after diagnosis (Dent et al. 2007).

Moreover, TNBC patients had relatively large tumours (> 2cm) and a high rate of lymph node invasion (54%). Statistically, as the tumour size increases, the rate of node invasion also increases, but this relationship was not seen among the TNBC patients. A major problem with triple negative tumour studies is that TNBC patients and experimental methods are limited by small sample sizes and short follow-up times. To some extent, the reason for that is the determination of TNBC phenotype relies on IHC, ICC staining of tumour slides and breast cancer cell line respectively. Furthermore, the triple negative tumour category is composed mostly of BL cells; it is therefore possible to classify with accuracy the majority of BL breast cancer and TNBC using IHC markers (Dent et al. 2007).

Nielsen et.al (2004) found that BL breast cancer was composed almost entirely of TNBC based on a comparison of IHC profiles. Using their definition basal-like carcinomas are

negative for ERα, Her2 and positive for either cytokeratin or EGFR. Abd-EL-Rehim DM (2004) emphasises the expression of an additional high molecular weight protein as positive or negative could be used to identify these carcinomas. Therefore, a combination of immunostaining for ERα and PgR would correctly classify the majority of breast cancers. Thus, by using a group of pathological markers, it could possible to correctly diagnose and assess the prognosis of the triple negative category of breast cancer, which exhibits a distinct pattern of recurrence. Recent trends in breast cancer have led to a proliferation of such studies attempting to go beyond the one bio-marker approach.

The knowledge gained through genetic and transcriptomic profiling of cancer tissue and cell lines or cell lines panels is also growing and provides experimental models to study specific subgroups of breast cancer. This trend is likely to have the greatest impact on improving the outcome for breast cancer patients. In addition, this may prove to be an important enabler of the concept of using combination therapies for advanced breast cancer (McClelland et al. 2001).

Data from literature indicate that the diagnosis of specific types of breast cancer might be associated with a different outcome if compared with other breast cancer subtypes. Additionally, the recognition of certain special types of invasive breast carcinoma might allow the identification of women with extremely good or extremely poor prognosis and may have therapeutic consequences. Recent developments in the study of the pattern of protein expression in triple negative tumours by ICC have been made, and the reasons are (**I**) the ICC method is particularly useful in identifying the cell type and the origin of metastasis to

find the site of the primary tumour and (**II**) the ICC method is widely available and has been used in many diagnose of the tumour site and the measurement of the stage and grade of tumours has improved.

1.16 Breast Cancer Metastasis and CRISPR-Cas9

As previously discussed breast cancer is a heterogeneous disease and comprises several distinct entities. Several reports have detailed the effects of the role of Epithelial-Mesenchymal Transitions (EMT) on breast cancer which is the principle mechanism associated with the generation of cancer steam-cells development of treatment resistance and the initiation and progression of metastasis. EMT is the principle mechanism involved in metastasis and tumour invasion. Most tumours contain tumour initiating cells (TICs), which help to self-renew and re- generate all the cell types within a tumour, for instance, (TICs) are found in breast cancer women and indicate their intrinsic therapeutic resistance. Additionally, gene transcription reveals that TICs identified with “claudin-low” intrinsic molecular subtype of breast cancer and a high expression of markers that linked with EMT (Creighton et al. 2010).

The literature review defines the EMT as a process, in which cells acquire molecular alterations, such as dysfunctional cell–cell adhesive interactions, junctions and more spindle-shaped morphology. These molecular alterations may promote cancer cell progression and invasion into the surrounding microenvironment. Targeting specific molecular pathways, such as Notch, Wnt, and TGFß is associated with the development

and EMT in the TIC subpopulation. Two novel concepts have emerged in cancer biology; first, the role of the so-called ‘‘cancer stem cells’’ in tumour initiation, second, the involvement of an EMT in the metastatic dissemination of epithelial cancer cells. Moreover, by studying the mammary tumour progression model, scientists have shown that cells possessing both stem and tumourigenic characteristics of ‘‘cancer stems cells’’ can be derived from human mammary epithelial cells that lead to activate the Ras-MAPK pathway (Morel et al. 2008). In the immortalised human mammary epithelial cells model, (EMT) Induces and increases the ability of cells to form mammospheres, and in the expression of stem cell and (TIC) markers. Additionally, (EMT) was first recognized as a crucial feature of embryogenesis, which converts epithelial cells into mesenchymal cells through profound disruption of cell-cell junctions and extensive reorganisation of the actin cytoskeleton (Mortazavi et al. 2008). Wellner (2009) explained that EMT is governed by complex networks and is influenced by signals from the neoplastic microenvironment.

Additionally, in vitro model studies showed that a variety of cytokines, including TGFb and growth factors like Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF) or Fibroblast Growth Factors (FGFs), can trigger EMT after activation of their cognate receptors in specific cell types. Moreover, growth factors transduce signals by activating the cognate receptor tyrosine kinases and their central downstream effector of Ras, which provides a rationale for the cooperative effect of Ras and TGFb in EMT promotion (Gregory et al. 2008). The experimental model of breast cancer progression discovered that the introduction of an activated version of Ras constitutes the initial event, which sensitises the mammary epithelial cells to EMT. Weinberg (2007) reported that the tumourigenicity of

experimentally transformed mammary epithelial cells is highly dependent upon the cell type of origin. When exposed to microenvironmental signals, these cancer stem cells would display motility capacities due to EMT.

The expression of intracellular adhesion molecules and other characteristics of an epithelial phenotype is thus lost. This subtype of breast cancer is highly motile and possesses steam-cell like properties such as a high degree of resistance to radiation and chemotherapy (Pradhan et al. 2010, Guo et al. 2008). As a result of epithelial-to-mesenchymal transition, a complex set of changes in the cancer cell, such as tumour metastasis occurs through changes in the cytoskeleton, integrin signalling and protease expression, which further promote invasion (Sulzmaier and Ramos 2013). Clearly, once tumour cells move across the vascular endothelium into new tissues, alterations in the cell death pathways protect the cells from apoptosis and permit the growth of metastasis (Kang et al. 2010). Florian J (2013) reported the analysis should include iso-form specific gene silencing using multiple shRNA/siRNA that target different sequences or genetic deletion or CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat) to determine which isoforms function in migration-invasion and metastasis in a specific cancer.

1.17 The Role of Genetic Alteration and Mutation in TNBC

Recent studies of tumour lymphocytic immune infiltrates in breast cancer have suggested an improved prognosis associated with increasing levels of Tumour Infiltrating Lymphocytes (TIL). This type of breast cancer may be immunogenic for several reasons for

example, TNBCs have a significant number of genetic mutations and the immune system may see the aberrant proteins encoded by these mutations as foreign. In addition, TNBC is associated with a prognostic gene signature (Stanton 2015).

The recent trends in the analysis of the Cancer Genome Atlas Network (2012) have led to a proliferation of studies on the molecular heterogeneity of breast cancer associated with somatic mutations and stromal micro environmental elements. This trend in molecular biology has permitted researchers to inquire into the heterogeneity of breast cancer, revealing that this disease requires the interconnection of several signalling pathways the cellular microenvironment and innate characteristic, which plays an essential role in outcome and patient treatment responses (Eroles et al. 2012).

1.18 The Ways of Genetic Manipulation

Improvements in molecular analysis have allowed researchers to sequence all human genomes and cancer genomes. Additionally, the rapid and sophisticated development in molecular biology may be approaching the ability to individualise diagnosis and cancer treatment (Russnes et al. 2011). Currently, advances in the Next- Generation Sequencing (NGS) use parallel sequencing to generate hundreds of millions of short (36 to 150) bp DNA reads (Mardis 2011, Meyerson et al. 2010).

Furthermore, the field of genome engineering holds enormous potential for applications in cancer research and biotechnology, such as in genome editing, and have led to a proliferation of studies that programme sequence specific endonuclease, which facilitates

precise editing of endogenous genomic loci (Ding et al. 2013, Soldner et al. 2011). Improved understanding of the mechanism of DNA repair has enabled the development of improved site-specific genome editing techniques, including Zinc- Finger Nucleases (ZFN), Transcription Activator- Like Effector Nucleases (TALENs) and the RNA-guided CRISPR/Cas9 nuclease system (Wood et al. 2011, Sanjana et al. 2012, Cho et al. 2013). ZFN and TALENs technologies rely on a strategy of tethering endonuclease catalytic domains to modular DNA–binding proteins for inducing targeted DNA cleavage at specific loci (Ran et al. 2013).

Subsequently, DNA Double-Strand Breaks (DSBs) at specific loci are re-ligated and repaired by the error-prone Non-Homologues End Joining (NHEJ) pathway or by alternative major DNA repair pathway. The homology-directed repair (HDR), which creates mutations (insertion or deletion) or provides a defined modification at a target locus in the form of conventional dsDAN or a single stranded DNA oligonucleotides (ssDNA). Both the NHEJ and HDR are active in dividing cells, but the level of efficiency can differ widely, depending on the cell type, genomic locus and repair template (Saleh-Gohari and Helleday 2004).

Genetic engineering and gene targeting technologies are able to selective knockout any gene of interest, thereby playing important roles in gene therapy and disease-modifying. These technologies permitted the generation of mammalian models that express specific human disease-associated proteins by replacing the normal gene with one containing a

specific mutation (Matano et al. 2015). ZNFs and TALENs use engineered sequence-specific protein DNA binding domains fused to a nuclease.

The DNA binding domains are identified and designed to provide the target specificity and the nuclease introduces double stranded DNA breaks in the neighboring sequence. Consequently, the double stranded breaks can be repaired either by blunt-end, NHEJ to introduce random mutations, or by homologous DNA repair by adding an engineered fragment of donor DNA with homology on either side of the DNA break and encoding a specific point mutation or insertion (such as cre recombinase, green fluorescent protein) (Chauvin 2018).

CRISPR (clustered, regularly interspaced, short, palindromic repeats)/Cas (CRISPR-associated) systems are RNA-based bacterial defense mechanisms designed to recognize and eliminate foreign DNA from invading bacteriophage and plasmids (Wang et al. 2013); with the main role of Cas/9 endonuclease is directed to cleave a target sequence by a guide RNA (gRNA). CRISPR/Cas9 is a cheaper genome-editing solution than TALENs. CRISP technology has two primary drawbacks.

First, CRISPR is more susceptible to off-target effects the unintentional modification of non-targeted genes, while, TALENs is a highly specific gene editing technique because cleaving activity requires a TALEN pair to dimerise before the FOK1 nuclease creates a DSB (Kopischke et al. 2017). By contrast, only one CRISPR gRNA is required to engage in DSB. What CRISPR lacks in specificity, it makes up for in simplicity. Additionally, the double nikase modification of the technology provides the needed high specificity.

Second, CRISPR-Cas9 can target DNA sequences with only a 70% probability of success, given a random stretch of DNA sequence while TALENs technology can, in theory, edit any portion of the genome (Stephens and Barakate 2017). Evidence suggests that CRISPR has captured the attention of the research community through the ability to study; the effectiveness of gene-editing to target cells and thereby gives CRISPR a competitive advantage relative to TALENs.

1.19 The Microbial Adaptive Immune System and the Cas9 RNA-Guided Nuclease

Recently genome editing using the bacterial clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system has emerged as the most efficient technology. This uses RNA guided nuclease to cleave foreign genetic elements (Cong et al. 2013, Garneau et al. 2010). There are three types of CRISPR system type I CRISPR, type II CRISPR and type III CRISPR. These can vary, depending on specific proto-spacer adjacent motif (McClelland et al. 2001) requirements such as *S.thermophilus* (5´-NNAGAA) for type I CRISPR, *S.pyogenes* (5´- NGG PAM for type II CRISPR and 5´- NGGNG for type III CRISPR) (Jinek et al. 2012).

However, type II CRISPR adapted from *S. pyogenes* is the most widely used representing a system that is highly specific, easy to design, efficient for a variety of cell types and organisms (Brouns et al. 2008) (Figure 1-6).

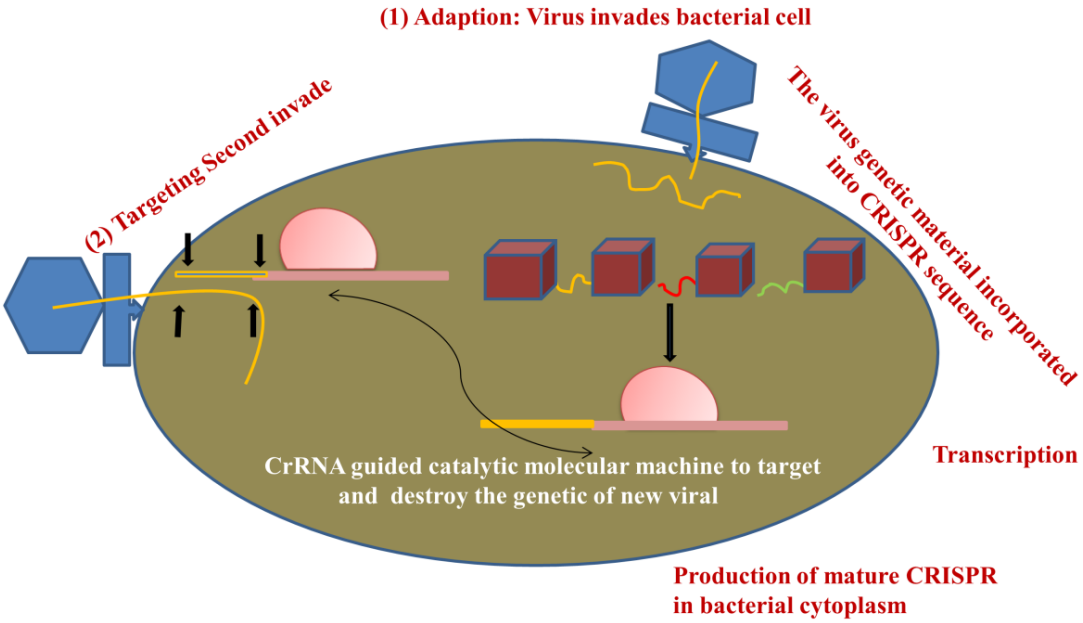


Figure 1-6 Summary of type II CRISPR /Cas9 system.

(A) The microbial adaptive immune system uses RNA-guided nuclease to break down foreign genetic segments by incorporating into the CRISPR array and is transcribed as short cr-RNA in the cell cytoplasm, which complementary binds the foreign genetic segment according to Watson -Crick base pairing. Plural, cr-RNA transcribes from CRISPR array as dose trans- activating crispr-RNA (tracr-RNA). (B) The two RNA structures (cr-RNA and tract-RNA) direct the CRISPR /Cas9 to associate and introduce double-strand (dsDNA) breaks in target DNA.

1.20 Prokaryotic CRISPR System and Control of Horizontal Gene Transfer (HGT)

There is a large volume of published research describing the Prokaryotic CRISPR system as an adaptive immune system. By evolving numerous systems, prokaryotes control mobile genetic transfer of which the best known are restriction modification system and CRISPR/Cas9 (Strotksaya et al. 2015).

The transcription results from the incorporation of viral or plasmid DNA (mobile genetic element) with prokaryotic genetic material re-ligated to long pro-cr-RNAs, which is processed into short cr-RNA in the cytoplasm. The short cr-RNAs is complementary to the incorporated viral or plasmid DNA, according to Watson-Crick base paring. The CRISPR array consists of short repetitive sequences termed repeats, flanking similarly sized sequences termed spacers. Additionally, the flanking repeats act as molecular handles for recognition by Cas9 proteins which recognise the Proto-spacer-Adjacent Motif (PAM) (McClelland et al. 2001).

This is known as CRISPR/Cas9 type II. The Cas9 forms a complex after the complementary pairing between the repeat cr-RNA sequences with trans-activating (tract-RNA). The complex structure facilitates the processing of the CRISP array into discrete units and allows the Cas9 nuclease to cleave target dsDNA at specific sites on the genome (Jinek et al. 2012). The sg-RNA pairs can guide Cas9 to nick both DNA strands of the target loci to create a DSB which increases the specificity of target recognition (Ran et al. 2013). To identify the target DNA, the latter is always associated with the PAM (McClelland et al. 2001) and mutation in this sequence results in failure of the interaction between PAM and Cas9. Genome editing, using the type II bacterial clustered regularly interspaced short palindromic repeat and the CRISPR-associated Cas9 is highly efficient in human cell lines. CRISPR-Cas9 genome editing in primary human cells is more difficult and the reasons also remain unclear.

However, the transfection rates, promoter activity, exonuclease activity, interferons production may all be contributory factors (Hendel et al. 2015). Many researchers suggest that one way to improve the genome editing is to define Cas-9 as mRNA or protein rather than through a DNA expression plasmid, which creates a simple and complete RNA or RNAP-based delivering method for the CRISPR-Cas9 system (Dellinger et al. 2011).

The field of genetic engineering is being rapidly revolutionised by CRISPR-Cas9 and the technology allows researcher to easily alter and manipulate the organism genome and the recent trend is to use (CRISPR –Cas9) for the development of the next–generation model of human cancer, for example, CRISP-Cas9 has been applied in cell culture systems and in-vivo; according, this technology can be harnessed for rapid and precise engineering for both of Loss-Of-Function (LOF) and Gain-Of-Function (GOF) mutations in tumour suppressor genes, oncogenes, the modulation of cellular transformation and drug responses. The modularity and power of the CRISPR-Cas9 has led to systematical engineering of both LOF and GOF mutations in model human olorectal cancer (CRC). However, the introduction of independent mutations associated with human (CRC) did not fully reproduce the tumourigenic and metastatic characteristics of the human disease. This suggests that secondary genetic and/or epigenetic events are needed for complete malignancy (Matano et al. 2015).

Moreover, the flexibility of CRISPR-Cas9 provides a way to investigate combinatorial vulnerabilities in cancer cells and test epistatic relationships and synthetic lethal interactions systematically. Additionally, this technique allows for generating endo-genome

conditional alleles relying on the site-specific recombinases tagging endo-genome alleles and intergenic non-coding DNA elements (Mansour et al. 2014, Wang et al. 2013). CRISPR-Cas9 has been shown to be efficient in cells and in-vivo after using it to trigger two distant DSB in the same chromosome or in different chromosomes, which then led to inversion, deletion or translocation of the target sequences (Blasco et al. 2014).

Recent exploiting of CRISPR-Cas9 includes carrying out of high-throughput CRISPR screens using Cas9 nuclease and dCas9 effectors for the identification of genes that participate in biological phenotypes (Chen et al. 2015). David Sabatini and Eric Lander (2014) created a library of ≈ 73,000 sgRNA and utilized to target human genes to screen for genes involved in the DNA mismatch repair (MMR) pathway and also for genes that disrupt the resistance to the topoisomerase II A (top2A) poison etoposide. Furthermore, Feng Zhang (2014) created a library ≈ 65,000sgRNA targeting human genes which screened and identified relevant genes in both cancer cell lines and pluripotent steam cell. Thus, high-throughput screen utilising CRISPR-Cas9 can be used to uncover genes mediating a specific biological phenotype such as mechanisms of therapeutics as well as in-vitro and in-vivo screens to uncover genes involved in metastasis and tumour progression.

1.21 Cancer and Wound Healing

Previously, tumours have been defined and described as wounds that do not heal and consist of an inflammatory phase followed by epithelial cell proliferation and tissue remodelling. During tissue injury, replenishment of epithelial cell loss is ensured by the

proliferation of these stem cells and their progeny in response to pro-inflammatory cytokines. This means that the inflammatory processes and inflammatory microenvironment that occurs during wound healing is associated with the pathological state of many tumours and Cancer Stem Cells (CSC) (Arnold et al. 2015).

Many studies have identified CSC in solid tumours in the breast, brain and colon. These are thought to be a subpopulation of tumour cells that continually undergo differentiation to populate the heterogeneous tumour. The process of inflammation in the tumour microenvironment may contribute to tumour aggressiveness and treatment resistance by shifting the equilibrium between differentiating and de-differentiating tumour cells (Singh et al. 2004, Al-Hajj et al. 2003, Schatton et al. 2008).

One stage of wound healing is re-epithelisation of the damaged tissue when epithelial cells undergo EMT and migrate to the edges of the wound, however, the failure of this process results in improper-tissue re-modelling. The impaired wound healing is typical of disorders, such as diabetes mellitus, pressure necrosis and vasculitis (Singer and Clark 1999, Eming et al. 2007). Wound healing responses are linked to tissue injury have been shown to promote the growth of breast cancer: injection of fluid into the wound close to the tumour site in mice bearing syngeneic breast cancer xenografts resulted in increased inflammation, and has been linked to tumourigenesis tumour progression and metastasis in many different cancers (Stuelten et al. 2008). In addition, environmental stimuli, such as

chemokines secreted from the tumour cells are able to aid pro-tumour inflammatory cells and help to shape the pro-tumour immune-responses (Qian and Pollard 2010).

Most of the open access literature on the wound healing assay is focused on monolayer tissue; tumour structures are built on cell-cell contacts in three dimensional (3D) space. The close physical contacts to their neighbours enable tumour cells to move collectively and attach to a substrate. This is exemplified by the multicellular spheroid migration assay, which is a better model to the in-vivo situation. There are many different types of cells (fibroblasts, myofibroblasts, pericytes, endothelial cells and immune cells) that together comprise the tumour stroma and influence the invasive potential of cancer cells; all these interactions are essential to recapitulate the in-vivo situation as close as possible (Gaggioli et al. 2007).

The capacity of stem cells to differentiate enables the cyclic development, involution and development of mammary glands after pregnancy and lactation (Farnie and Clarke 2007). Several researchers have documented that small fragment rodent ducts or terminal end buds could develop into an entire and functional mammary tree. Moreover, these fragments have shown evidence that pluripotent stem cells give rise to both luminal and myoepithelial cells of the mammary gland after transplantation into many fat pads of a synergetic host (Ormerod and Rudland 1986).

A stem cell can be defined as an attractive candidate as an origin of cancer. There is a similarity between cancer cells and stem cells that include their ability to differentiate,

however, the mechanism of differentiation is highly controlled and regulated in the stem cells and is the direct opposite to the mechanism found in cancer cells. Additionally, many epithelial tissue experiments have shown that breast stem cells are capable of regeneration as a functional mammary gland system when the pattern of X-chromosome inactivation throughout the ductal and lobular epithelial is observed. Further studies showed that contiguous patches and epithelial with inactivation of the same X-chromosome were present in the human breast which clearly suggest that the cells within the patch had been derived from the same system of stem cells (Novelli et al. 2003).

Pardal clark (2003) revealed that the long life span of stem cells allow them to accumulate the mutations and epigenetic changes in normal highly regulated pathways, increase the malignancy within the cells and promote the activation of the self-renewal pathways. Regulatory pathways of stem cells that are dysregulated in cancer are Wnt, Hedgehog, N OTCH, LIF, TGF ß and EGFR, Prolactin, Estrogen and Progesterone regulated pathways. These pathways have formed the focus of much work as potential targets for therapeutic intervention and/or adjuvant therapy. Notch signalling pathway has been found to play a role as a mediator of cell-cell communication in many tissues (Stylianou et al. 2006) and is shown to assist in self renewal, cell fate, apoptosis, proliferation and migration (Politi et al. 2004).

To date, there are disagreement amongst researchers about the correlation between cell division and cancer risk, for either stem or non-stem cells. Another point of debate is the relative contributions of the intrinsic mechanisms and the extrinsic factor to tumorigenesis.

To illustrate the above, the strong mutagen, such as the radiation from nuclear fallout sources can impact on the lifetime risks for all cancers. In this case, it is clear that the proportion of cancer risk caused by intrinsic errors would be small, however, the current studies of cancer risks highlight the number stem-cell-division as contributing intrinsic factor as well (Tomasetti and Vogelstein 2015).

Many cells can play roles in increasing malignancy by secreting tumour-enhancing products. Additionally, vascularisation has long been recognised as playing supportive role. Adipocytes secrete collagen IV, which is subsequently cleaved to give a c-terminal fragment that promotes tumour growth. In the mouse model, the genetic ablation of collagen IV induced the aspersion of the Polyoma Middle T oncogene (PyMT) model of breast cancer in the mammary, which resulted in a reduced tumour burden and an inhibition of tumour progression(Allen and Louise Jones 2011) . Moreover, fibroblasts and hematopoietic cells (mast and macrophages) have been shown to enhance tumour cell growth especially, for those that are isolated from the tumour (Iyengar et al. 2005). Additionally, matrix metalloproteinase 9 (MMP9) that is synthesied by mast cells is involved in the reduction of the progression of benign tumour to malignancy.

1.22 Hypothesis and Research Questions

The main aim of the project has been to elucidate recently identified molecular pathways that contribute to metastasis in TNBC. Several protein candidates show differential expression between metastatic and non-metastatic tumours. We hypothesize that

overexpression of a specific group of proteins increases the metastatic propensity of TNBC. These proteins are Scribble, STAT1 and EGFR.

In particular, we aim to test whether deregulation of Scribble (by overexpression, loss or abnormal phosphorylation) combined with over-expression of EGFR contributes to invasion and metastasis and affects the aggressiveness of breast tumours. If our hypothesis is true tumours that over-express EGFR and deregulate Scribble might be targeted with specific inhibitors and better diagnostic tools can be developed based on measuring the abundance, localisation, and phosphorylation of these proteins. The present study is motivated by the need to take into consideration that the TNBC is not one disease; it is a group of diseases. TNBC represents heterogeneous subtypes of BCs, generally correlated with an aggressive clinical course and where targeted therapies are currently limited. To test our hypothesis, we will pursue the following research questions.

The first research question is to profile the expression of the chosen protein candidates in a group of breast cancer cell lines, such as MDA-MB-231, MDA-MB-468, ZR, MDA-MB-453 and MCF-7 enriched for those representing TNBC features. This will be assessed to define the cell lines that most closely capture individual examples of the heterogeneous TNBC phenotypes and use them as effective tools for drug discovery and/or biomarkers development.

The second research question is to develop quantitative assays Lc-MS/MS WB, ICC and IHC for detection of the proteins Scribble, CD74, STAT1, EGFR and MX1, which should

provide higher sensitivity and specificity for TNBC diagnosis than is afforded with single protein analysis. The third research question is to analyse breast tumour tissue samples and quantify the candidate group of proteins in a collection of TNBC tissue samples. The final research question is addressing how using CRISPR/Cas9 to modulate expression levels of selected targeted proteins in TNBC cell lines could assess the effect on wound healing.

The hypothesis tests if the specific group of proteins Scribble, STAT1 and EGFR correlate with the aggressiveness of TNBC and whether the study of this group of proteins provides higher sensitivity and specificity for TNBC diagnosis than is afforded with single proteins along with targeting some of the proteins in the group with inhibitors or monoclonal antibodies which can be an effective therapeutic strategy for tumours which over-express the group.

Summary and Context of Study

Triple negative breast cancer (TNBC) is increasingly recognised as a serious, worldwide public health concern. TNBC has the ability to recur after treatment and appears to be greatest in the first few years. Breast cancer of the triple negative phenotype is a dynamic disease that evolves with time and as a function of therapy. Moreover, the phenotypical changes of the metastatic TNBC represent a unique and a heterogeneous tumour cell population with special biological features that permit travel to distant sites and the establishment of a clinical disseminated disease. Triple-negative tumours do not express the nuclear hormone receptors such as estrogen-receptor ERα, progesterone receptor PgR nor the epidermal growth factor receptor Her2. However, in breast cancer, these three biomarkers are used clinically to guide treatment (Luo et al. 2010).

There are many phenotypical changes associated with malignant transformation, such as cell proliferation, adhesion and migration mediated or initiated by proteins linked with a plasma membrane which make these central in the biological process and a potentially effective drug target (Harvey et al. 2001). A combination of diverse proteins Scribble, STAT1 (Greenwood et al, 2012) and EGFR (Yang et al, 1996) are correlated with increased migration and invasion in breast tumour tissue and cultured cell lines. Previous studies utilising clinical proteomics have reported that overexpression of a set of proteins: STAT1, CD74, Mx1 and dysregulation of Scribble is associated with increased invasion and increased expression of membrane proteins involved in cell adhesion and cancer metastasis in TNBC (Metodieva et al. 2013, Greenwood et al. 2012). The main objective of

this study has been to use methods, such as in-gel digestion and mass spectrometry, western blotting, immunocytochemistry, CRISPR/Cas9, and wound healing assay to further elucidate the mechanisms involving these proteins that contribute to metastasis, using triple negative breast tumour sample and breast cancer cell lines. Our results could provide better clinical therapies in the future, could serve as the basis of screens to identify and stratify TNBC patients in the clinical setting and for the retrospective evaluation of the efficacy of known treatment agents with this tumour type.

Chapter 2 Materials and Methods

2.1 Cell Culturing

Breast cancer cell lines MDA-MB-435, MDA-MB-231, MCF-7, ZR, MDA-MB-468 and embryonic kidney cell line HEK293. Breast cancer cell lines and HEK293 were grown on RPMI-1640 (Roswell Park Memorial Institute -1640, Thermo Fisher Scientific) and/or Dulbecco's modified eagle medium DMEM (Dulbecco's Modified Eagle Medium from Lonza) supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific) at 37˚C and 5% CO_2 and split after 48 hr. The cells were grown to approximately 60%-80% confluency monolayer, detached, washed with phosphate-buffered saline PBS and stored at -80˚C until needed for analysis. This study was relied on low tissue culture passage (< 50) with 80% viable and (60%-80%) confluent monolayer on the day of transfection. HEK 293 cells are popular for their ease of growth and transfection, making them a common cell culture in cancer research. However, the tumourigenicity of the HEK 293 cell line reached 100% when the passage exceeded 65 (Stepanenko and Dmitrenko 2015).

Cells were passaged three times per week. Next, cells were collected using 0.05% trypsin (Sigma-Aldrich) and incubating at 37℃ for three minutes. Trypsin was inactivated with an equal volume of FCS (Sigma-Aldrich) containing medium and the cells were collected by centrifugation at 800 rpm for five minutes. Transfection plasmid p AC152-dual-d Cas9VP64-sgExpression (p AC152) (addgene) was used into MDA-MB-468, MCF-7 and HEK293 (Cheng et al. 2013).

2.2 Wound Healing Assay

The main purpose of the wound healing assay in Hek293, MDA-MB-468 and MCF-7 is to detect the alterations of cell motility caused by CRISPR/Cas9 mediated overexpression of the target genes. Cells were seeded uniformly to culture plates (Thermo Fisher Scientific, UK) with an artificial wound carefully created by using pipette tip p-200S (Thermo Fisher Scientific, UK). Cells were split from 80-100% confluent monolayer tissue culture dishes. Cells were seeded into 12- well- cell culture plate 1 ml/well (Thermo Fisher Scientific, UK). In order to achieve a 100% confluent cell monolayer on the day of assay, each well was supplemented with complete DMEM (Dulbecco's Modified Eagle Medium from Lonza), (10% Fetal Bovine Serum). 12 well-culture plates (Thermo Fisher Scientific, UK) were incubated in 37˚C. Next, to make a single scratch line of each well, yellow pipette tip was used. A clear single line across each well was appeared. Old media was aspirated by a pipetting- vacuum and confluent monolayer culture was rinsed with 1 ml PBS to remove the detached and scratched cells. Each well was supplemented with complete DMEM (Dulbecco's Modified Eagle Medium from Lonza) and imaged the plate by Nikon E clips scanning microscope. The measurement was repeated to obtain time (0, 24, 48, and 72) hours. Directly, after each measurement, the plate was re-incubated.

2.3 Protein Sample Preparation

Proteins extracted from breast cancer cell lines (as described above) by lysis buffer containing 2% SDS, 50 mM Tris-HCl (Sigma-Aldrich), pH 6.8, protease and phosphatase inhibitors. Extraction conditions were carried out on ice-container to prevent proteolysis. Proteins concentrations were evaluated by a short quantitative analysis based on color intensity. Spot 1 µL from protein extracted on a nitrocellulose paper, and were stained by Amido dye. Methanol/Acetic acid v/v was used as destained solution. Based on color intensity, recommended the concentration of SDS loading buffer (2x or 5x). The volume of DTT reagent (Dithiothreitol-Sigma-Aldrich-GE17) (10 mM -15 mM), 1 µl Iodacetamide per sample and keep it in dark place for 30 minutes. All samples proteins were heated at (95 - 100° C) for 5 minutes and centrifuged 30 seconds at 4°C in 14000 RPM. 15 -18 µl per samples was loaded into 10% SDS-PAGE. Gel was running in Tris-Glysin-SDS buffer for 20 minutes in 80 V and then to 40-45 minutes in 150V. Gel was stained with Ethanol-based Coomassie Blue G-250 stain (Sigma-Aldrich).

2.4 Western Blotting

2.4.1 SDS-PAGE Gel Electrophoresis

1D-10% SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was used with ten candidate's proteins samples from different breast cancer cell lines (as described above). 10% Resolving gel and Stacking gel were prepared according to these concentrations in (Table 2-1,Table 2-2).

Table 2-1 Preparing 10% Resolving gel

Chemicals	Volume
1.5 M Tris-HCl, pH 8.8	2.5 ml
30% Acrylamide mix	3.3 ml
Milli-Q H2O	Up to 10 ml
APS	100 µl
TMEDA	10 µl

Table 2-2 Preparing 4% Stacking gel

Chemicals	Volume
0.5 M Tris-HCl, pH 6.8	2.5 ml
30% Acrylamide mix	1.3 ml
Milli-Q H2O	Up to 10 ml
APS	100 µl
TMEDA	10 µl

2.4.2 Protein Staining, Blocking, Blotting and Specific Detection

1D-SDS-PAGE gel was immersed in Ethanol-based Coomassie Blue stain for 15 minutes, next, Microwave was used for fast binding of the dye to un-transferred proteins for 20 seconds, de-stain with 90% Methanol **-** 5% Acetic acid V**/**V overnight at room temperature. Proteins were separated by SDS-PAGE and electrophoretically transferred to a PVDF-membrane. The PVDF- membrane (Millipore) was blocked with 5% nonfat dry milk in 1xTBS Tris-buffer saline 1 hour in the rocker. The PVDF- membrane was incubated with the appropriate primary antibody mouse monoclonal 1/1000 Ab(s) [anti-Scribble (C-20), Santa Cruz, Biotechnology, Inc., Santa Cruze, CA, USA), anti-EGFR (1005), Santa Cruz, Biotechnology, Inc., Santa Cruze, CA, USA), anti-STAT1(C-136), Santa Cruz,

Biotechnology, Inc., Santa Cruze, CA, USA and diluted according to the manufacturer's instructions. Next, three washes in PBS-Tween-20, while the secondary antibodies 1/2000 (Abcam, Cambridge, UK) were diluted at 1/2000 with 0.1% Tween-20 for 1hr at room temperature. Proteins were visualised using the ODYSSY infrared Imaging System (LI-COR Bioscience).

2.4.3 Gel Electrophoresis Prior to Mass Spectrometry

1D-10% SDS-PAGE (10% resolving gel and 4% stacking gel) was used with ten protein samples from different breast cancer cell lines (breast cancer cell lines MDA-MB-435, MDA-231, MCF7, ZR, MDA-468 and HEK293 (human embryonic kidney)). Eight proteins samples were tested to measure the protein concentration based on color intensity as brief quantitative analysis for each sample. Gel slices were cut into small pieces (1-2 mm) and subjected to in- gel digestion as described by (Metodiev et al. 2012).

Gel slices were de-stained by 50% Methanol (Sigma-Aldrich) for 5 times (until the pieces of gel are cleared from stain, one hour/time) 300 µL from a mix solution of 25 Mm NH_4HCO_3 in 50% acetonitrile and dehydrated in 100% acetonitrile before drying. The band of protein interest was excised and subjected to the in-gel tryptic digestion, and the tryptic peptides were subsequently extracted and analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Samples of gel slices were stored at -80˚C.

2.5 Statistical Analysis of Protein Abundance

Statistical analysis was performed using the Wilcoxon-Mann-Whitney test in R-software programme for statistical computing and graphics. For comparison of Mean (M) and Standard Deviation (SD) (Adam et al. 2002) (each M values three replicate measurements +/- SD). Results were considered statistically significant when P-value < 0.05. Results were tabulated into Microsoft Excel worksheet and exported into the R-programming language. Variances in antibody specificity, experimental and biological conditions between tissue types, and even researchers may generate some modifications from the original procedure of immunostaining method.

2.6 Immunocytochemistry

2.6.1 Acidic Treatment of Coverslips

Small round 22 mm diameter glass coverslips (Thermo-Fisher) were used and placed in 1M HCl overnight at room temperature. Next, these coverslips were deipped in H_2O for 30 minutes and with 100% Ethanol for 10 minutes until completely dry. Finally, they were sterilised by dry heating at 200° C for 2 hours and placed in 24 well-plates (Thermo-Fisher).

2.6.2 Seeding and Cell Adhesion Assay

Immunocytochemistry was performed on acid treating glass coverslips. MDA-MB-468, HEK293 and MCF-10A were grown as 70% - 80% monolayer cultures. After harvesting,
1 ml of complete media was added over each coverslip in the 24-well-plates (Thermo-Fisher). Followed by, 2 ml (approximately $5X10^6$) of cell suspensions from each cell line.

The 24-well-plates were incubated at 37℃ in a humidified CO_2 incubator for 48 hours. Cells were examined under a light microscope. Finally, the culture medium was aspirated from each well and gently rinsed by 1X PBS at room temperature.

2.6.3 Cell Fixation

The cells were fixed on a coverslip by dropping 100% methanol (chilled at -20 ℃) on the edges of the wells for 20 minutes at room temperature, so as to avoid the possibility of losing the cells. They were aspirated and washed by ice-cold 1x PBS for 1 minute. Quenching was performed with a solution of 0.3% H_2O_2/ Methanol for 20 minutes at room temperature. To permeabilise the cells, fresh PBS-TritonX-100 was used for 30 minutes and washed twice with 1X PBS for 5 minutes each.

2.6.4 Permeabilization

To permeabilize the cells, fresh solution of 0.3% Triton X-100 (Sigma-Aldrich) in PBS was used for 5 min which is the most popular detergent for improving the penetration of the antibody.

2.6.5 Blocking and Incubation

The unspecific bind was blocked in 1X PBS, 0.05% Tween-20, 2% Serum, 1% BSA. Next, cells were incubated with primary Ab(s) 1/1000 [Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA] at 4℃ overnight or 2hrs at room temperature in a humidified container. They were afterwards washed three times with 1X PBS, 5 min each. Followed by, incubation for 1 hr with secondary Ab(s) 1/2000 [Santa Cruz Biotechnology, Inc, Santa

Cruze, CA, USA] in 1X PBS, 0.05% Tween, 1% PBS in dark container. DAB (3, 3-diaminobenzidine, Sigma-Aldrich) was prepared and used immediately according to supplier instructions for 6 min. The DAB (3, 3- diaminobenzidine, Sigma-Aldrich) solution was decanted and washed with 1X PBS-1% BSA. For counter staining, hematoxylin (Sigma-Aldrich) was used for 10 second and dehydrates in 95% ethanol and Absolute ethanol for 2 minutes each time. Next, cells were fixed in Xylene (Sigma-Aldrich) for 5 minutes. In attempt to standardise the immunocytochemistry detection of EGFR, STAT1 and Scribble, I evaluated two normal cell lines (MCF-10A, Hek293 and MDA-MB-468) for sensitivity and specificity of three commercially antibodies anti-Scribble, anti-STAT1 and anti-EGFR.

2.6.6 Mounting and Visualization

The DAB (3, 3- diaminobenzidine, Sigma-Aldrich) solution was decanted and the cells were washed with 1X PBS-1% BSA. For counter staining, hematoxylin was used for 10 second and dehydrated in 95% ethanol and Absolute ethanol for 2 minutes each time. Cells were fixed in Xylene for 5 minutes and a drop of mounting medium was added to the glass slide. Each coverslip was picked up and placed with the cell side face down.

2.7 Statistical Analysis of Immunocytochemistry Images

Stain intensity and the proportion of immunostaining positive cells were assessed by light microscope. Intensity of staining (IS) was evaluated and graded by a semi quantitative approach used to assign an H-score to ICC of MDA-MB-468, HEK293 and MCF-10A slides

on a scale of 0-no colour reaction (0=no positive cells), 1= mild reaction (1=<10% positive cells), 2= moderate reaction (10-50% positive cells), 3= strong reaction(51-80% positive cells). The H-score was used, i.e. based on a predominant staining intensity, or complex, including the sum of individual H-score for each intensity level seen. The percentage of cells at each staining intensity level is calculated and finally the H-score is assigned the following equation (Hirsch et al. 2003).

[1*(% cells 1+) +2* (% cells 2+) +3*(% cells 3+)]

Statistical analysis was performed using Student's t-test in R-software program language and software environment for statistical computing and graphics; as an inferential statistical test that determines whether there is a statistical difference between means in two unrelated groups. Results were tabulated into Microsoft Excel worksheet and exported into the R-programming language for comparison of M and SD (Adam et al. 2002). For each M value two replicate measurements were calculated. Results were considered statistically significant when P-value < 0.05.

2.8 CRISPR Reagent Description

2.8.1 CRISPR/Cas9 guide to design forward and revers oligo

To obtain and design the promoter target sequence of human (Scribbe, EGFR and STAT1, the target sequence was copied from a genome browser called Ensemble (www.ensembl.org/Homo_sapiens/Gene/Sequence?db=core;g=ENSG00000180900;r=8:1

43790920-143815379), which includes tools such as BLAT, BLAST, BioMart and variant effect predictor for vertebrate genome.

Essentially, this genome browser annotates genes, computes multiple alignments, and predicts regulatory function and collects disease data, as well, sequence variation and transcriptional regulation, (http://www.ensembl.org/index.html). Next, the copied sequence was pasted on CRISPR/Cas9 oligo selection server at MIT (http://crispr.mit.edu) and the unique genetic region was selected after the name and detailed address was field. Then, the highlight sequence was pasted and submitted. The complete analysis of display information about oligo pairs for double nickaes targeting and for WT Cas/9 targeting were received after a while time. The appropriate double nickase were selected and the sequences were copied and pasted into a new file such as excel spreadsheet. Finally, the oligo sequences were sent to Eurofins Genomics for synthesis.

For designed reverse oligo sequences and according to Western-Crick system, I edited the sequence to add (CACC) to the 5′ end of each forward oligo while for each reverse oligo I added (AAAC). The oligo sequencing was sent for synthesis. Select Human genome was used to acquire the promotor target sequence for STAT1 and EGFR. I followed the instructions on the Ensemble Genome Browser to extract the 1000bp sequence upstream of the transcriptional start site and loaded into the E.CRISP tool (htt://www.e-crisp.org/ECRISP/desigine crispr.html). It is important to have highly specific sequences without off-target probability after comparing the 1000bp sequence against the entire human genome. On the above web-site, the highest score <100% identity to other genome

target was selected. The pair of oligonucleotides encoding corresponding sgRNA was ordered from Eurofins Genomics. Additionally, I added flanking site for ligation into restriction (BbsI) to the oligonucleotides ordering. I then selected the sgRNA targets with Cas9 based on three criteria, (**I**) Maximisation of on-target activity (**II**) Maximisation of off-target activity (**III**) avoidance of homo polymer stretches (e. g AAA.GGG and GC content).

Table 2-3 Forward and Reverse oligonucleotides for different DNA gene targets

Oligonucleotides Name	Direction	Sequences (5´- 3´)
STAT1-1	**Forward**	**CACCGATTATACCAGAAGGAACGT**
	Reveres	**AAACACGTTCCTTCTGGTATAATC**
STAT1-2	**Forward**	**CACCAATGGAGAATGCTTAGTCAC**
	Reveres	**AAACGTGACTAAGCAATTCTCCATT**
STAT1-3	**Forward**	**CACCCACCACAACTGAGAAGGCAT**
	Reveres	**AAACATGCCTTCTCAGTTGTGGTG**
STAT1-4	**Forward**	**CACCTTCGAAAGTTCGGCTGGCTG**
	Reveres	**AAACCAGCCAGCCGAACTTTCGAA**
EGFR-1	**Forward**	**CACCTCGGTGCCATTATCCGACGC**
	Reveres	**AAACGCGTCGGATAATGGCACCGA**
EGFR-2	**Forward**	**CACCCGTCGGATAATGGCACCGAC**
	Reveres	**AAACGTCGGTGCCATTATCCGACG**
EGFR-3	**Forward**	**CACCCGCGGGACCTAGTCTCCGGC**
	Reveres	**AAACGCCGGAGACTAGGTCCCGCG**
EGFR-4	**Forward**	**CACCATTTGGCTCGACCTGGACAT**
	Reveres	**AAACATGTCCAGGTCGAGCCAAAT**

2.8.2 Seeding Cells

Human breast carcinoma cell line MDA-MB-468, human breast adenocarcinoma cell line MCF- 7and human embryonic kidney cell line Hek293 were treated by trypsin (Sigma-Aldrich) to de-attach and dissociated to single cancer cell. Cells were counted by standard trypan blue exclusion. Cells were seeded as 4 x 10^4 / cells in 0.5 mL of competent DMEM

(Dulbecco's Modified Eagle Medium from Lonza) for a single well at the day of transfection and the cell density were 60%-80% confluent monolayer.

2.8.2.1 Transfection

Human tumour cell lines (MDA-MB-468, MCF-7 and Hek293) were used in transfection processes. Transfection procedure is influenced by many factors such as the viability and health of cancer cell line. The quality of quantity plasmid DNA (p AC150-dual-dCas9VP64-sgExpression (p AC150), transfection reagent such as Lipofectamine 3000 (Invetrogen) and (FuGENE HD), the number of passage, the degree of confluent monolayer and the competent of media were measured and monitored to have a successful transfection.

2.8.3 Transfection of MDA-MB-468, MCF- 7 and Hek293 by FuGENE-HD

Plasmid p AC152-dual-d Cas9VP64-sgExpression (p AC152) was transfected into MDA-MB-468, MCF- 7 and Hek293 by Fugene –HD. According to the instructions and the nano-drop-spectrometry reading of plasmid DNA concentration for STAT (STAT1, STAT2, STAT3 and STAT4) and EGFR (EGFR1, EGFR2, EGFR3 and EGFR4), the Fugene- HD transfection protocol was followed with some modifications. To prepare the concentration of plasmid DNA of STAT and EGFR, I divided the nano-drop-spectrometry reading of plasmid by the maximum DNA plasmid reading from Fugene –HD. The mixture was vortex for 1 minute and incubated for 5 minutes at room temperature. Fugene-HD was prepared 7:2 transfection reagent to DNA ration, 7 µl of Fugene-HD was added to plasmid DNA + Opti/MEM media and incubated for 20 minutes at room temperature to form DNA-Fugene –

HD complex. The mixture was added to the suspension of MDA-MB-468, MCF-7 and Hek293 cells, separately. Cells were incubated at 37°C with 5% CO_2 overnight. The transient transfection protocol was followed and fresh selective culture media with G-418 (Type of antibiotics for the selection and maintenance the stably transfected with plasmid expressing puromycin, resistance) was added after 24 hours to select the expression of transfected antibiotic resistant.

2.8.4 Transfection of MDA-MB-468, MCF- 7 and Hek293 by Lipofactamine

To transfected plasmid p AC152-dual-d Cas9VP64-sgExpression (p AC152) into MDA-MB-468, MCF- 7 and Hek293, lipofactamine 3000 reagent was used for 6 well-plate according to the instructions and the nano-drop-spectrometry reading of plasmid DNA concentration for STAT (STAT1, STAT2, STAT3 and STAT4) and EGFR (EGFR1, EGFR2, EGFR3 and EGFR4).

2.8.5 Transfection Efficiency Measurement

Green Fluorescent Protein (GFP) was used to assess the transfection efficiency in the MDA-MB-468, MCF-7 and Hek293. GFP was used in the complex competent of transfection reagent mixture. Cells were visualised via florescent microscope for quantitative assessment of protein expression, morphology and viability.

2.8.6 Harvesting of Transfection Hek293, MDA-MB-468 and MCF-7

Selective conditions (resistant cells to G-418) were based to harvest the transfection Hek293, MDA-MB-468 and MCF-7 cell line. The sensitivity of transfection were tested and checked after 48hr under resistance to G-418 and adherent cells were treated by trypsin. To increase the selection of stable expression, cells were cultivated into DMEM (Dulbecco's Modified Eagle Medium from Lonza) with G-418. Suspension cells were plated on tissue culture plate 10 cm. Cells were continuously fed every 24-48 hours to outgrowth of resistance cells (three weeks with G-418 medium to avoid contamination with non-resistance cells. Negative control (cells were with empty oligonucleotides plasmid).

2.8.7 Protein Sample Preparation of Transfection Hek293, MDA-MB-468 and MCF-7

To prepare lysate from Hek293, MDA-MB-468 and MCF-7, solutions and buffer were ice-box conditions. Cells were washed with ice-cold PBS. Next, cells were treated by lysis buffer (1 ml/100 mm tissue culture dish). Adherent cells were transferred into pre-cold tubes and mixed for 30 minutes at 4^{o}C. Cells were centrifuged for 20 minutes at 12,000 rpm. The cell suspension was aspirated to test the protein concentration. A drop of cell suspension was applied on nitrocellulose paper. Next, the nitrocellulose paper was treated by organic dye Black Amido Stain (Sigma-Aldrich) for 5 minutes and washed for 2 minutes. The concentration of dye is proportional to the concentration of protein. To denaturation, the mixture was boiled at 95 -100° C for 5 minutes protein suspension.

2.8.8 Cloning and Plasmid Construction of STAT1 (STAT1-1, STAT1-2, STAT1-3, STAT1-4) and EGFR (EGFR1, EGFR2, EGFR3, EGFR4)

The simplified CRISPR cloning procedure was adapted from various sources with some modifications. Each pair of oligonucleotides was annealed at 95° C for 5 minutes in a thermocycler. The annealed oligonucleotide tubes were incubated at room temperature for 1 hour. Followed by digestion step, 1 µg of plasmid was mixed with 2 µl of 10x Buffer (provided with the enzyme), then, MilliQ H2O was added up to final concentration of 20 µl. Next, 1 µl of BbsI (Thermo Fisher Scientific) was added to the digest plasmid mixture tube and incubated at 37° C for 1hour. Finally, the ligation components were added to the digest plasmid mixture tube which included, 2.5 µl of 10x T4 Ligase Buffer, 1 µl annealed oligonucleotides (10 µM stock, 0.4 µM final concentrations) and 1.5 µl T4 DNA Ligase.

The reaction was incubated at 37°C for 1 hour. The results of cloning and constructing were confirmed by DNA sequencing (Source Bioscience Sequencing Company).

2.8.9 Generation of Plasmid

To facilitate Cas9-mediated geneium cleavage, two forms of guide RNA were used, first, p X260 (or p X334), *S.Pyogenes* Cas9 or Cas9 D10A nickase) + CRISPR RNA array + tracrRNA. This plasmid consists of three expression cassettes and digested by BbsI.

The pair of annealed oligos were designed based on the target site sequence (30bp) and flanked on the 3´end by a 3bp NGG PAM sequences of annealed oligos design (Cong et al. 2013) (Figure 2-1).

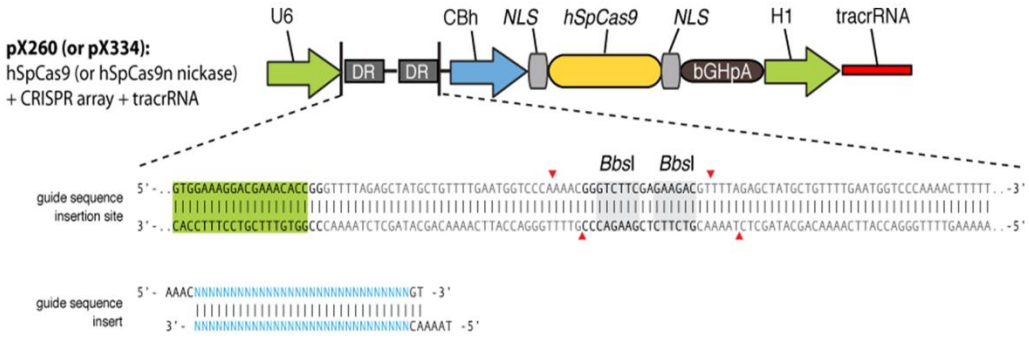


Figure 2-1 p X260(or p X334), *S.Pyogenes* Cas9 or Cas9 D10A nickase) + CRISPR RNA array+tracrRNA. Adapted from ((Cong et al. 2013).

Second, p X330 or(p X335), *S.Pyogenes* Cas9 (or CasD10A nickase+chimeric guide RNA containing 85 of tracr RNA. The structure of this plasmid contains two expression cassette (hspCas9+the chmiric guide RNA and digested by BbsI. A Pair of annealed oligos were cloned into the RNA guide. The design of oligos was based on the target site sequence (20bp) and flanked on the 3′end by a 3 bp NGG PAM sequences. The plasmid backbone contained a longer fragment of the tracRNA (+ 85nt). The longer the chimeric guide RNA the more efficient and accurate is the targeting (Cong et al. 2013).

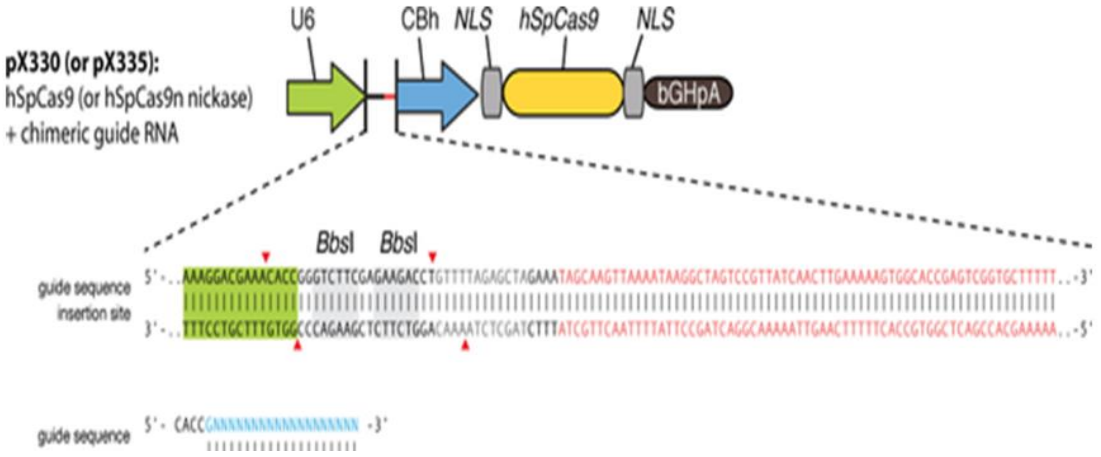


Figure 2-2 p X330 or(p X335), *S.Pyogenes* Cas9(or CasD10A) nickase+chimeric guide RNA. Adapted from (cong et al. 2013).

2.8.10 Annealing and Cloning of STAT1, EGFR and Scribble Oligo

First step to anneal of oligonucleotides, a pair of oligonucleotides were annealed 1 µl from oligoforword and oligorever). The mixture was annealed in the thermocycler at 95˚C for 5 mins and up to 8 µl of H2O was added. The annealed mixture was left at room temperature for 1 hr. The second step was digested by BbsI (Thermo Fisher Scientific) by mixing 2 µl 10 X buffer enzyme with 1µl plasmid and 17 µl H2O + 1 µl BbsI at 37˚C for 1 hour, while the ligation components (2.5 µl 10 X TU ligase buffer+ 1µl anneal (from first step) +1.5 µlT4 DNA ligase were added to digest product (from second step). The mixture was left for 1hr at 37˚C and the steps of protocol were applied separately for each protein.

2.8.11 Restriction Digests of Plasmid Backbone

The plasmid p AC152 backbone was digested by the restriction enzyme (Bbsl) with high fidelity and effects and to recognise the nucleotides sequence (GAAGAC) sites. In particular, the site around the sgRNA target region at 37˚C (Figure 2-3).

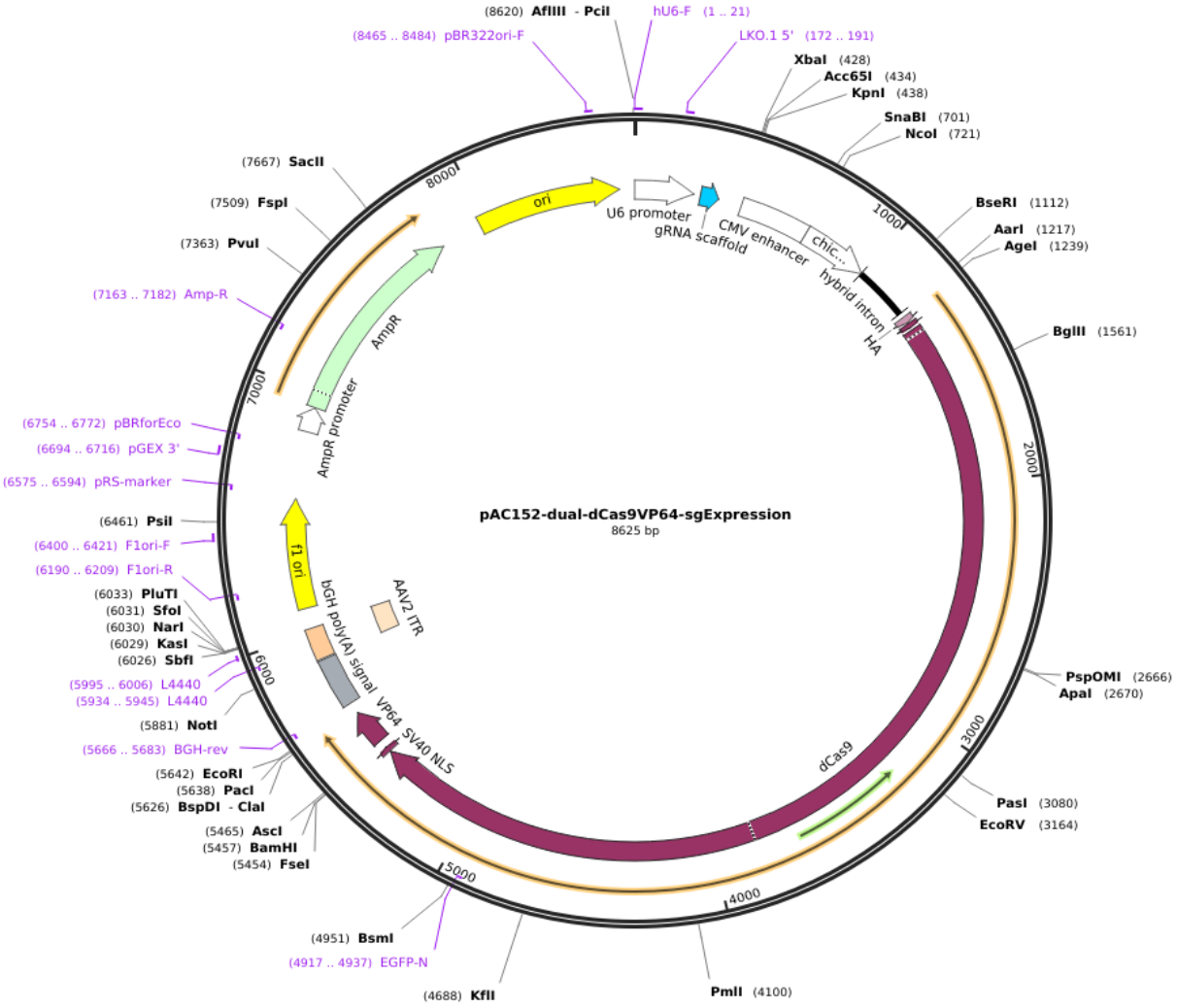


Figure 2-3 pAC152-dual-dCas9VP64-sgExpression

Dual expression constructs expressing both dCas9VP64 and sgRNA from separate promoters. Adapted from (Cheng et al. 2013).

2.8.12 The Microbiology laboratory Techniques

2.8.12.1 Bacterial Strain and Media Preparation

Media (Tryptone Yeast extract, Sigma-Aldrich) were supplemented with appropriate antibiotics concentration (Ampicillin 100, Sigma-Aldrich). Agar was added to re-constitute at source concentration 2XYT (1.5% Agar, Sigma-Aldrich) and swirl to mix. Media was sterilised by autoclave. After agar was cooled, Ampicillin was added to media at concentration 100 mg/ml and swirled. Tryptone Yeast extract agar was poured into 10 cm Petri dish and left to solidify.

2.8.12.2 Antibiotic Preparation

Ampicillin stock was prepared by adding to sterile distilled water 100 µL of a 100 mg/mL ampicillin stock (1000X stock). The stock concentration (100 mg/mL) was stored at 4℃. This concentration was enough to inhibit the formation of peptidoglycan cross- links in cell wall synthesis.

2.8.12.3 Transformation Bacterial Strain (Competent E.coli)

Five µl from reaction mixture (CRISPR cloning step) was transformed to 50 µl E.coli competent cells and mixed gently and negative control (E.coli competent cells, *Escherichia coli* strain was grown in 2XYT broth). Followed by, incubation on ice for 30 mins. Negative and positive tubes were exposed to heat shocking at 42℃ for 45℃ seconds and retained on ice-box for 5 minutes, 500 µL of 2XYT broth was added to each tube and incubated in a shaker at 37℃ at 250 rpm for 30 minutes.

Followed by centrifugation at 10000 rpm for 2 minutes and plated onto 2XYT agar (1.5% Agar) / Ampicillin (100 µg/ml) to transfer the assembled reaction into a competent cells *E.coli* strain. Cells were thawed once and the digest product was added to *E.coli* competent. The mixture was incubated on ice for 5 minutes. Next, the mixture was heated (Heat shock) at 42° C for 30 seconds and returned to ice immediately at 2 minutes to prepare starter culture for mini-prep, since colonies were picked by a sterile pipette tip. The selected colonies were inoculated into 5 ml 2X YT broth / Ampicillin (100 µg/ml). The culture was placed on shake for approximately 16 hr at 37° C at 250 rpm, as recommended by the (manufacturer's instructions).

2.8.12.4 Colony and Plasmid Extraction

A single colony was picked from agar/Ampicillin plate by sterile toothpick and inoculated in 5 ml 2x YT broth/ Ampicillin(100 µg/ml) for 16 hour at 37°C shaker incubator. Plasmid extraction was performed by using (Thermo Scientific Gene JET plasmid Miniprep Kit) and combined with buffer from 2x 2ml of culture in the resuspension buffer and processed as per procedure. The remaining culture was stored at -80°C as Glycerol stock.

2.8.12.5 Sequence of sg RNA Cloning

To verify the correct insertion of the validation sg RNA, the concentration of plasmid DNA extraction was read by a drop-spectrometry (100 ng/µl) and the dilute primer was calculated which mainly depends on the number of samples and evenly divided the dilute concentration of primer. The DNA extraction tube was sent to Source Bioscience Company for sequencing. The sequencing results were compared to the sg RNA oligonucleotide

report to check that the 20 sg RNA target sequences was properly inserted between the promoter and the rest of the sg RNA oligonucleotide Validation of STAT1, EGFR by DNA Sequencing was relied on Sequence primer –LKO seq (5´-GACTATCATATGCTTACCGT-3´) and the thermocycler cycling conditions was read as cycler number 1, condition 95˚C, 5 minutes for annealing the reaction.

2.8.12.6 Culture and Plasmid DNA Extraction

DNA extraction and mini prep were used to pick and inoculate the single colony in 5 ml 2x YT broth/ Ampicillin (100µg/ml). The culture was kept at 37˚C at 250 rpm for 16 hours. Next, 2x2 ml of culture was transferred into 2 sterile Eppendorf tubes and centrifuged at 14,000 rpm for 5 minutes. Supernatant was aspirated and the cell pellet was left (Thermo Scientific Gene JET plasmid Miniprep Kit) portal was used to extract DNA in this study.

Chapter 3 Results Mass Spectrometry and Proteomic Study of Human Cancer Cell Lines and TNBC Human Breast Tumour Tissue

3.1 Introduction

A large and growing body of literature have shown that there are more than 1,300,000 cases and 450,000 death from breast cancer each year around the world (Network 2012). The Estrogen receptor group and HER2/ERBB2 group amplified are great clinical success stories because of the effectiveness of the Endocrine therapy and therapeutic targeting of EGFR2 while the TNBC group have only the chemotherapy option and is in need of new targeted therapies (Paik et al. 2004). Mass spectrometry and proteomics are essential promising approaches for cancer diagnosis and cancer biomarker discovery (Diamandis 2004). The ability of mass spectrometry to measure the mass-to- charge ratio with high accuracy providing spectra of very high resolution and the development of tandem mass spectrometry enhanced this technique use in proteomics tandem mass spectrometry identification and is even able to obtain de novo protein sequence information (Aebersold and Mann 2003). Many researchers have pioneered to use of mass spectrometry as a paradigm shift in cancer diagnostic depends on complex differences between proteomic patterns in serum or tissue between patients with or without cancer identified by bioinformatics. The mass spectrometry approach can be used to study protein fragments produced by cancer cells or their microenvironment that enter in the general circulation. The concentration (abundance) of these proteins / fragments could be analysed by mass spectrometry and the obtained data can be processed with a mathematical algorithm to

discover biomarkers (Petricoin et al. 2002). Data from both high protein intensity and proteins critical for biological function such as membrane receptor kinase were explored by proteomic and mass spectrometry.

Conventional clinical and molecular biology laboratories use gas-chromotograph mass spectrometry (GC-MS), automated immunoassay and high pressure liquid chromatography diode array detector (HPLC-DAD) technique to perform general unknown screening (GUS) analysis. In proteomics, in-gel digestion together with mass spectrometry analysis combine the classic and the modern biochemistry strategies, which allow for targeted and proteome wide analysis.

In this study, protein samples from transfected human breast cancer cell lines are used to fractionation by 1D-SDS-PAGE and protein samples are reduced and alkylated. Pre-fractionation of protein mixtures before and following enzymatic digestion is required prior to mass spectrometric analysis to improve the depth analytical and decrease the protein sample complexity.

Protein mixtures are separated into several fractionations by SDS-PAGE or other appropriate techniques such as membrane proteins enrichment or column chromatography. In polyacrylamide gel electrophoresis, the gel is molecular sieve and helps to separate proteins from low to high molecular weight removes buffer components interfering with downstream mass spectrometric analysis. Additionally, the advantage of in-gel digestion procedure is the ability for targeted analysis of specific gel bands or spot of a particular

molecular weight and iso- electric point and is useful for investigating proteins and differ under certain cellular condition (In-gel trypsin digestion/M.M. Lab).

This approach also allows for protein visualisation colorimetric estimation of protein concentration prior to protein digestion (Paulo et al. 2012). The major purpose of the mass spectrometry and proteomics analysis in this chapter is to measure the levels of our candidate proteins and to assess the levels of systematic variation in protein abundance, which is associated with the different sample sources either from tumour tissue (26) or tumour cell lines in a particular analysis.

3.2 Methods

To start and pursue the questions of the hypothesis and to investigate the answers; the first research question was to profile the expression of the chosen protein candidates in a group of breast cancer cell lines, such as MDA-MB-231, MDA-MB-468, ZR, MDA-MB-453 and MCF-7, which is enriched for those representing TNBC features. This is assessed to define the cell lines that most closely capture individual examples of the heterogeneous TNBC phenotypes and use them as effective tools for drug discovery and/or biomarkers development. I used the human breast cancer cell lines in Table 3-1

Table 3-1 Human breast cancer cell lines

Cell Line	Tissue of Origin	Cell Media	Supplements
MDA-MB-231	Pleural effusion from female with a metastatic mammary adenocarcinoma	DMEM	5% fetal bovine serum
MCF-7	Mammary gland, breast; derived from metastatic site: pleural effusion	DMEM	10% fetal bovine serum
MDA-MB-468	Mammary gland/breast; derived from metastatic site: pleural effusion	DMEM	10% fetal bovine serum
MDA-MB-453	Mammary gland/breast; derived from metastatic site.	DMEM	10% fetal bovine serum
ZR	Breast	RPMI-1640	10% fetal bovine serum
HEK293	embryonic kidney	DMEM	10% fetal bovine serum

Proteins were extracted from human cell lines by lysis buffer (2.2) and prior to mass spectrometry, western blotting (2.3) has been used and one dimension 10% SDS-PAGE was used with ten candidate's proteins samples from different breast cancer cell lines. (2.3.1), and procedure for protein staining, blocking, blotting and specific detection (2.3.2), while the second part of this study rely on the data from human triple negative breast tumour tissue (26 samples of ER -, PgR – and ErbB2 - tumoures) were collected under Local Research Ethics Committees (LREC) and National Health Service (NHS) from Histopathology Department, Broomfield Hospital, Broomfield, Chelmsford, United Kingdom. The R software for statistical computing was used for data analysis.

The correlation between (x, y) was determined using the following equation:

x <- log (as.numeric(data[1,-c(1,2)])).

The boxplot (x, y) was generated using the following equation:

> boxplot(log(as.numeric(x[a,b:c])), log(as.numeric(b[a,b:c])), names =c("a", "b"), ylab="Log(a, name)").

3.3 Results

I used simple statistical analysis to evaluate the development of a quantitative tumour proteomics methodology and its implementation to study a type of breast cancer known as triple negative receptor for the three bio-markers such as ER^-, PgR^- and $Her2^-$ that is used as a clinical guide treatment. Quantitative proteomics technique developed in the course of the study use label-free mass spectrometry and size-based protein separation combined with bioinformatics and allows an unlimited number of tumour samples to be analysed and compared in a highly automated manner.

3.3.1 Quantitative In-Gel Digestion and Mass Spectrometry Profiling of Breast Cancer Cell Lines

Quantitative detection on polyacrylamide gel electrophoresis was used to extend the range over which proteins of different molecular masses can be effectively separated before western blotting. The method is based on a principle that is very similar to the analysis of

gene expression from triple negative breast tumours. The relative abundance of the proteins is calculated from the number of MS/MS spectra and from ion intensities.

Prior to mass spectrometry, I analysed breast cancer cell lines MDA-MB-435, MDA-231, MCF7, ZR, MDA-468, and HEK293T by in-gel digestion method and LC-MS/MS. Nine protein samples were separated by 1D-SDS/PAGE and sliced into segments according to protein ladder which shows the MW in kDa for each protein (Figure 3-2).

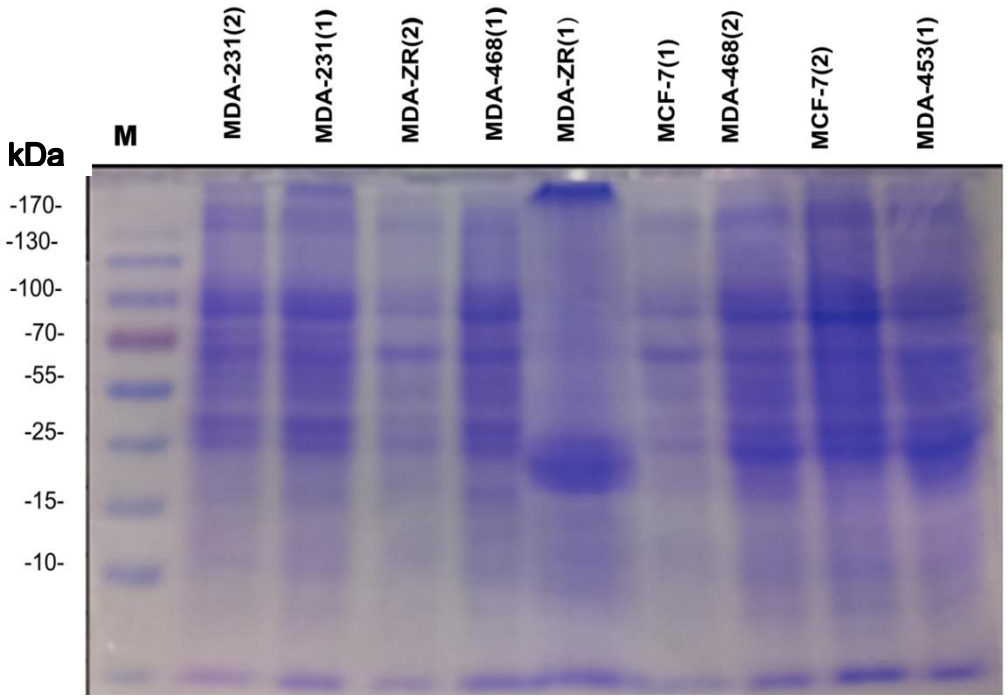


Figure 3-1 1D SDS-PAGE gel electrophoresis of total proteins samples extracted from breast cancer cell lines.

The image represents a pre-fractionation of protein mixtures by 1D SDS-PAGE gel electrophoresis. Fractionation of protein samples (9) from cellular and plasma membrane proteins (MDA-MB-435 (1), MDA-MB-231 (Baron et al. 2018), MDA-MB-231 (2) membrane, MCF-7, MCF-7(2) membrane, ZR, ZR (2) membrane, MDA-MB-468, MDA-MB-468 (2) membrane). Protein samples are reduced and alkylated. Protein extracts were prepared from breast cancer cell line (MCF-7, MDA-MB-468, MDA-MB-453 and ZR). Proteins were separated on 10% SDS-PAGE gel using the slab gel form and the SDS-discontinuous buffer system. 10% polyacrylamide gel was run at 150 V and stained with Coomassie blue. The left side of the gel is the protein marker with molecular weight (MWs) between 10-170 kDa.

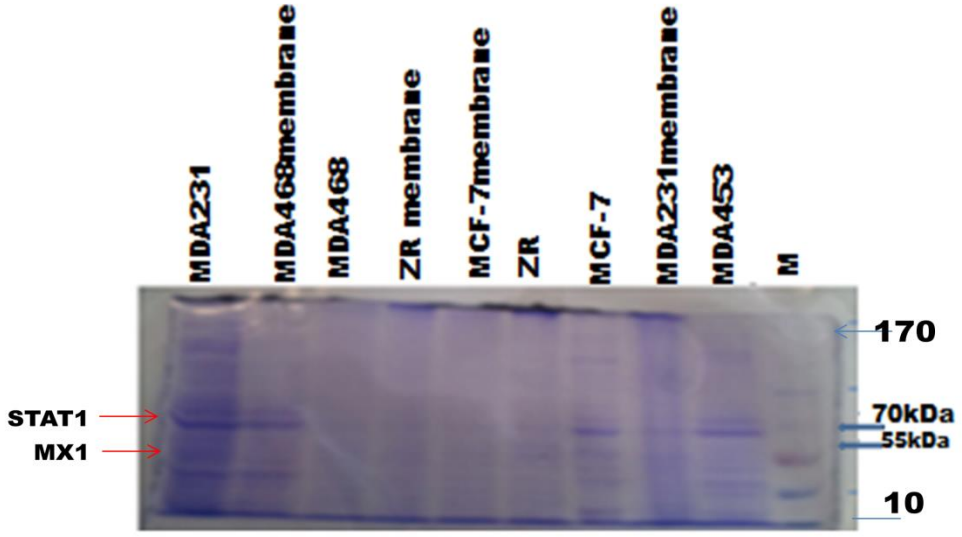


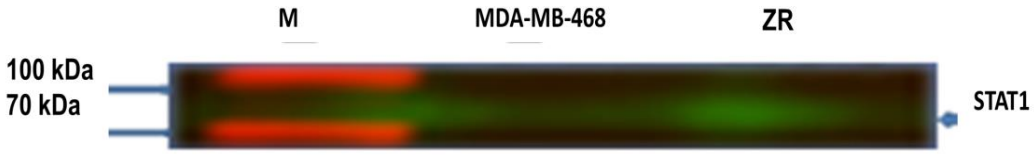
Figure 3-2 1D SDS-PAGE gel electrophoresis of protein extraction (cellular and plasma membrane proteins) for targeted analysis of STAT1 and MX1

Polyacrylamide gel electrophoresis image of nine identical samples extracted from breast cancer cell lines (MCF-7, MDA-MB-468, MDA-MB-453 and ZR and MDA-MB-231). 1D SDS-PAGE gel was separated by the slab gel form and the SDS-discontinuous buffer system. The gel was run at 150 V and stained with Coomassie blue. The right side of gel represents the molecular weight mass (protein ladders 10-250 kDa). Proteins were visualised after electrophoresis. Gel slices corresponding to the expected positions of 70-100 kDa for STAT1 and the 55-70 kDa position for MX1 were excised, digested and analysed by mass spectrometry.

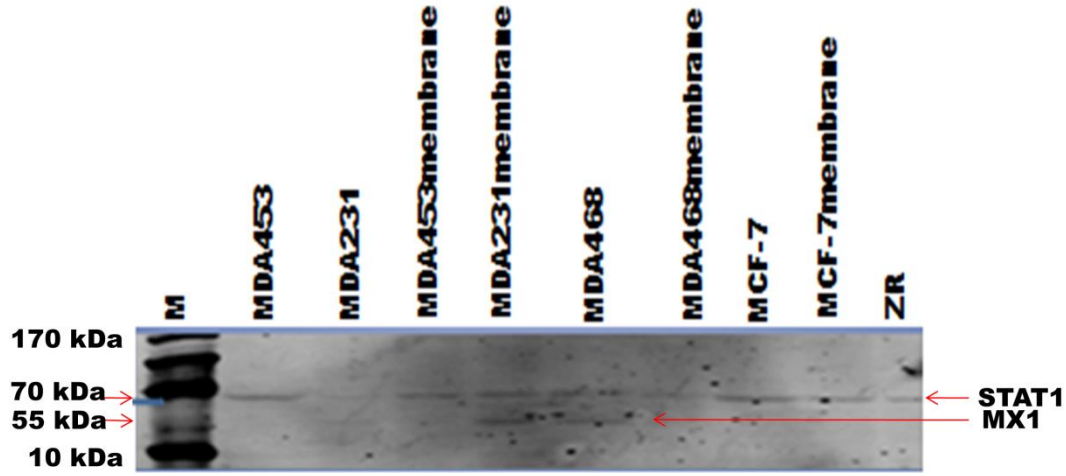
3.3.2 Measurement of Protein Expression Levels in Breast Cancer Cell Lines

Mixture of ten identical samples of plasma membrane cellular proteins extracted from breast cancer cell line (MCF-7, MDA-MB-468, MDA-MB-453 and ZR) and mammary denocarcinoma cell lines MDA-MB-231 were spotted on nitrocellulose membrane.

Protein concentration for above cell lines were evaluated by a short quantitative analysis based on colour intensity. Appendix B.13.14 and 15



A)



B) Figure 3-3 Detection of STAT1 in protein fractions

(A) and (B) Images represent western blotting analysis of proteins (cellular and plasma membrane were extracted from transfected breast cancer cell line (MCF-7, MDA-MB-468, MDA-MB-453 and ZR) and mammary adenocarcinoma cell lines MDA-MB-231. Samples were resolved on 10% polyacrylamide gel (1D-SDS-PAGE/ gel electrophoresis), and transferred to a PVDF membrane. Two PVDF membranes were incubated with anti-STAT1 (1:1000) and then with a secondary antibody (1:2000). Membranes were visualized with Odyssey Classic Infrared Imaging System. In the first Western blot image (above) the green single bands show STAT1 detection in MDA-MB-468 and ZR cell lines, based on the molecular weight mass (protein ladder 10-250 kDa/ image left side). The white and black image shows the combined detection of STAT1 (70 kDa) and MX1 (55 kDa) in a panel of breast cancer cell lines.

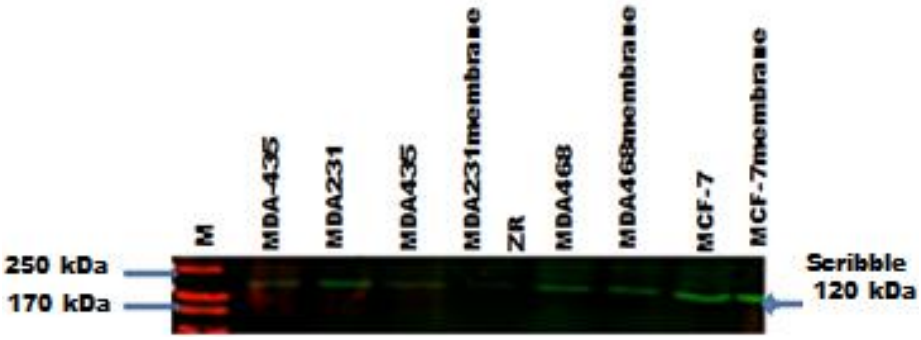


Figure 3-4 Detection of Scribble in a panel of breast cancer cell lines

The image represents western blotting analysis of nine samples. Proteins (cellular and plasma membrane) were extracted from transfected the breast cancer cell line (MCF-7, MDA-MB-468, ZR and MDA-MB-231). Samples were identically prepared, resolved on 10% polyacrylamide gel and fractioned using (1D-SDS-PAGE/ gel electrophoresis), followed by a transfer to PVDF membrane. The PVDF membrane was blocked and incubated with anti-Scribble (1:1000) and then with a secondary antibody (1:2000). The Western blot image showes the green single bands of Scribble detection in the breast cancer cell lines, based on the molecular weight mass in kDa (protein ladder 250- 10 kDa/ image left side)

Western blot analysis illustrated with the above figures showed varying abundance of the three studied proteins in the different breast cancer cell lines. Scribble was detected in the membrane fractions and STAT1 and MX1 were mostly detected in the soluble fractions.

3.3.3 Protein Overlaps Seen Between Breast Tumour Tissue and Breast Cancer Cell Lines

Breast cancer cell lines have been used to study the biology of cancer and to provide pre-clinical data guiding treatment pathway. Breast tumour tissue and breast cancer cell lines results significantly show that one of candidate proteins (STAT1, EGFR and Scribble) are

shared by breast cancer cell lines such as MDA-MB-468 and human breast tumour tissue. Figure 3-5, Figure 3-10). However the similarity between cell lines both TNBC and non-TNBC are more than the similarity between tissue and cell line of the same type of breast cancer. LC-MS/MS analysis of the samples provides crucial advantage over western blotting as it allowed us to obtain quantitative information for many hundreds of proteins in addition to the specific targets that are studied by western blot. Bioinformatics and statistical analysis of the data obtained from the LC-MS/MS analysis revealed that the set of proteins over-expressed in the panel of breast cancer cell lines compared to non-cancer cells are particularly enriched for proteins involved in the maintenance and regulation of epithelial tissue polarity, mitogen - activated protein kinase of the ERK family pathway and cell cycle.

From this analysis, one particular protein that stood out was EGFR as significantly more abundant in the MDA-231- breast cancer cell line. Scribble is well detected in most of the breast cancer cell lines that were used in this study such as, breast cancer cell line (MCF-7, MDA-MB-468 and ZR), mammary adenocarcinoma cell lines MDA-MB-231, but not in MDA-MB-435. STAT1 and MX1 stood out in MDA-435 and MDA-468 respectively. Label-free quantitation of EGFR, STAT1, Scribble, MX1 were analyzed statistically and the results from this analysis are presented in figures Figure 3-5-Figure 3-10) the values plotted on each graph are the mean of three replicate analyses. The number of cells was normalised using abundant heat shock proteins GRP94.

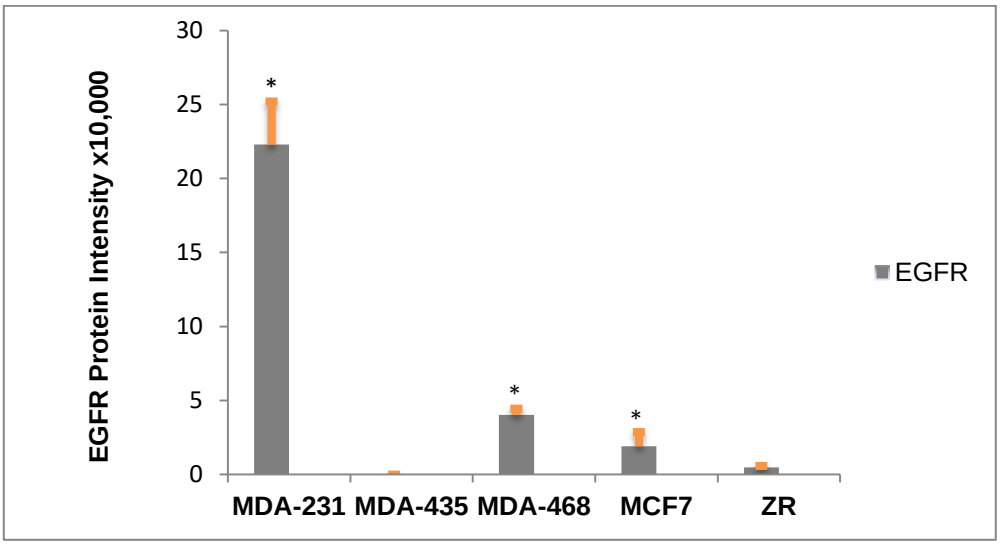


Figure 3-5 EGFR protein intensity in breast cancer cell lines

The bar chart given elucidates information on the EGFR protein intensity (y= EGFR protein Intensity) in different tumour cell lines (x= MDA-231, MDA-435, MDA-468, MCF7 and ZR). Protein identification data were assessed using Student's t-test with ($p< 0.05$). The values plotted on the EGFR graph are the mean of three replicate (n=3) and standard deviation is given by vertical bars. * indicates the level of EGFR protein in MDA-231, MDA-468 and MCF7 cell lines compared with MDA-435 and ZR cell lines

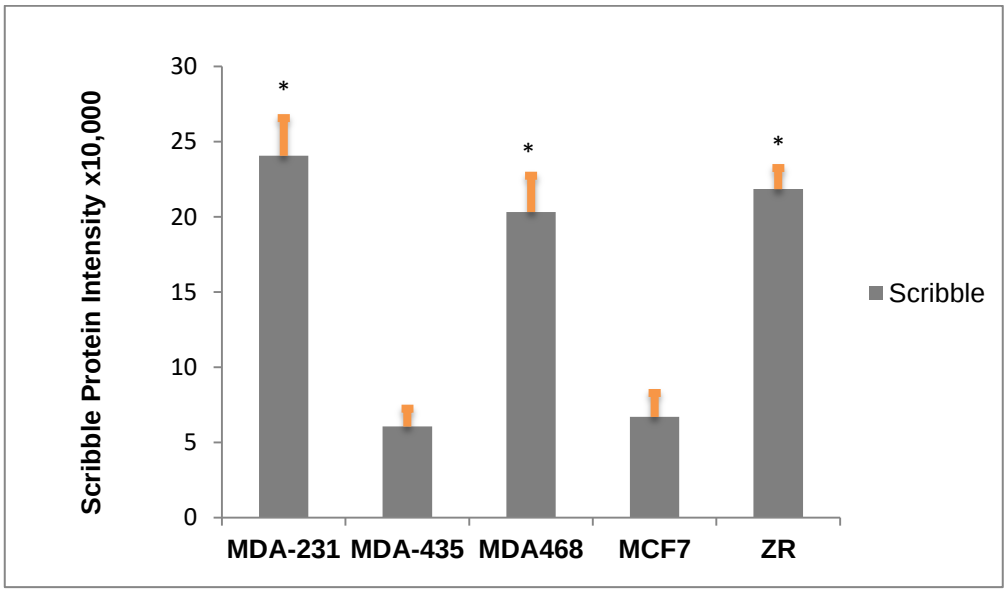


Figure 3-6 Scribble protein intensity in breast cancer cell lines

The bar chart given elucidates information on the Scribble protein intensity (y= Scribble protein Intensity) in different tumour cell lines (x= MDA-231, MDA-435, MDA-468, MCF7 and ZR). The values plotted on the Scribble graph are the mean of three replicate (n=3) and standard deviation is given by vertical bars. Protein identification data were assessed using Student's t-test with ($p < 0.05$). * indicates the level of Scribble protein in MDA-231, MDA-468 and ZR cell lines compared with MDA-435 and MCF7 cell lines

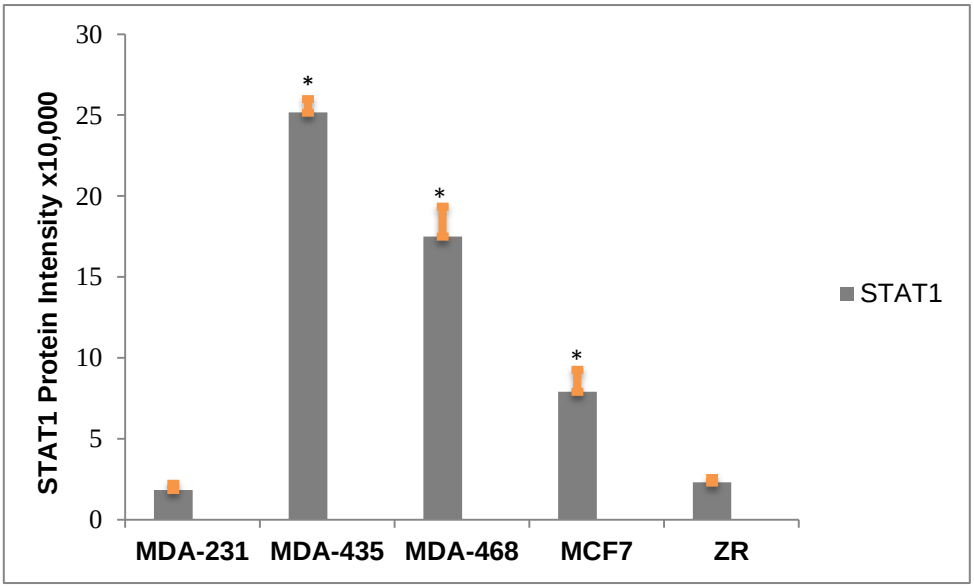


Figure 3-7 STAT1 protein intensity in breast cancer cell lines

The bar chart given elucidates information on the STAT1 protein intensity (y= STAT1 protein Intensity) in different tumour cell lines (x= MDA-231, MDA-435, MDA-468, MCF7 and ZR). Protein identification data were assessed using Student's t-test with ($p< 0.05$). The values plotted on the STAT1 graph are the mean of three replicate (n=3) and standard deviation is given by vertical bars. * indicates the level of STAT1 protein in MDA-435, MDA-468 and MCF7cell lines compared with ZR and MDA-231cell lines

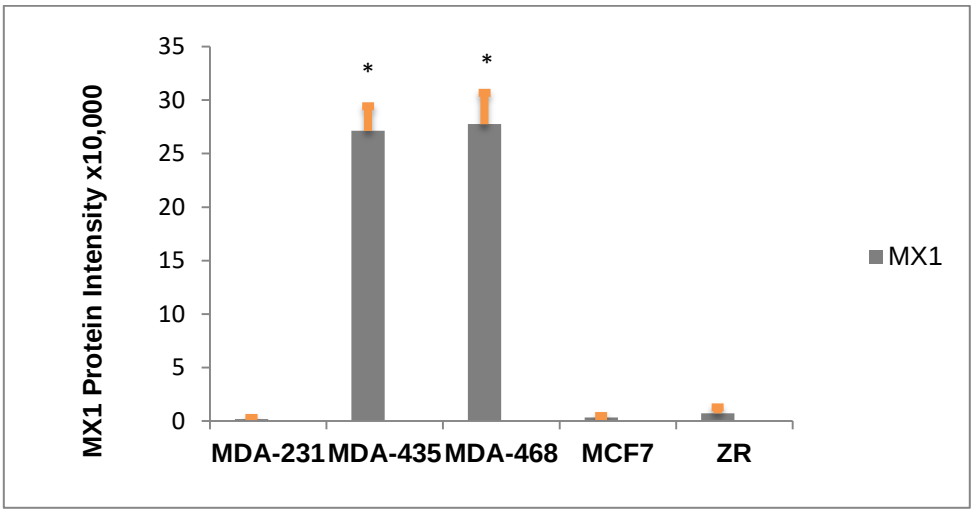


Figure 3-8 MX1 protein intensity in breast cancer cell lines

The bar chart given elucidates information on the MX1 protein intensity (y= MX1 protein Intensity) in different tumour cell lines (x= MDA-231, MDA-435, MDA-468, MCF7 and ZR). Protein identification data were assessed using Student's t-test with ($p < 0.05$). The values plotted on the MX1 graph are the mean of three replicate (n=3) and standard deviation is given by vertical bars. * indicates the level of MX1 protein in MDA-435, MDA-468 cell lines compared with ZR, MCF7 and MDA-231cell lines

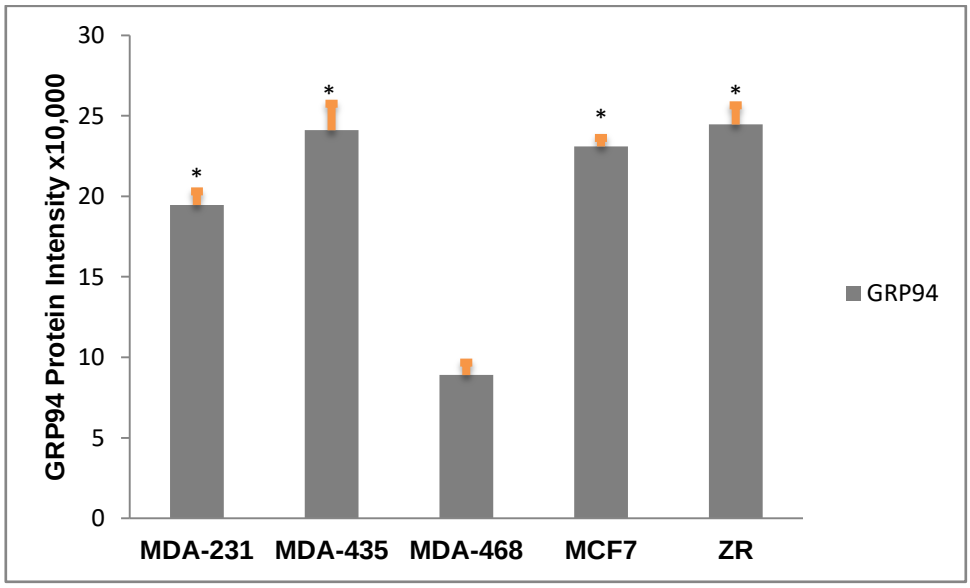


Figure 3-9 GRP94 protein intensity in breast cancer cell lines

The bar chart given elucidates information on the GRP94 protein intensity (y = GRP94 protein Intensity) in different tumour cell lines (x= MDA-231, MDA-435, MDA-468, MCF7 and ZR) and is shown as an example of a nuclear protein that can be used for normalization to cell number. The values plotted on the GRP95 graph are the mean of three replicate (n=3) and standard deviation is given by vertical bars. Protein identification data were assessed using Student's t-test with ($p < 0.05$). * indicates the expression level of GRP94 in MDA-435, ZR, MCF7 and MDA-231cell lines compared with MDA-468 cell line

In this experiment LC-MS/MS enabled simultaneous detection and quantitation of the chosen protein targets (EGFR, Scribble, Stat1, and MX1) in a single run, which takes 90 minutes to complete. Protein samples were separated into membrane and soluble fractions and analysed in triplicate to allow statistical analysis. Label-free quantitation was performed using MaxQuant as described in Greenwood et al (2012). Bar charts were prepared in MS Excel.

3.3.4 Quantitative Proteome Profiling of TNBC Tumour Tissue

Triple negative (ER -, PgR -, ErbB2 -) tumours were collected under LREC and NHS (Alldridge et al. 2008). Twenty-six tumour tissue samples were collected from Histopathology Department, Broomfield Hospital, Broomfield, Chelmsford, United Kingdom.

All patients are female and all tumours are TNBC. All tumours are grade three, which represents invasive ductal carcinoma with high-risk. Thirteen tumours are classified as high-risk because the patients developed distant metastases and died within five years of diagnosis, while patients with low-risk tumours have survived more than five years without relapse and without distance metastasis. Tumour samples were used to generate proteomic profiles and identify proteins which correlate with good survival G1 (low-risk and non-metastatic) and poor survival G2 (high-risk and metastatic). Furthermore, they were used to allow association between clinical outcome and the expression of EGFR, STAT1, MX1 and Scribble (Table 3-2).

Table 3-2 Breast cancer patient information

Cancer No	Age	Tumour grade	Lymph node Positive	Recurrence or death within 5 years
1	71	3	0	0
2	34	3	1	0
3	51	3	0	0
4	49	3	0	0
5	56	3	0	0
6	71	3	0	0
7	71	3	0	0
8	60	3	1	0
9	73	3	0	0
10	81	3	0	0
11	58	3	1	0
12	36	3	0	0
13	53	3	0	0
14	71	3	1	1
15	62	3	1	1
16	79	3	0	1
17	66	3	1	1
18	62	3	0	1
19	55	3	0	1
20	75	3	0	1
21	58	3	1	1
22	57	3	0	1
23	73	3	1	1
24	51	3	1	1
25	44	3	0	1
26	71	3	0	1

The more significant correlation is with the triple negative tumours from G2 poor survival patients (high-risk and metastatic) and over-expression of EGFR protein (Table 3-3), (Figure 3-10). Strong evidence of the over-expression of EGFR protein in the metastasis TNBC was found in the Mann-Whitney-Wilcoxon test by R-software program and box plotted; showed ($p-< 0.05 = 7.692e-07$). Clearly, the hypothesis that EGFR correlates with poor prognosis is significant at the p-value < 0.05 level. A positive correlation was found between two proteins (STAT1 and MX1) with p-value = 0.01976 in (Figure 3-11).

Table 3-3 Mean and Standard Deviations for tumour breast tissue G1&G2

Proteins	Mean for G1	STDEV for G1	Mean for G2	STDEV for G2
Scribble	3741215	4684700	5354969	2921883
STAT1	45155792	32376279	44667331	88258634
EGFR	13522892	8917523	3.69E+08	9.2E+08
MX1	43195815	48785735	54318508	88658068
GRP94	6.94E+08	4.46E+08	5.40E+08	4.59E+08

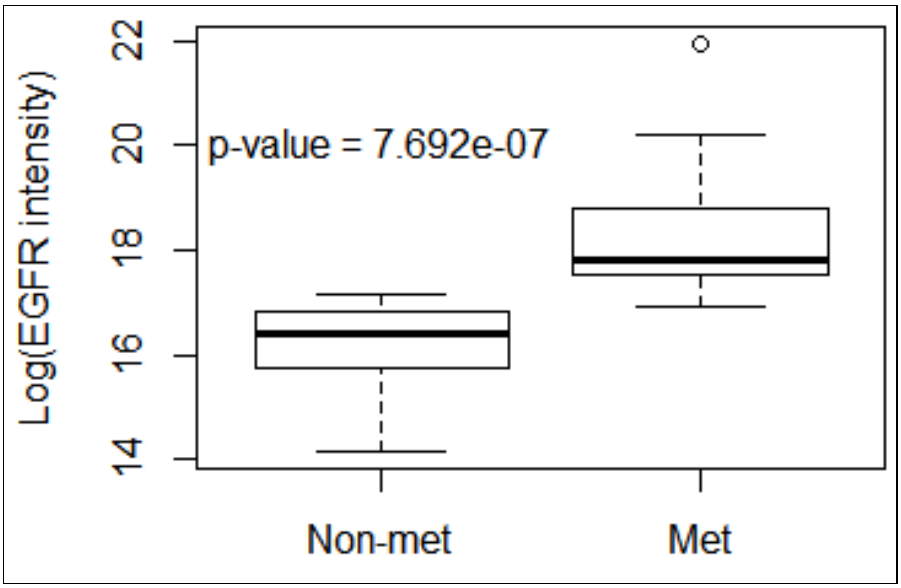


Figure 3-10 The heterogeneity of EGFR protein in metastasis and non-metastasis tumour

Boxplots and a Mann-Whitney test were performed for statistical analysis, to show and compare the level of EGFR protein intensity in human breast metastatic (Met) tumour in patients with poor survival and non-metastatic (Non-met) from patients with good survival. The Mann-Whitney-Wilcoxon Test in R- shows that p-value = 0.0000007692 (7.692e-07).

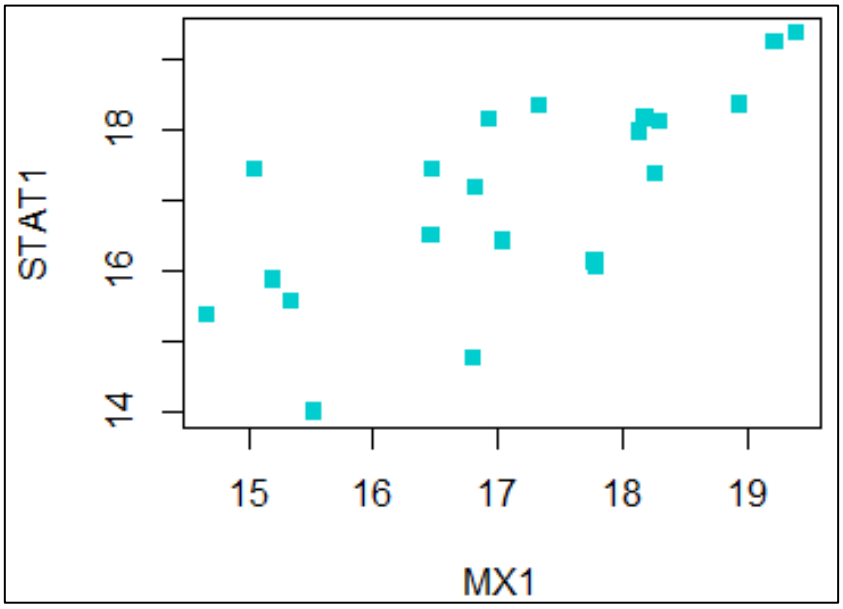


Figure 3-11 The correlation between MX1 and STAT1.

Shows the positive correlation between STAT1 and MX1 with R^2 =0.8322. Each plot represents the log of numeric data from patients with metastatic (poor survival) of TNBC in a R- program.

No significant correlations were found between STAT1 and EGFR or Scribble. Furthermore, no clinical significance greater than the EGFR protein was observed from the other candidate proteins (

Figure 3-12 and Figure 3-13)

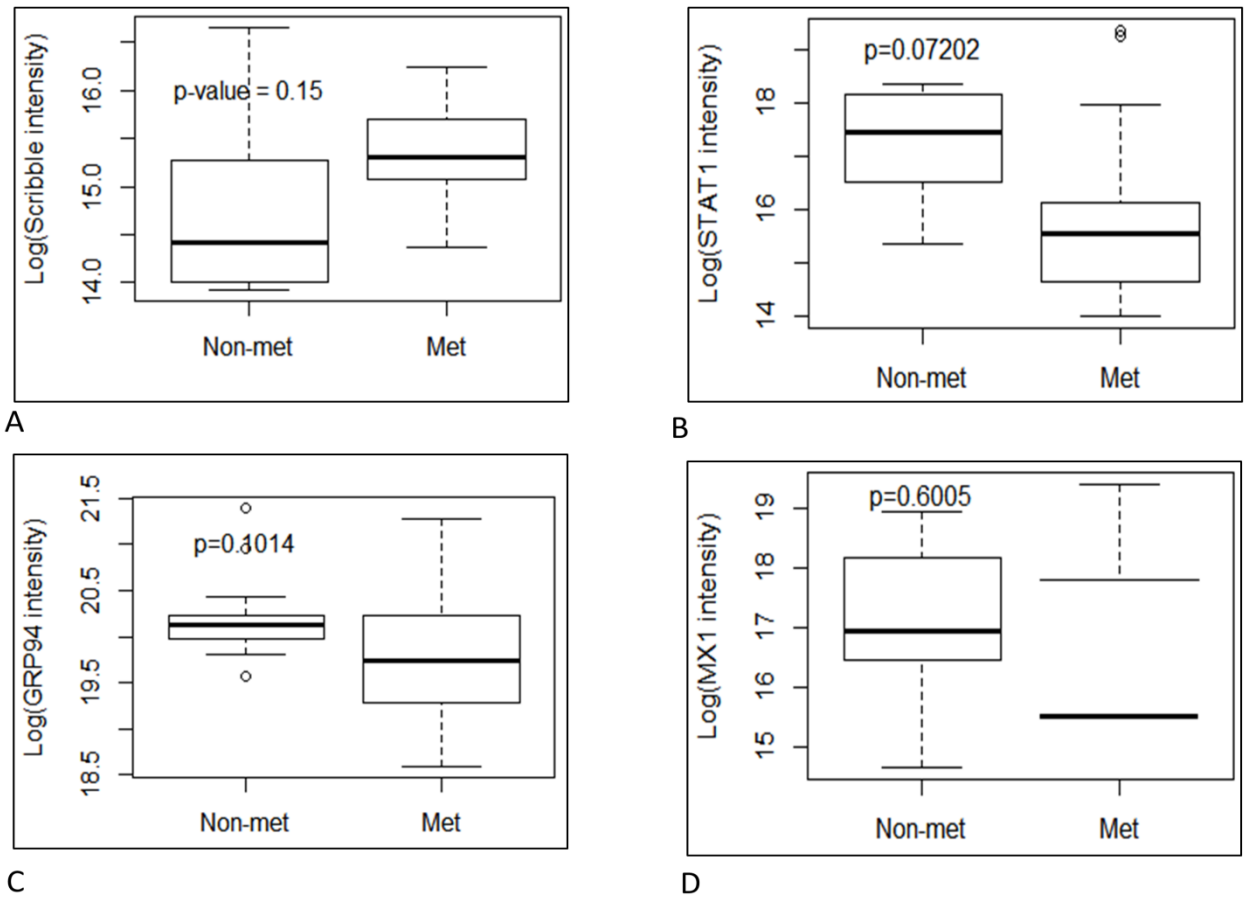


Figure 3-12 Candidates protein intensity in human breast non-metastasis and metastasis tumour tissue

Illustrates the Boxplots were performed for statistical analysis, to show and compare the level of Scribble(A),STAT1(B), GRP94(C) and MX1(D) protein intensity in human breast metastatic (Met) tumour in patients with poor survival and non-metastatic (Non-met) from patients with good survival. The Mann-Whitney-Wilcoxon test in R- soft program shows that p-values for each these proteins.

Additionally, no significant correlations were found between MX1 and EGFR or Scribble.

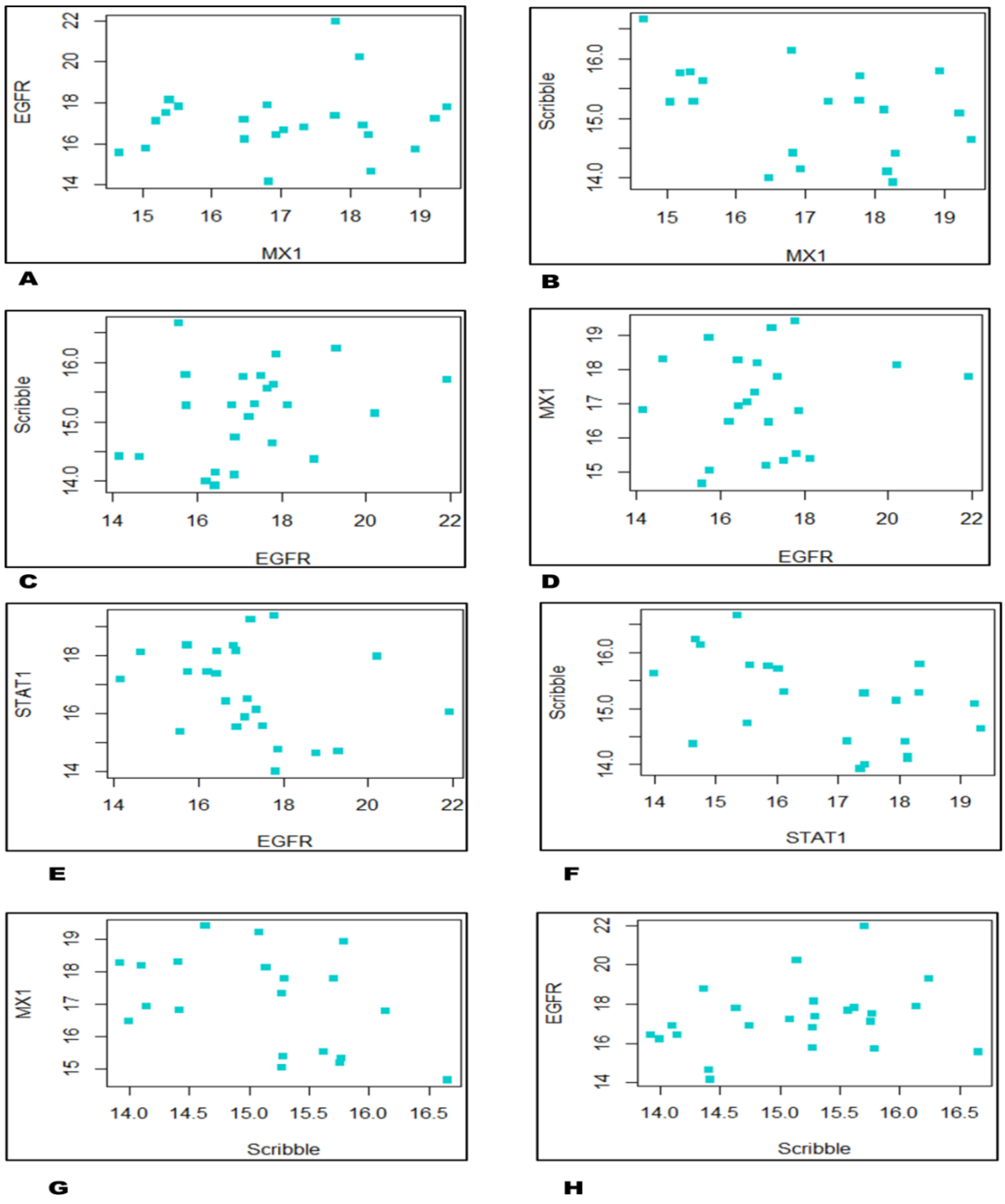


Figure 3-13 The correlation between candidates proteins.

The figure illustrates the XY Scatter plot. It represents the log of numeric data from patients with metastatic (poor survival) of TNBC in R-program and R^2 value is provided to calculate the correlation between (A) EGFR and MX1 with R^2 = 0.0002 (B) Scribble and MX1 with R^2 =0.037. (C) Scribble and EGFR with R^2 = 0.0148. (D) MX1 and EGFR with R^2 = 0.0479. (E) STAT1 and EGF with R^2 = 0.04272. (F) Scribble and STAT1 with R^2 = 0.0667. (G) MX1 and Scribble with R^2 = 0.01653.

3.4 Discussion

TNBC is increasingly recognised as a disease that lacks the rationale-based therapy and molecular markers to assist classifications, diagnosis, prognosis and thus the selection of therapeutic options (Cleator et al. 2007). Breast cancer is a dynamic disease that evolves with time and as a function of therapy with special biological properties that permit travel to distant sites and establishment of clinically disseminated disease. The metastatic breast cancer cell frequently differs from primary breast cancer in properties such as the cell receptor status. The tumours of this subtype do not express the nuclear hormone receptors ERα, PgR, and the epidermal growth factor receptor Her2. With improvements in the systematic treatment of TNBC, nodal status, tumour size and Androgen receptor expression are the most useful prognostic markers; yet, the survival rate of these patients has not improved significantly over the past two decades. My approach relies on the assumption that the over-expression of a specific group of proteins (Scribble, CD74, STAT1, EGFR and MX1) increases the malignancy and metastatic propensity of TNBC. This assumption is based on the correlation between specific patterns of protein expression in human breast tumour tissue and breast cancer cell lines (MDA-MB-435, MDA-231, MCF7, ZR, and MDA-MB-468) with the metastasis process.

Table 3-4 Summary of most significant protein expression results obtained in studies of breast cancer cell lines and tumour tissue.

Breast cancer cell lines and HEK293	Tumour tissue samples
Scribble stronger signal in MDA468 than in HEK293.	EGFR overexpressed in node-positive TNBCs.
EGFR stronger staining in MDA468 cells	STAT1 and MX1 expression correlated.
STAT1 and EGFR expression seem correlated in CRISPR/Cas9 experiments	

In proteomic development, there has been an impressive emergence of mass spectrometry-based technologies applied towards the study of proteins and the elucidation of the proteomics of a specific disease (Semmes et al. 2006) which provide promise for better diagnosis, prognosis and therapeutic intervention. Clinical proteomic platforms are quickly becoming very powerful and useful tools in cancer research because they do not only promote an early detection of the disease, but can also be used for determining the cancer risk stratifying the disease state and for grading the monitored response to therapy.

In grade 3 tumours, the orignisation of tumour tissue and tumour cells appear not normal and tend to grow rapidly and spread faster compared with poorly differentiated tumours with 2 grades. The lower the grade, the less aggressive the breast cancer). Conversely, higher grades indicate a more aggressive breast cancer (Pereira et al. 1995). The breast cancer tumor grade is based on both cytological and architectural features of the breast cancer specimen and the pathologist report (Jo et al. 2009). Tumour grade, tumour size and the number of affected positive nodes are prognostic factors that indicate the state of advancement of a tumour and early diagnosis. The patient cancer death rarely results from failure to control local disease; the outcome is usually determined by the development of metastatic disease. Thus, metastasis is the master reason for the resultant and consequent mortality of patients with cancer (Martin et al. 2013).

One fundamental issue (pitfalls) before considering using human tissue for clinical research, includes the patient consent, therapeutic reasons and tissue specimen annotation (Grizzle et al. 2011). Small fragments of breast tumour tissue are a heterogeneous population, containing fibroblast, epithelial cells and unknown cell type. The fibroblasts can extend growth to the epithelial cells and the disadvantage of the outgrowth, is that it may give rise to a rapid genetic proliferation of normal cells (Speirs et al. 1998). The combination of human tumour tissue and tumour cell lines cannot extrapolated to all patients, because, this combination may not represent the natural history of distant metastasis as recurrences after chemotherapy and radiation treatment. The breast tumours are often treated with a chemotherapy or radiation system before surgery; such therapy system may destroy selective cell population (normal and neoplastic cell type) and affect the metastatic lesions which differ from the original disease.

Mass spectrometry and proteomics provide a quantitative data set of breast tumour proteomes, which allows us to study and compare the abundance of all identified proteins. Variability in the specimens may however affect the utility of the data obtained in small scale studies.

Membrane extraction techniques were used in this study to attempt to increase the analytical power of the proteomic approach and discover which proteins are associated with important signal transduction and migration and metastasis, as well as, evaluate the potential of using these proteins as biomarkers. Our candidate proteins (EGFR, STAT1, and Scribble) are well expressed in tumour cell lines and tissue specimens and EGFR specifically was overexpressed in the metastatic human tumour tissue. This finding has important implications for understanding the role of these proteins in metastasis. It would be beneficial to determine if these candidate biomarkers are expressed heterogeneously in the tumour tissue, as it contains tumour cell population with capacity to self-renew and differentiate which create a heterogeneous tumour cell population (Rosen and Jordan 2009, Nowell 1976).

As mentioned, this group of proteins (Scribble, STAT1, EGFR and Mx1) were targeted and measured by accurate, high-resolution mass spectrometry. Previously, this set of proteins was suggested to have an essential role in metastasis and to be an enhancer of the survival of tumour cells. The data analysis obtained from this initial experiment demonstrates that my quantitative method is very reproducible. The data yielded by this approach provide convincing evidence that, for example, the highest expression level of EGFR was in one of

the metastatic cell lines MDA-MD-231, while the other metastatic cell line model do not express EGFR. The analysis of clinical tissue samples showed that the expression of the four markers is very heterogeneous. Treating the data as originating from two homogeneous groups, a high-risk group of metastatic cancers, and a low-risk group of non-metastatic cancers did not result in statistically-significant p-values when the Student's t-test was applied to calculate statistics.

Thus statistical analysis illustrated the over-expression of EGFR in the high-risk group of the 26 tumours samples of TNBC patients with p-value < 0.05 [Fisher's Exact Test p-value = 0.00003269 [3.27E-05] and Pearson's Chi-squared test p-value = 0.002928] and the positive correlation between [STAT1 and MX1 with p-value = 0.01976] that the basis of the evidence currently available, it seems fair to suggest that this group of proteins has the potential to become a multimodal bio-marker to assist classifications, diagnosis, prognosis and the selection of therapeutic options. Furthermore, the findings observed in this study confirm the previous studies, Metodieva et al (2013) showing a decreased Scribble expression in MDA-MB-435, the only cell line that expresses CD74. Scribble protein acts as a scaffold protein and is associated with the β catenin/cadherin complex. It is phosphorylated on multiple canonical MAPK sites. A deficiency in Scribble protein results in the loss of epithelial apical-basal polarity manifested in the maldistribution of apical polarity and adherence junctions to the basolateral cell surface and abnormal cell growth (disorganisation).

We have used the one dimensional SDS-PAGE method and an approach that combines this method with mass spectrometry for the detection and the identification of proteins. 1D-SDS-PAGE is a highly sensitive, specific, accurate, and reproducible technique in which, proteins are separated according to their molecular weight.

Accumulating evidence has highlighted increased STAT1 activation with increased tumour progression in multiple types of cancer, such as breast cancer (Hix et al. 2013). It seems possible that these results are due to the essential role that STAT1 and MX1 play in breast cancer development, which in our analysis is highly correlated. The observed correlation between STAT1 and MX1 might be explained by the known mechanisms by which STAT1 activated by IFN, stimulates the expression of anti-viral immunity (Weaver and Yang 2013). Our results agree with Zhi-Ming's group report which studied varying levels of STAT1 expression (Luo et al. 2010). In addition, STAT1 has been associated with an anti-tumour effect. However, accumulating evidence has explained an increased STAT1activation with a more rapid tumour progression in multiple types of cancer, such as breast cancer (Hix et al. 2013). As summarized in the introduction, the loss of STAT1 is associated with breast cancer development based on data using STAT1-/- models. This part of our research suggests that increased STAT1 levels correlate with the increased invasion, migration and survival of invasive breast cancer cells. This seemingly contradictory and confusing roles of STAT1 in tumour progression; underscore the fact that many functions essential in normal cell biology such as proliferation, differentiation, adhesion, cell-cell interactions motility and migration are linked with each other and are highly relevant for stages of tumourigenesis

starting by precancerous lesion which includes enhancing metastasises of malignancies (Braun et al. 2009).

Prior studies that have noted the importance of GRPs family, GRP95, in breast tumour cells survival. Therefore, expression status of GRP95 could be part of the intrinsic biology of cancer cell lines due to its function in exhibition of both tumor and stem cell characteristics (Nami et al. 2016), such as, heat shock chaperons protein, which are charged with regulation of protein machinery and modulation of endoplasmic reticulum homeostasis (Wu et al. 2017). GRPs are essential regulator of ER function due to their function in protein translocation, folding and assembly, targeting malfolded protein for degradation, ER Ca^{2+} binding and controlling the initiation of ER stress sensors (Luo and Lee 2013). GRP95 was used as normalising factor as it is very abundantly detected in the membrane fractions of all breast cancer cells studied. Its abundance also did not show much variability across the cell lines and tumour tissue samples studied. STAT1 and breast cancer must be interpreted with caution as the immune system in humans does not function as in STAT-/- mouse models in STAT1 – overexpressing or STAT1- deleted breast cancer cell lines, in-vitro and in-vivo, despite to the fact that the source for these cell lines is human.

In this research, proteomic data from human tumour tissue (node-negative and node-positive samples) and breast cancer cell lines revealed a relative abundance variability of the set of proteins (EGFR, STAT1 and Scribble). There are several possible explanations for this result; the tumour tissue and breast cancer cell line are enriched for proteins involved in cytokine signaling, mRNA processing, growth factor signaling and the cell cycle.

Protein abundance variability can arise through differential gene expression, regulation of turnover: if degradation is inhibited while translation is unaffected the protein abundance would increase.

Alternatively, the rate of translation could be increased in response to certain signals. This level of control is different compared to post-translational modifications that change the activity of the protein but not its abundance. To get the full picture of protein abundance regulation we would need to measure mRNAs together with translation rates, together with rates of degradation. This is not possible with the technology of today. Measuring mRNA is possible but has its own problems when a cell line model such as in breast cancer cell line and Hek293 are considered. Since, the harvesting time may reduce the expression of housekeeping genes and proteins involved in mRNA stability. This in turn may either directly or indirectly affect the mRNA half-lives. Therefore, care must be taken in extrapolating data collected up to 48-72 h.

Our data suggest that the study of this selected set of proteins can be developed by increasing the number of breast cancer cell lines and focus on additional candidate protein targets. Since, other cell lines would enrich the variety of membrane, cellular and secreted proteins and make the model more similar to the breast tumour tissue. Protein abundance data were analysed by proteomics, which is a powerful way to study also the posttranslational modifications. However, the heterogeneity of the human tissue can provide data which are irrelevant to the tumor cells. However, post-translationally modified proteins such as phosphoproteins actually could be one of the most unstable types of molecules in tissue specimens.

Thus, very rapid processing of specific tissue to maintain phosphoproteins may be necessary for some types of tissue and/or research. Investigators should evaluate the stability in tissue of the molecular features they are studying and should be aware that such stability may vary among specific tissues. The results of this study showed that the challenges presented by poor antibody and inefficient sensitivity of detection by WB can be addressed by using LC-MS/MS, for example, WB experiments did not show a clear band for EGFR owing to probably insufficient sensitivity, which was then addressed by using mass spectrometry. The present study was designed to identify molecular pathways that contribute to metastasis in TNBC. Our candidate proteins (EGFR, STAT1 and Scribble) show differential expression in breast cancer cell lines and tumour tissue. It seems possible that these finding are due to the essential role of EGFR as downstream signaling steps for regulating cell growth in epithelial tumour. Katherin (2007), also found that EGFR has different expression data profiles in breast tumour subtypes.

Another possible explanation for high expression of EGFR in metastasic tumour tissue is the EGFR-RAS-MEK pathway signatures in-vivo, as well; stimulation of EGFR leads to active the signaling molecule of STAT1 and STAT3 of STAT family. This activation was constitutive in transformed squamous epithelial cells. Links to pathway signatures, receptor dimerization of EGFR leads to activation of the intrinsic protein kinase activity. EGFR and HER2 are overexpressed in breast cancer and this is linked with a more aggressive clinical behavior (Hoadley et al. 2007). EGFR can also be activated constitutively by mutations, but our study did not aim to detect such events. In addition to receptor activation and as mentioned above, STAT1 are also activated by many growth factor receptors with intrinsic

tyrosine kinase activity. Regulation of JAK-STAT pathway is achieved by STAT1 binding to target gene promoters in the nucleus (Aaronson and Horvath 2002).

Patterns in expression levels of Scribble may also be explained by the fact that Scribble has been implicated in JAK-STAT pathway also (Greenwood et al. 2012). Scribble is a scaffold protein that is involved in the cell polarisation and migration and in the development of breast epithelium (Zhan et al. 2008).

Explaining differential expression of STAT1 in breast tumours is complicated. The increased STAT1 abundance (relative to the baseline) could come in some tumours from infiltrating immune cells. These would overexpress STAT1 in response to cytokines. In other tumours it coud be the tumour cells themselves that overexpress STAT1, again, it could be in response to cytokines secreted by the immune cells or other cells, including some tumor clones bearing specific mutations or epigenetic abnormalities. The human tumour tissue is associated with state of chronic inflammation and the immune system can inhibit and even reduce established tumour growth (Yu et al. 2009). The STAT protein family has a regulator role of cancer-associated inflammation and influence interactions between tumour cells and their immune microenvironment; and determines whether the inflammation promotes or inhibits cancer. Moreover, the dual role of STAT family can acts as transducer of signals (cytoplasm) and transcription factors (nucleus) (Darnell et al. 1994). Thus, STAT expression can be seen as a marker of chronic inflammation in the tumour. Another possible explanation for the correlation between immunity system (involvement) and STAT1 is that, tumour associated macrophages (innate immunity cells) have a promoter role through

secreted angiogenic, metastatic and growth factors (Matsukawa et al. 2005). As well, myeloid lineage of Hematopoietic Stem Cells (HSCs) and Myeloid-Derived Suppressor *Cells (MDSC*) directly contribute to tumour growth as suppressors of anti-tumour immunity (Ostrand-Rosenberg and Sinha 2009). However, other innate immune system cells, such as natural killer cells and dendritic cells, can attack tumour cells when appropriately activated (Trinchieri 2003). As mentioned in introduction chapter, tumour-cell proliferation and survival, tumour angiogenesis and metastasis are the hallmarks of cancer, for thus simultaneous activation of proto-oncogenes and oncogenic signalling pathways with inactivation of tumour-suppressor genes are vital and crucial processes in malignant transformation and progression. The weak role of immune cells in tumour microenvironment is promoted to oncogenesis activity. Signal transducer and activator of transcription protein family (e.g STAT1, STAT3 and STAT3) are constitutively activated (dual role) in tumour cells and in immune cells of the tumour microenvironment. Several studies in multiple cancer models have implicated constitutive STAT1 activation as tumour promoting factor, for example, STAT1 overexpression in human squamous carcinoma cells was found to induce pro-survival genes and resistance to genotoxic stress. STAT1 overexpression in breast cancer may have tumour – promoting rather than tumour- suppression role (Hix et al. 2013).

Patients data was collected (Table 3-2), under LREC and NHS Trust were collected under Local Research Ethics Committees (LREC) and National Health Service (NHS) from Histopathology Department, Broomfield Hospital, Broomfield, Chelmsford, United Kingdom, The pathologist report mentioned that all tumours are grade three, which represents an invasive ductal carcinoma with high-risk. The first group (thirteen tumours) is classified as a

high risk group because the patients have node positive tumours developed distant metastases subsequently. The second group (thirteen tumours) patients with low-risk tumours have survived without relapse and no distant metastasis. The clinical information was collected based on the aim of this study and the analytical methods, such as 1D – SDS/PAGE and mass spectrometry. WB is a validation experiment following the proteins profiles data. Tumour samples were matched the Grade III triple negative (ER− /PgR− /ErbB2−), size and grouped by their nodal status (first group had at least 2-postive nodes and the second group was negative).

All methods are accurate for the research aims; however, these methods include limitations. It was not possible to investigate a large enough collection of tissue, because human triple negative breast cancer frequency is low. Therefore, part of proteomics data analysis did not yield statistically significant results in the correlations between the candidate proteins and nodal status.

3.5 Conclusion

The results presented in this chapter have highlighted potentially interesting differences in abundance of several candidate proteins (Scribble, STAT1 and EGFR), between invasive and none invasive tumours and across a panel of breast cancer cell lines. A clinical proteomic analysis for breast cancer cell lines as model and human tumour tissue demonstrated to be reproducible with a significant p-value. We demonstrated the over expression of EGFR in 26 tumour samples of TNBC patients with p-value < 0.05 [Fisher's

Exact Test p-value = 0.00003269 [3.27E-05] and Pearson's Chi-squared test p-value = 0.002928] in the metastatic group and the positive correlation between [STAT1 and MX1 with p-value = 0.01976].

This finding suggests that proteomics could be good approach for elucidation of the mechanisms of TNBC. In addition, results of this chapter seen in the context with earlier scientific published consideration, in which, STAT1 supports CD74 overexpression and dual role in tumour immunity (as mentioned above). Scribble is connected with the cadherin/catenin beta complex and is implicated in cell polarisation and migration of epithelium tissue. EGFR is associated and involved in the STAT family pathway and aggressiveness to breast tumour tissue. All these results are valuable clues towards determining which proteins might be exploitable for the clinical categorisation of TNBC, for example, tumours that overexpress EGFR and deregulate Scribble might be targeted with specific inhibitors. Further, these results reveal the data from mass spectrometry is essential to establish various biomarkers for early metastasis and provide a better understanding of the mechanism of metastasis and aggressiveness of TNBC.

Chapter 4 Results of Immunocytochemistry of STAT1, EGFR and SCRIBBLE Proteins in Human Breast Cancer Cell Lines

4.1 Introduction

Immunocytochemistry staining (ICC) is together with immunological and biochemistry reaction to identify discrete cell culture component through interaction of target antigens with specific antibodies with a visible label. Additionally, it is capable to visualise the distribution and localisation of specific cellular and sub-cellular components within the normal and cancer cell. It is essential to recognise the importance of Immunocytochemistry and analyse the localisation (plasma membrane, cytoplasm and nucleus) and the expression pattern of a group of protein candidates. The ICC method is one of the more practical ways to identify the cell type and the origin of metastasis.

This research analysed the expression patterns of three proteins (EGFR, Scribble, and STAT1) by using immunocytochemistry to assess their relevance in TNBC. This study used commercially available antibodies and human adenocarcinoma breast cancer cell lines MDA-MB-468 and human kidney cell line HEK293. Immunocytochemistry staining for each protein was performed on fixed 70%-80% confluent monolayer of human breast cancer cells and HEK293 cells. Fixed breast cancer cell lines were treated with different concentration of primary antibodies (anti-EGFR, anti-STAT1, anti-Scribble) to determine the optimal concentrations.

From the pattern of expression for each protein on the breast cancer cell line slide (small round 22 mm coverslip), it can be seen that the EGFR expression was found in eight slides in 80% rate of expression in breast cancer cell lines MDA-MB-468. These results show that the prognostic value total abundance in tumour cells of the studied group of proteins (EGFR, Scribble, and STAT1) can be further enhanced by investigating the spatial patterns of proteins expression. It supports the idea that the three protein expression profiling may represent a clinically practical approach to assess triple negative breast cancer heterogeneity and prognosis in various stages and possibly to stratify patients for targeted therapies.

ICC is used to anatomically visualise the localisation of a protein expression in the cells. It allows researchers to evaluate and determine which sub-cellular compartments are expressing the protein. In addition, it is valuable tool for the determination of cellular contents from separate cells. Antibodies represent an essential tool for demonstrating both the presence and the sub-cellular localization of any protein. It was clearly shown that MX1 is over-expressed in metastatic TNBC (Greenwood et al. 2012), as well as, CD74 (Metodieva et al. 2013).

This chapter complements results previously reported in chapter three (Mass spectrometry and proteomic study of human cancer cell lines and TNBC human breast tumour tissue) and will possibly provide a more proper understanding of the mechanism of metastasis and aggressiveness of TNBC, in order to improve the way of diagnosis and target therapies. Here, we showed it was possible to visualise the distribution and localisation of Scribble, STAT1, EGFR protein expression in a cellular component within an adenocarcinoma breast

cancer cell, such as cell-cell junction, plasma membrane as well as, in the subcellular location such as cytoplasm.

4.2 Methods

The second question of our hypothesis is to determine the cellular location of the candidates proteins, for detection of candidates proteins Scribble, STAT1 and EGFR, which provide higher sensitivity and specificity for TNBC diagnosis than is afforded with single protein analysis. This study used Immunocytochemistry procedure was performed on acid treated glass cover slips (2.6.1). Breast cancer cells were grown as monolayer cultures (2.6.2). Cells were fixed by methanol 100%2.6.3) and permeabilised by PBS-TritonX-100 for 30 mins 2.6.4). The unspecific binding was blocked in 1X PBS, 0.05% Tween-20, 2% Serum, 1% BSA. Next, cells were incubated with primary Ab 1/1000 [Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA], and secondary Ab (s) 1/2000 goat-anti-rabbit IgG –HRP-SC-2030 and goat-anti-mouse IgG –Sc-2005 (2.6.6).

The Antigen retrieval step has been done by heating the citrate buffer PH= 6. The coverslips were removed from the buffer and immersed in PBS for 3 min. In order to block the endogenous peroxidase, the cells were treated with 0.3% H_2O_2 in Methanol for 30 mins. The heating step is not necessary for EGFR and Scribble proteins. For counter staining, hematoxylin was used for 10 seconds and dehydrated in 95% ethanol and Absolute ethanol for 2 mins each time.

The mathematical equation is determined for each cell in a fixed field by one method; the percentage of cells at each staining intensity level is calculated. Finally the H-score is assigned the following equation (Hirsch et al. 2003).

[1*(% cells 1+) +2* (% cells 2+) +3*(% cells 3+)]

Statistical analysis was performed using Student's t-test in R-software program; as an inferential statistical test that determines whether there is a statistical difference between means in two unrelated groups. Results were tabulated into Microsoft Excel worksheet and exported into the R-programming language for comparison of M and SD (Adam et al. 2002). For each M value two replicate measurements were calculated. Results were considered statistically significant when P-value < 0.05. Cells were detached, stained and counted under low magnification on the BX41 Olimpus microscope and magnification power was x40 objective.

4.3 Results

4.3.1 Cellular Localisation of EGFR, STAT1 and Scribble

This chapter is part of our research aimed at studying the mechanism of metastasis in TNBC. ICC was used to study the pattern of protein expression and localisation in the human breast carcinoma cell line MDA-MB-468 (triple negative breast cancer cell line). The morphology of adherent monolayer culture of breast carcinoma cell line on the glass coverslip can be identified by cell-cell junction, plasma membrane and cytoplasmic characteristics. Furthermore, ICC was used to determine the cellular localisation using the (DAB - 3, 3-

diaminobenzidine), which develop the brownish colour as a product of peroxidase reaction as positive result given that our group of proteins is expressed in the human breast carcinoma cell lines MDA-MB-468.

This research found that the expression pattern of EGFR, STAT1 and Scribble in the human breast carcinoma cell line (triple negative breast cancer cell line MDAMB468) differ significantly from the expression patterns observed in HEK293 cells p-value < 0.05 [Student's *t*-test for EGFR p-value= 2.643e-10, STAT1 p-value = 7.727e-07 and Scribble p-value= 7.115e-08]. These results are valuable clues toward understanding the mechanisms of TNBC metastasis and to determine, which assessed the clinical categorisation of this type of breast cancer. Optimisation of the cell fixation, blocking, antibody incubation, and antigen retrieval steps ensured strong and specific signal. EGFR protein expression was found in cell surfaces (external and internal of plasma membrane). The same method to detect Scribble was used to examine the presence of a brown detection chromogen on the edge of the hematoxylin stained on the external side of the cell membrane, peripheral membrane and cell projection adjunction as subcellular locations. The results from all slides revealed a heterogeneous pattern of staining in the human breast carcinoma cell line MDA-MB-468 (triple negative breast cancer cell line) EGFR, STAT1, Scribble status were identified as positive based on the staining by immunocytochemistry (Table 4-1, Table 4-2).

Table 4-1 The Concentration of primary antibodies

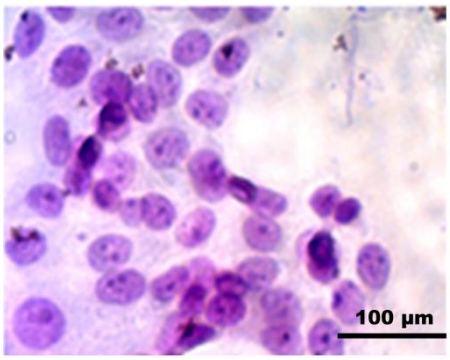
Primary Ab(s)	Conc /experiment	Name of the cell line	Incubation time
Anti-Scribble Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA	1:1000 1:500 1:330	MDA-MB-468 Hek-293 MCF-10A	Overnight at +4° C
Anti-STAT1 Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA	1:500 1:330	MDA-MB-468 Hek-293 MCF-10A	Overnight at +4° C
Anti-EGFR Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA	1:500 1:330	MDA-MB-468 Hek-293 MCF-10A	Overnight at +4° C

Table 4-2 The Concentration of secondary antibodies

Secondary Ab(s)	Conc /experiment	Name of the cell line	Incubation time
Goat-anti-mouse-IgG–Sc-2005 Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA	1:2000 1:1000 1:500	MDA-MB-468 Hek-293 MCF-10A	2 hour 1 hour 1 hour
	1:2000 1:1000 1:500	MDA-MB-468 Hek-293 MCF-10A	2 hour 1 hour 1 hour
Goat-anti-rabbit-IgG–HRP-Sc-2030 Santa Cruz Biotechnology, Inc., Santa Cruze,CA,USA	1:2000 1:1000 1:500	MDA-MB-468 Hek-293 MCF-10A	2 hour 1 hour 1 hour

4.3.2 EGFR Protein Expression

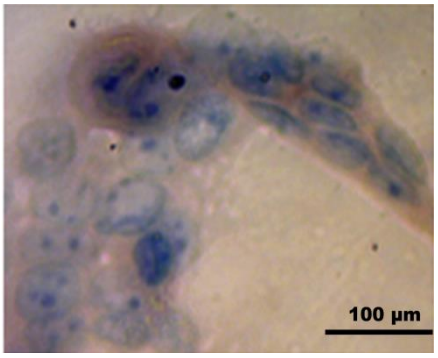
A positive ICC staining was defined as the presence of a brown detection colour on triple negative breast cancer cell (MDA-MB-468). We observed high levels of EGFR expression and the proportion of positively stained in plasma membrane was notably higher than in the cytoplasmic localization (Figure 4-1-Figure 4-2).



A

Figure 4-1(**A**) Image of negative control of EGFR protein in MDA-MB-468

Immunocytochemical image of negative control of EGFR protein in MDA-MB-468, Cells were grown in DMEM with 10% FBS. Immunostaining was performed after 48 hrs and stained by Hematoxlin. Magnification power was x40 objective. The image bar was 100 µm.

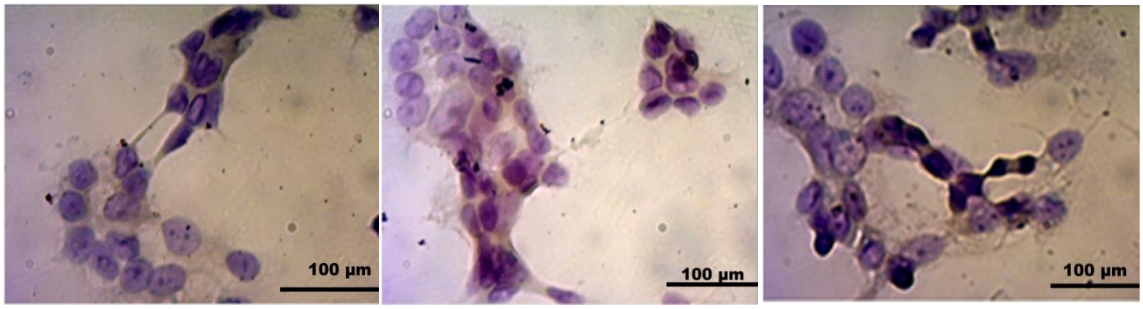


B

Figure 4-2 (**B**) EGFR protein localisation in MDA-MB-468

Detection of EGFR protein in MDA-MB-468 (B) and (C) represent images of EGFR protein expression detected with anti-EGFR antibody and stained by Hematoxylin in immunocytochemistry. Magnification power was x40 objective. The image bar was 100 µm.

This study consistently observed stronger (+2, +3) immunostaining in sub-cellular localisation of EGFR expression in breast carcinoma cultured cell with a strong immunostaining grad (+1 and +2) showing in the membrane (M) and cytoplasm (C) in MDA-MB-468 (Figure 4-3).



B **C** **D**

Figure 4-3 (**B, C, D**) EGFR protein localisation in MDA-MB-468

Immunocytochemical detection of EGFR protein in MDA-MB-468 Image (D), (E) and (F) represent the positive pattern of EGFR protein expression after treatment with anti-EGFR antibody and stained by Hematoxylin. Magnification power was x40 objective. The images bar was 100 µm.

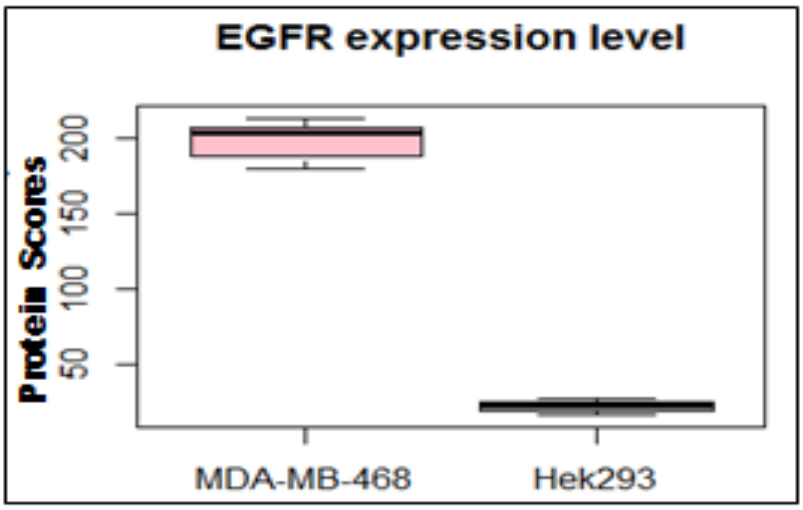


Figure 4-4 The EGFR expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of EGFR protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R- program software shows that p-value = 2.643e-10

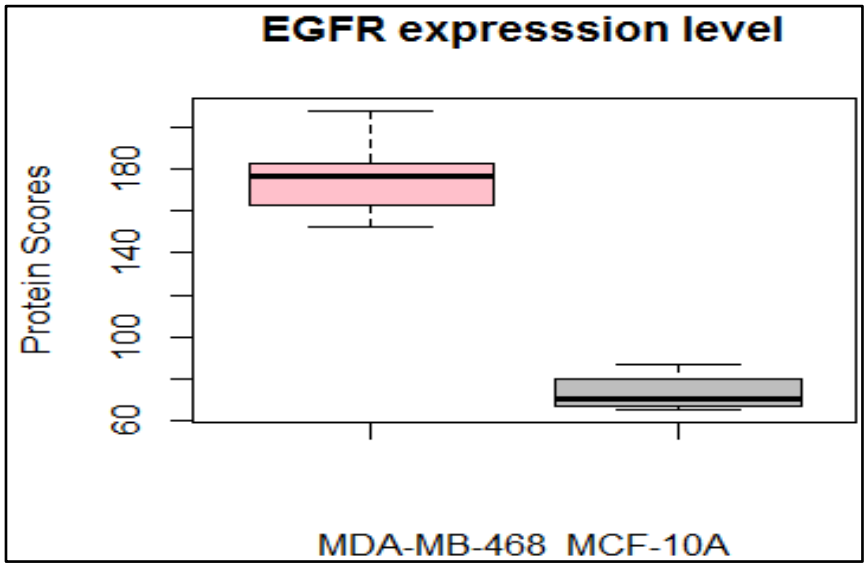


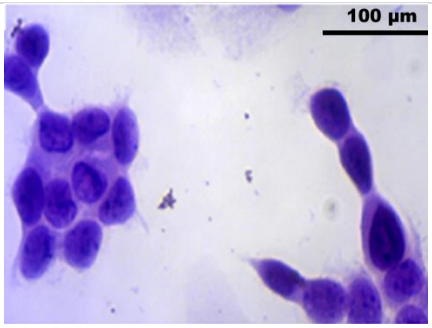
Figure 4-5 The EGFR expression level in MDA-MB-468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of EGFR protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human breast cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value =2.915e-07.

4.3.3 STAT1 Protein Expression

The role of Signal Transducer and Activator of Transcription 1 (STAT1) as a family of transcription factors and a pro-immune and anti-tumour transcription factor in the tumour suppressor is vital. To show the positive ICC staining on the human breast carcinoma cell line MDA-MB-468, we observed high immunoreactivity expression of STAT1 in cytoplasmic localisation (C) and weak staining in areas of plasma membrane

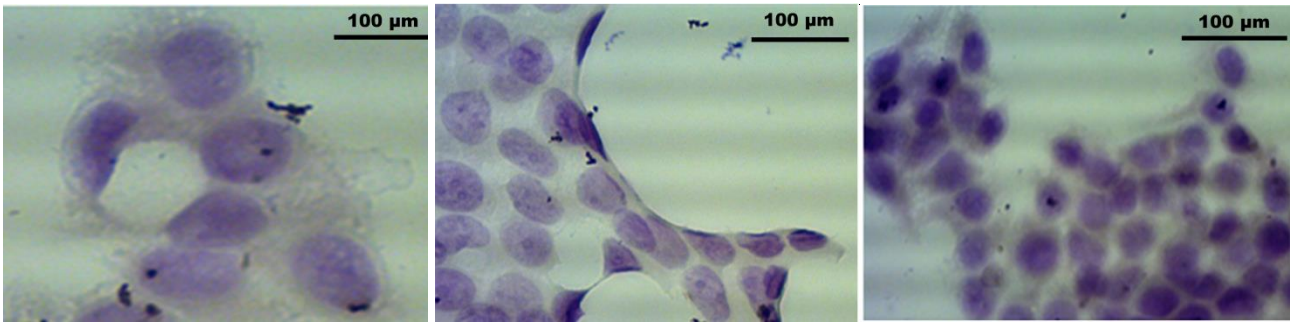
Figure 4-6).



A

Figure 4-6 (**A**) Image of STAT1 negative control in MDA-MB-468

Immunocytochemical detection of negative control STAT1 protein expression in the human breast carcinoma cell line MDA-MB-468. Cells were grown in DMEM with 10% FBS. Immunostaining performed after 48 hr and stained by Hematoxlin. Magnification power was x40 objective. The image bar was 100 µm.



B **C** **D**

Figure 4-7 (**B, C, D**) STAT1 protein localisation in MDA-MB-468

Immunocytochemical detection of STAT1 protein expression in the human breast carcinoma cell line MDA-MB-468. Images (B) and (C) represent a positive pattern of STAT1 protein expression in MDA-MB-468 after treatment with anti-STAT1 antibody and stained by Hematoxylin. Magnification power was x40 objective. The images bar was 100 µm.

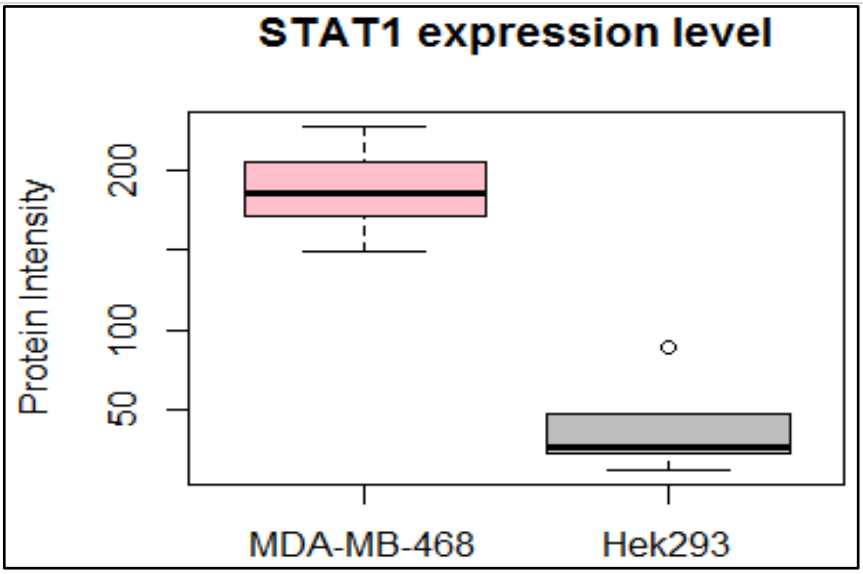


Figure 4-8 The STAT1 expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of STAT1 protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R- program software shows that p-value = 7.727e-07.

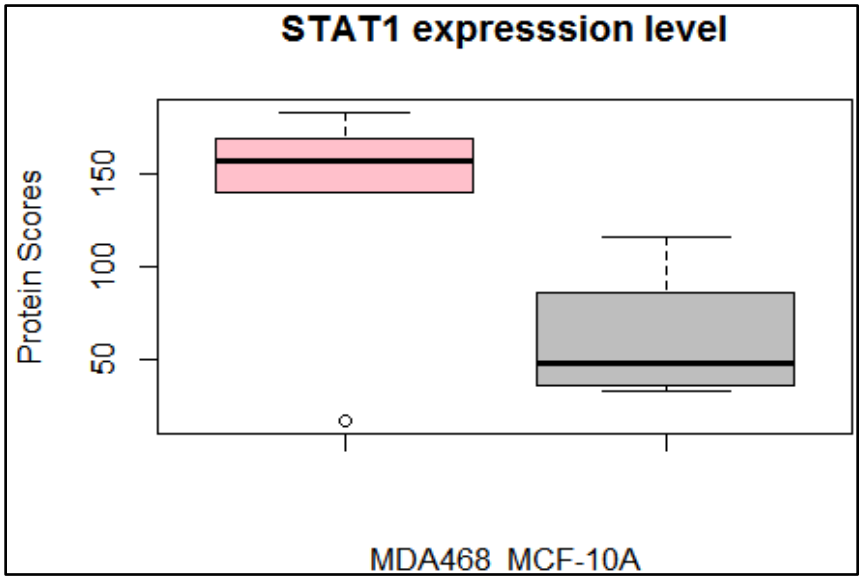
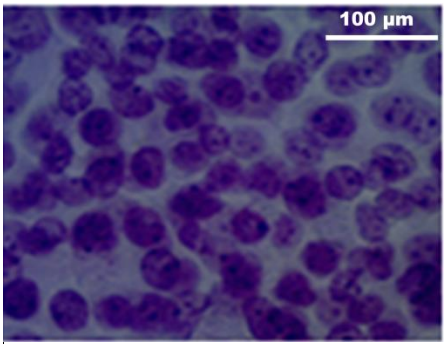


Figure 4-9 The STAT1 expression level in MDA-MB-468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of STAT1 protein intensity in (MDA-MB-468-PINK) and normal human breast cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value = 0.005924.

4.3.4 Scribble Protein Expression

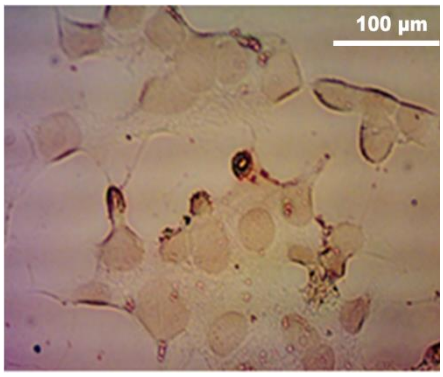
The pattern of Scribble protein expression in the triple negative breast carcinoma cells (MDA-MB-468) is indicated by the presence of a brown colour as immunoreactivity staining. We observed positive staining levels of Scribble protein expression in the areas of peripheral plasma membrane (
Figure 4-10).



A

Figure 4-10 (**A**) Image of Scribble negative control localisation in MDA-MB-468

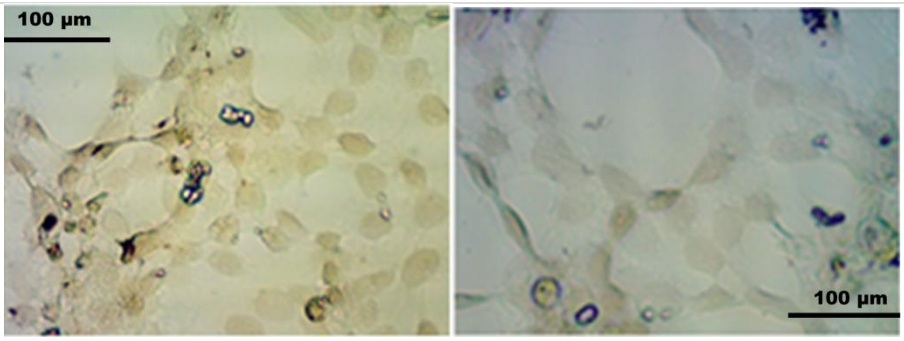
Immunocytochemical image of Scribble protein negative control localisation in MDA-MB-468.Cells were grown in DMEM with 10% FBS. Immunostaining performed after 48 hrs and stained by Hematoxlin. Magnification power was x40 objective. The image bar was 100 µm.



B

Figure 4-11 (**B**) The Scribble protein localisation in MDA-MB-468

Immunocytochemical detection of Scribble protein in the human breast carcinoma cell line MDA-MB-468. Image (B) represent a positive pattern of Scribble protein expression after treatment with anti-Scribble antibody and stained by Hematoxylin. Magnification power was x40 objective. The image bar was 100 µm.



C **D**

Figure 4-12 (**C, D**) The Scribble protein localisation in MDA-MB-468

immunocytochemical detection of Scribble protein in human adenocarcinoma breast cancer cell line MDA-MB-468. Images (C), (D) and (E) represent of a strong positive pattern of Scribble protein expression after treatment with anti-Scribble antibody and stained by Hematoxylin. Magnification power was x40 objective. The images bar was 100 µm.

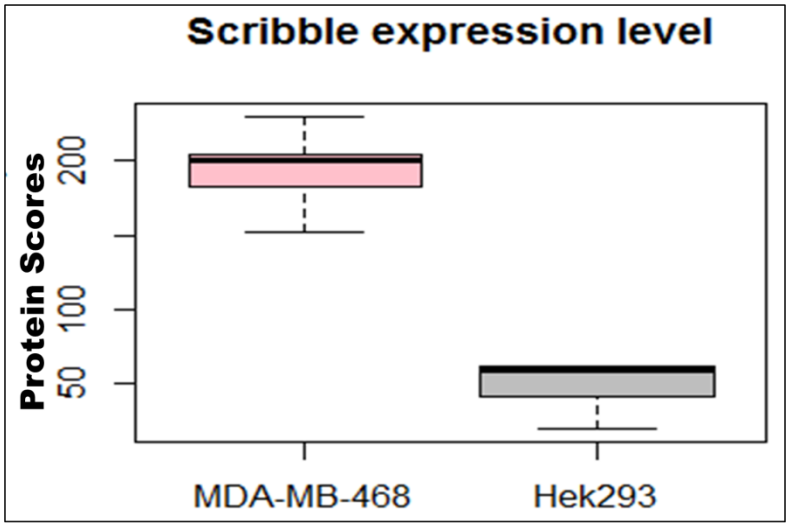


Figure 4-13 The Scribble expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of Scribble protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R- program software shows that p-value= 7.115e-08

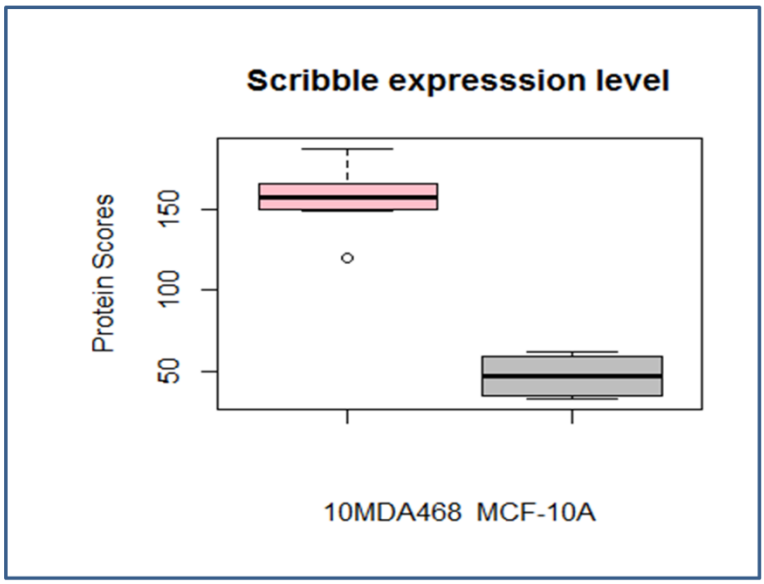
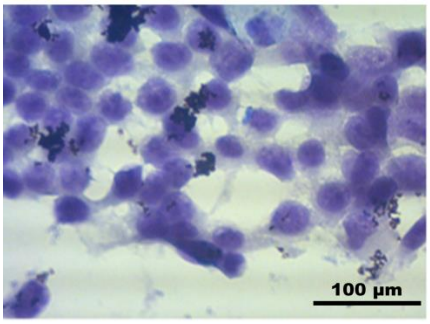


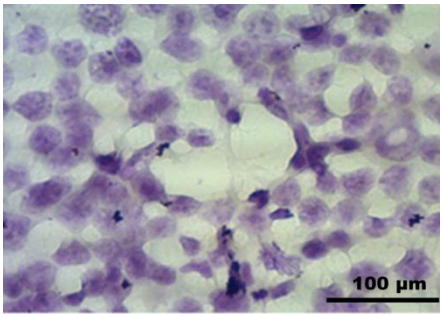
Figure 4-14 Scribble expression level in 10MDA-468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of Scribble protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value= 4.144e-07

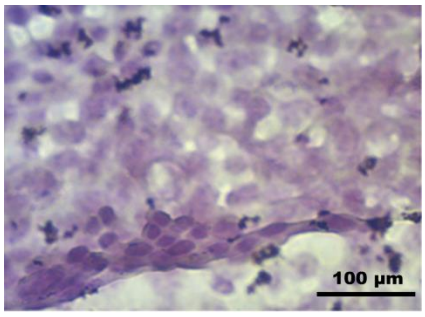
4.3.5 EGRF, Scribble, STAT1 Protein Expressions in Hek293 Cell Line



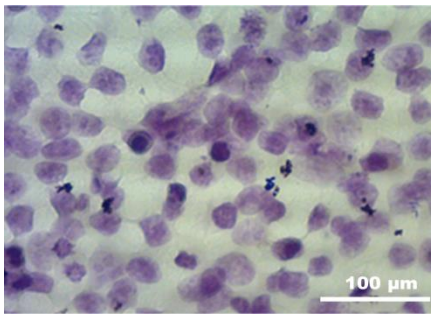
A-Negative Control of Hek293



B- EGFR
Positive Control of Hek293



C- Scribble



D- STAT1

Figure 4-15 Image of negative and positive Hek293 normal human cell line

A) Immunocytochemical image of negative control in Hek293. Cells were grown in DMEM with 10% FBS. Immunocytochemistry method performed after 48 hrs and stained by Hematoxlin. The morphology of group of cells (monolayer surface). Magnification power was x40 objective. The images bar was 100 µm. B) Immunocytochemical detection of EGFR protein (B), Scribble protein (C) and STAT1 protein (D) in the normal human cell line Hek293. Images (B), (C) and (D) represented a negative pattern of EGFR, Scribble and STAT1 respectively. Proteins expression in Hek293 after treatment with anti-EGFR, anti-Scribble and anti-STAT1 antibody and stained by Hematoxylin. Magnification power was x40 objective. The images bar was 100 µm.

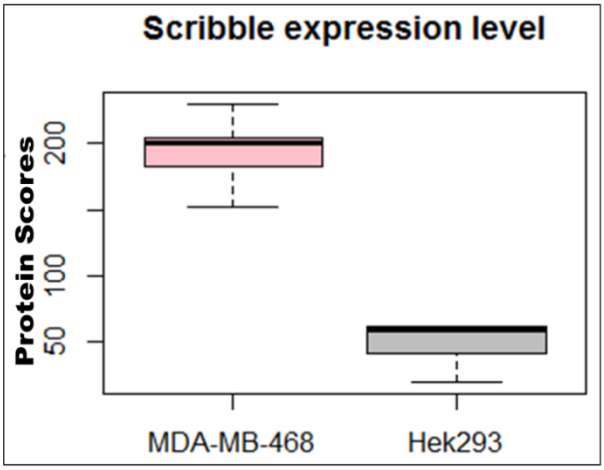


Figure 4-16 The Scribble expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of Scribble protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R- program software shows that p-value= 7.115e-08

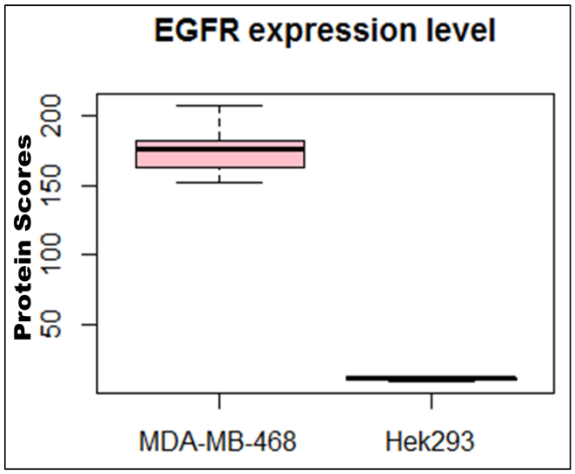


Figure 4-17 The EGFR expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of EGFR protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R-program software shows that P-value= 2.643e-10

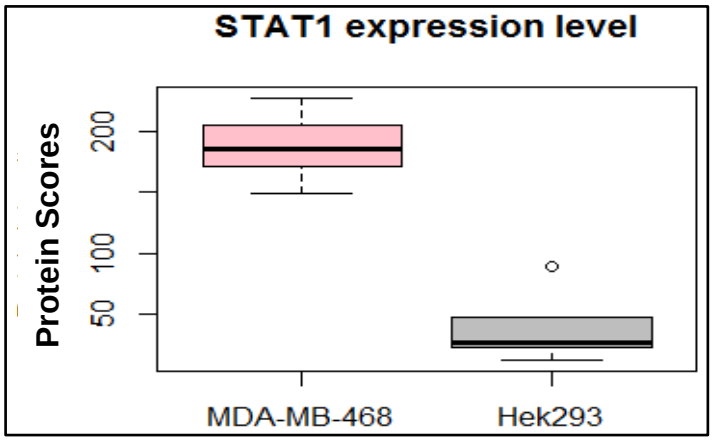
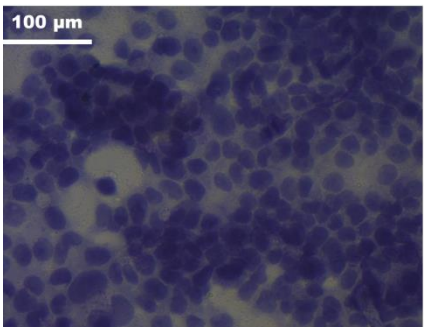


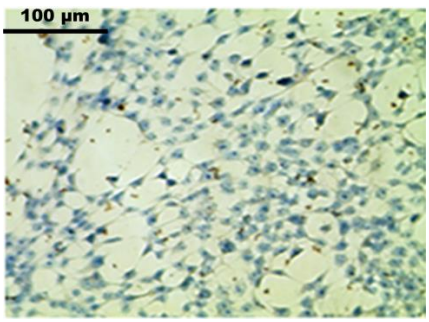
Figure 4-18 The STAT1 expression level in MDA-MB-468 and Hek293

Boxplots and Student's t-test were performed to compare the level of STAT1 protein intensity in the human TNBC cell line (MDA-MB-468-PINK) and normal human cell line (Hek293-GRAY). Student's t-test in R- program software shows that p-value = 7.727e-07

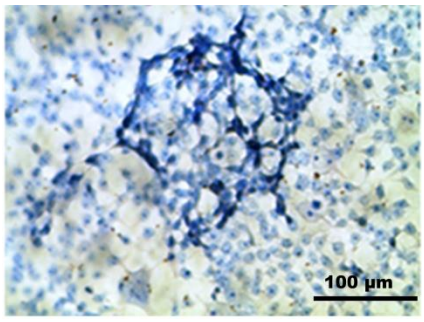
4.3.6 EGRF, Scribble, STAT1 Protein Expressions in MCF-10A



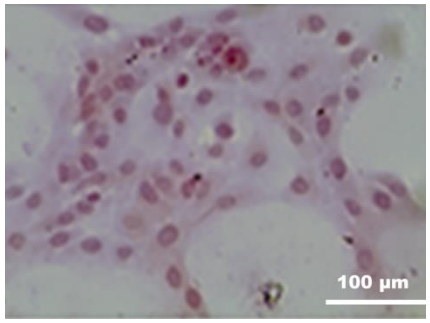
A- Negative control of MCF-10A



B-EGFR
Positive Control of MCF-10A



C- Scribble



D- STAT1

Figure 4-18 Image of negative and positive MCF-10A normal breast human cell line

A**)** Image of negative control of MCF-10A normal human breast cell line.
Immunocytochemical image of negative control of normal human breast cell line MCF-10A. Cells were grown in DMEM with 10% FBS. Immunocytochemistry method performed after 48 hrs and stained by Hematoxlin. The morphology of group of cells (monolayer surface). Magnification power was x40 objective. The images bar was 100µm. B), C) and D Image of detection of candidate proteins in MCF-10A normal human breast cell line (B) EGFR, (C) Scribble and (D) STAT1 in the normal human breast cell line MCF-10A. Images (B), (C) and (D) represented a expression pattern of Scribble, STAT1 and EGFR respectively. Proteins expression in Hek293 after treatment with, anti-Scribble, anti-STAT1and anti-EGFR antibody and stained by Hematoxylin. Magnification power was x40 objective. The images bar was 100 µm.

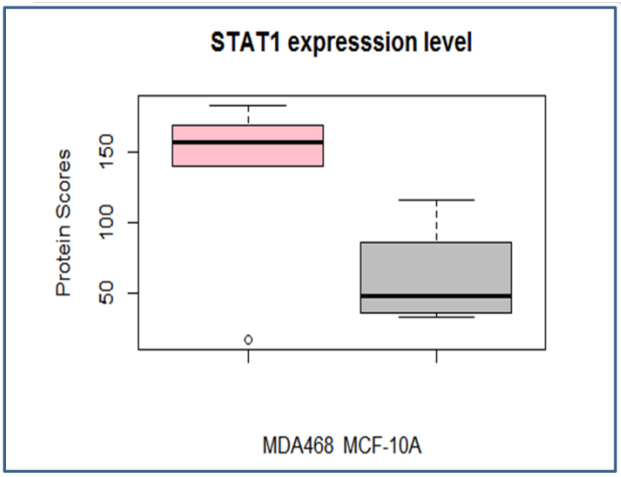


Figure 4-19 The STAT1 expression level in MDA-MB-468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of STAT1 protein intensity in (MDA-MB-468-PINK) and normal human breast cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value = 0.005924.

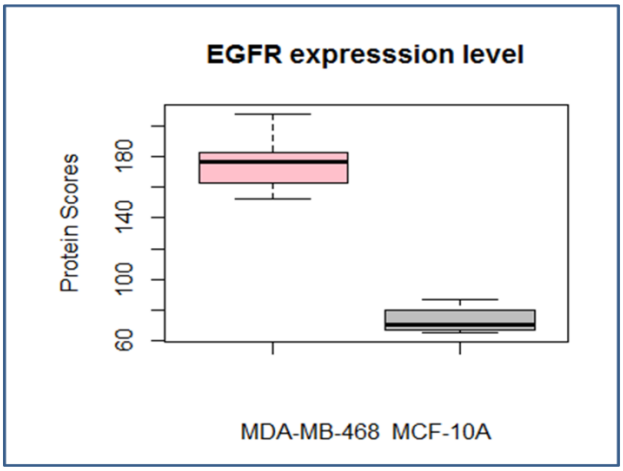


Figure 4-20 The EGFR expression level in TMDA-MB 468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of EGFR protein intensity (MDA-MB-468-PINK) and normal human breast cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value =2.915e-07.

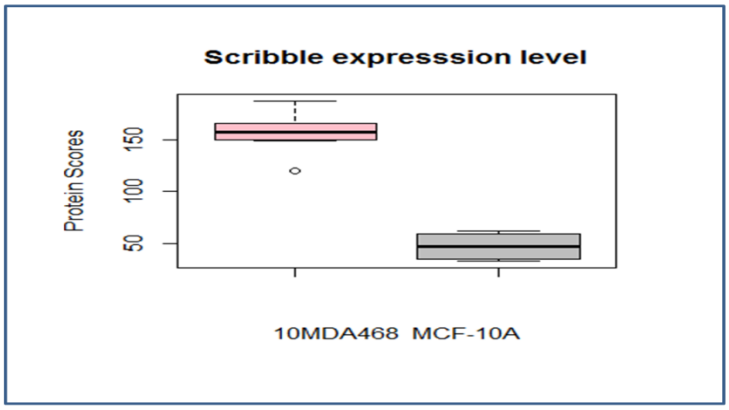


Figure 4-21 Scribble expression level in 10MDA-468 and MCF-10A

Boxplots and Student's t-test were performed to compare the level of Scribble protein intensity in(MDA-MB-468-PINK) and normal human cell line (MCF-10A-GRAY). Student's t-test in R- program software shows that p-value= 4.144e-07.

Table 4-3 Cell lines characters

Cell Line	Cell Source	Cellular Morphology	STAT1	EGFR	Scribble
Hek293	embryonic kidney	Epithelial (spindle)	0 , +1	0	0
MCF-10A	Mammary gland and breast cell	Longitudinal epithelial	0,+1	0,+1	0,+1
MDA-MB-468	Mammary gland and breast from metastatic site	Mixed round and epithelial	+2,+3	+3	+2,+3

Human tumour-derived cell lines grown in vitro are essential cell-models to search in cancer development, therapeutic response and resistance to anticancer drugs. However, using cell line in clinical research has been criticised due to the difference between the property of cell line and primary tumour cells. This study develop the quantitative method to show the site of expression of (STAT1, EGFR and Scribble) as well as, to identify the cancer cell lines that are more clinically relevant for biological experiments, when compare with in-vitro tumour tissue.

4.4 Discussion

Targeted treatments consist of strategies that are directed at a distinct molecular target. These strategies are differentiating malignant from benign cells and are important for the growth and progression of the cancer. Most targeted treatments strategies have been directed towards nuclear or surface receptors of cancer cells. The results of this report indicate that cellular and sub-cellular location of protein expression is essential to prognosis and diagnosis. This information is obtained by using methods such as ICC, which is antibody–based method. By contrast, cytotoxic treatment is not classified as a targeted treatment because many of its specific targets have not been defined. Nuclear receptor (i.e. oestrogen receptor [ERα] or progesterone receptor [PgR]) positive breast cancer and HER2 positive breast cancer, currently, account for 75-80% and the remaining 20-25 % of breast cancers are in a so called receptor-negative or triple-negative category as defined by absent expression of these three proteins.

A combination of diverse proteins Scribble, STAT1 (Greenwood et al, 2012) EGFR (Yang et al, 1996) are correlated with increased migration and invasion in breast tumour tissue and breast cultured cell lines. Previous studies utilising clinical proteomics have reported that overexpression of a set of proteins: STAT1, CD74, Mx1 and dysregulation of Scribble is associated with increased invasion and increased expression of membrane proteins involved in cell adhesion and cancer metastasis in TNBC (Metodieva et al. 2013, Greenwood et al. 2012).

Several studies have documented that TNBC is characterised by unique molecular profile with distinct pathways for metastasis, aggressive behavior, and lack of approved targeted therapies (Anders and Carey 2008). Studies of gene expression suggested that TNBC arises from basal cells of the mammary epithelium and is associated with high mitotic activity and invasive tumours in younger patients and in premenopausal women (Greenwood et al. 2012).

Moreover, it contains a population of stromal cells with characteristics of fibroblasts. This clearly explains why these cancers give rise to an outgrowth (Seemayer et al. 1979, Gogoi and Borgohain 2015). Prognosis remains poor and this is due to two factors: first, shortened disease free interval between the adjuvant and neoadjuvant treatment. Second, more aggressive clinical course in the metastatic setting (Ismail-Khan and Bui 2010).

4.4.1 The Molecular Heterogeneity in Triple Negative Breast Cancer

The present study was designed to investigate the expression pattern of EGFR, STAT1 and Scribble in TNBC cells; also, we needed to tailor the approach to the heterogeneous nature of triple-negative subtypes. Thus, we used ICC in the human triple negative breast cancer cell line MDA-MB-468 (Figure 4-1,

Figure 4-6 and

Figure 4-10) and normal human source cell line HEK293 (Figure 4-15) because in prior studies, we have noted the importance of ICC as an accurate and reliable method that provides a key to detect special types of invasive breast carcinoma as well as, might allow

to identify the breast cancer cell lines with good or poor prognosis which may have therapeutic consequences.

However, the specificity and sensitivity of ICC is limited by the specific activity of the antibodies used in the different steps of the procedure.

In order to better understanding the mechanism of metastasis, exploiting the molecular heterogeneity in TNBC had led to the development of new potential therapeutic targets. Such as the pathway of DNA damage response (i.e the development of poly (ADP-ribose) polymerase inhibitors, the use of alkylating agents, angiogenesis or epithelial /mesenchymal transition and deregulation of EGFR could offer a potential target for early development in TNBC patients. Gene expression clustering study of breast cancer did not identify subtypes of TNBC. Immunostaining method identifies triple negative breast cancer based on one or/and two proteins characteristics, however, in ability to detect larger panels of proteins reflects the limited resolution of the present Immunostaining system (Choi et al. 2012).

4.4.2 The Expression and Localisation of STAT1, Scribble and EGFR in Hek-293 and MDA-MB-468

Strong evidence for the expression pattern of our target proteins EGFR in (Figure 4-2, Figure 4-3) with p-value < 0.05 (Figure 4-4,Figure 4-5), STAT1 in (Figure 4-7) with p-value < 0.05 (Figure 4-8, Figure 4-9) and Scribble in (Figure 4-11, Figure 4 12) with p-value < 0.05 (Figure 4 13, Figure 4 14) show that the three proteins are much more abundant in MDA-MB-468 cells than in the Hek293 cells

Possible explanation for this might be that most of the physiological changes in breast cancer cell are mediated by molecular alterations at the protein level (overexpression of proto-oncogene) including cancer specific changes arising from the tumour cell, again this result suggests the candidate proteins panel can be utilised to provide bio-markers that can guide treatment decisions on the molecular level.

One benefit in using cell lines panels in proteo-genomic investigations of the heterogeneity of TNBC is that the expression profiles of breast cancer cell lines are not contaminated with normal epithelial or stromal cells and the cluster analysis of gene expression would have clearer meaning compared to similar analysis of tumoure tissue samples (Kuperwasser et al. 2004, Hong Liu et al. 2004). Moreover, a cell line may derive from subpopulation of tumour cells that are selected because they grow well (Al-Hajj et al. 2003). The observed expression patterns or different localisation could be attributed to the different triple negative breast cancer derived cell. Proteins expression can be obtained from clinical tumour tissue but it will less pure and in many instances contaminated with non tumour cells (Adam et al. 2002).

The results of this chapter agree with the findings of other studies and the possibly explanation by Hanahan and Weinberg (2000) in which they discuss the fundamental differences of the regulation of signalling pathways controlling proliferation between normal and cancer cells. In normal cells, careful control is exerted on the production, release of growth-promoting signals and progression through the cell division and the growth of cells, thereby ensuring a homeostasis of cell number thus, maintenance of the normal tissue

architecture and the function. In contrast, in cancer cells the deregulation of these signals makes cancer cells masters of their own destinies.

Furthermore, these signals are conveyed in large part by growth factors that bind cell-surface receptors which contain intracellular tyrosine kinase domains (Hanahan and Weinberg 2000, Lemmon and Schlessinger 2010).

The signal proceeds to emit signals via branched intracellular signalling pathways that regulate progression of the cell cycle as well as, in cancer cell size (cell growth);and these signals influence cell survivals and energy metabolism. However, the precise identities and cellular location of the proliferation signals operating with in normal tissues where poorly understood. Among plausible explanation for these finding is that the mechanisms controlling the release of mutagenic signals are complicated which is due to the fact that the growth factors signals controlling cell number and position within the tissues are thought to be transmitted in a temporally and spatially regulated fashion from once cell to its neighbours.

Moreover, these growth factor signals are regulated by sequestration in the peri-cellular space and extracellular matrix and the actions of a complex network of protease. This finding corroborates the ideas of Perona (2006) and Witsch et.al **(**2010**)** provided an explanation as to the understanding of the mitogenic signalling in cancer cells is better understood ;by the fact that cancer cells can acquire the capability to sustain proliferative signalling in a number of alternative ways. Thus, we believe that the observed increase in the expression pattern of STAT1 and EGFR in MDA-MB-468 relative to the non- cancer

cells with p-value <0.05 could be explained with early studies of oncogene action showing that overexpression of such genes and the signals manifested in their protein products would result in correspondingly increase of the cancer cells proliferation and tumour growth.

By drawing on the concept of the TNBC cell line of this report, our results are in keeping with previous observational studies by Neve (2006) which has been able to show that the cell lines display the same heterogeneity in copy number and expression abnormality as the primary tumours and they carry almost all of the recurrent genomic abnormities associated with clinical outcome in primary tumours. In term of the cluster of basal-like and luminal expression subsets, the breast cancer cell lines do as primary tumour. However, the partitioning of genome aberrations between these subsets is different than that in basal-like and luminal primary tumours. It has conclusively been shown that the cell line collection exhibits heterogeneous responses to targeted therapeutic paralleling clinical observation, and the cell line collection mirrors most of the important genomic and resulting transcriptional abnormality found in primary breast tumour.

However, the possibility exists that a single malignancy may harbour two intrinsic phenotypes. The independent characteristics of breast cancer cell lines may be attributed to differences in the origin of the tumour of the tumour cells, but another possibility is that tumour cells may be arrested at different stages of epithelial cell development. In our study we use breast cancer cell lines and a panel of candidate proteins, which we suspect being involved in a novel mechanism linked to metastasis.

Cancer cell metastasis remains not fully understood. The molecular mechanism of metastasis is built on the sequential program that cancer cell penetrate the basement membrane, intravasate into blood and lymphatic vessels and continue on a trip in vasculature, extravasate into secondary sites and adapt to have a new host environment (Cheng et al. 2007, Fidler 2003).

A clear point of metastasis is associated with molecular changes including decreased cell-cell junction proteins and increased basement membrane degradation exemplified by E.Cadherin downregulation and matrix metalloproteinase and collagenases upregulation, respectively (Batlle et al. 2000, Maslow 1987). Increased expression of EGFR could be part of this mechanism as our results suggest: we find EGFR levels in tumour tissue to be correlated with clinical outcomes in TNBC, and we find EGFR to be overexpressed in breast cancer cell lines compared to non-cancer cell lines.

Prior studies that have mentioned the importance of others methods of localising of proteins in cells and the selection of the relative protein detection methods is essential, because, the proteomic technique measures quantitative changes in protein expression level in cell and tissue sample. In general, the ideal detection methods, which is associated with low as possible an optimal signal to noise ratio, mass spectrometry-compatible, and not too expensive (Westermeier and Marouga 2005). To date, no protein detection method assembly all these requests. This research believes that is important to select appropriate method, which is based on our hypothesis aims, proteomic lab producer and sample type. Results from this research were collected from combines 1D-SDS-PAGE and Nanoscale

liquid chromatography coupled to tandem mass spectrometry nanoscale LC/MS/MS (as mentioned in the methods and materials chapter). 1D-SDS-PAGE is reproducible, robust and advantage over conventional highest resolution protein technique (Alldridge et al. 2008), and nanoscale LC/MS/MS is powerful tool in the proteomics research. 1D-SDS-PAGE used as first step of fractionation by small pore size of SDS-PAGE prior to the nanoscale LC/ MS/MS.

It is powerful method, which provides information for the size of the separates proteins. Additionally, 2D-SDS-PAGE is high resolving power; this technique is now applied by many laboratories for protein research. Despite significant improvements, 2D-SDS-PAGE still suffers from a lack of reproducibility and from time-consuming manual interventions (Vollmer et al. 2003). The result of STAT1 and Scribble mass spectrometry (the first chapter) differs from the findings of ICC and this inconsistency may be due to lost isoforms and splice variants information of unseparated protein samples after detecting the protein peptides by MS/MS, as well, difficulty to obtain an accurate analysis of millions of peptides in a single LC run. Proteomic data of first chapter are processed by the massive number of proteins; in particular, more abundant proteins may inhibit the signal of lower abundance proteins

One of the issues that emerge from these results is use the appropriate protein fractionation technique to separate the wanted proteins from the rest, such as, size, shape, adsorption chromatography and substrates affinity to isoulate the protein of interest from cell and /or tissue sample.

Localising of proteins require simple and efficient protein purification methods that are amenable to high throughput, such as Biotinylation is an attractive approach for protein complex purification due to the very high affinity of avidin/streptavidin for biotinylated templates (de Boer et al. 2003). Based on, the extremely high affinity of Biotin that binds to streptavidin and avidin., these interactions are used to identify protein-protein interactions and post-translational events such as ubiquitylation (de Boer et al. 2003). Cellular proteins localisatiion may relate to the cytoplasm ubiquitin, which appears to play a visibly different actions, for a fraction, ubiquitin ligation is an obligatory step for the multi-enzyme energy-dependent proteolytic pathway that responsible for degradation of various short lived and abnormal proteins (Haas et al. 1987).

In contrast, ubiquitin ligation to some target proteins may serve regulatory roles to external sites on receptor of plasma membrane proteins (Baker and Board 1987). However, Biotinylation is rapid and unlikely to disturb the natural function of the molecule due to the small size of biotin. Prior results in this chapter have noted the importance of analytical fractionation of proteins by biochemical approach, such as chemical modified of bead surfaces to enable a protein investigation and monitor formation. For example, became feasible when using mixed bead substrates method consisting of unmodified glass beads and enzyme-modified beads, this method observed decrease autolysis of native tissue (Tucker et al. 2011). In situ digestion, enzyme-modified beads are applied in order to perform in situ digestion peptides from proteins were identified and localised. As mentioned above, method of protein localisation in cell, such as fractionation, biotynation and beads, are essential for mass spectrometry data validation, as well, the sensitivity to measure the

abundance of group of proteins is an essential value to study protein networks and pathways

4.4.3 The Expression Pattern of Scribble Protein

The underlying mechanism of a disease such as TNBC is quite complicated in that often multiple deregulated proteins are involved. So, one of our aims is to develop quantitative assays LC-MS/MS, WB, ICC and IHC for detection of panels or combination of the proteins Scribble, STAT1 and EGFR, that should provide higher sensitivity and specificity for TNBC diagnosis than is afforded with single protein analysis. This chapter found that Scribble shows strong positive staining (+2, +3) and was detected in subcellular location such as cell-cell junction, nuclear membrane and membrane Figure (13, 14,15) and the p-values (p-value= 7.115e-08(0.00000007115)) and (p-value=5.952e-09(0.000000005952)), Scribble is often implicated in contexts of morphogenesis cycle, the deregulation of proliferation and the polarity pathways. All these are required to induce the neoplastic growth during carcinomas development. Furthermore, it provides an ideal platform for better understanding of the role of cell polarity during initiation and progression of carcinoma.

The obtained results might be an indication that the expression and localisation of Scribble in the triple negative human breast cancer can be used to detect EMT. EMT is an essential developmental process by which cells of an epithelial organ lose epithelial characteristic and acquire a mesenchymal phenotype with fibroblast-like morphology cytoskeleton reorganisation, increased motility, invasiveness and metastatic compatibility and the lack of

epithelial cell junction proteins (Sarrió et al. 2008, Gupta and Massagué 2006, Savagner 2001).

Scribble localizes at the basolateral membrane and this is required for its ability to inhibit growth G1 to synthesis S transition in the cell cycle (Nagasaka et al. 2010). A possible explanation for these results may be the lack of cell polarity and subsequently tissue disorganization as each; biological function is achieved by the special structural organization of epithelial cells within those tissues. So, the integrity of their architecture is essential, however, it remains unclear whether a loss of cell polarity is a consequence or one of the many causes of human cancer.

The later is in agreement with Lixing Zhan (2008) who provided insight for understanding how cell polarity regulated cell transformation and the steps leading to initiation of carcinoma; through proposal that the transformation of epithelial cells should move toward a deregulated morphogenetic process instead of a simple increase in cell number. Moreover, there are two essential issues in Lixing's approach .First; he considers both regulators of cell number and cell structure are critical components of the oncogenic process. Second, it accounts that in a pathological state the number of cells is increased during normal tissue remodeling such as wound repair. These normal conditions lead to an increase in the cell number and are coupled to a normal morphogenesis program that results in restoration of glandular organisation and normal function.

Both of the cell proliferations and morphogenesis cycle repeat throughout life and that is called homeostasis is the morphogenesis cycle. In general, therefore, it seems that the disruption of the polarity pathways induces dysplastic growth by deregulation the morphogenesis cycled without directly affecting cell proliferation while the lack of the changes in polarity pathways induced increase in cell number that create a hyperplasia status. This results may explain the relatively good correlation between Scribble and the loss of cell polarity, Royer and Lu (2011) emphasised that the majority of human cancer are initiated from epithelial tissue and display loss of cell polarity and as consequence tissue disorganization. Accordingly, numbers of studies reports decreased expression or loss of Scribble in primary tumours from human patients (Cavatorta et al. 2004).

Previous studies found that the epithelial tissue of breast organ converts from normal regular morphology to de-regulate and loose cell proliferation control when the mutation of a Scribble gene (Scribble protein) occurring (Downward 2003, Rakha et al. 2008). Scribble protein plays vital role in the maintenance and regulation of epithelial tissue polarity (Bilder and Perrimon 2000). With respect to this study, it was found that there are close connections between growth factor signalling and the mechanisms of cell transformation results from hyper activation of the mitogen signalling pathway (Bernards and Weinberg 2002). As well as, the epithelial tissue of breast organ converts from normal regular morphology to de-regulated and lose control on cell proliferation when Scribble gene is mutated (Downward 2003, Rakha et al. 2008).

In our ICC experiments, we find Scribble to be more abundant in MDA468 compared to HEK293 cells. It is properly localized to the membrane in the cancer cells. This could mean several things: MDA468 is a non-metastatic cells line and as such have not undergone EMT, therefore Scribble is not affected. On the other hand overexpression of Scribble could also mean disruption of polarity as it would not be properly localised and this could explain why in the cancer cell line Scribble is overexpressed compared to the non-cancer cell line. A more trivial explanation is that in the experiments with HEK293 cells staining of Scribble was less efficient for unknown reasons. This could be rulled out if a combined ICC and LC-MS/MS analysis is carried out and the LC-MS/MS results show less Scribble in HEK293 compared to MDA468.

4.4.4 The Expression Pattern of EGFR Assessed by Proteomics and ICC

The findings observed in this report mirror those of the previous studies (early part of this project) that have examined the expression pattern of EGFR in human breast cancer cell lines MDA-MB-468 and metastasis human breast tumour. In this study, we used TNBC cell line MDA-MB-468 as described by Lu et al. (2010). The different expression sites are shown (Figure 3-5, Figure 3-10, Figure 4-2, Figure 4-4, Figure 4-5). It is becoming increasingly difficult to ignore the role in breast cancer of EGFR as a member of the type I transmembrane receptor tyrosine kinase [RTKs] of the ERBB/HER family, which includes ERBB2/HER2, ERBB3 and ERBB4 (Yarden and Pines 2012). Accordingly, multiple researches have focused on EGFR as prognostic and therapeutic markers (Cheang et al. 2008).

Human proteome is complex and traditional antibody-based and target directed analysis is limited to known proteins. On the other hand, proteomics is able to detect, compare and identify hundreds of unknown proteins simultaneously (Lu et al. 2010). Recently, proteomic techniques, such as mass spectrometry coupled with powerful bioinformatics tools enabled high through-put; discovery of new proteins. This field evolves rapidly to meet the formidable challenge of protein diversity in tumour tissues and cell lines These results match those observed in earlier studies for Susan cleator (2007), breast cancer was sub classified on the basis of cellular morphology and the presence of several receptors, named ERα, PgR and the ERBB2, identified by IHC. Such classifiers have become useful in terms of predicting prognosis and guiding treatment recommendation.

Like many other RTK, EGFR has important roles in proliferation and differentiation of normal cells and malignant transformation (Eccles 2011). It has been well-established that EGFR is a major oncogenic factor and a promising therapeutic target of certain types of cancer, for example, EGFR is highly expressed in more than 50% of TNBC while its role and therapeutic potential in breast cancer is poorly understood (Burness et al. 2010).
Most breast cancer clinical trials with EGFR inhibitor as single agents have turned out to be disappointing. Yi et al.(2013) pinpoints that the phosphoinositide 3-kinase (P13) / AKT pathway inhibitors the (P13K, AKT) enhance the anti-proliferative effects of inhibiting EGFR in susceptible triple negative breast cancer cell line MDA-MB-468

The question remains whether EGFR is a valid target when many of the EGFR tyrosine-kinase inhibitors in metastatic breast cancer have at most 5% response rate. At the

European Sociality (ES) for medical oncology meeting in October 2010 an EGFR–targeted therapy was reported. Overall, a response rate of 20% was seen in patients who received the combination therapy with EGFR inhibitor compared to therapy without EGFR inhibitor response rate of 10.3%. These exciting clinical data suggested that EGFR is an important target for TNBC. In addition, they seem agree with other research which found that EGFR expression had a significant prognostic value of TNBC patients (Cho et al. 2011).

4.4.5 The Expression Pattern of STAT1

STAT1 staining is shown (Figure 4-7) with p-value in (Figure 4-8, Figure 4-9). To detect STAT1 the protocol was modified to enable penetration the cell membrane. As one of our interesting set of proteins it was recently discovered that STAT1 regulated DNA repair pathways and can act as a predictive marker for breast cancer chemotherapy and radiotherapy resistance (Khodarev et al. 2004). STAT1 overexpression has been demonstrated in several human cancers including head and neck cancer (Buettner et al. 2002)and breast cancer (Greenwood et al. 2012). In terms of cancer, STAT1 has been associated with anti-tumour effect, however accumulating evidence has explained increased STAT1 activation with increased tumour progression in multiple types of cancer such as breast cancer (Hix et al. 2013).

Thus, the present research makes several noteworthy contributions to the molecular complexity and heterogeneity of TNBC and has led to study a group of proteins (EGFR, STAT1 and Scribble) that might be of great value for the development of new approaches for diagnosis, prognosis and targeted therapies. This molecular sub classification has

implicated several biological processes as potential therapeutic targets; the DNA damage response, the deregulated proliferation angiogenesis, EMT and immune deregulation (Irshad et al. 2011). We show the expression pattern of STAT1 that agree with Ming' group report which studied varied levels from STAT1 expression and is claiming that STAT1 has been associated with anti-tumour effect.

However, accumulating evidence has linked increased STAT1 activation with increased tumour progression in multiple types of cancer such as breast cancer (Hix et al. 2013). Regarding to ICC results (images in Figure 4-7) and overexpression of STAT1 in the human TNBC cell line MDA-MB-468, we demonstrated similar patterns have been reported on breast cancer and oral squamous cell carcinoma by Widschwendter (2002). For this, we do not have specific explanation, but we have considered the following possibility as agreeing with Widschwendter .A (2002), that STAT1 has been demonstrated as tumour suppressor functioning in many cancer cell types including breast cancer.

In terms of tumour suppressor, STAT1 effects by modulating the transcription of the pro-apoptotic host and anti-proliferative genes, such as membrane proteins of the death receptor family and tumour necrosis factor-related apoptosis ligand (Choi et al. 2003, Bernabei et al. 2001)

Thus, to investigate our candidate proteins panels for the clinical categorisation of this type of breast cancer. We show that a panel of three proteins can stratify TNBC into well separated groups using WB, ICC and/or mass spectrometry enables proteomics. This

could serve as the basis of a strategy to identify TNBC patients more responsive to specific therapies in the clinical setting and for the retrospective evaluation of the efficacy of treatment agents on this tumour type.

4.5 Conclusion

The findings of this report provide insights for studying the group of proteins (STAT1, Scribble and EGFR) that in the long run could provide a better understanding of the mechanism of metastasis and aggressiveness of TNBC.

Results obtained from ICC experiments with cultured cells, explains that our Candides proteins (EGFR, STAT1 and Scribble) and several other proteins from mass spectrometry obtained data are linked with increased the metastatic propensity. The molecular pathway considerable for increasing the malignancy cell behaviour associate in signaling pathway of JAK/STAT, EGFR receptor pathway and Scribble involved in JAK/STAT signaling, as well, the positive feedback loop between CD74 and STAT1. Togeather, all these signaling pathways can be applied to develop a new diagnostic treatments. This study has shown that ICC of candidate proteins (STAT1, EGFR, and Scribble) is quantitative measurement of protein abundance that applies for validation of proteomic data set. One possible implication of this is that proteomic profile of patient can as a period of personalised medicine, which helps to integrate into clinical indices and active disease model to permit patient data management.

Overall, the semi-quantitative method ICC demonstrated to be reproducible with a significant p-value for STAT1, Scribble and EGFR in studies that compare their expression in MDA-MB-468 to Hek293. Our findings provide clinically useful information about tumour biology, clinical behaviour and a valuable clue to determining what proteins might be exploitable for the clinical categorisation of TNBC.

Chapter 5 Results of CRISPR/Cas9 and Human Breast Cancer Cell Line Model

5.1 Introduction

Despite many therapeutic options, 15-20% of breast cancer is TNBC patients succumb to the disease due to tumour relapse and acquired therapy resistance. However, TNBC developing effective treatments remain challenging due to the lack of a common vulnerability that can be explored by targeted approaches (Iskit et al. 2016). Within TNBC, a heterogeneous group of molecular subtypes have been identified with various biological pathways that may serve as differential targets for therapy (Shaitelman et al. 2017). Additionally, breast cancer is the most common cancer in the UK with nearly 54,000 new cases diagnosed each year (Evans et al. 2016). Many studies characterised breast cancer on the basis of expression of PgR-ER and HER2 and discovered wide variation in survival in the ER-, PR- and HER2- subtype (Parise and Caggiano 2014). TNBC shows greater resistance to conventional systemic chemotherapy and tends to exhibit a more aggressive pattern of disease (Foulkes et al. 2010).

The primary goal of CRISPR/Cas9 and human breast cancer cell line model was to utilise the novel CRISPR/Cas9 system to augment over-expression of STAT1 and EGFR in BC(a) human breast carcinoma cell line MDA-MB-468 and human embryonic kidney cell line Hek293 and test how the over-expressed STAT1 and EGFR affect wound healing. Our research was relying on a new investigation discovering that a subgroup of TNBC overexpress STAT1 and other cytokine induced genes (Greenwood et al. 2012).

In recent studies, genome editing technologies have allowed for endogenous regulation of human genes to be selectively manipulated and the effect of this manipulations studied (Cheng et al. 2013, Gilbert et al. 2013). Human breast carcinoma cell line MDA-MB-468 used in our CRISPR/Cas9 editing project is extracted from a pleural effusion of female patients with metastatic adenocarcinoma of breast ; additionally, both MDA-MB-468 and MCF-7 have a high growth rate and are poorly immunogenic (Wang et al. 2013, Cailleau et al. 1978b).

Earlier research discovered that overexpression of STAT1 has been associated with invasion in TNBC together with EGFR overexpression and deregulation of Scribble. Also, STAT1 has been associated with IFN gamma responses, which have been shown to play an essential role in immunoediting of cancer cells. The pleiotropic effects of cytokines such as interferon result from the combined effects of a large number of cellular proteins. Interferon- inducible proteins are involved in the regulation of cellular RNA and protein metabolism. Greenwood et.al (2012) treated MDA-MB-231 cell with INF- gamma to measure overexpression of STAT1 and other interferon–induced genes in occurs in TNBC as a results of immunoediting and the results showed an increase that the migration and invasion associated with increased STAT1 abundance and proportionally increased abundance of CD74; concluding that interferon gamma can induce coordinated overexpression of Stat1 and CD74 in patients with advanced breast cancer.

CRISPR/Cas9 system was used to increase the expression of the target genes, not to change DNA sequence and started by constructing site specific 24 oligonucleotides encoding guide RNA and cloning them into dual expression vector harboring dCas9 fused to the transcription activation domain of VP64. Studies have found that robust endogenous gene activation is achieved by fusing the Cas9 to an activation domain of the appropriate transcription factor and targeting the product fusion protein by sgRNA to the promoters of target genes. Four different sgRNA (designated STAT1, STAT2, STAT3, and STAT4) were designed to target the STAT1 promoter at an individual location and four different sgRNA (designated EGFR1, EGFR2, EGFR3, EGFR4) were designed to target EGFR promoter (Figure 5-1).

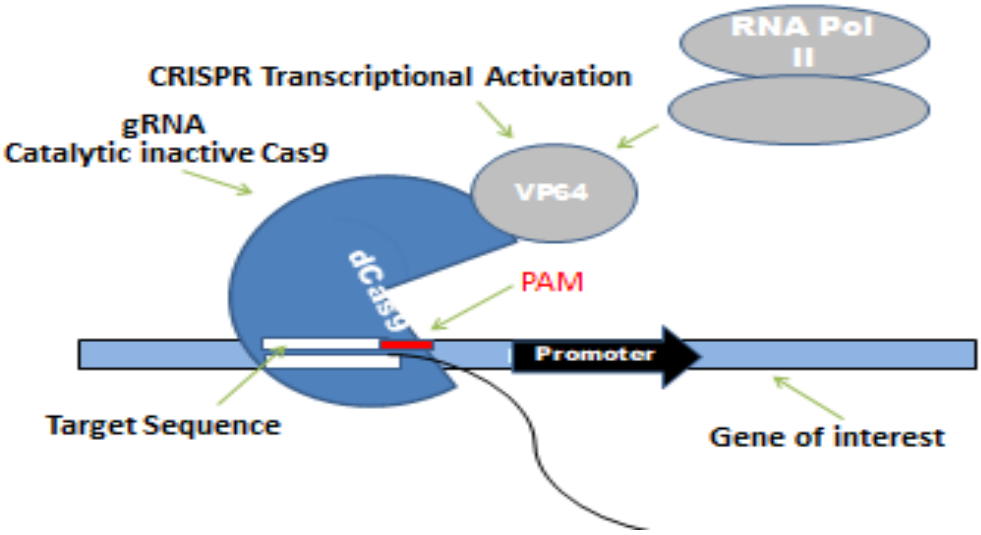


Figure 5-1 Activation of endogenous genes by the CRISPR/Dcas9 genome editing system CRISPR transcriptional activation domain (VP64) and catalytic inactive Cas9 are fused to enhance the expression of gene of interest (adapted from Addgene 2015)

5.2 Methods

5.2.1 CRISPR/Cas9- Guide oligonucleotides design (Forward and Revers Oligonucleotide).

To address the aim of the overexpression of STAT1 and EGFR a site-specific guide (gRNA) was designed and cloned into p AC152-dual-dCas9VP64- sg Expression vector. We copied the promoter sequences from Ensemble and pasted on CRISPR/Cas9 oligo selection server at MIT (crispr.mit.edu). The unique genetic region was selected after the name and email address were filled in. Next, the sequences were pasted and submitted to server. The complete analysis was provided with display information for oligonucleotides pairs of targeting sequence and Cas9 targeting after (10-15) minutes. The appropriate oligonucleotides that ensure highest specificity (lowest number of off-target genes) were selected and the sequences were copied and pasted into a new Excel spread sheet. Finally, the oligonucleotides were sent for synthesis (2.8.1). Bacterial strain and media preparation Trypton Yeast Extract (Sigma-Aldrich) was used and supplemented with appropriate antibiotics (2.8.12.2).

Escherichia coli strain was grown in 2XYT broth (2.8.12.3). Followed, the heat shock procedure was used to have competent cells. A single colony was selected and inoculated in 2XYT broth / Ampicillin (100µg/ml, Sigma-Aldrich) for 16 hr. at 37˚C. Plasmid extraction was performed using plasmid DNA isolate with the Thermo Scientific Gene JET plasmid / Miniprep Kit. The sequence validation of sgRNA cloning was verified by Source Bioscience Company (2.8.12.5).

Human breast carcinoma cell line MDA-MB-468, human breast adenocarcinoma cell line MCF-7 and human embryonic kidney cell line HEK293 were grown in the cell culture medium DEMEM (Gibco) and supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific). Cells were maintained and incubated in 5% CO_2 incubator with p H at 37˚ C and 95% humidity rate; transfection procedure depended on a low tissue culture passage (< 50).

Cells were collected using 0.05% trypsin (Sigma-Aldrich) and incubated at 37℃ for three minutes. Transfection plasmid p AC152-dual-d Cas9VP64-sgExpression (p AC152) was used into MDA-MB-468, MCF-7 and HEK293 and described in (2.8.3, 2.8.4). According to the instructions and the nano-drop-spectrometry reading of plasmid DNA concentration for STAT (STAT1, STAT2, STAT3 and STAT4) and EGFR (EGFR1, EGFR2, EGFR3 and EGFR4), plasmid p AC152-dual-d Cas9VP64-sgExpression (p AC152) was transfected into MDA-MB-468 and Hek293 by Fugene–HD and Lipofactamine 3000 Reagent. Green fluorescent protein (Clontech) was used to assess the transfection efficiency (2.8.5).

Resistant cells to (G-418) were harvested from the transfection Hek293, MDA-MB-468 and MCF-7 cell line (2.8.6). To increase the selection of stable expression, cells were cultivated into DMEM media with G-418. Suspension cells were plated on tissue culture plate 10cm. To prepare protein sample of transfection Hek293, MDA-MB-468 and MCF-7, cells were wished with ice-cold PBS and treated by lysis buffer (1 ml/100 mm tissue culture dish). Cells were centrifuged for 20 mins at 12,000rpm. Cell suspension was aspirated to test the protein concentration (2.8.7).

CRISPR cloning and plasmid construction of STAT1 (STAT1-1, STAT1-2, STAT1-3, and STAT1-4) and EGFR (EGFR1, EGFR2, EGFR3, and EGFR4) were adapted from various sources with some modifications. Each pair of oligonucleotides was annealed at 95° C for 5 min in a thermocycler (2.8.10). The annealed oligonucleotide tubes were incubated at room temperature for 1 hour. This was followed by, the digestion step, 1 µg of plasmid was mixed with 2 µl of 10x Buffer. MilliQ H2O was then added up to final concentration of 20 µl. Next, 1 µl of BbsI (Thermo Fisher Scientific) was added to the digest plasmid mixture tube and incubated at 37° C for 1hour. The ligation components were added to the digest plasmid mixture tube which included, 2.5 µl of 10x T4 Ligase Buffer, 1µl annealed oligonucleotides (10 µM stock, 0.4 µM final concentrations) and 1.5 µl T4 DNA Ligase. The reaction was incubated at 37℃ for 1 hour. The results of cloning and constructing were confirmed by DNA sequencing (Source Bioscience Sequencing Company). After the targeting experiment of human promoter of EGFR and STAT1, the validation and the quantification of STAT1 and EGFR overexpression were achieved by wound healing assay (2.2), WB (2.4) and Proteomic (2.4.3).

5.3 Results

5.3.1 Transformation

5.3.1.1 Confirmation of Plasmid and CRISPR/dCas9n Construct

To facilitate multiplex site specific targeting of the promoters of STAT1 and EGFR,

24-nucleotide sequences for each targeted site were designed and cloned into plasmid p AC152. This plasmid contains a fusion of catalytically-dead Cas9 (dCas9) with the transcription activation domain of VP64.

To validate the presence of the sgRNA sequences for STAT1 and EGFR in the p AC152 plasmid, plasmid DNA from positive colonies was sent for sequencing (Source Bioscience). Primer LKO sequence (5´-GACTATCATATGCTTACCGT-3´) was used for sequencing after transformation in competent E.coli and extraction of the plasmid. All positive colonies were sent to Source Bioscience for sequencing and positive clones were identified thorough comparing of the sequence complementary to the target DNA. Cloning was highly efficient, which is to be expected as it uses the golden gate system-the restriction enzyme BbsI cuts outside of its recognition sequence and once an insert is ligated the plasmid molecule would not be cut by BbsI, while reclosed empty plasmids are cut repeatedly until an insert is ligated (Table 5-1). DNA sequencing results from source bioscience is available in the Appendix (App B1, 2, 3, 4, 5 and 6). Below table (Table 5-1) shows the confirmation results after the DNA from positive colonies were sent for sequencing (highlighted (red) nucleotides represent the corresponding sgRNA insert).

Table 5-1 sgRNA sequences that have been cloned into p AC 152

sgRNA	DNA Sequencing Results (5´- 3´)
STAT1-1	NNGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGATTATACCAGAAGGAACGTGTTTTA
STAT1-2	NNGANNNNTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCAATGGAGAATGCTTAGTCACGTTTTAG
STAT1-3	NNGATTTNCTTGGCTTTNTATATCTTGTGGAAAGGACGAAACACCCACCACAACTGAGAAGGCATGTTT
STAT1-4	NNAAANNNNNNTNNNNNTTNNNNTATCTTGTGGNNNNGACGAAACACCTTCGAAAGTTCGGCTGGCTGGTTTTAGAG
EGFR-1	NNNTTGNNTNNNNATATNNNGTGGAAAGGACGAAACACCTCGGTGCCATTATCCGACGCGTTTTAG
EGFR-2	NNNTTTNCNNGNNNNNNTATATCTTGTGGANGGACGAAACACCCGTCGGATAATGGCACCGACGTTTNNN
EGFR-3	NNNNTNNNNNNTNNNANNTCNNNNGGANNNGACGAAACACCCGCGGGACCTAGTCTCCGGCGTTTTAG
EGFR-4	NNCNNNNNCNNGGCTTTATATATCTTGTGGAAGGACGAAACACCATTTGGCTCGACCTGGACATGTTTTA

5.3.1.2 CRISPR/Cas9- mediated transcription reprogramming of STAT1and EGFR.

We used pAC152 for constructing guides sgSTAT1 and sgEGFR; and the templates for producing guides sgSTAT1 and sgEGFR were constructed in pAC152. We designed the strand primer for cloning of each candidate gene individually and tested whether STAT1 and EGFR could be reprogramed to be overexpressed in-vivo. We tested the effect of expression of sgSTAT1 and sgEGFR in Hek293, normal human cell line and human breast cancer cell lines MDA-MB-468 and MCF- 7. We used p AC152- dual-dCas9-VP64-sgeExpression for constructing guides for sgSTAT1 and sgEGFR. Prior to transfecting the cells, the DNA concentration was measured by nanodrop-spectrometry and the reading data for each plasmid was higher > 200 ng/ µl (Table 5-2,Table 5-3).

We tested the transfection efficiency in Hek293, normal human cell line and second in human TNBC cell lines (MDA-MB-468) by using green florescent protein (Figure 5-2). Transfected cells (p AC152-dual-dCas9VP64- sg Expression) were revealed under the fluorescent microscope in green color. Also, WB results confirmed the overexpression of STAT1, while the result for EGFR from these cell lines was inconclusive.

Table 5-2 Plasmid concentration p AC152-dual-d Cas9VP64-sgExpression after incorporating STAT oligonucleotides

STAT	The Concentration Reading
STAT1	268 ng/ µl
STAT2	200 ng/ µl
STAT3	300 ng/ µl
STAT4	500 ng/ µl

Table 5-3 Plasmid concentration p AC152-dual-d Cas9VP64-sgExpression after incorporating EGFR oligonucleotides

EGFR	The Concentration Reading
EGFR1	457 ng/ µl
EGFR2	210 ng/ µl
EGFR3	267 ng/ µl
EGFR4	362 ng/ µl

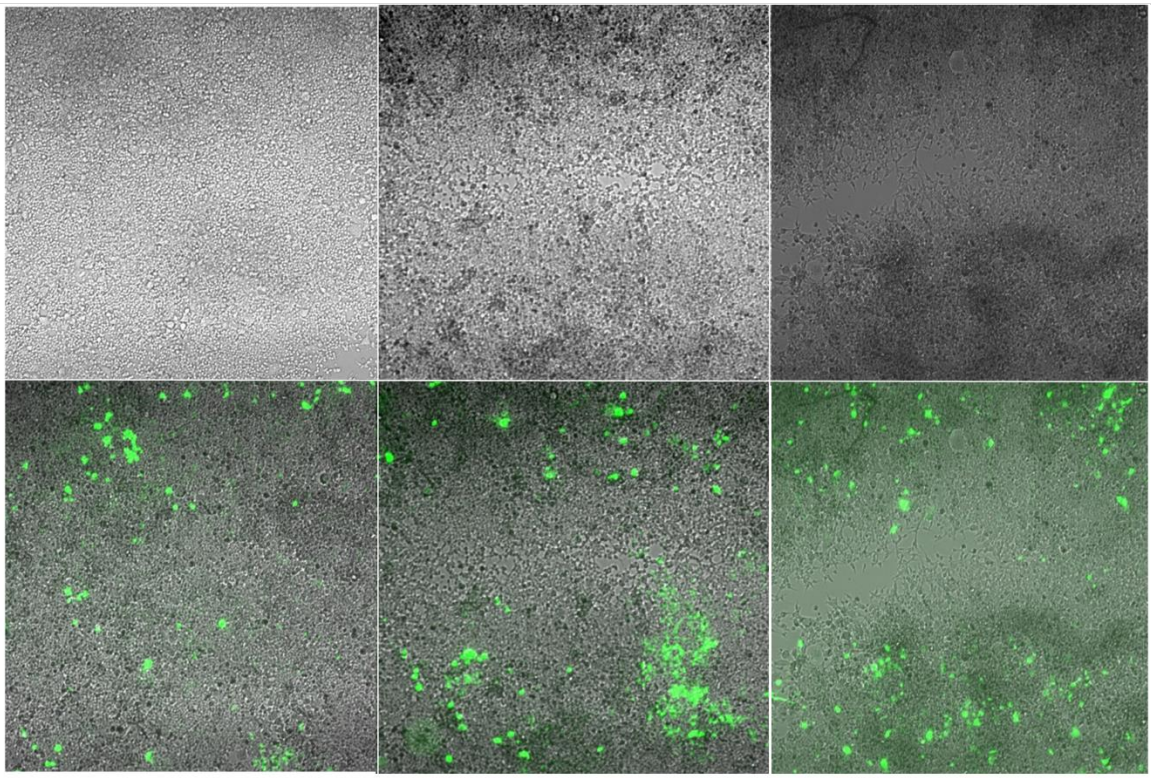


Figure 5-2 GFP expression by florescent microscopy in sgSTAT1 and sgEGFR transfected Hek293 cell line

Images show the relative levels of GFP expression in sgSTAT1 and sgEGFR transfected Hek293 cell line. Cells were co-transfected with four different dCas9-sgSTAT1 (four different sgRNA designated STAT1-1, STAT1-2, STAT1-3, STAT1-4 were designed to target the STAT1 promoter) and four different dCas9-sgEGFR (four different sgRNA designated EGFR1, EGFR2, EGFR3, EGFR4 were designed to target EGFR promoter) at individual construction. GFP (Green Fluorescent Protein) was used in control p AC152, p AC152-EGFR and p AC152-STAT1 to indicate that the transfection was successful. After 48 – 72 hour of transfection, the green fluorescent color was reflected under florescent microscope and the expression level for sgSTAT1 and sgEGFR was observed in different amount compared with the negative control group (empty plasmid p AC-152).

5.3.1.3 Post-Transfection of Hek293, MDA-MB-468 and MCF-7

After adding of G-418 (Type of antibiotic for the selection and maintenance the stable transfection with plasmid expressing puromycin resistance) to the culture medium of (Hek293, MDA-MB-468 and MCF-7), microscopic inspection shows stably transfected cells can be selected according to resistance to G-418 as a selected marker. The morphology of cell culture is shown as a group of cells in a poly clonal population of stable expression cells and green fluorescence revealed abundant expression (Figure 5-3).

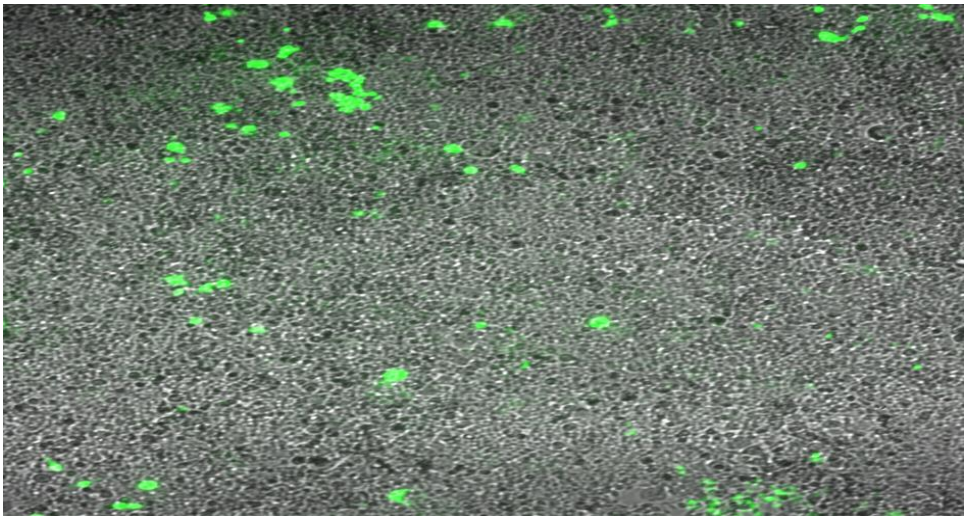


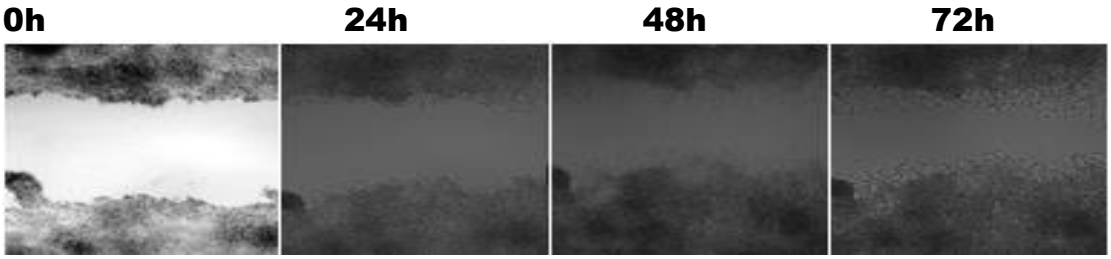
Figure 5-3 Hek293 cells transfection transiently

The fluorescent microscope image of breast carcinoma cell line MDA-MB-468 shows the morphology of poly clonal cells culture with green fluorescence as a positive control for transfection by Fugen-HD reagents.

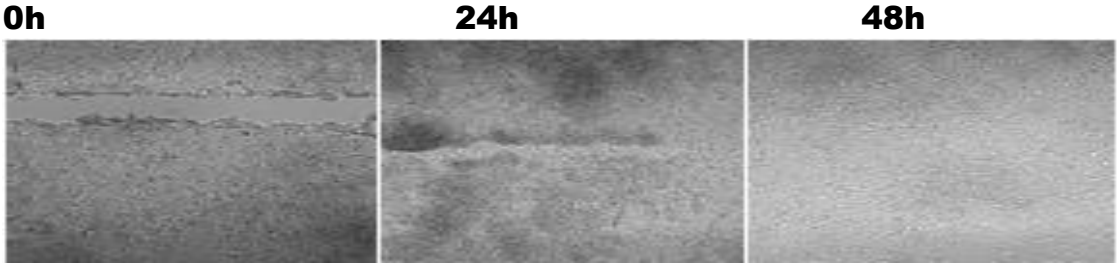
5.3.2 Wound Healing Assay

The main goal of wound healing assay is for monitoring migration of homogenous cell populations after transfection. Wound healing assays have been adapted to measure migration of individual cells in the reaching edge of the scratch. Confluent human Hek293 cell line culture was scratched and immediately imaged at 5X magnification at (0 hr). The image shows the in-vitro scratch to track migration of Hek293 cell in the leading edge. Cells were positively transfected with plasmid p AC-152-sgSTAT1, p AC-125-sgEGFR (separately but with a GFP plasmid to track transfected cells) and compared with control cells were transfected with empty plasmid p AC153 and GFP plasmid. Recovery of cell-free area Hek293- CRISPR-Cas9-sg-STAT1 was based on the migration rate and the time in the reaching edge of the scratch. As a result, transfected cells showed that the positively transfected cells migrated at a slower rate compared with control transfected cells in the leading edge of the scratch in the same dish (Wound Healing Images of CRISPR/Cas9-sg-STAT1

). Wound healing assay in Hek293- CRISPR-Cas9-sg-EGFR showed positive transfected cells migrate at the same rate as the surrounding un-transfected cells (control) in the leading edge of the scratch (Figure 5-6).

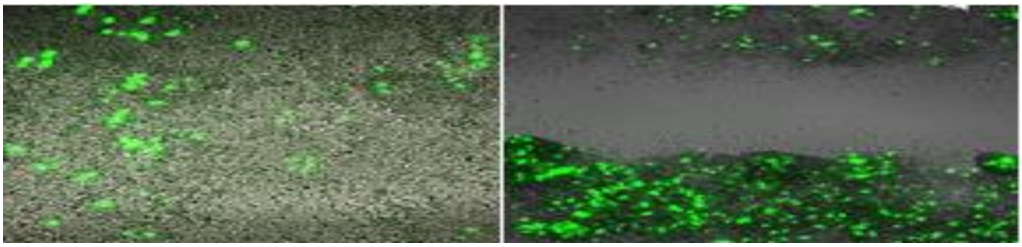


Hek293-CRISPR/dCas9-sg STAT1



Hek293-CRISPR/dCas9-Control

A)



48h 72h

Hek293- CRISPR/dCas9-Control Hek293-CRISPR/dCas9-sg STAT1

B)

Figure 5-4 Wound Healing Images of CRISPR/Cas9-sg-STAT1

Images show the wound healing assay at (0, 24, 48, and 72 hour) in different characteristic between (Hek293-CRISPR/Cas9-sg-STAT1) and control transfected with empty plasmid p AC-152 (Hek293- CRISPR/Cas9-control). Migration shows reduced wound healing by 48 hrs and eventually complete healing of the wound at 72 hrs. Control image have more diffuse wound margin indicating cell migration at 24 hrs and showing complete healing at 48 hrs. B) Fluorescence imaging of transfected Hek293 cells was showing the in-vitro scratch to track migration of individual cells in the leading edge of the scratch but only cells which were positively transfected with p AC-152-sgSTAT1 and GFP. A and B Image analysis show the positive transfected cells migrated at a slower rate in sgSTAT1 compared with the empty plasmid in the leading edge of the scratch in the same dish.

To measure the cell-free area after cell layer wounding, the manual analysis was performed to show the percentage of the open wound area (72, 48, 24 hours relative to 0 hour) values were quantified manually representing three replicates.

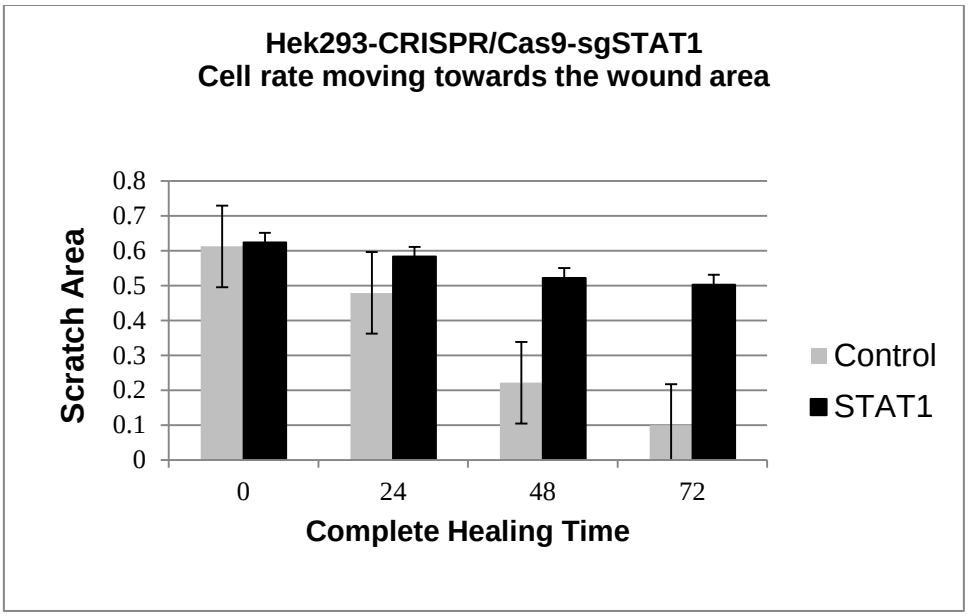
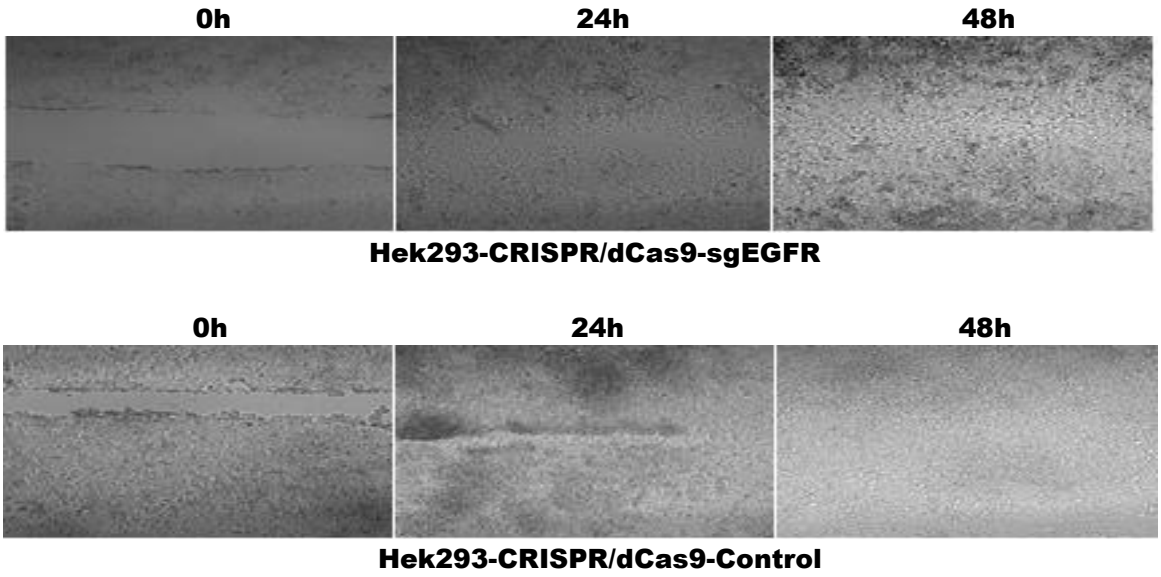
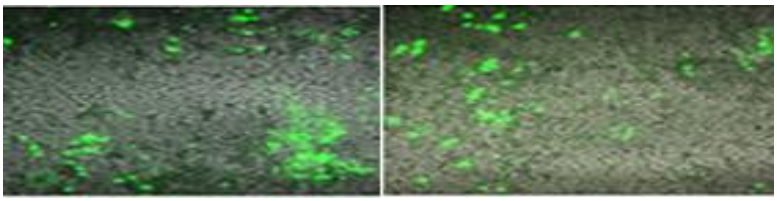


Figure 5-5 The migration time of Hek293-CRISPR/Cas9-sg-STAT1

In-vitro scratch was performed to evaluate the migration of transfected Hek293-CRISPR/Cas9-sg-STAT1 cells. The wound healing capacity of cells was monitored with automated Nikon microscope at 0 hour intervals up to 24, 48 and 72 hours. Cell migration data was calculated manually by the rate of cells moving towards the scratched area. The average migration results were determined in three independent experiments and the p-value (>0.05) is calculated using the t-test (0.015258)



A)



B)

Figure 5-6 Wound Healing Images of CRISPR/Cas9-sg-EGFR

A) Wound healing assay using Hek293 human cell line. Cells migrate to cover the cell-free area after transfected with plasmid bearing Hek293-CRISPR/Cas9-sg EGFR and control cells (Hek293-CRISPR/Cas9-control). Images acquired at 0, 24, 48 hours. Transfected cell free area recovery at 48 hours, while control cells completely recovered at the same time point. B) Fluorescence imaging of transfected Hek293 cells was showing the in-vitro scratch to track migration of individual cells in the leading edge of the scratch (positive transfected cells with p AC-125-sgEGFR and GFP). As marked by green florescent protein, cells were transfected with a control (Hek293-CRISPR/Cas9-control) and did not affect cell migration. Positive transfection cells were marked by fluorescence green color. Image analysis show that the positively transfected cells migrated at similar rate as the control at 48 hours in the leading edge of the scratch in the same dish.

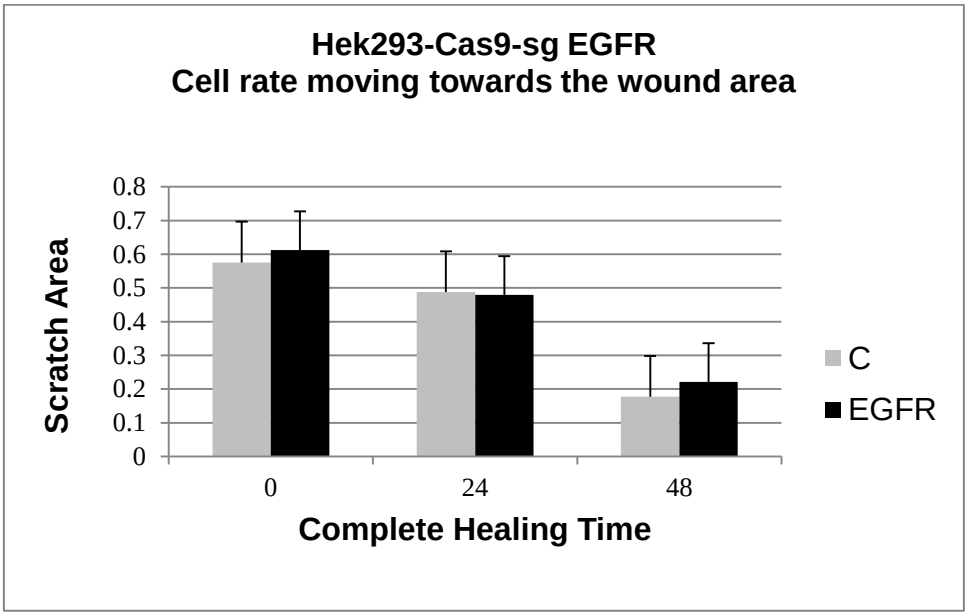


Figure 5-7 The migration time of Hek293-CRISPR/Cas9-sg- EGFR

In-vitro scratch was performed to evaluate the migration of transfected Hek293-CRISPR/Cas9-sg-EGFR cells. The wound healing capacity of cells was monitored with automated Nikon microscope at 0 h intervals up to 72 hrs. Cell migration data was calculated manually by the rate of cells moving towards the scratched area. The percent migration results were determined in three independent experiments and the p-value (> 0.05) is calculated using the t-test (0.028966).

5.3.3 The Quantitative Measurement of the Transfection

5.3.3.1 Western Blotting

Western blotting is one of the most common methods for verifying protein expression and additional information on protein localisation respectively (Figure 5-8 , Figure 5-9).

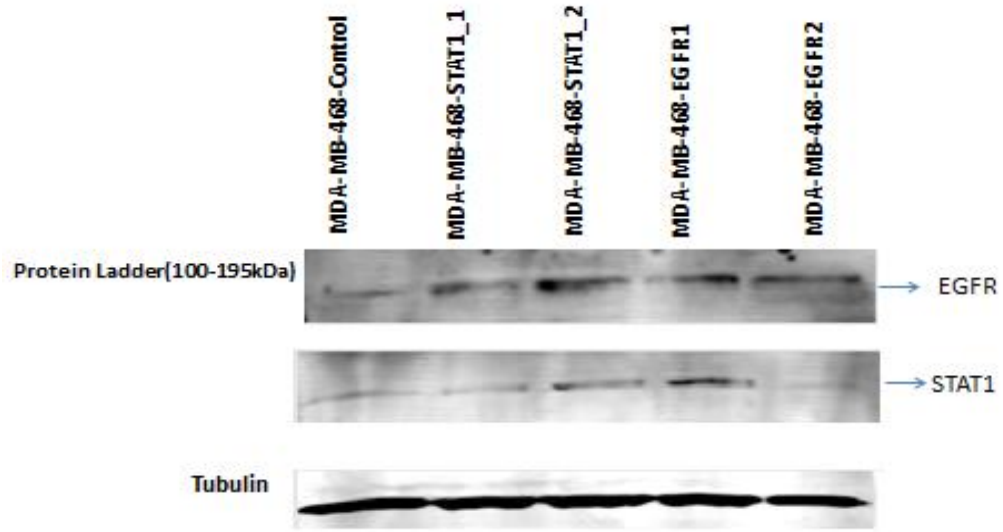
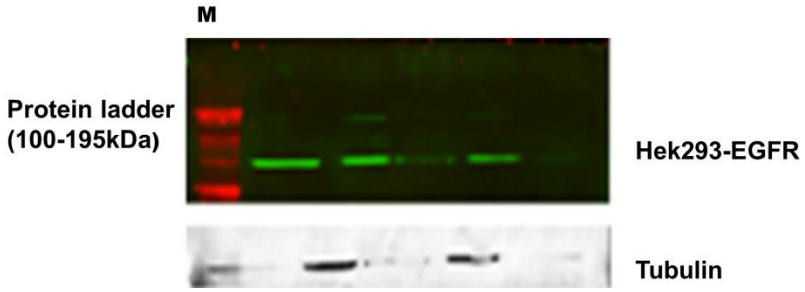
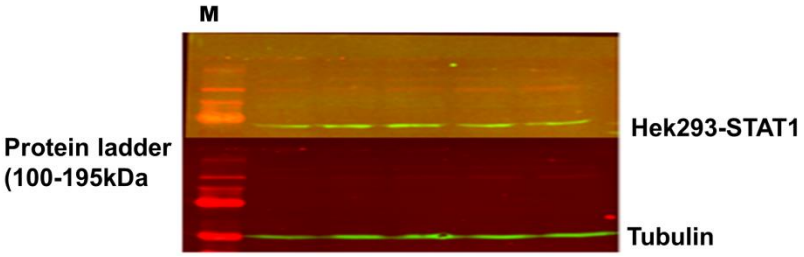


Figure 5-8 Western blot analysis of proteins from transfection breast cancer cell line

The figure showing protein samples extracted from breast cancer cell lines MDA-MB-468 after transfection with plasmid bearing p AC-152-sgSTAT1 and p AC-152-sgEGFR individually. The extraction proteins were fractionated using the 1D-SDS-PAGE followed by a transfer to PVDF membrane. PVDF membranes were incubated with anti-STAT1 and anti-EGFR then with a secondary antibody (1:2000). PVDF membrane was re-propping by Tubulin. The left side of the gel contained control (cells transfected with empty plasmid-p AC-152. Protein ladder was between 10-250 kDa. Image shows detection of STAT1 and EGFR (100-195 kDa).



A)



B)

Figure 5-9 Western blot analysis of proteins that extracted from transfection Hek293 cell line

Western blot analysis shows proteins samples (A-image- EGFR and B-image-STAT1) which were extracted from transfected Hek-293 with plasmid pAC-152-sgSTAT1 and p AC-152-sgEGFR individually. PVDF membranes were incubated with anti-STAT1 and anti-EGFR then with a secondary antibody (1:2000). PVDF members were re-probing by tubuin. Protein ladder was (100-195 kDa). Green bands show detection of STAT1 and EGFR (100-195 kDa).

The concentration of STAT1, EGFR and Tubline bands were determined after transfecting Hek-293 with plasmid p AC-152-sgSTAT1, p AC-125-sgEGFR (separately) and compared to the Hek-293 control transfected with empty plasmid p AC-152 (Table 5-4).

Table 5-4 The Quantities concentration of proteins abundance after transfected Hek293 cell line

Hek293	C	STAT1-1	STAT1-2	EGFR1	EGFR2
STAT1	14	28	29	22	19
EGFR	42	62	77	92	109
Tubulin	24.22	26.69	31.51	30.85	32.08

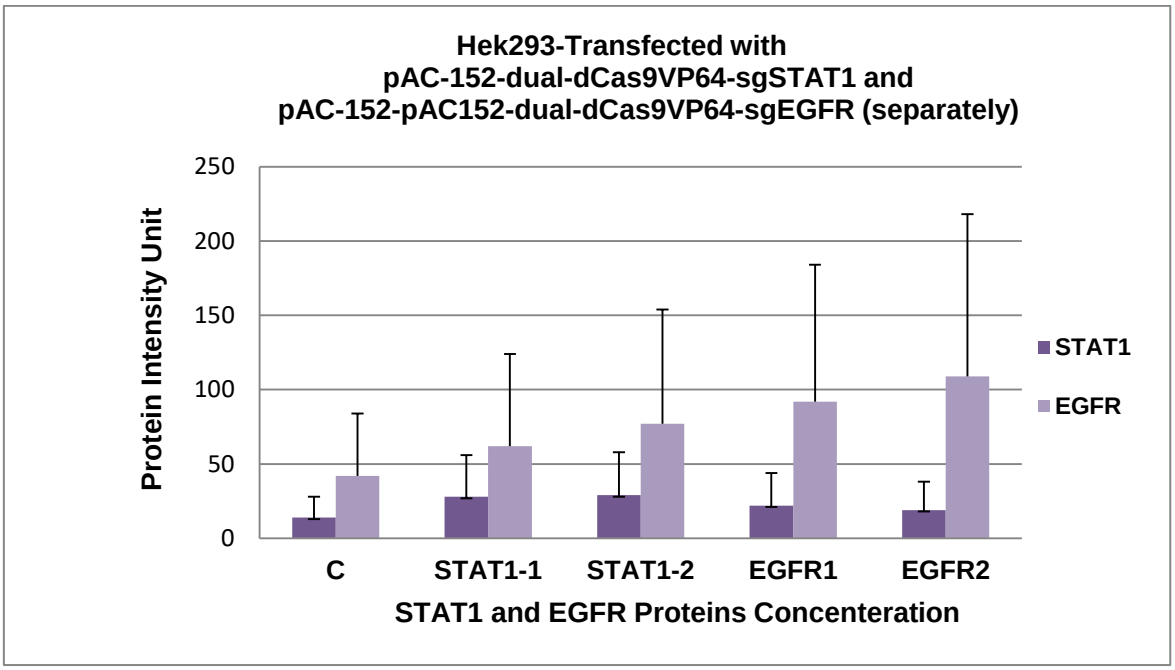


Figure 5-10 The quantity concentration of candidate proteins after transfection of Hek-293

Bar chart shows the concentration (arbitrary unit) for each protein after transfection with plasmid p AC-152-sgSTAT1 and p AC-125-sgEGFR (separately) in Hek293 cell line. Each bar is mean of two blot replicates.

5.3.3.2 Quantitative Proteome Profiling of MDA-MB-468 TNBC Cell Line to Investigate the STAT1 Overexpression

Validation of dCas9-sgSTAT1 constructs encouraged a LC-MS/MS experiment to screen the target protein expression (STAT1 and EGFR) in MDA-MB-468 triple negative breast cancer cell line. It was conducted to detect if the cell line express the STAT1 and EGFR at a higher level than control. Bioinformatics data (Excel sheet in Appendix B) showed that MDA-MB-468 triple negative breast cancer cells over- expressed of STAT1, while EGFR was only weakly detected, which might indicate that the EGFR gene is mutated or epigenetically silenced negating the effect of dCas9-VP64 as illustrated in (Figure 5-11). The results show that the STAT1 expression is clearly increased and successfully receptive to CRISPR/Cas9- sg STAT1.

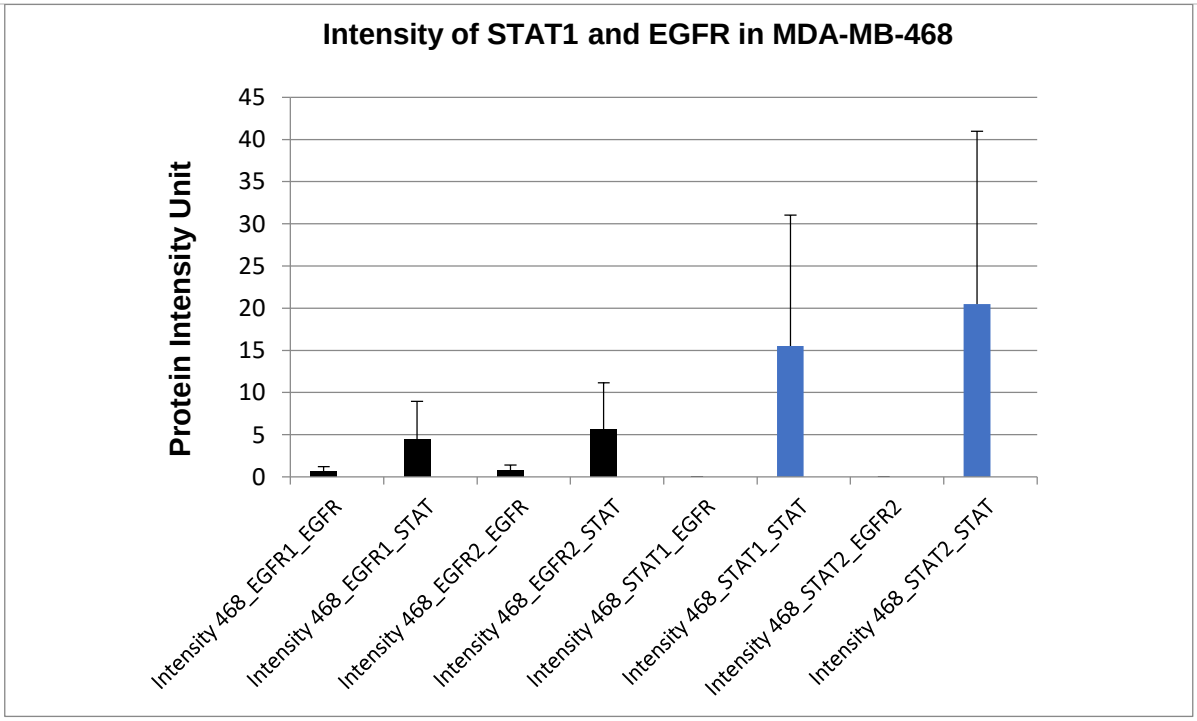


Figure 5-11 Proteomic data of the intensity of STAT1 and EGFR in transfection MDA-MB-468 breast cancer cell line

Statistical analysis showed the STAT1 are significantly over-expressed in transfected (p AC-152-sgSTAT1) breast cancer cells, while transfected cells with (p AC-125-sgEGFR) normally express and complete the lack of expression. Legend: the first identifier gives the gene targeted in the CRISPR experiment. The second identifier gives the protein quantified by LC-MS/MS. Thus STAT1_STAT denotes the amount of STAT1 protein in cells transfected with sgSTAT1. Similarly, EGFR_STAT gives the amount of STAT1 protein but in cells transfected with sgEGFR plasmids.

5.4 Discussion

The primary goal of the CRISPR/Cas9 and human breast cancer cell line model was to determine and utilise the novel CRISPR/Cas9 system to over- express the two candidate proteins STAT1 and EGFR in BC(a) human breast carcinoma cell line MDA-MB-468, human breast adenocarcinoma cell line MCF- 7 and human embryonic kidney cell line Hek293. Our present approach which relies on a new investigation of much that has been observed in earlier studies, discovering of molecular pathways that contribute to metastasis in TNBC and, on several protein candidates (Scribble, STAT1 and EGFR) showing different expression between metastatic and non-metastatic tumours and, on a subgroup of TNBC over-expression of STAT1 and other cytokine induced genes. In particular, we aim to test when deregulation of STAT1 (by over-expression, loss or abnormal phosphorylation) combined with over-expression of EGFR which contributes to invasion and metastasis. If our primary hypothesis is true the tumours with over-expression might be targeted with specific inhibitors and better diagnostic tools can be developed based on measuring the abundance and phosphorylation of these proteins.

In most recent studies, genome editing technologies have allowed for the endogenous regulation of human cell line genes (Cheng et al. 2013, Gilbert et al. 2013). Moreover, human breast carcinoma cell line MDA-MB-468 is extracted from a pleural effusion of female patients with metastatic adenocarcinoma of the breast ; as well as, both MDA-MB-468 and MCF-7 which rapidly develop growth with poor immunogenic (Wang et al. 2013, Cailleau et al. 1978b).

This system is a relatively in-vitro manipulation to create a cancer model cell- line. We used the CRISPR/Cas9 system constructing a site specific cloning of a 24 oligonucleotide sgRNA into dual expression vector harboring dCas9 in MDA-MB-468 and MCF-7.

CRISPR/Cas9 technique can be harnessed rapidly and precisely engineered for both LOF and GOF mutations in tumour suppressor genes, oncogenes, the modulation of cellular transformation and drug responses (Matano et al. 2015). This technique also allows for generating endo-genome conditional alleles based on the site-specific recombinases and tagging endo-genome alleles and intro-gating non-coding DNA elements (Mansour et al. 2014, Wang et al. 2013). Human breast carcinoma cell line MDA-MB-468 is extracted from a pleural effusion of female patients with metastatic adenocarcinoma of the breast . MDA-MB-468 tumour cells develop rapidly, according to the morphological, Cytogenetics, immunological reaction and tumourgenicity in mice as well (Wang et al. 2013, Cailleau et al. 1978b).

This is the first time that CRISPR/Cas9 (reagents description and producers) has been used to construct a site specific cloning of a 24 oligonucleotides sgRNA into dual expression vector harboring dCas9 as a relatively in-vitro manipulation system for STAT1 and EGFR. Studies have found that robust endogenous gene activation is approached by fusing the Cas9 to an activation domain by construction with sgRNA. Additionally, four different sgRNA (STAT1-1, STAT1-2, STAT1-3, STAT1-4) were designed to target the STAT1 promoter an individual location and four different sgRNA (EGFR1, EGFR2, EGFR3, EGFR4) were designed to target EGFR promoter. In the current approach, we also

employed the quantitative analysis by Mass Spectrometry and proteomic profiling for the target proteins STAT1 and to detect increased expression levels.

Research prior to this study, discovered that overexpressed STAT1 has been associated with anti-tumour effects, as well as that STAT1 overexpression in breast cancer may be associated with tumour – promoting rather than tumour- suppression role (Hix et al. 2013). In addition, STAT1 has been associated with IFN gamma and have been shown to play as essential role in immuno- editing of cancer cells. The pleiotropic effects of interferon result from its effects on the expression of a large number of cellular proteins; interferon inducible- proteins, some of those proteins have been revealed to participate in new pathways of regulation of cellular RNA and protein metabolism. Moreover, Greenwood et.al (2012) treated MDA-MB-231 cell with INF- gamma to measure if overexpression of STAT1 and other interferon –induced genes in TNBC has immun- editing effects and the results showed an increase in the migration and invasion associated with increased STAT1 abundance and proportionally increased abundance of CD74.

As a heterogeneous disease, breast cancer relies on multiple signalling pathways for cancer cells to acquire a proliferative capacity, which requires them to gain the ability to produce growth factors that encourage their own proliferation in an autocrine manner or/and activation of paracrine growth factors, by surrounding normal stroma cells or/and by an increase in the amount of receptor on cell surface or by alterations of the proteins structure that consistently activates the signalling pathways dependent upon its ligand (Eroles et al. 2012).

Furthermore, the membrane receptors, such as a HER-2, to form homo or hetero - dimer membrane receptor- complexes, which activate three major signalling pathways Ras/ Raf/ MAPK, JAK/STAT and PI3K/AKT/ m TOR. All these pathways participated in cancer-related cellular functions (migration capability and cell survival).

The results, as obtained from biological function assays such as wound healing and the molecular assay such as WB and Mass spectrometry, clearly illustrated the synchronisation of STAT1 and EGFR expressions in breast cancer cell line that reflects that cells are effectively applicable for dCas9-sg STAT1 targeting and dCas9-sg EGFR individually. In reference or turning now to the experimental evidence on WB, the results obtained indicated a surprising robust synchronized activation of STAT1 and EGFR expression by the corresponding sgRNA constructs- sgSTAT1 seemed to activate EGFR expression and vice versa- sgEGFR seemed to activate STAT1 expression. Such results have not previously been described. They are very intriguing and if a replicate experiment confirms them further studies should definitely be performed to elucidate the mechanism of this synchronisation. The results of wound healing assay illustrated that targeting the promoter of STAT1 leads to almost complete inhibition of migration and invasion.

This could be due to increased amount of STAT1 protein or could be a direct consequence of the association of Cas9-VP64 with the promoter of STAT1. More experiments are needed to determine which one of the two, or whether both hypotheses are true. If blocking of the promoter is the cause, this would suggest an interesting potential therapeutic

approach in which Cas9 could be used as gene therapy to block invasion of TNBC cells by targeting STAT1 promoter in-vivo.

Alternatively, STAT1 overexpression could be related to reduction and inhibition of the migration of breast cancer cells. In general, it is becoming increasingly difficult to ignore the confusing evidences for both oncogenic and tumour suppressor activity of STAT1, with new insights of the STAT Family (Shuntao et al. 2006, Wu et al. 2015). Knowledge from the open literature review that STAT1 has been demonstrated as a tumour suppressor function in many cancer cell types such as breast cancer and melanoma by modulating the transcription of a host of pro-membranes of the death receptor family (Inos, Fas, FasL) and tumour necrosis factor-related apoptosis ligand (Wen et al. 1992). However, many review articles highlighted that STAT1 may be oncogenic such as in Leukaemia initiating cells (Wu et al. 2015, de Prati et al. 2005).

It stills an open question in what context STAT1 acts as a tumour suppressor and in what as an oncogene. It is fully possible that this functional dichotomy is regulated by factors such as other oncogenes expression (EGFR) or dysregulation of other tumour suppressors (Escribá et al. 2008). Therefore, we carried out additional experiments to confirm the clinical feasibility of profiling and manipulating STAT expression in cancer cells trying to put it in the context of EGFR overexpression. We used CRISPR/Cas9 to manipulate expression and wound healing or scratch assays as functional read out. The in-vitro scratch assay is suitable for studies on the effects of gene expression, cell matrix and cell-cell interaction on cell migration. It mimics cell migration, and is easily quantified by

comparing the images of treated and control migrating cells (Liang et al. 2007). However, care must be taken as changing in atmospheric and thermostatic conditions can rapidly affect cell growth, particularly when grown in small volume well plates (96 well-plate) or/and when the plates are transported from the 37˚C incubator to the microscope and back again (Vergara et al. 2014).

Gene expression can be manipulated in multiplex targeting experiments by fusing transcription activating or inhibiting domains to proteins binding to corresponding gene promoters, which facilitates the modulating of endogenous gene expression (Cheng et al. 2013); CRISPR/Cas9 could be used to activate multiple genes either individually or simultaneously. Catalytically dead Cas9 targeted genomic region up and downstream of the transcription start site allows for specific and sustainable gene expression level alterations in in-vitro tumour tissue culture (Braun et al. 2016). EGFR is overexpressed in some TNBC, the reason for this is un-clear, but it may relate to non-receptor tyrosine kinase Src which is active in breast cancer cells and the strong cooperative relation between Src and EGFR in breast cancer. The cooperative pathway of Src and EGFR is involved in the malignant process which extends to overexpression of Src substrate that has a role in invasion and metastatic potential of breast tumour cells (Hynes 2000). Clinical application of the conventional gene editing approach utilising Cas9 is problematic because of significant off-target cleavage by CRISPR/Cas9 in humans.

It is an essential complication. Thus, human cancer gene therapy its off-target the human cancer by CRISPR/Cas9 off - target activity should be investigated wisely and thoroughly

before clinical application (Baltimore et al. 2015). Our results however suggest that blocking the promoter of target protooncogenes with a catalytically dead Cas9 might be a safer alternative to editing. Further studies are needed to explore this opportunity. In our research, we used lipid-mediated transfection to deliver CRISPR/Cas9 components to cancer cell line which is the common method for nucleic acid delivery to cells. In clinical research, researchers normally involve encapsulation or complexing of nucleic acid which can undergo interactions between the positively charged lipid head group and the negativity charged phosphate backbone consequently, the cellular up take is mediated by endocytosis and a micropinocytosis mechanism (Gori et al. 2015). If we are to continue with the exploration of the CRISPR /Cas derived model described in this chapter, the studies need to move to the stage where an animal model should be developed. Animal models of cancer provide different means to determine the cause of and treatments for tumour; thus representing a source of a massive potential for cancer medicine and research, for example, the experiment of modelling cancer in mice has developed to the extent the method that researchers can both observe and manipulate a complex disease pathway.

However, the human tumour tissue models are limited in design and technology development and there are many limitations expected and developed in using human tumour tissue as models, such as, tumor heterogeneity, complications and costs of invasive biopsies, and patient comfort. Therefore, using human tumor tissue should be explored to limit tumour biopsies and patents discomfort, costs and inconvenience.

Furthermore, the mechanisms that control the maintenance and survival of tumourgenic cells are unclear. The reason is the pre-supposed lack of appropriate model system.

However, there is the continuing manner that the breast cancer cell- line continues to share many of the molecular and genetic features of the primary breast cancer from which they derived; this approach is needed, to use the essential value for tumour patients adapted from this modern biotechnology. Re-plating of single cell clones is recognized as inefficient, a poor yield for clones and a low capacity to expand and to propagate. Moreover, a single cell clone derived from the same cancer cell -line do not have equal metastatic or tumourigenic characteristics (Chenwei Li et al. 2007). Thus, a potential development of us would be to extend the CRISPR/Cas approach to be used in an animal model. We could use our constructs to generate stable cancer cell lines expressing the sgSTAT1 and sgEGFR constructs under the control of an inducible promoter. These can then be used to produce a xenograft mouse model and test how inducing of suppressing the expression of STAT1 and EGFR or blocking the promoters of STAT1 affects tumour growth and metastasis.

One limitation of the CRISPR/Cas9 chapter approach resides potentially in the unwanted genomic modifications at the target site. One of the main challenges of using the CRISPR-Cas9 editing technique is off-target effects, where Cas9 enzymes cut or otherwise affect the function of wrong genes. Another limitation is the use of only one breast cancer cell line.

5.5 Conclusion

The following conclusions can be derived from our CRISPR/Cas9 research, it appears that when the EGFR promoter is targeted with the dCas9-VP64 construct, this not only leads to a direct effect on EGFR expression but also increased the expression of STAT1 as suggesting some form of functional interaction between EGFR and STAT1. Furthermore, when the STAT1 promoter was targeted this lead to increase in EGFR expression.

Chapter 6 General Discussion

As mentioned in the literature, the data on health care indicates that 29% cancer cases are referred to breast cancer alone; TNBC represents approximately 10–15% of all breast cancers and patients with TNBC have a poor outcome compared to the other subtypes of breast cancer (Chavez et al. 2010). Other studies show that TNBC accounts for 12–17% of breast cancers (Pareja et al. 2016).

TNBC women are Her2 negative, progesterone negative and oestrogen negative with the majority of triple negative patients having a poor diagnosis for this type the first line of treatement is cytotoxic chemotherapy (Parisi et al. 2018). As stated before, the main purpose of this thesis is to contribute to the elucidation and the identification of the molecular pathways that contribute to metastasis in TNBC; one approach to pursue the main purpose of this thesis is to search protein candidates 1.22), which show differential expression levels between metastatic and non-metastatic tumours, as those are likely to carry the molecular functions required for increasing the metastatic propensity.

In terms of analytic technology, the transcriptional activation can be detected by RNA-seg or microarrays but methods working directly on protein level might be better suited for such discovery projects. Such protein expression profiling methods could use antibodies, such as in immunohistochemistry (no results show), immunocytochemistry (Figure 4-3, Figure 4-7,

Figure 4-12) and Western Blotting.

These are the most common methods to detect the protein expression when a single protein or just a few proteins need to be measured. Alternatively, proteomics could be used to measure thousands of proteins in an unbiased manner. The most interesting finding was shown (Table 3-3 Mean and Standard Deviations for tumour breast tissue G1&G2Figure 3-10, Figure 3-10, Figure 3-11). Using the qualitative data analysis from this study, much has been learned and this provides a better understanding about metastatic and non-metastatic TNBC. In particular, this study aimed to test whether deregulation of Scribble or STAT1 (by over-expression, loss or abnormal phosphorylation) combined with over-expression of EGFR contributes to invasion and metastasis which affect the aggressiveness of breast tumours.

Some molecular research explained that the deregulation in Her-2-neg- pathway might lead to sustained proliferative signalling; the reason is the membrane receptors, which belong to the HER-2 family (after forming–homo or/and heterodimer) have the ability to initiate the intracellular tyrosine kinase function and activate the three major signalling pathways Ras/Raf/MAPK, JAK/STAT and PI3K/AKT/ m TOR (Konecny et al. 2003, Chen et al. 2016) . It can be clearly seen, in the literature that these pathways are participating in cellular function such as growth and cell survival, proliferation, division, metabolism migration capability and apoptosis.

More molecular biology information allows for the classification of breast cancer; however, these current results from genome editing system (CRISPR/Cas9-sgEGFR and sg-STAT1) are not complete and not applicable to clinical practice.

Further research is needed to elucidate the mechanisms underlying the observed results, which should allow translation to clinical developments. Nevertheless, this study has provided valuable information about the differences between metastatic and non-metastatic TNBC that could lead to a better understanding of the mechanism in TNBC. Historically studies of the molecular biology of signalling pathways have contributed to our understanding of the processes of the formation, maintenance and expansion of tumours, as the new knowledge about HER2, estrogen receptor, IGF1R, PI3K/AKT, mTOR, AMPK and angiogenesis pathways has allowed researchers to discover and develop new target therapies that are being tested in ongoing clinical trials (Polivka Jr and Janku 2014). Studies on signalling pathways studies have revealed important signalling molecules, such as BAD (Bcl-2-associated death promoter), GRB2 (growth factor receptor-bound protein2), IRS1 (insulin receptor substrate 1), MDM2 (murine double minute 2), mTOR (mammalian target of rapamycin), PDK1(3-phosphoinositide-dependent protein kinase 1), PI3K (phosphoinositide-3 kinase), PIP2 (phosphatidylinositol bisphosphate),PIP3 (phosphatidylinositol triphosphate), PTEN (phosphatase and tensin homologue deleted on chromosome ten), RAPTOR (regulatory associated protein of TOR) (Eroles et al. 2012).

Furthermore, comparison of the protein profiles collected from breast tumours and cancers cell lines (results in chapter three) and by using the analytical methods of protein technology (2.32.4, 2.4.1and 2.4.3) has showed that this approach has the potential to identify breast cancer bio markers and facilitate suitable drug development. Protein expression pattern differences between metastatic and non-metastatic tumours provide specifics, which need to be taken into consideration (Figure 3-12, and Figure 3-13).

This study also demonstrates that protein expression in tumours is very heterogeneous with groups of tumour cells strongly expressing some proteins and other groups not expressing them at all. Thus, the results obtained with methods such as mass spectrometry or WB need to be further validated by IHC or ICC, that then gives spatial resolution and show in which cells and even exactly where in the particular cell (cellular location) that the protein is expressed in the tumour tissue sample.

In the past, it was recognised that the ICC technique has the ability to detect the Ag in the sub cellular location in tumour cell lines. Throughout this study, particular attention has been paid to reproducibility and statistical significance of the results. This has been demonstrated in the corresponding figures and tables by providing statistical metrics such as the observed correlation between expressions of different target proteins, the error bars in the results of proteomics (3.3.33.3.4) and CRISPR/Cas9 data analysis.

Error bars demonstrate wide variability in tumour protein expression, which is expected because of the proteomic heterogeneity of tumour tissues, which is now a well-established fact. It has been shown that error bars can be used as a direct manipulation interface for controlling, probabilistic algorithms and approximate computation. However, when the error bars overlap it does not clearly show whether or not a statistical significant difference exists (Cumming et al. 2007). For this reason formal statistical tests, such as the Student's t-test and the Fisher exact test have been computed and the corresponding p-values reported.

Table 3-1 Human breast cancer cell lines were used in this study as this permits high throughput drug or RNA interference screening to allow rapidly development into tumours and thereby showing susceptibility and drug resistance. However, the rapid rate of mutation over time may represent limitations in the ability to extend tissue culture. An implication of this is the possibility that integrating proteomics data with tumour heterogeneity remains a major challenge. The examination of inter-and intra-tumour heterogeneity is required for the development of treatment; for example, one tumour consists of multiple colonies, these colonies may well have different functions and constructions of the various characteristics of a tumour. Thus, one dominates a targeted clone, while other minor clones within a tumour may continue to grow and metastasise (Jhan and Andrechek 2017, Tabassum and Polyak 2015).

The heterogeneous nature of breast cancer has important implications for physicians and their patients. There are many phenotypic changes associated with the malignant transformation, such as cell proliferation, adhesion and migration, mediated or initiated by a large variety of proteins, some of them linked to the plasma membrane, which makes these central in the biological process and potentially effective drug targets (S Harvey et al. 2001). Previous research conducted in Metodiev's group identified a group of proteins (EGFR, Scribble, STAT1, MX1 and CD74) as potential markers and targets for TNBC; however, what is not yet clear is what their individual and combined impact is on TNBC. By exploring these proteins further, we can try to identify gaps in the literature and design experiments that could help fill these gaps. Additionally, many researchers have made

noteworthy contributions to understand several risk factors linked with TNBC. Unfortunately to date, many causes remain unknown. Moreover, developments in proteomic analysis have enabled researchers to use a large scale approach to compare groups of TNBC tumours, which creates a possible strategy for the identification of novel target proteins. Essentially, in order to develop the ICC, this study needs to detect the target protein by using a specific Ab. Alternatively; WB involves harvesting protein and separating by electrophoresis before staining with the specific Ab 2.12.4). The ICC results (Figure 4-5 The EGFR expression level in MDA-MB-468 and MCF-10AFigure 4-14 Scribble expression level in 10MDA-468 and MCFprovide further information about protein localization, which requires a more specific (Ab) than WB. Clearly we can see that WB is preferable for the verification of our candidate protein expression (Scribble, STAT1 and EGFR). EGFR protein can be used as early prognosis bio-markers and diagnosis of breast cancer; and also as response to treatment monitoring therapy and/or as targets for new therapy. Additionally, the EGFR tyrosine-kinase inhibitors can be used for TNBC as a potential clinical target (EGFR–targeted therapy) (Burness et al. 2010).

Alternatively, mass spectrometry following in-gel digestion is used and this offers several benefits over in solution digestions. In-gel digestion can target and identify specific gel bands in specific molecular weights, which assess and investigate proteins that differ under certain cellular condition. We used this approach in our quantitative experiments to measure STAT1 and EGFR in breast cancer cell lines. This study used CRISPR/Cas9; the new and revolutionary genome editing system, which helped to create a model of STAT1 and EGFR overexpression of human breast cancer cell line. Our results agree with the

Martin Jinek (2012) report that bacterial protein, Cas9 can be expressed and localised to the nucleus of human cells. It assembles with single guide RNA (sgRNA) to edit target genes in-vivo; and also to produce a generation of a complex double stranded break and stimulates NHEJ repair in genomic DNA at a site complementary to the sgRNA sequence. Furthermore, the RNA sequence can be extended at the 3´ end, which enhances DNA target activity in-vivo (Table 5-1). Additionally, Martin Jinek (2012) and his colleague explained that experiments relied on the use of the extract from transfected cells showing the sgRNA assembly into Cas9, which is the limiting factors for Cas9 mediated DNA cleavage. These reasons are why we were encouraged to use CRISPR/Cas9 and the feasibility sgRNA programmed genome editing in human cells. We elected to use a modified CRISPR/Cas9 method, which does not aim to induce breaks, but instead modulates the activity of the target gene.

The most interesting finding in this study was that the CRISPR/Cas9 system could provide the way to revolutionary applications in human gene therapy by selectively interfering with the activity of the target gene promoters. The results (presented in chapter 5) were facilitated by using type II bacterial CRISPR/Cas9 system, which could be used to guide future genome editing experiments in normal and cancer human breast cell lines.

To reprogram STAT1 and EGFR, we used appropriate sgSTAT1 and sgEGFR, which are easily designed by online tools; such, RNA-guided genome editing offers distinct advantages, owing to the simplicity of the sgRNA design and the ease of cloning of the synthetic oligos by using the Golden Gate mechanism (table 1-5, figure 5-2 and 5.3.1.3).

It is primarily valued as a cost-effective and rapid mechanism for targeting gene promoters in large scale research efforts.

Additionally, research prior to this study, has shown that over-expression of STAT1 has been associated with anti-tumour effects. As mentioned in the literature STAT1 over-expression in breast cancer may be diagnosed with a tumour–promoting rather than a tumour- suppression role (Hix et al. 2013). It must be noted that cancer cell invasion assays (5.3.2 that use in-vitro cancer models where cells are cultured in 2D substrates),used in such studies, are unable to completely model the tumour microenvironment and to address the effect of the microenvironment on the invasiveness of cancer cells (Toh et al. 2009, Fidler 2002).

It is estimated that 90% of cancer death results from metastasis, whenever, a woman diagnosed with breast cancer who also has a positive-lymph node disease. This means the tumour has spread from the original breast tumour to the underarm lymph nodes on the side of the breast cancer. The lymph node status is determined in the early-stage as invasive breast cancer based on the stage from I to III with lymph node involvement (Seyfried and Huysentruyt 2013, Onda et al. 1997).

This approach discovered a surprising synchronization interaction between EGFR and STAT1. It appears that when the EGFR promoter is targeted with the dCas9-VP64 construct, this not only leads to a positive effect on EGFR expression, but also increased the expression of STAT1. Conversely, when the STAT1 promoter was targeted this lead to

the increase in expression of EGFR; and also to robust activation of STAT1 expression with respect to potential clinical applications. Usually, more robust clinical responses are seen when targeted therapies are combined with radiotherapy, chemotherapy or other target compounds. However, several decades of sustained effort to develop targeted therapies has shown the inhibition of cancer relevant pathways leads to surprising results due to the enormous complexity of the signalling pathways in the cancer cell, for example, the role of some proteins, such as tyrosine phosphatases (PTP) as a tumour suppressor has been redefined in tumour biology as being involved in the inhibition of growth factor receptors and JAK/STAT signalling. Molecular signalling pathways studies show that PTP inhibition can represent a therapeutic approach, as the loss of PTP function predisposes cancer cells to the effects of the immune system. Thus, it suggests that development of highly specific allosteric PTP inhibitors might open a new era in cancer drug development (Wiede and Tiganis 2017).

The signal transducers of the tyrosine kinase type are well established oncogeneses in breast cancer; PTP serves to attenuate oncogenic protein tyrosine kinase signalling and are often mutated or not expressed in cancer (Kairouz and Daly 2000). Signalling pathways and functions of tumour phosphatases suppressor such as tyrosine specific phosphate are inactivated by EGFR, Janus-activated kinase, JAK and STAT1 in the context of the dependent. In some contexts, it inhibits tumour development and in others, these inhibitors are ineffective on tumour-immune system interactions. Another possible explanation for this might be that the non-receptor tyrosine kinase Src is hyper-active in breast cancer and

there is cooperation between Src and EGFR, which contributes to malignancy (Hynes 2000).

Clearly, we can see that the cooperation between Src and EGFR is involved in the process of malignancy, which extends the overexpression of Src substrate that has a role in the invasion and the metastatic potential of breast tumour cells. Our hypothesis is built on the overexpression of a group of candidate proteins that contributes to the propensity of metastasis and the invasion of TNBC. Additionally, the therapeutic combinations directed at multiple molecular targets may prove to be more efficient than monospecific therapy in the treatment of breast cancer.

The pathway that STAT1 activates in the mammary tumour remains unclear. The activation might be promoted by a tumour-intrinsic mechanism mediated by tyrosine kinase supported by the association of IFN-Y. This is produced by immune cells and marker genes for infiltrating immune cells; particularly, tumour tissue with high STAT1 levels. However, the associations between the expression of IFN-Y and STAT1 transcript in mammary tissue are significant and support the tumour prognoses, which appears to be non-redundant (Tymoszuk et al. 2014). In terms of gene expression, the rationale to use breast tissue was owing to the fact that breast tissue is one of a few tissues that can be induced to undergo dramatic shifts in structure and function. Additionally, breast cancer cells provide an example of loss of structure and altered gene expression, which can be used for the comparison of the genotype and phenotype. The genetic alterations in the breast cancer progression from non-malignant to metastasis have revealed hundreds of

coding mutations in multiple cancer derived genes. These mutations have the ability to change the gene expression (i.e. changing the proliferation and adhesion of the genes involved and the signalling pathway such as Wnt and MAPK). When considering the generation of cell-based models of cancer transfection by plasmid DNA cancer and normal cell lines are indispensable. The crucial experimental steps and non-viral vectors are generally less efficient in delivering DNA and initiating gene expression when compared with viral vectors. Additionally, it is known that the transfection efficiency of DNA is different between cell lines, which are used in experimental research (Horibe et al. 2014).

It can therefore be assumed that the high expression of GRP94 (Figure 3-9) supports more strongly the literature that points to GRP94 as a secreted chaperone, membrane protein and molecular chaperone, that directs folding and/or assembly of secreted chaperones unlike other ubiquitous luminal chaperones (Marzec et al. 2012). GRP94 is a selective chaperone to those much shorter and to other ubiquitously expressed endoplasmic reticulum ERα chaperones. Also, sequence analysis shows that the promoter of GRP94 is highly similar to that of other ERα stress inducible promoters characterized by the presence of a considerable number of CCAAT-like motifs flanked by GC-rich regions (Chang et al. 1989). However, many plasma membrane receptors are secreted product such as MHC class I proteins, that are not required for GRP94 activity for proper folding and/or traffic to the cell surface; this is demonstrated by knockout and the knockdown cells (Randow and Seed 2001). GRP94 is an abundant luminal constituent and has the ability to form complexes with many proteins. Additionally, GRP94 is often found in multiple tumours

as a highly overexpressed protein and many publications have implied it is a candidate marker for monitoring tumour progression (Ma and Hendershot 2004, Ni and Lee 2007).

In this study, we used selected protein candidates extracted from cultures of different breast cancer cell lines such as MDA-MB-231, MDA-MB-468, ZR, MDA-MB-435 and MCF-7 and quantitative detection by WB, Proteomic and ICC. This evaluation was carried out for EGFR, STAT1, and MX1. However, the specificity and sensitivity of an ICC is limited by the specific activity of the antibodies used in the different steps of the procedure. Coverslip-slide preparation from breast cancer cell line MDA-MB-468 uses monoclonal Ab in a different concentration (primary anti-Scribble and secondary) with a high quality technique. The reasons for studying the pattern of protein expression in the triple negative tumour by the ICC, is (**I**) to identify the cell type and origin of the metastasis, in order to find the site of the primary tumour and (**II**) to diagnose a site location and measure the stage and grade of tumours. These two points have been highlighted. In this study, our results showed it was possible to visualize the distribution and localisation of Scribble protein expression in a cellular component within an adenocarcinoma breast cancer cell, such as cell-cell junction, plasma membrane and in the subcellular location such as cytoplasm. Formerly, it was recognized that adenocarcinoma breast cells can be defined as a pathological subtype of breast cancer and as an invasive ductal carcinoma.

Furthermore, it contains a population of stromal cells with characteristics of many fibroblasts and clearly explains that these cancers give rise to outgrowth (Seemayer et al.

1979, Gogoi and Borgohain 2015). Previous studies found that the epithelial tissue of the breast organ converts from normal regular morphology to de-regulate and lose cell proliferation control when mutation in the Scribble gene (Scribble protein) occurs (Downward 2003, Rakha et al. 2008). Many studies in gene expression analysis suggested that TNBC arises from basal cells of the mammary epithelium and is associated with high mitotic activity and invasive tumours in younger patients and in premenopausal women (Greenwood et al. 2012).

EGFR expression was found in metastatic cell lines such MDA-MB-231 and MDA-MB-468 and not in MDA-MB-435. This result can be explained by the derivative history of MDA-MB-435 and the patient from whom the cell line was derived. Anderson Cancer Center found extensive infiltrating carcinoma in the breast and two of eight axillary positive-lymph nodes, following the mastectomy and axillary lymph node dissection for breast cancer (Cailleau et al. 1978a, Chavez et al. 2010). Consistent with this, the cDNA expression data analysis found that MDA-MB-435 clustered with melanoma cell lines and not with other breast cancer cell lines (Ross et al. 2000), which raised two probabilities. The first probability is that the breast cancer cells are undifferentiated and express markers of melanocyte differentiation. Most research has indicated that primary breast tumours can express melanocyte related genes and found similar melanocyte related gene expression in the MDA-MB-435 cell line (Montel et al. 2009). The second probability is that the MDA-MB-435 melanoma cell line has in an unknown way contaminated and overgrown the culture of the original cell line (Sellappan et al. 2004, Rae et al. 2007).

The existences of a heterogeneous cell population within breast tumour tissue and cell lines that have a higher tendency to intra vassate and eter into the blood stream; are therefore increasing the likelihood of cancer metastasises. Moreover, there is evidence showing that a more invasive phenotype is a result of not only the intrinsic genetic variation, but also in a different tumourigenesis. The reason for the outcome of cancer metastasis depends on multiple interactions between metastatic cells and homeostatic mechanisms. Therapy of metastasis is targeted not only against tumour cells, but against a host of micro environmental factors that support the progressive growth and survival of metastatic cancer cells.

Scribble protein plays an essential role in the maintenance and regulation of epithelial tissue polarity (Bilder and Perrimon 2000). With respect to this study, it was found that there are close connections between growth factor signalling and the mechanisms of cell transformation, which results from hyperactivation of the mitogen signalling pathway (Bernards and Weinberg 2002). Most of the physiological changes in cancer are mediated by molecular alterations at the protein level; such disease specific changes arising from the tumour cell can be utilised to provide bio-markers that can guide treatment decisions at the molecular level.

Moreover, our results of immunocytochemistry findings provide an opportunity to develop more clinical therapies in the future which could serve as the basis to identify TNBC patients in the clinical setting and for the retrospective evaluation of the efficacy of known treatment agents on this tumour type.

Genes and pathways that protect cancer cells from diverse stresses linked with the malignant state represent a second class of potential vulnerabilities. Generally, in the comparison between normal and cancer cells, the cancer cell depends on cytoprotein pathways as they experience elevated levels of mitotic and DNA- damage-related-stress (Luo et al. 2009). In terms of genetic definition, many cancer studies of malignant and pre-malignant cells are required to highlight the specific features of the oncogenic state that recognise cells to the inhibition of individual stress response pathways and CRISPR technology to interrogate them (Gilbert et al. 2014). The data obtained about the effect of co-overexpression and deregulation of EGFR, Stat1 and Scribble can provide valuable clues toward understanding the mechanisms of TNBC metastasis and to determine which proteins are potentially exploitable for the clinical categorisation of this type of breast cancer.

There are many phenotypic changes associated with malignant transformation, such as cell proliferation, adhesion and migration, mediated or initiated by proteins linked with the plasma membrane, which make these central in the biological process and a potentially effective drug target (Harvey et al. 2001). A combination of diverse proteins Scribble, STAT1 (Greenwood et al, 2012), EGFR (Yang et al, 1996) are correlated with increased migration and invasion in breast tumour tissue and cultured cell lines.

Proteomic data in this study with approaches (ICC and CRISPR/Cas 9) has enabled to understand the image of the molecular pathways of breast cancers. This allows the response of biomarkers to be characterised more accurately than before. In the future, patients could be treated according to the molecular portrait of their tumor biomarker expression, maximising the therapeutic benefit that each patient receives. Further researches with rapid and efficient analysis of proteins technologies and large sample size are expected to expedite the translation of basic research findings into clinical applications, also by providing biomarkers for diagnosis, early detection, and novel therapies. Although more work remains to be done, we believe that the proteomic–based approaches (combined with Immunocytochemistry, CRISPR/Cas9) detailed here already represents advance step toward defining a useful comprehensive inventory of candidate proteins (STAT1, EGFR and Scribble). The data presented should be of value for future studies on the range of biological roles performed by these proteins.

Chapter 7 General Conclusion

TNBC has the ability to recur after treatment, and normally has the great risk reoccurrence within the first few years following treatment. This research took into consideration that the TNBC is not one disease; it is a group of diseases and that TNBC represents heterogeneous subtypes of breast cancer with limited target therapies. Returning to the hypothesis and research questions 1.22): The aim was to elucidate and identify the molecular pathways that contribute to metastasis in TNBC. Several protein candidates (Scribble, STAT1 and EGFR) were shown to have differential expression patterns between metastatic and non-metastatic tumours.

Furthermore, this study was undertaken to design and develop quantitative assays such as western blotting, immunohistochemistry, protein interaction screens, and protein phosphorylation site analysis by mass spectrometry and molecular genetics. Returning to the hypothesis/question posed at the beginning of this study. It is presently possible to state that the expression profile of a group of breast cancer cell lines (enriched for those representing TNBC features, such as MDA-MB-231, MDA-MB-468) were assessed to define the cell lines that most closely capture individual examples, regarding the chosen protein candidates, of the heterogeneous TNBC phenotypes, and use them as effective tools for drug discovery and/or biomarkers development.

Additionally, the present study delivers several noteworthy contributions with a potential to improve diagnosis. First, clinical proteomic analysis for breast cancer cell lines were used as a model and breast human tumour tissue were demonstrated to be reproducible with a significant p-value ($p<0.05$) of the over expression of EGFR in 26 tumour samples in the metastatic group. Additionally, a positive correlation between STAT1 and MX1 (p-value = 0.01976) was obtained, which provides valuable clues towards determining which proteins might be exploitable for the categorisation of TNBC. This study provides insight for studying the group of proteins (STAT1, Scribble and EGFR), and extending the investigation in the future could provide a better understanding of the mechanism of the metastasis and the aggressiveness of TNBC. We investigated a high-risk group of breast cancer that lacks the benefits of specific therapy with the intention to contribute to the development of such therapies in the future. The results obtained support the idea that the expression profile of the three proteins could represent a clinically effective approach to assessing TNBC heterogeneity and prognosis in various stages and possibly to stratifying patients for targeted therapies.

Moreover, the phenotypical changes of the metastatic TNBC may represent a unique and a heterogeneous tumour cell population with distinctive biological features that permits travel to distant sites and the establishment of a clinically disseminated disease. The resulting evidence from this research provides insights for studying the group of proteins (STAT1, Scribble and EGFR), which could provide a more complete understanding of the mechanism of the metastasis and aggressiveness of TNBC. The results revealed that using CRISPR/Cas9 to modulate expression levels of selected targeted proteins (STAT1

and EGFR) in MDA-MB-468, MCF-7 and Hek293 and assess the effect on wound healing, was a promising approach. Expression of the candidate proteins was manipulated by using CRISPR/Cas9 technology and the effect on cell migration and invasion was assessed by wound-healing assays.

Targeting STAT1 promoter with CRISPR/Cas9 shows inhibition of the closing the wound (migration) in the wound healing assay. Thus, one possible development in the future could be a therapeutic approach to target promoters for specific genes in a similar manner to prevent the invasion of tumour cells. Further studies in this direction might provide valuable clues towards understanding the mechanisms of TNBC metastasis and determining what proteins might be exploitable for the clinical categorisation of this type of breast cancer. The CRISPR/Cas9 technique allows the creation of cell and animal models of cancer and provides different means to determine the cause of, and treatments for, tumours. The data obtained relating to the effect of co-overexpression and deregulation of EGFR and STAT1 can provide valuable clues towards understanding the mechanisms of TNBC metastasis and to help determine which proteins might be exploitable for the clinical categorization of this type of breast cancer.

Together, proteomic data, Immunocytochemistry and CRISPR/Cas9 technologies are expected to play a key role in the study of human cancers as they provide invaluable resources to define and characterise regulatory and functional pathways of genes and proteins within cells. Additionally, proteomics provide tools to identify and exploit

biomarkers that may predict response to anti-cancer treatments and has the capacity to revolutionise the way that patients with cancer are treated.

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Appendix (A)

Monitoring and Optimizing Western Blotting Protocol for Scribble, EGFR, STAT1, MX1 and CD74

This protocol has been modified from (M .Metodiev's Lab) protocol. The transfer buffer should acts as an electrically conducting medium in which proteins are soluble and dose not interfere with binding of the proteins to the membrane, for this purpose, we classified the monitoring and optimizing protocol into before and after Trans –blot-electrophoretic. Prepare running buffer contains (Tris, Glycine, SDS1%) and transfer buffer (Tris Base and Glycine) before transferring process add 20%Absolute Methanol and prevent stored the transfer buffer with Methanol for a long period may cause deteriorate the p H measure.

Resolving size and Proteomics of breast cancer Cell Lines

The slices were subjected to in-gel digestion protocol (with some modifications) and subsequent analysis by Nano-LC/MS/MS. During the courses of each individual LC/MS/MS run data were recorded table (App.A1). Table (App.A2) illustrates the name of breast cancer cell lines that used in-gel digestion prior to mass spectrometry. Marker or protein ladder was used (170-10) kDa to compare with gel electrophoresis before cutting (over or under the red band).According to the reader of Marker the band in approximately size 70-100 kDa denotes to STAT1 while the band in approximately size 55-70 kDa denotes to MX1.The total number of samples was 30 test tubes.

Table (App.A.1) shows the name and the description of protein as different measurement after mass spectrometry reading. Also, illustrates each protein where intensity in different breast cancer cell line.

Name of breast cancer cell line	The Size band according to the Marker	Describe the band location according to the Marker	The band name	Tube number
MDA-MB-435-1	≈70-100	Over the red band	STAT1	1
MDA-MB-435-1	≈55-70	Approximately on and under the red band level	MX1	2
MDA-MB-231-1	≈70-100	over the red band	STAT1	3
MDA-MB-231-1	≈55-70	Approximately on and under the red band level	MX1	4
MDA-MB-231-2	≈70-100	Over the red band	STAT1	5
MDA-MB-231-2	≈55-70	Approximately on and under the red band level	MX1	6
MDA-MB-468-2	≈70-100	Over the red band	STAT1	7
MDA-MB-468-2	≈55-70	Approximately on and under the red band level	MX1	8
ZR-2	≈55-70	Approximately on and under the red band level	MX1	9
ZR-2	≈70-100	Over the red band	STAT1	10
MDA-MB-468-2	≈55-70	Approximately on and under the red band level	MX1	11
MDA-MB-468-2	≈70-100	Over the red band	STAT1	12
MDA-MB-435-2	≈70-100	Over the red band	STAT1	13
MDA-MB-435-2	≈55-70	Approximately on and under the red band level	MX1	14
MDA-MB-468-1	≈55-70	Approximately on and under the red band level	MX1	15
MDA-MB-468-1	≈70-100	Over the red band	STAT1	16
MDA-MB-435-2	≈70-100	Over the red band	STAT1	17
MDA-MB-435-2	≈55-70	Approximately on and under the red band level	MX1	18
ZR-1	≈55-70	Approximately on and under the red band level	MX1	19
ZR-1	≈70-100	Over the red band	STAT1	20
MDA-MB-468-1	≈55-70	Approximately on and under the red band level	MX1	21
MDA-MB-468-1	≈70-100	Over the red band	STAT1	22
ZR-2	≈55-70	Approximately on and under the red band level	MX1	23
ZR-2	≈70-100	Over the red band	STAT1	24
MDA-MB-MCF7-2	≈55-70	Approximately on and under the red band level	MX1	25
MDA-MB-MCF7-2	≈70-100	Over the red band	STAT1	26
MDA-MB-435-1	≈70-100	Over the red band	STAT1	27
MDA-MB-435-1	≈55-70	Approximately on and under the red band level	MX1	28
MDA-MB-MCF7-1	≈55-70	Approximately on and under the red band level	MX1	29
MDA-MB-MCF7-1	≈70-100	Over the red band	STAT1	30

Table (App.A.2) shows proteins description (gene and protein name) and illustrates each protein where intensity in different breast cancer cell line.

Protein Name	Gene Name	Protein Descriptions	Intensity of breast cancer cell lines
EGFR, Proto-oncogene-c-ErbB-1, Receptor tyrosine-protein kinase erbB-1	EGFR; ERBB1	Isoform1, Isoform2, 3 and 4 of EGFR; Uncharacterized protein; Isoform 3 of EGFR 72 KDa.	MDA-231,MDA-435,MDA-468, MCF7 and ZR
Protein LAP4;Protein scribble homolog; SCRIB protein	CRIB1;KIAA0147;LAP4,SCRB1; SCRIB;VARTUL	Isoform 3 of Protein Scribble homolog, Isoform1, Protein Scribble homolog; Isoform 2 of Protein Scribble homolog	MDA-231,MDA-435, MDA-468, MCF7and MDA-ZR
Signal transducer and activator of transcription 1-alpha/beta;	STAT1	STAT1,alpha/beta;83 kDa protein; Isoform Beta of Signal transducer and activator of transcription 1-alpha/beta	MDA-231,MDA-435,MDA-468, MCF7 and ZR
Protein Mx1;c DNA,FLJ35689 clone SPLEN2019379,highly similar to INTERFERON-REGULATED RESISTANCE GTP-BINDING PROTEIN MXA.	MX1	Interferon-induced GTP-binding protein Mx1; highly similar to INTERFERON-REGULATED RESISTANCE GTP-BINDING PROTEIN MXA	MDA-231,MDA-435,MDA-468, MCF7 and ZR
GRP94;HSP90B1;TRA1	Endoplasmic; Uncharacterized protein	MDA-231,MDA-435,MDA-468, MCF7 and ZR	

Table (App.A.3) shows statistical analysis, Mean, Standard Devas ion SD. The intensity of each protein was analyzed in different breast cancer cell line. The experiment was performed in triplicate.

Protein	**breast cancer Cell lines**									
	MDA-231		**MDA-435**		**MDA-468**		**MMCF7**		**ZR**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
STAT1	18405333.3	2755112	251583333	8497978.2	174976667	18471016	79071333.3	13672938	23009666.7	2732930
MX1	**1918166.67**	**1307839**	**271343333**	**23100438**	**277543333**	**29238273**	**3524666.67**	**1517950**	**7374366.67**	**5565119.5**
EGFR	**222986667**	**29110346**	**0**	**0**	**40260333**	**4394128**	**19118000**	**9630270**	**4844333.33**	**1049443.8**
Scribble	**240620000**	**25134190**	**605523333**	**11996944**	**203210000**	**24090100**	**66962000**	**16010814**	**218420000**	**14032017**
GRP95	**1946366667**	**85346724**	**2410633333**	**166113646**	**890500000**	**77522470**	**3210500000**	**53307035**	**2446366667**	**119770169**

Table (App.A.4). Triple negative tumour tissue (26 samples from TNBC patients).

Illustrates the bioinformatics data for Scribble, STAT1, EGFR, MX1 and GRP94 from tumour breast tissue G1 which represents a good survival. B. Illustrates the bioinformatics data for the same set of proteins from triple negative breast tumour tissue G2 which represents a poor survival

A

	SCRIB	STAT1	EGFR	Mx1	GRP
1	1824100	28249000	1409800	20267000	6.1E+08
1	1332100	76144000	21605000	78763000	4.76E+08
1	17163000	4669800	5744500	2355600	3.15E+08
1	1809900	73224000	2285000	89216000	5.53E+08
1	1113800	34897000	13575000	85735000	5.68E+08
1	0	14561000	28587000	14162000	4.39E+08
1	1393200	75886000	13832000	22709000	1.97E+09
1	0	13349000	16949000	25167000	4E+08
1	1203100	37260000	11015000	14286000	1.26E+09
1	4308400	91563000	20129000	33595000	5.42E+08
1	4299500	37174000	6966600	3427400	5.52E+08
1	7219200	92340000	6809700	1.68E+08	7.5E+08
1	6969500	7708500	26890000	4002600	5.84E+08

B

	SCRIB	STAT1	EGFR	Mx1	GRP
2	10187000	2543700	58915000	19755000	4.99E+08
2	4322200	0	75647000	4829400	1.18E+08
2	3766300	62682000	6.05E+08	75457000	3.76E+08
2	6623700	9109600	3.39E+09	53408000	2.38E+08
2	11310000	2359800	2.42E+08	0	3.62E+08
2	2266400	2.53E+08	53089000	2.67E+08	9.8E+08
2	4402000	9970000	34936000	52762000	4.63E+08
2	5761200	0	47342000	0	9.74E+08
2	1734800	2240600	1.43E+08	0	6.09E+08
2	3551200	2.26E+08	30592000	2.23E+08	1.76E+09
2	6091300	1191000	54506000	5548100	1.5E+08
2	7069300	5751700	40228000	4601100	1.84E+08
2	2529200	5516900	21900000	0	3.05E+08

Appendix (B)

Figure. App.B.1
The order was started from notch; MCF-7STAT1_1, MCF-7STAT1_2, MCF-7EGFR1, MCF-7EGFR2 and MCF-7Control

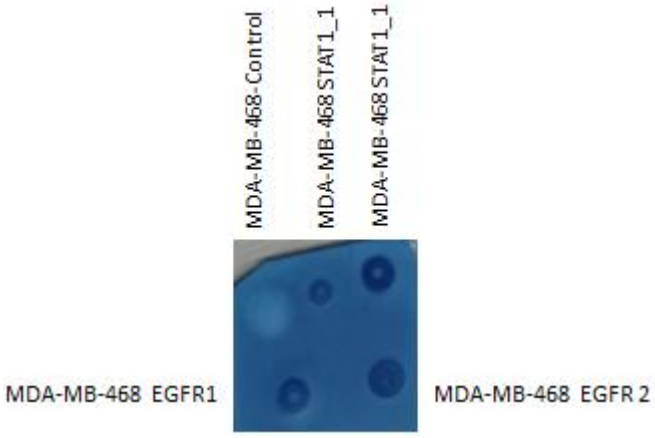

Figure App.B.2
The order was started from notch 468-control, 468-stat1-1, 468-stat1_2, 468-EGFR1 and 468-EGFR2

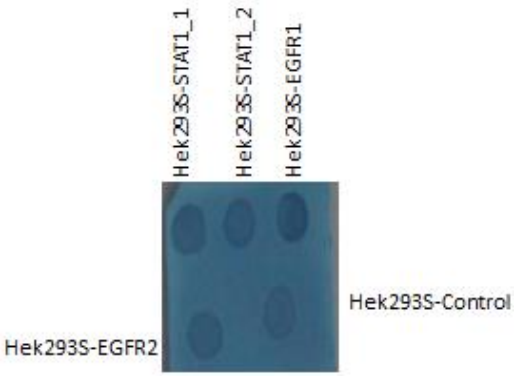

Figure App.B. 3

The order was started from notch Hek239 stat1-1, Hek239 stat1_2, Hek293 EGFR1, Hek293 EGFR2 and Control –Hek293

Figures (App.B.1, App.B.2 and App.B. 3) Quantitative analysis of ten identical samples of plasma membrane and cellular proteins extracted from breast cancer cell lines MDA-MB-435, MDA-MB-435 membrane, MDA-231, MDA-MB-231 membrane MCF7, MCF7 membrane, ZR, ZR membrane, MDA-468, MDA-468 membrane. 1µL from each protein was spotted on nitrocellulose paper and then stained by Amido dye. Methanol/Acetic acid v/v was used as de-stain solution. The more stain, the higher the protein concentration.

Table(App.B) shows the wound healing sources and diameter area.

Items	Wounding Healing Sources	Areas	EqDiameter
1	Hek_contr_plate_1_0h.tif	906.05	33.96
2	Hek_contr_plate_1_24h.tif	157.36	14.15
3	Hek_contr_plate_2_0h.tif	787.9	31.67
4	Hek_contr_plate_2_24h.tif	179.87	15.13
5	Hek_contr_plate_3_0h.tif	862.91	33.15
6	Hek_contr_plate_3_24h.tif	213.2	16.48
7	Hek_contr_plate_4_0h.tif	991.4	35.53
8	Hek_contr_plate_4_24h.tif	140.57	13.38
9	Hek_Stat_2_0h.tif	2839.38	60.13
10	Hek_Stat_2_1_72hh.tif	243.17	17.6
11	Hek_Stat_2_24hh.tif	904.91	33.94
12	Hek_Stat_2_48hh.tif	381.43	22.04
13	Hek_Stat_2_72hh.tif	339.44	20.79
14	Hek_Stat_3_1_0h.tif	3247.37	64.3
15	Hek_Stat_3_1_1_24h.tif	2650.34	58.09
16	Hek_Stat_3_1_1_48h.tif	1663.4	46.02
17	Hek_Stat_3_1_1_72h.tif	1378.01	41.89
18	Hek_Stat_3_1_1_72hDIC.tif	1463.14	43.16
19	Hek_Stat_3_1_24hh.tif	2670.42	58.31
20	Hek_Stat_3_2_0h.tif	3466.43	66.43
21	Hek_Stat_3_2_24hh.tif	2699.24	58.62
22	Hek_Stat_3_2_48hh.tif	1806.13	47.95
23	Hek_Stat_3_2_72hDIC.tif	1430.04	42.67
24	Hek_Stat_3_2_72hh.tif	1343.2	41.35
25	Hek_EGFR_plate_2_1_48hDICh.tif	172.87	14.84
26	Hek_EGFR_plate_2_2_48hDIIC.tif	424.44	23.25
27	Hek_EGFR_plate_1_48hDIC.tif	241.61	17.54
28	Hek_EGFR_2_24h.tif	599.15	27.62
29	Hek_EGFR_plate_1_0h.tif	1789.32	47.73
30	Hek_EGFR_plate_1_24h.tif	439.49	23.66
31	Hek_EGFR_plate_2_0h.tif	2408.2	55.37
32	Hek_EGFR_plate_2_1_0h.tif	2236.21	53.36
33	Hek_EGFR_plate_2_1_24h.tif	681.05	29.45
34	Hek_EGFR_plate_2_2_0h.tif	3044.43	62.26
35	Hek_EGFR_plate_2_2_24h.tif	576.34	27.09
36	Hek_EGFR_plate_2_3_0h.tif	2616.32	57.72