Epithelial-to-Mesenchymal Transition: what is the impact on breast cancer stem cells and drug resistance

Paraskevi Mallini

Student No: 110548117

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Abstract

Breast cancer is fatal mainly due to the development of metastatic disease and the resistance to current in use chemotherapeutic options. The role of cancer stem cells (CSCs) has become one of increasing interest over the last few years, as they seem to be involved in the acquisition of drug resistant and more aggressive cell phenotypes. Side population (SP) cells are a putative CSC population identified by their ability to efflux the DNA binding dye Hoechst 33342 due to the expression of members of the ABC transporter family of proteins. This efflux ability is also thought to confer on these cells the ability to efflux chemotherapeutic reagents. Epithelial-to-Mesenchymal Transition (EMT) has been shown to regulate the function of several CSC populations and it is a process that is predominantly associated with metastasis. Hypoxia has been shown to activate several EMT related signalling pathways.

The aims of the present study were to investigate the effects of Transforming Growth Factor-β1 (TGF-β1) and hypoxia driven EMT on the breast cancer stem cells (BCSCs), including SP and CD44+ cells of the MDA-MB-231 (ER-/PR-/HER2-) and MCF-7 (ER+/PR+/HER2-) breast cancer cell lines. The TGF-β receptor expression was also assessed in both cells lines and I confirmed the activation of the TGF-β signalling pathway when these were induced to undergo EMT. Both MDA-MB-231 and MCF-7 cells were found to express TGFB-RI, but MCF-7 cells had low expression of TGFB-RII. Interestingly, both TGF-β1 and hypoxia-induced EMT resulted in the loss of MDA-MB-231 SP phenotype, while TGF-β1 significantly reduced and hypoxia significantly increased the MCF-7 SP population. Changes on the CD44+ cells were found to be non significant.

My data suggest that autocrine TGF-β1 production might be responsible for the reduction of the SP population in both cell lines and that targeting the SP population through the TGF-β signalling pathway in hormonal responsive breast cancer patients may be promoted by the cooperative effect of hypoxia and TGF-β treatment. Most importantly, I concluded that resistance to chemotherapeutic treatment due to SP presence can be further induced by hypoxia as seen by the increased MCF-7 SP numbers compared to hypoxia alone. Understanding the regulatory mechanisms of SP cells by EMT could enable the identification of new therapeutic targets in certain groups of breast cancer patients for overcoming metastasis and drug resistance.

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Abbreviations

Adenomatous Polyposis Coli	APC
Aldehyde dehydrogenase 1	ALDH1
3-Amino-9-EthylCarbazole	AEC
ATP-binding cassette	ABC
basic Helix-Loop-Helix	bHLH
Blood–Brain Barrier	BBB
Breast Cancer Stem Cells	BCSCs
Cancer Stem Cells	CSCs
Casein Kinase 1	CK1
Cell Adhesion Molecules	CAMs
Cobalt (II) chloride hexahydrate	$CoCl_2$
complete DMEM	cDMEM
Control of Substances Hazardous to Health	COSHH
Costal-2	COS-2
cyclin-dependent protein kinases	cdk
Delta-like	Dll
3,3'-Diaminobenzidine Tetrahydrochloride	DAB
Dishevelled	Dvl
Ductal carcinoma in situ	DCIS
Epidermal Growth Factor	EGF
Epithelial Hyperplasia Lacking Atypia	EHLA
Epithelial-to-Mesenchymal Transition	EMT

Estrogen Receptor	ER
Estrogen Responsive Elements	ERE
Extracellular Matrix	ECM
Fine Needle Aspirates	FNAs
Fluorescent-Resonance Energy Transfer	FRET
Forkhead box	FOX
Forward -scattered light	FSC
Frizzled	Fz
Fumitremorgin C	FTC
Fused	FU
Glycogen Synthase Kinase 3	GSK3
Hormone Replacement Therapy	HRT
Human Epidermal Growth Factor Receptor	HER2
Human Mammary Epithelial Cells	HMEpC
Human Mammary Epithelial cell line	HMLE
Hypoxia-Inducible Factors	HIFs
Hypoxia Responsive Elements	HRE
Immunocytochemistry	ICC
Infiltrating Ductal Carcinomas	IDCs
Inflammatory Breast Cancer	IBC
Invasive Ductal Carcinoma	IDC
Invasive Lobular Carcinoma	ILC
Interleukin-6	IL-6
Kruppel-like factors	KLF

Lobular carcinoma in situ	LCIS
Low-Density Lipoprotein Receptor related protein 6	LRP6
Lysil Oxidase	LOX
Mammary Stem Cells	MaSCs
Mammosphere Forming Efficiency	MFE
Matrix Metalloproteases	MMPs
Membrane-Spanning Domain	MSD
Mesenchymal-Epithelial Transition	MET
messenger RNA	mRNA
Metastasis-Associated protein 3	MTA3
Metastatic Breast Cancer	MBC
Multi-Drug Resistance	MDR
Namru murine Mammary Gland	NmuMG
National Centre for Biotechnology Information	NCBI
non SP	NSP
non template control	NTC
Normal Goat Serum	NGS
Not Otherwise Specified	NOS
Nottingham Prognostic Index	NPI
Nucleoside-Binding Domain	NBD
Oxygen-Dependent Degradation	ODD
Patched receptor	PTC1
per-aryl hydrocarbon receptor nuclear translocator	ARNT
Phosphate Buffered Saline	PBS

Phosphorylated Smad 2 and 3	p-smad 2/3
Polymerase Chain Reaction	PCR
Progesterone Receptor	PR
Progesterone	PG
Progesterone Responsive Elements	PRE
Prolyl-Hydroxylase	PHD
Protein Kinase A	PKA
Quantitative Real-time PCR	qPCR
Reactive Oxygen Species	ROS
Red Blood Cell	RBC
Reverse Transcription Polymerase Chain Reaction	RT-PCR
ribosomal RNA	rRNA
Scarff-Bloom-Richardson	SBR
Short Tandem Repeat	STR
Side Population	SP
Side-scattered light	SSC
Smoothened	SMO
Suppressor of Fu	SUFU
T Cell Factor/Lymphoid Enhancer Factor	TCF/LEF
Terminal End Buds	TEBs
Tumor-Associated Macrophages	TAMs
Tumour Node Metastasis	TNM
transfer RNA	tRNA
Transforming Growth Factor-β1	TGF-β1

Transmembrane Domain	TMD
Tris/Borate/EDTA	TBE
Tris Buffered Saline	TBS
Tyrosine Kinase Inhibitors	TKIs
urokinase-type Plasminogen Activator	uPA
von-Hippel-Lindau	VHL
Whey Acidic Protein	WAP

Chapter 1: Introduction

1.1 Cancer

Cancer is defined by the excessive and uncontrolled proliferation of cells leading to the formation of tumors. It is caused due to the accumulation of genetic mutations affecting the signalling pathways that are associated with self-renewal, proliferation and apoptosis in normal cells. This results in cells characterized by an increased ability to self-renew and divide infinitely. Cancer cells also evade the cellular mechanisms that control growth suppression as they acquire mutations in essential tumor suppressor genes. Additionally, they gain access to the angiogenic processes that sustain tumors by providing them with nutrients and oxygen. Finally, cancer cells activate signalling pathways that are responsible for invasion and metastasis and they become resistant to cell death and immune response (Hanahan and Weinberg, 2011).

1.2 Epidemiology of breast cancer

Breast cancer is the most common type of cancer in women affecting over 1.4 million of them worldwide every year. In the UK it accounts for 1/5 of deaths in women aged 40-50 and the annual incidence in women aged 50 and over is 3/1,000 while in women aged 65-69 it is 4/1,000 (Dixon, 2012). Breast cancer accounted for approximately 11,556 deaths in women and 77 in men in the UK in 2010 (Cancer Research UK Website). Overall, the annual number of new breast cancer cases has increased two times in the last 30 years with more than 12,000 deaths each year, although death rates have decreased by a fifth over the last 10 years (Dixon 2012). The survival rates are usually higher in women aged 50-60 compared to younger or older women (Dixon 2012).

In breast cancer it is rarely the primary tumor that is fatal; fatality is often due to the development of MBC. The 5-year relative survival rate is significantly higher (9 out of 10) in women diagnosed with stage I breast cancer compared to those diagnosed with stage IV (1 out of 10) (Britton *et al.*, 2011). Additionally, almost 30% of early breast cancer patients eventually develop MBC for which no cure exists. The first-line chemotherapeutic agents that are currently used include anthracyclines and/or taxanes, but these are often effective only for the first 6-10 months of the treatment, as in 90% of MBC patients multi-drug resistance (MDR) occurs. Second-line agents have not been shown to have any significant long term benefit (Coley, 2008).

1.3 Breast cancer risk factors

Age: The incidence of breast cancer increases with age and it is most prevalent after menopause.

Geographical location: There is a 3-fold to 4-fold difference between the incidence of breast cancer in Far Eastern and Western countries. Environmental factors contribute to the pathogenesis of breast cancer to a higher extent than genetic factors as shown by the rates of breast cancer in migrants from Japan to Hawaii which adjust to the ones in the host country within one or two generations (McPherson et al 2000, Dixon 2012).

Breast density: Density of the breast decreases with age, which influences detection by mammography. Breast density is not increased with tamoxifen treatment, as opposed to hormone replacement therapy (HRT). According to 42 studies comparing breast cancer risks in women with high and low density breasts, it has been concluded that there is a 4 to 5-fold difference (Mandelson *et al.*, 2000; McCormack and dos Santos Silva, 2006; Boyd *et al.*, 2007; Bertrand *et al.*, 2015).

Age at menarche and menopause: Women that start menstruating early or stop late in life are more likely to develop breast cancer. In fact, women who experience menopause after the age of 55 have twice as high a risk compared to those who experience menopause before the age of 45 (Dixon 2012).

Age at first pregnancy and birth: Nulliparous women or women whose age at first birth is higher than 35 have an increased risk of breast cancer. There is also a 2-fold risk increase in women that have their first child at the age of 30 compared to those that have a first child at the age of 20 (McPherson *et al.*, 2000; Dixon, 2012).

Family history: In Western countries familial breast cancer due to a strong genetic predisposition accounts for almost 5% of breast cancer cases. In these families, breast cancer is inherited as an autosomal dominant with variable penetrance. This means that it can be inherited from individuals within the same family to both sexes without the patients experiencing breast cancer themselves. In women with a strong family history it is likely that they will develop the disease before the age of 50 (Dixon, 2006). In a recent study familial breast cancer was found to be a very heterogeneous disease comprised of 10 different subtypes. This classification was based on the discovery of

variation in copy number or expression of at least 10 different genes (IGF1R, KRAS, EGFR, CDKN2B, BRCA2, RB1, ATM, SMAD4, NCOR1 and UTX) (Curtis *et al.*, 2012). Most importantly, mutations in the BRCA1 and BRCA2 genes, located on the long arms of chromosomes 17 and 13, respectively are associated with a substantial number of familial breast cancer cases. These genes are large and mutations at any site in them could be involved in the pathogenesis of the disease (Dixon, 2006). Genetic testing for mutations in these genes is now routinely performed in high-risk women and surgical risk-reduction is becoming a common preventive option (Dixon 2012).

Lifestyle: Obesity has been found to increase the risk for breast cancer two times in postmenopausal women, while other lifestyle options, such as alcohol intake or smoking have been shown to slightly contribute to the development of the disease. Interestingly, each year of breast feeding is thought to reduce the risk by 4.3% (Dixon 2012). The short term use of oral contraceptives appears to have no major impact on breast cancer risk. However, there is a slight increase in women who have taken oral contraceptives for 10 years and in those who begin taking them before the age of 20. The risk of breast cancer in women 10 or more years after cessation of oral contraceptives falls to normal population levels. Women receiving HRT seem to have an increased risk for breast cancer by 2.3% each year, while risk rates become normal almost immediately after the cessation of HRT. The combination of both progesterone and estrogen HRT can lead to a 2-fold increase of breast cancer after 5 years or more of use. HRT affects the sensitivity and specificity of breast screening, since it increases breast density. It has also been reported that women taking HRT were more likely to be diagnosed with larger tumors and node positive forms of the disease (Dixon 2012).

1.4 Anatomy of the breast

The adult human breast has a heterogeneous anatomical structure and is composed of different types of tissue, mainly fatty and glandular tissue. It contains 14-18 lactiferous lobes and each lobe consists of 20-40 lobules that converge to the nipple through a network of ducts that are responsible for the milk production and storage (Figure 1.1). Each nipple is surrounded by a circular and pigmented area called the areola, which is responsible for lubricating the nipple during lactation. The process of lactation is stimulated due to the excretion of the hormone prolactin normally during late pregnancy and after birth. Breast tissue is drained by a number of lymphatic vessels that lead to

axillary, supraclavicular and internal mammary lymph nodes. These lymph nodes are common sites of metastasis in breast cancer (Elston, 1998).

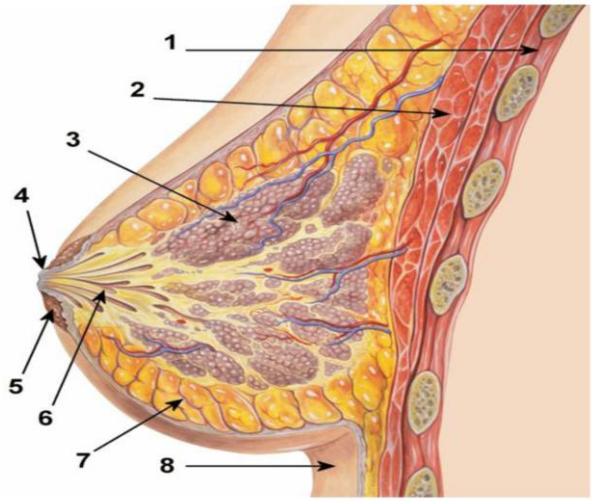


Figure 1.1: Anatomy of the breast. 1. Chest Wall 2. Pectoralis muscles 3. Lobules 4. Nipple 5. Areola 6. Milk duct 7. Fatty tissue 8. Skin (Jütte *et al.*, 2014)

1.5 Normal mammary gland development and cell types in the breast

The mammary gland consists of an extensive tree-like network of branched ducts expanding from an epithelial bud. The developing epithelial ducts ultimately give rise to the mature ductal tree through cellular processes that are regulated by hormonal stimuli (Sternlicht, 2006). The mammary gland is mainly characterized as having two different cell types; a) luminal cells which are further divided into ductal and alveolar luminal cells with the former constituting the inner lining of the ducts and the latter forming the alveolar units during pregnancy and b) myoepithelial cells, which are located at the basal surface of the epithelium (Figure 1.2). There are three stages of human mammary gland formation; a) the embryonic stage when the basic structure is developed b) the pubertal stage at which the ducts elongate and branch through the mammary fat pad to form terminal end buds (TEBs) and c) the pregnancy stage that is characterized by alveolar differentiation and tertiary ductal branching in preparation for lactation. TEBs are composed of many epithelial layers and they are located in the end of the ducts. Their differentiation results in the development of the ducts, which consist of both luminal epithelial and myoepithelial cells. In addition, cap cells at the end of TEBs also

contribute to epithelial plasticity in normal conditions (Micalizzi *et al.*, 2010) (Figure 1.2).

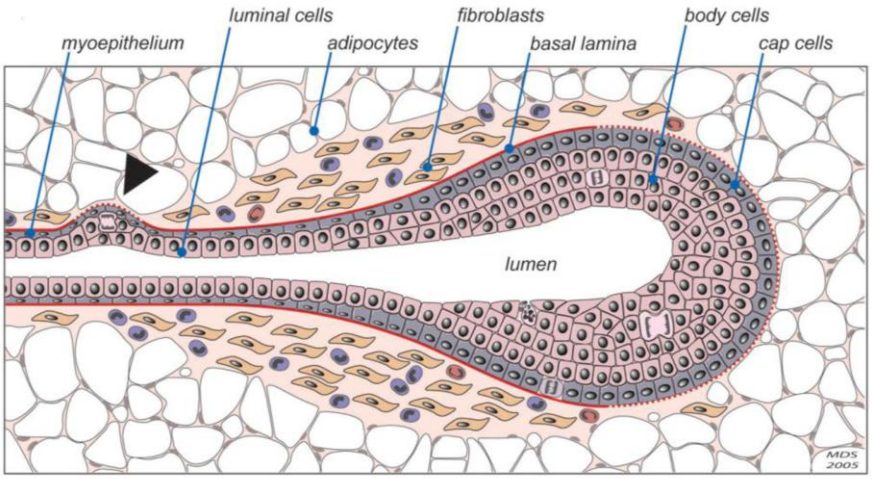


Figure 1.2: Schematic representation of a TEB consisting of luminal cells forming the inner lining of the ducts and myoepithelial cells the basal surface of the epithelium. Cap cells at the end of TEBs contribute to epithelial plasticity in normal conditions (Sternlicht, 2006).

1.5.1 Normal mammary gland stem cells

The dynamic expansion of the mammary gland during puberty and pregnancy, implicates the existence of mammary stem cells (MaSCs) which differentiate in order to generate mature epithelial structures of either the luminal or myoepithelial origin (Visvader, 2009). Deome and colleagues were the first to demonstrate that different parts of the mammary gland at different stages postnatally could give rise to mammary epithelial outgrowths (DeOme *et al.*, 1959), while other studies supported the concept that the mammary gland consists of cells with different degrees of differentiation (Chepko and Smith, 1997; Smith and Chepko, 2001). Several experimental approaches have been used for the identification of putative MaSCs populations, including cell surface marker profiling, mammary fat pad transplantation assays and cell culture techniques to test self renewal and differentiation capacity (Petersen and Polyak, 2010). However, the origin and lineage of MaSCs has not been fully understood with several studies suggesting that these cells are restricted to the basal cell population (Stingl *et al.*, 1998; Shipitsin *et al.*, 2007; Eirew *et al.*, 2008; Lim *et al.*, 2009) and another indicating

that that both the luminal and basal cell compartments are composed of MaSCs (Keller *et al.*, 2012). The diversity of the data generated from these studies could be due to different methods used for the dissociation of breast tissue, cell culture and the assessment of stem cell properties. Additionally, the plethora of cell surface markers that have been suggested increased the need for the performance of functional assays to confirm the stemness of potential MaSC populations. For instance, BrdU labelling allowed the detection of slow cycling cells which retained the dye for longer and they were characterized by the absence of differentiation markers, Sca-1 expression and the SP phenotype (elaborated in following section) with a luminal origin and a high regenerative capacity (Welm *et al.*, 2002).

1.6 Histological breast cancer classification

Breast cancer occurs in the epithelial cells that line the terminal duct lobular unit. Breast cancer is divided into ductal and lobular, depending on whether is arises from the ducts or the lobules, respectively. 80% of breast cancer cases have ductal origin and 20% lobular. Cancers that remain within the basement membrane are characterized as in-situ or non-invasive, whereas cancers that invade in the surrounding adjacent tissue are classified as invasive. Some invasive breast cancer subtypes may have particular cellular morphology and growth patterns (tubular, mucinous, medullary and papillary) and they usually have a better prognosis. All the other types not belonging to these special types are known as not otherwise specified (NOS) (Dixon 2012).

Ductal carcinoma in situ (DCIS)

DCIS is the most common non-invasive type of breast cancer which remains in the milk duct and does not invade in the surrounding breast tissue. There is a 30% possibility of recurrence or future new disease within the first 5-10 years after the initial diagnosis in a conserved breast, however, radiotherapy reduces this to less than 5% when resection margins are clear (Breast Cancer Website). DCIS is further subdivided into Comedo, Cribiform, Micropapillary, Papillary and Solid DCIS depending on microscopic morphology (Malhotra *et al.*, 2010).

Lobular carcinoma in situ (LCIS)

LCIS arises within the lobules at the terminal ends of the ducts and is not invasive. This is usually diagnosed in women before menopause who undergo a biopsy for some other

reason, since it is asymptomatic and cannot easily be detected by mammogram. LCIS is extremely rare in men (Breast Cancer Website).

Invasive Ductal Carcinoma (IDC)

This is the most common invasive form of breast cancer, accounting for almost 80% of invasive cases. In IDC the abnormal cells break through the basement membrane of the milk duct and invade to other areas of the breast, and can also metastasize to other organs of the body. The risk for IDC increases with age and it is also the commonest type in men (Breast Cancer Website).

Invasive Lobular Carcinoma (ILC)

ILC is the second most prevalent type of invasive breast cancer in which cancer spreads locally beyond the lobules ultimately to the lymph nodes and possibly to other parts of the body. It usually occurs later in life (early 60's) than the IDC and HRT before or during menopause has been suggested to increase the risk for ILC (Breast Cancer Website).

Paget's disease

Paget's disease is a rare form of non invasive breast cancer that affects the nipple. Cancer cells accumulate in the nipple ducts and then they expand to the nipple surface and the areola. Most importantly, more than 97% of patients with Paget's disease have invasive or non invasive breast cancer deeper within the affected breast (Breast Cancer Website)

Inflammatory Breast Cancer (IBC)

IBC is a rare but aggressive type of breast cancer and as its name implies it mimics inflectional. It starts with reddening and swelling of the breast with or without the presence of a lump. Symptoms worsen rapidly requiring prompt treatment. A combination of chemotherapy, surgery, radiation therapy and targeted therapies is currently being used for the management of IBC (Breast Cancer Website; Dixon, 2006).

1.7 Molecular classification of breast cancer

Although the above described classification system is commonly used and it has prognostic value the molecular classification of breast cancer is more useful for

predicting the patient's response to different therapeutic options. There are four different molecular breast cancer subtypes depending on their hormone receptor status (estrogen receptor (ER), progesterone receptor (PR) or their human epidermal growth factor receptor (HER2) status. These subtypes are: 1) Luminal A (ER+/PR+/HER2-), 2) Luminal B (ER+/PR+/HER2+), 3) HER2 (ER-/PR-/HER2+) and basal like or triple negative (ER-/PR-/HER2-) (Breast Cancer Website; Iwata, 2012). More recently, the claudin low subtype has also been identified. All these subtypes have differences in overall survival and disease free-survival rates with the triple negative one being associated with the shortest rates. What is more, Luminal A and B types have very distinct clinical outcomes (Malhotra *et al.*, 2010).

1.7.1 The role of the estrogen receptor in the normal mammary gland and breast cancer

Estrogen exposure promotes the proliferation of epithelial cells leading to branching morphogenesis during puberty, pregnancy and the menstrual cycle. Almost 2/3 of breast cancer patients express higher levels of ERα and ERβ. Increased exposure to estrogen due to early menarche or late menopause or hormonal treatment can also increase the risk for breast cancer (Clemons and Goss, 2001). Patients with this phenotype usually have a better prognosis and response to endocrine treatment, which involves anti-estrogen drugs, such as tamoxifen or aromatase inhibitors which reduce the levels of estrogen (Dixon 2012) (Ikeda and Inoue, 2004). Binding of estrogen to ERα and ERβ leads to the formation of homodimers or heterodimers, which in turn bind to the estrogen responsive elements (ERE) of target genes via their DNA binding domain (Glass and Rosenfeld, 2000). The list of estrogen-regulated target genes is increasing, however, the exact mechanisms that induce tumor growth have not been fully elucidated (Ikeda and Inoue, 2004). Estrogen signalling can be either ligand independent through the AF-1 region in the N-terminal domain or ligand dependent through the AF-2 region in the E-domain in the ER genes (Glass and Rosenfeld, 2000).

1.7.2 The role of the progesterone receptor in breast cancer

Progesterone (PG) is involved in the regulation of many reproductive processes in women, including the establishment and maintenance of pregnancy and ovulation. The biological actions of PG are mediated through the nuclear ligand-activated progesterone receptors PR-A and PR-B. Upon binding of PG to the inactive receptor complex with HSP90 and immunophilins, the receptors become activated by dissociating from this and form dimers. They can then bind to progesterone responsive elements (PRE) in

target genes and activate or suppress their expression (Leonhardt and Edwards, 2002). In the mammary gland ER signalling induces the expression of PR, whereas PG inhibits ER synthesis (Conzen, 2008). PR expression is usually associated with ER expression in breast cancer and ER+/PR+ patients have the best response to hormone therapy. Cases of ER-/PR+ breast cancer are rare (Dixon 2012).

1.7.3 The role of the HER2 in breast cancer

The HER2 receptor is a transmembrane tyrosine kinase belonging to the epidermal growth factor (EGF) or HER receptor family of proteins consisting of 4 members; HER1, HER2, HER3 and HER4. Although the ligands of these receptors have not been completely characterized, it has been shown that signalling mediated through the HER2 receptor is the strongest. The effects of the HER signalling depend on the dimerization partners, with the PI3K/Akt, phospholipase Cγ, mitogen-activated protein kinase and STAT pathways being the most commonly activated pathways. In normal breast tissue HER2 signalling is thought to play a role in cell proliferation, motility, apoptosis and adhesion, while HER2 overexpression (mainly caused due to the amplification of the HER2 gene) has been found to promote carcinogenesis by inducing the hyperactivation of the PI3K/Akt and MAPK pathways (Ross *et al.*, 2003). Breast cancer patients with these features have worse prognostic outcomes, early recurrence rates and reduced response to hormone therapy, as HER overexpression has been correlated with ER-negativity (Ross *et al.*, 2003) (Dixon 2012).

1.8 Staging of invasive breast cancer

The extent of many cancers can be assessed by the Tumour Node Metastasis (TNM) system, which takes into account the size of the primary tumor (T), the status of the lymph nodes (N) and the presence of distant metastases (M). However, this system does not take into account the biological properties of the tumor, including hormonal status, which would allow the detection of early tumors and identify the patients who might benefit from endocrine treatment (Escobar *et al.*, 2007). There are also histological grading systems, including the Scarff-Bloom-Richardson (SBR) and the Nottingham Prognostic Index (NPI). The SBR system uses information about a) tubule formation b) nuclear grade and c) the mitotic rate. Each of the categories gets a score between 1 and 3; with "1" meaning that cells look mostly normal, and a score of "3" meaning that the cells and tissue have an abnormal appearance. The scores for the three categories are then added, resulting in a total score of 3 to 9. There are three grades according to the total score, as described in Table 1. The most commonly used system is the NPI system

which considers a) the size of the lesion b) the number of the affected lymph nodes (0 =1, 1-3 = 2, >3 = 3) and c) the grade of the tumor (Grade I =1, Grade II =2, Grade III =3). It can be calculated using the following formula: NPI = (0.2 x size) + stage + grade. The total score can be interpreted as shown in Table 2 (Haybittle *et al.*, 1982).

SBR score	SBR grade	Degree of differentiation
3-5	Low	Well differentiated
6-7	Intermediate	Moderately differentiated
8-9	High	Poorly differentiated

Table 1.1: The SBR scoring system for breast cancer

Score	5-year survival
>/=2.0 to </=2.4	93%
>2.4 to </=3.4	85%
>3.4 to </=5.4	70%
>5.4	50%

Table 1.2: The NPI scoring system for breast cancer

1.9 Diagnosis

The introduction of the national screening programme in the UK in the late 1980s has revealed a huge number of undiagnosed cases of breast cancer. It is performed for women at the age 47-73 every year and it leads to the detection of 8.1/1,000 new cases per annum (Dixon 2012). Mammography is the most efficient method of screening for breast cancer in the UK and is normally offered to women over 47 or younger women with a family history of breast cancer, previously diagnosed with a benign but atypical lesion or those that have been treated with mantle radiotherapy as a young adult or child. The aim of the screening process is to detect the disease at its early stages and thus reduce the rates of morbidity and mortality. Patients with an abnormality detected often require further mammography or ultrasonography, clinical examination and needle or core biopsy (Breast Cancer Screening NHS Website; Dixon, 2006)

1.10 Treatment

Surgery is the primary treatment option for most patients with breast cancer. Breast-conserving surgery is the operation for the removal of the tumor (lumpectomy) or the

part of the breast tissue within which the cancer is (partial mastectomy). In some cases, the whole breast needs to be removed (total mastectomy). Selective lymph node dissection for biopsy is carried out at the same time. Chemotherapy can be used before surgery in order to reduce the size of the tumor and render it operable (neoadjuvant therapy). In addition to chemotherapy, radiation or hormonal therapy for patients whose tumors express hormonal receptors can be used as a post-surgery treatment (adjuvant therapy). Hormonal therapy involves the administration of anti-estrogen therapy to patients both as adjuvant therapy or to those with metastatic disease. Post-menopausal women with hormone-dependent breast cancers are usually treated with aromatase inhibitors, which prevent the enzyme aromatase from converting androgen to estrogen. Targeted therapy is an approach that aims to treat particular molecular targets within the tumor without causing harm to normal cells. Monoclonal antibodies, such as trastuzumab or pertuzumab to cancer cells combined with chemotherapy and tyrosine kinase inhibitors (lapatinib for HER2 positive subtypes or PARP inhibitors for triple negative cancers) are used as part of targeted therapy, where specific molecules are used to block or inhibit growth factor receptors on breast tumor cells (Dixon, 2006).

1.11 Theories related to the cellular origin of cancer

Cancer is caused due to the accumulation of genetic mutations affecting the signalling pathways that are associated with self-renewal, proliferation and apoptosis in normal cells (Al-Hajj *et al.*, 2004; Britton *et al.*, 2011) Although many of these mutations have been successfully identified, the origin and differentiation status of the cell populations responsible for these transforming events have not yet been elucidated for most human cancer types (Polyak and Hahn, 2006). Two models have been suggested to explain the cellular origin of cancer:

1) *The Stochastic Theory*; which claims that every single cell can potentially become cancerous in the appropriate microenvironment (Dick, 2003). Given the clonality of tumors, cells can be induced to undergo malignant transformation by acquiring different combinations of mutations. This results in the generation of tumours consisting of heterogeneous cell populations with a variable degree of differentiation and proliferative potential. Differentiated cells have a shorter life span and they rarely proliferate, therefore they are unlikely to accumulate a sufficient number of mutations in order to become neoplastic. (Polyak and Hahn, 2006). In addition, the use of cancer cell lines or patient-derived cells in culture requires a large number of cells in order to form tumours

in most experimental or even xenograft models leading to very low efficiency (Masters, 2000; Al-Hajj *et al.*, 2003).

2) *The Hierarchy (Cancer Stem Cell) Theory*; which supports the hypothesis that CSCs are more likely to generate a tumour, because of the fact that they have a longer life span and ability to self-renew. This means that CSCs asymmetrically divide resulting in one daughter cell that retains its stem cell properties and one committed progenitor. Consequently, fewer mutations are required for neoplastic transformation (Waterworth, 2004). Cohnheim was the first to introduce the concept of CSCs in 1875, suggesting that tumor formation is caused by stem cells that are misplaced during embryonic development (Cohnheim, 1875). Furthermore, many signalling pathways that regulate normal stem cell function have been found to be mutated in human cancers, including the Wnt, Notch, Bmi-1, TGF-β, Hedgehog and others. CSCs (Crowe *et al.*, 2004; Polyak and Hahn, 2006) are also good candidates for tumor formation due to their relationship with their microenvironment, known as the stem cell niche (Spradling *et al.*, 2001).

Although there is an increasing number of studies that focus on the role of CSCs in cancer, their origin remains poorly understood. It is believed that CSCs presumably arise either from the de-differentiation of differentiated cells or from the acquisition of mutations in normal stem cells. However, the exact molecular mechanisms that are involved in these processes have not been completely defined. This is of great clinical importance, given that conventional chemotherapy targets the bulk of the tumor cells but fails to target slow cycling cells, such as CSCs (Reya *et al.*, 2001) (Figure 1.3). Therefore, the identification and targeting of CSCs could enable the more effective prevention and management of metastasis and drug resistance in cancer.

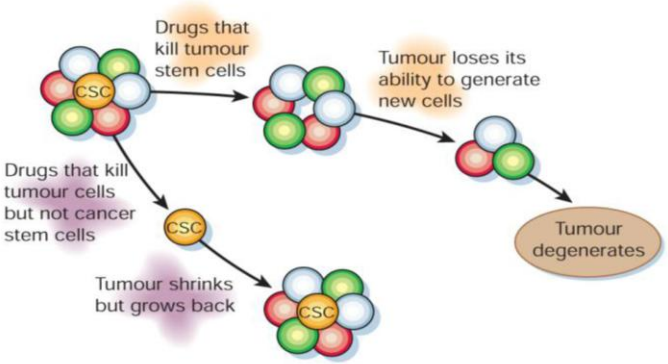


Figure 1.3: The cancer stem cell theory and its relationship with treatment failure. Most current therapies aim to target the bulk of the tumor cells leaving the CSC populations

intact. If CSCs can be targeted more effectively tumours could eventually degenerate , since these are thought to be responsible for tumor recurrence (Reya et al., 2001).

1.12 Evidence for the existence and methods for identification of breast cancer stem cells (BCSCs)

In recent years it has been suggested that metastasis occurs early in primary tumor development (Gunasinghe *et al.*, 2012). In breast cancer, BCSCs can be isolated using several methods (Gangopadhyay *et al.*, 2013). One of these methods is testing the mammosphere forming ability of single cells in non-adherent culture conditions (Dontu and Wicha, 2005). These have been found to promote tumorigenesis in immunodeficient mice. They are thought to contain tumor-initiating cells, including BCSCs and thus they are used to test self-renewal capacity (Ponti *et al.*, 2005). The identification of BCSCs can also be based on the use of specific markers, such as CD44, CD24, CD49f and ALDH1. Cells expressing these markers are sorted and used for further analysis of their clonogenicity, proliferation, self-renewal capacity, differentiation and tumorigenic ability (Britton *et al.*, 2011) In fact, cells that expressed high levels of CD44 and low levels of CD24 were found to be present in eight out of nine patients with breast cancer. The tumorigenic ability of these CD44+/CD24- cells was demonstrated in immunocompromised mice in which a few cells were sufficient to form new tumors, while a high number of cells with alternative profiles failed to do so. Al-Hajj et al. also showed that CD44+/CD24- cells were able to give rise to new tumorigenic and non tumorigenic cells (Al-Hajj *et al.*, 2004).

Aldehyde dehydrogenase 1 (ALDH1) is an enzyme responsible for the oxidation of intracellular aldehydes. It has been suggested that it is involved in self-renewal of cells at early differentiation stages by oxidizing retinol and thus converting it to retinoic acid (Duester, 2000). Moreover, ALDH1 activity has been identified in murine and human hematopoietic and neural stem cells, suggesting it has a role in stem cell function (Armstrong *et al.*, 2004). ALDH1 expression has been detected in both normal and cancer human mammary stem cells (Ginestier *et al.*, 2007). While ALDH1+ tumor cells derived from human breast cancer cell lines have a higher ability for mammosphere formation in culture and increased tumorigenicity *in vivo* compared to ALDH1- cells (Deng *et al.*, 2010).

ALDH1 has been reported to be inversely correlated with the survival rate of breast cancer patients. Interestingly, CD44+/CD24-/ALDH1+ cells have also been found to be

more tumorigenic than CD44+/CD24- cells (Ginestier *et al.*, 2007). However, a recent study aimed at revealing the distribution of CSCs markers in different breast cancer subtypes suggested that ALDH1 was present in a low number of patients, but was significantly associated with basal-like tumors, high tumor grade but not with poor clinical outcome as suggested in previous studies. The authors also indicated that luminal breast cancer cell lines are enriched with CD44-/CD24+ cells, basal/mesenchymal with CD44+/CD24- and basal/epithelial with CD44+/CD24+ (Ricardo *et al.*, 2011). Zhu and co-workers proposed that BCSCs can also be sorted based on the CD44+/CD24-/EpCAM+ phenotype (Zhu *et al.*, 2012).
Additionally, several functional assays have been used for identifying the properties of putative BCSC populations. One of these approaches utilizes their ability to retain bromodeoxyuridine or H3-thymidine for a longer time compared to cycling cells, since BCSCs are slow cycling cells that remain inactive in the G0 phase (Kenney *et al.*, 2001; Smith, 2005). Another method involves the use of the lipophilic dye, PKH26 and it is also based on the quiescence of BCSCs (Pece *et al.*, 2010). Moreover, ALDH1+ cells can be detected by their high aldehyde dehydrogenase activity using the ALDEFLUOR assay (Ginestier *et al.*, 2007). Finally, SP cells are sorted due to their property to export the Hoechst 33342 dye resulting in a less intensively stained population, as described in more detail in the following sections (Goodell *et al.*, 1996).

1.13 ATP-binding cassette (ABC) transporters: structure and normal function

The ABC superfamily is one of the largest families of proteins encoded in the human genome. All members of this family are characterised with two distinct domains: the hydrophobic transmembrane domain (TMD) or the membrane-spanning domain (MSD) and the nucleoside-binding domain (NBD). Although ABC transporters differ in the arrangement of these domains, a common ABC transporter contains two MSDs and two NBDs (e.g the MDR P-gp protein, ABCB1 transporter). However, some others have only one MSD and one NBD (e.g BCRP, ABCG2) or three MSDs and two NBDs (e.g MRP2, ABCC2). TMDs are composed of alpha helices and most ABC transporters have 12 alpha helices (6 per monomer). ABC transporters also have a highly conserved cytoplasmic ATP-binding cassette (ABC) sequence motif consisting of the Walker A and B sequences, which are common in all ABC transporters and a C upstream of the B sequence, which is specific for each member of the family (Toyoda *et al.*, 2008). Two NBDs can be joined via binding to ATP, which causes conformational changes, and the reversal of these can allow the transporters to efflux substrates by utilizing ATP

hydrolysis (Figure 1.4). There are 48 characterized ABC transporter genes in the human genome, which are responsible for several functions in normal tissue. Of note, genetic disorders in 18 of them are linked with Mendelian diseases, such as cystic fibrosis caused by a mutation in the ABCC7/CFTR gene and adrenoleukodystrophy, caused by mutations in ABCD1 (Dean, 2009). Several ABC transporters are distributed in tissues all over the human body contributing to the protection of these tissue cells from xenobiotics. These transporters are normally expressed at essential pharmacological barriers, such as the brush border membrane of intestinal cells or the epithelium that contributes to the blood–brain barrier (BBB) (Gottesman *et al.*, 2009).

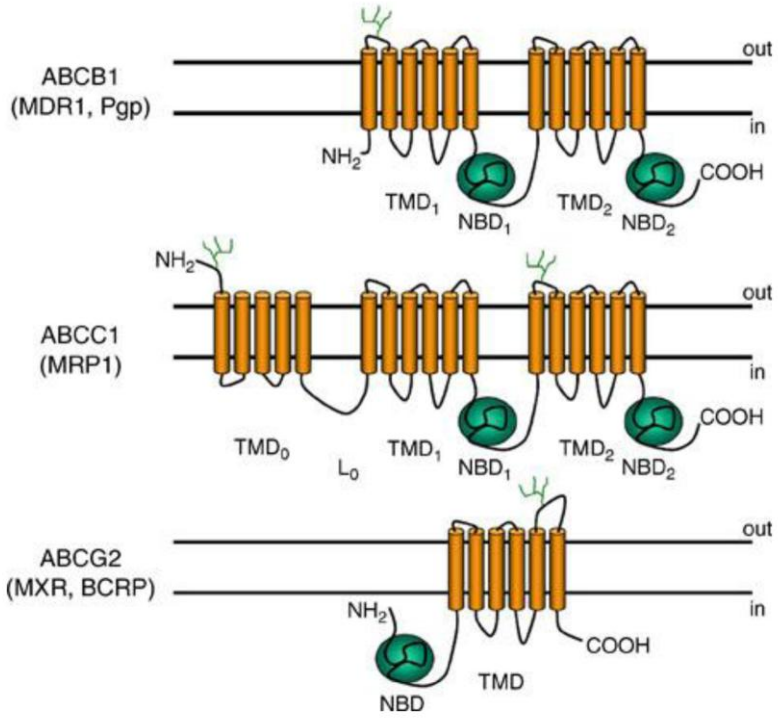


Figure 1.4: Structure of the ABCB1, ABCC1 and ABCG2 transporters. Transmembrane Domain: TMD, Nucleoside-Binding Domain (NBD), L_0: linker region between TMD_0 and TMD_1 in ABCC1 (Szakács et al., 2008).

1.13.1 ABC transporter expression in normal stem cells

It has been reported that many stem cell populations are characterized by the expression of high levels of ABC transporters. In fact, it has been found that hematopoietic stem cells highly express ABCG2 and/or ABCB1, but they stop expressing these once they become differentiated (Gottesman *et al.*, 2002; Kim *et al.*, 2002b; Scharenberg *et al.*, 2002). However, the expression of ABC transporters does not seem to be essential for the survival, growth and maintenance of stem cells, since gene silencing of ABCG2, ABCB1 and ABCC1 has not affected the viability and fertility of mice and their stem cell populations (Zhou *et al.*, 2002; Jonker *et al.*, 2005). Nevertheless, these mice had increased sensitivity to certain substances, including vinblastine, ivermectin, topotecan and mitoxantrone, suggesting the loss of these transporters results in the loss of protection they provide against toxins and xenobiotics (Schinkel *et al.*, 1994).

1.13.2 Contribution of ABC transporter expression to the SP phenotype

SP cells, which have been found to express high levels of ABC transporters, can be sorted due to their ability to expel Hoechst 33342 dye, which binds to AT-rich regions in the minor groove of the DNA. The Hoechst fluorescence intensity is indicative of the DNA content, chromatin structure and cell cycle stage. Additionally, it has been reported that less differentiated cells have a higher Hoechst efflux activity (Goodell *et al.*, 1996). The term SP is based on the observation, that these cells appear as the less intensively stained population in flow cytometry analysis to one side on a density plot (Golebiewska *et al.*, 2011).

The mechanism by which Hoechst is transported has been controversial. It has been thought that this is achieved through both ABCB1 and ABCG2, since SP populations seem to disappear when verapamil is used to block their activity (Golebiewska *et al.*, 2011). It should be noted that verapamil is a calcium channel inhibitor that binds with a higher affinity to ABCB1, while it is considered less specific for the inhibition of ABCG2 (Britton *et al.*, 2012). In 2001 Zhou et al demonstrated that ABCB1 only partially contributes to the SP phenotype in bone marrow cells, while ABCG2 expression is directly associated and can be considered the only essential molecular determinant for this (Zhou *et al.*, 2001) Additionally, it has been shown that ABCG2-null mice were characterized with the presence of reduced hematopoietic SP numbers compared to the wild-type mice, while these were also more sensitive to mitoxantrone (Zhou *et al.*, 2002). The SP assay is useful for the identification of stem/progenitor cells in several tissues, especially when there is lack of cell surface markers for serving this

purpose. However, it should be noted that although the SP population is enriched with stem cells, the SP phenotype is not exclusive to stem cells and the expression of ABC transporters is not limited to the stem cell compartment in most tissues (Golebiewska *et al.*, 2011)

1.13.3 ABC transporter expression and the presence of SP cells in normal breast tissue

It has been reported that ABCG2 is expressed in the mammary gland and during lactation in particular. In fact, an estrogen responsive element is present in the ABCG2 promoter (Ee *et al.*, 2004). What is more, ABCG2 expression in the breast is thought to be involved in the secretion of riboflavin (van Herwaarden *et al.*, 2007), while several other ABC transporters participate in the lipid and cholesterol transportation during lactation as well (Farke *et al.*, 2008). However, knockdown of ABCG2/ABCB1A/ABCB1B in mice resulted in the loss of murine mammary gland SP cells, suggesting that the SP phenotype can be attributed to the expression of other transporters apart from ABCG2 (Jonker *et al.*, 2005). SP cells have been isolated from both murine and human breast tissue (Alvi *et al.*, 2002; Clayton *et al.*, 2004). It remains questionable whether SP cells on the mammary gland are considered a stem cell population or a more-restricted progenitor cell population. Nevertheless, human mammary gland cells have been shown to give rise to both luminal and myoepithelial lineages (Clayton *et al.*, 2004), which were able to form branching structures in matrigel and had a high mammosphere forming capacity *in vitro* (Clarke, 2005; Clarke *et al.*, 2005).

1.13.4 ABC transporter expression in CSCs

CSCs are resistant to chemotherapy partially due to the high expression of ABC transporters. It has been suggested that they can lead to MDR tumors by generating more cells with these properties (Dean *et al.*, 2005). The ability of SP cells to efflux the Hoechst dye could explain their ability to also export cytotoxic drugs contributing to MDR. Patrawala and colleagues reported that 30% of human cancer cells and xenograft tumors have an SP population (Patrawala *et al.*, 2005) and several studies aimed to investigate their properties in comparison to the NSP population (NSP, the bulk cells minus the SP cell population) (Britton *et al.*, 2011). SP cells found in cancer cell lines have also found to have self-renewal (Szotek *et al.*, 2006) and asymmetric division (Patrawala *et al.*, 2005; Szotek *et al.*, 2006; Ho *et al.*, 2007) properties and express stem cell markers, such as Notch1 and Bmi-1 (Hirschmann-Jax *et al.*, 2004; Patrawala *et al.*,

2005; Ho *et al.*, 2007). Intriguingly, SP cells were also shown to be chemoresistant (Hirschmann-Jax *et al.*, 2004; Szotek *et al.*, 2006; Ho *et al.*, 2007), radioresistant (Wang *et al.*, 2007a; Woodward *et al.*, 2007) and have a higher invasive potential *in vitro* (Fuchs *et al.*, 2009) and increased metastatic potential *in vivo* (Steiniger *et al.*, 2008; Nishii *et al.*, 2009).

1.13.5 ABC transporter expression and the presence of SP cells in breast cancer cell lines

The role of ABC transporters in the existence of SP cells has been investigated for many breast cancer cell lines. In many studies ABCG2 expression was found to be significantly increased in the SP compared to NSP counterpart (Zhou *et al.*, 2002; Patrawala *et al.*, 2005; Steiniger *et al.*, 2008). SP cells were also shown to be more resistant to chemotherapeutic agents (Steiniger *et al.*, 2008; Yin *et al.*, 2008) such as mitoxantrone and carboplatin and they also seem to be radioresistant (Woodward *et al.*, 2007). All these properties of SP cells would support the idea that they might contribute to MDR and thus they could be good targets for the management of breast cancer. In the MCF-7 cell line, the use of Fumitremorgin C (FTC), a specific inhibitor of ABCG2, resulted in the depletion of the SP population and the same effect was observed with the use of siRNA inhibitors for ABCG2. These isolated SP cells were able to asymmetrically divide and give rise to both SP and NSP cells and they formed new tumors when injected in the mouse mammary gland (Rabindran *et al.*, 2000).

Moreover, the SP cells in breast cancer cell lines that were predominantly ER+/PR+/HER2+ were found to be present at a higher percentage and they were characterized with a higher colony forming efficiency *in vitro*, in comparison to the Basal A and B subtypes. HER2 expression was also significantly associated with the SP presence, since cell lines induced to express HER2 had an increased SP percentage. Notably, treatment with the HER2 inhibitors, tyrophostin AG825 and trastuzumab, promoted the reduction of the SP numbers and decreased tumor growth *in vivo* (Nakanishi *et al.*, 2010).

There are conflicting data regarding the presence of SP cells in the MDA-MB-231 cell line. To be more specific, many studies have reported that although there is high expression of ABCG2 in these cells, but they do not contain SP cells (Patrawala *et al.*, 2005; Christgen *et al.*, 2007; Yin *et al.*, 2008; Golebiewska *et al.*, 2011). However, Britton et al recently showed that SP cells can be isolated from both MDA-MB-231 and

MCF-7 cell lines and both SP populations had a higher ABCG2 mRNA expression level than the NSP cells. Conversely, ABCG2 protein expression was only higher in the MCF-7 SP cells and this was thought to result in a greater resistance to mitoxantrone than that of the MCF7 NSP, whereas no significant difference was observed in either population of the MDA-MB-231 cells (Britton *et al.*, 2012). In addition, SP cells isolated from the MDA-MB-231 did not have an increased invasive potential at one of the most common metastatic sites of breast cancer, the bone (Hiraga *et al.*, 2011).

1.13.6 ABC transporter expression and the presence of SP cells in breast cancer patients

Although the presence of SP cells has been identified in various breast cancer subtypes (Christgen *et al.*, 2007), Clarke et al. demonstrated that SP cells in normal breast tissue are ER-positive (Clarke, 2005). As a consequence, it has been suggested that SP cells can mainly be found in luminal breast cancer patients (Nakshatri *et al.*, 2009). The SP prevalence has been detected in clinical breast specimens derived from luminal breast cancer patients (Nakanishi *et al.*, 2010). Based on the clinical data and on the findings on breast cancer cell lines mentioned in the previous section, the authors concluded that HER2 signalling is essential for the regulation of the SP population in HER2+ breast cancer patients leading to the formation of aggressive and chemoresistant tumors (Nakanishi *et al.*, 2010).

However, in a more recent study Britton and colleagues also detected a high prevalence of SP in most Fine Needle Aspirates (FNAs) from patients with ER-negative or triple negative breast cancer subtypes and elevated protein expression of ABCG2 was significantly correlated with these cases by using IHC approaches. On the other hand, in some cases both ABCG2 and ABCB1 transcripts were detected in SP populations isolated from these patients, indicating that ABCG2 expression alone could not be used as a marker for the identification of SP cells in breast cancer. Patients with triple negative breast cancer have poorer prognosis outcomes and therefore they do not respond effectively to particular therapeutic approaches, such as endocrine therapy. The presence of SP cells in these individuals could be indicative of their clinical condition and would represent a robust target for treatment (Britton *et al.*, 2012).

1.14 EMT in normal embryonic development and oncogenesis

In all complex organisms there is a distinction between epithelial and mesenchymal cells. These different cell phenotypes arise early in normal development and organogenesis and their role is equally essential for these processes. Epithelial cells provide cell-cell adhesion contacts and they are attached to the basement membrane. Thus, they create an important barrier for the appropriate regulation of the internal environment. On the other hand, mesenchymal cells are motile and also responsible for supporting the structure of epithelial cells mostly by producing components of the extracellular matrix (ECM). However, the action of these cell phenotypes is not static, which means that they convert from one phenotype to the other depending on external or internal signals. This dynamic conversion is termed EMT and the reverse process is Mesenchymal-Epithelial Transition (MET). In EMT the epithelial cells lose their epithelial characteristics and acquire more mesenchymal properties by cytoskeleton rearrangements and alterations in adhesion, cellular structure and morphology. In fact, cell surface proteins, such as E-cadherin or integrins are replaced by mesenchymal markers, such as N-cadherin, vimentin or fibronectin. This leads to the detachment of epithelial cells from the basal membrane and these are then more capable of migrating to other sites (Micalizzi *et al.*, 2010). EMT mediates many early events during embryogenesis, such as gastrulation (Solnica-Krezel, 2005), neural crest formation (Tucker, 2004), palatogenesis (Nawshad *et al.*, 2004), heart valve formation (Mercado-Pimentel and Runyan, 2007), nephrogenesis (Chaffer *et al.*, 2007) and myogenesis (López-Novoa and Nieto, 2009). It is also known to contribute to wound healing and regeneration by providing cellular flexibility (Shook and Keller, 2003; Choi and Diehl, 2009).

In addition to the role of EMT in normal embryonic development, it is also involved in pathological conditions, such as fibrosis and cancer metastasis (López-Novoa and Nieto, 2009). In epithelial cancers, including breast cancer, metastasis is thought to occur by EMT. During this process, the epithelial cells lose their epithelial characteristics and acquire more mesenchymal properties as described above. As a result, epithelial cells are detached from the basal membrane and they are then more capable of migrating to other sites or they become more invasive and enter the blood and lymphatic systems (Britton *et al.*, 2011) (Figure 1.5).

1.14.1 EMT as a physiological process in normal mammary development

There are three stages of human mammary gland formation; a) the embryonic stage when the basic structure is developed b) the pubertal stage at which the ducts elongate and branch through the mammary fat pad to form TEBs and c) the pregnancy stage that is characterized by alveolar differentiation and tertiary ductal branching in preparation for lactation. TEBs are composed of many epithelial layers and they are located in the end of the ducts. Their differentiation results in the development of the ducts, which consist of both luminal epithelial and myoepithelial cells. Besides, cap cells at the end of TEBs also contribute to epithelial plasticity in normal conditions. However, these cells display features of epithelial plasticity, without losing their cell-cell contacts, but by losing their apico-basal polarity (Ewald *et al.*, 2008). In addition, TEB cells exhibit changes in their interaction with the ECM as shown by the secretion of extracellular proteases and the expression of a different panel of integrins and ECM receptors (Fata *et al.*, 2004). In general, TEB cells do not undergo all the changes that are observed in a complete EMT, but they exhibit certain signs of epithelial plasticity (Micalizzi *et al.*, 2010).

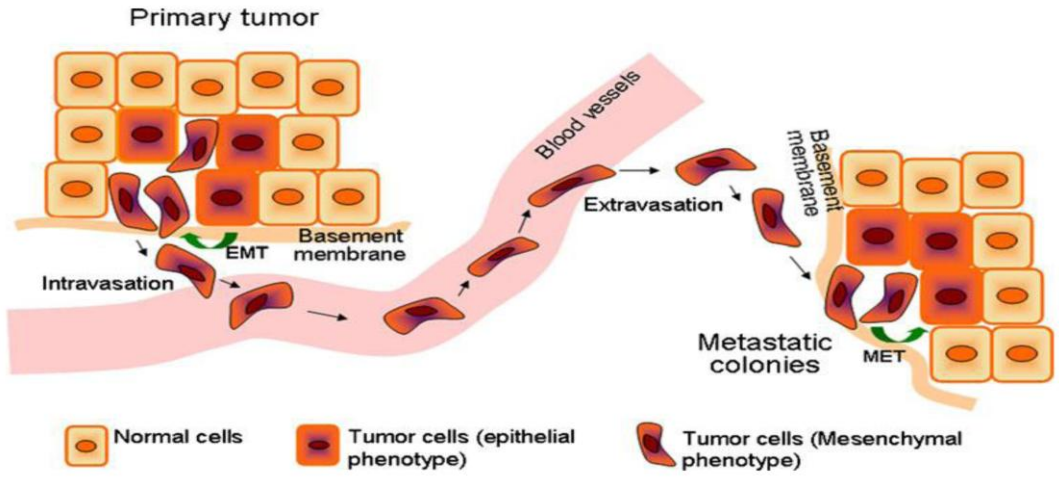


Figure 1.5: Schematic representation of the EMT and MET processes in cancer metastasis. Epithelial cells are detached from the primary tumor by losing their cell-cell contacts and acquiring a fibroblast-like morphology and a higher migratory potential. This allows them to enter the blood circulation and colonize at distant sites or organs. In order to form metastatic colonies the reverse process MET enables these cells to incorporate to the new tumor (Meng and Wu, 2012).

1.14.2 EMT in breast cancer progression and metastasis

The epithelial plasticity and EMT can also facilitate the local migration of tumor cells at early stages of breast cancer and their invasion to distant sites during the metastatic disease (Guarino *et al.*, 2007). Interestingly, EMT is thought to occur in a more local pattern rather than across the whole tumor (Carter *et al.*, 2006). One of the limitations for identifying EMT features is that cells that have undergone this process might morphologically resemble fibroblasts. Furthermore, the fact that most metastatic sites in many cancers are characterized by the presence of cells with an epithelial structure has contributed to the notion that EMT does not contribute to tumor progression. Nevertheless, it has also been suggested that EMT can be reversed in cells that have metastasized and colonized at distant sites (Micalizzi *et al.*, 2010) (Figure 1.6). Consistent with this hypothesis, cell fate mapping of epithelial tumor cells in the Whey Acidic Protein (WAP)-Myc transgenic mice has resulted in the conclusion that there is a direct relationship of EMT in the acquisition of an invasive phenotype in tumor cells in breast cancer (Trimboli *et al.*, 2008). Certain breast cancer subtypes have been correlated with the existence of EMT. In fact, basal or triple negative breast cancer patients have been found to have a more aggressive phenotype and poorer clinical outcome (Carey *et al.*, 2006). These patients and patients belonging to the claudin-low subtype have also been reported to express EMT markers, supporting the role of EMT in particular breast cancer patient subgroups, which can be reversible depending on signals from the local microenvironment (Sarrió *et al.*, 2008; Hennessy *et al.*, 2009). Another theory suggests that metastatic progression is a result of the co-operation of both EMT and non-EMT cells while each one of these populations alone is not sufficient to induce these effects (Tsuji *et al.*, 2009). What is more, EMT is thought to occur not only in single tumor cells, but also in a group of tumor cells leading to collective migration, as seen in a breast cancer xenograft model (Alexander *et al.*, 2008; Giampieri *et al.*, 2009). Strikingly, collectively migrated cells have been shown to lead to lymphatic dissemination by inhibiting the TGF-β signalling pathway that is involved in the induction of EMT and migration of single cells in a rat breast cancer model (Giampieri *et al.*, 2009).

Furthermore, EMT has been linked to the emergence of cancer-initiating cells, which have stem cell properties, also known as CSCs. The exact molecular events by which this happens are not clearly understood, but it has been suggested that this can be caused by either the conversion of epithelial cells to a more mesenchymal phenotype or the de-diferrentiation to a more progenitor or stem cell-like phenotype (Thomson *et al.*, 2005;

Frederick *et al.*, 2007). EMT also seems to contribute to drug resistance in the CSC populations and this explains the failure of existing chemotherapeutic therapies in basal/claudin-low breast cancer patients. The role of EMT in the BCSCs and drug resistance will be covered in more detail in following sections (Creighton *et al.*, 2009; Gupta *et al.*, 2009). Finally, EMT has also been found to directly suppress the immune system and therefore to impair the tumor surveillance mechanisms and promote the development of more aggressive and uncontrolled tumors (Kudo-Saito *et al.*, 2009).

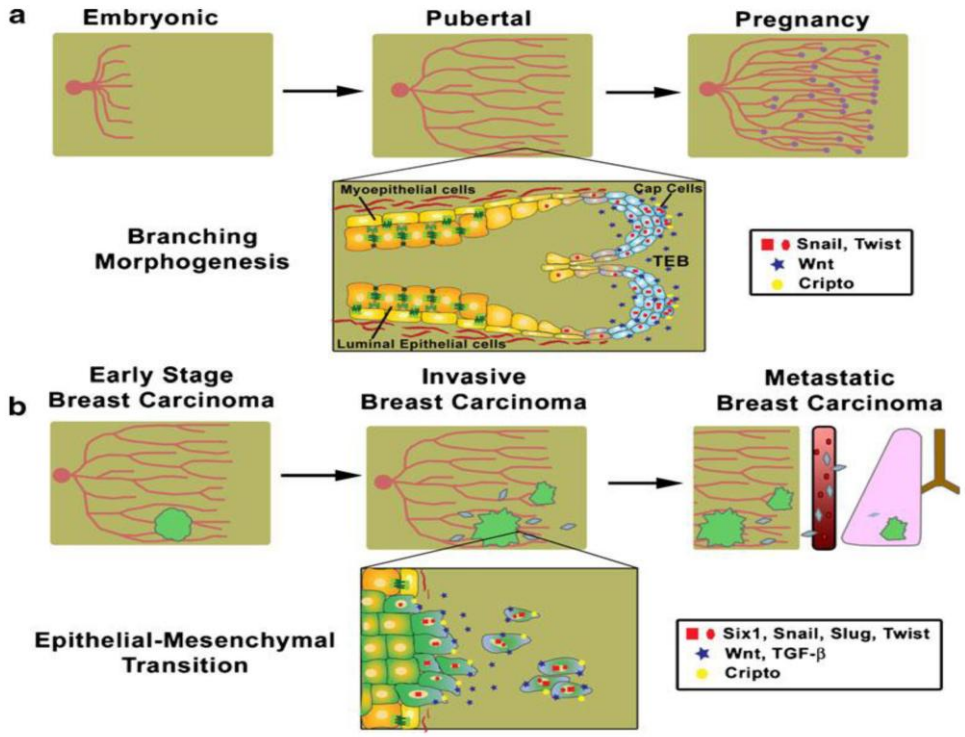


Figure 1.6: EMT in normal mammary gland development and in breast cancer metastasis. Mammary gland development begins during embryogenesis resulting in the formation of the basic structures of the ductal system. During puberty, these structures elongate leading to the extension of the ductal tree, while differentiation of precursor cells in the TEB into the luminal and myoepithelial cells also occurs. During pregnancy, branching morphogenesis is completed in preparation for lactation. In breast cancer the inappropriate expression of genes involved in normal EMT leads to the generation of motile and metastatic breast cancer cells (Micalizzi *et al.*, 2010).

1.14.3 Molecular regulation of EMT

The most critical and well studied EMT molecule is E-cadherin (CDH1). It belongs to the family of genes coding calcium dependent cell adhesion molecules (CAMs) and

plays an important role in the maintenance of epithelial tissues. Loss of E-cadherin expression is associated with increased invasiveness in cancer and is considered a hallmark in the process of EMT. It has also been shown that E-cadherin expression is re-established in cells that form secondary tumor colonies and undergo MET. A recent study demonstrated that the levels of E-cadherin were significantly higher in these cells compared to the cells from the primary tumor. Additionally, more than 50% of metastatic organs in breast ductal carcinoma showed increased expression of E-cadherin (Chao *et al.*, 2012). The underlying mechanisms controlling E-cadherin re-expression are not clear, but it may occur at the metastatic sites if the appropriate microenvironment and signals are provided, so that the migrating tumor cells can connect and incorporate with the target organs. For instance, E-cadherin promoter methylation was reversed in some breast cancer cells when co-cultured with normal hepatocytes (Wells *et al.*, 2008).

The transcription factors Snail, Slug and Twist are known to regulate the down-regulation of E-cadherin. In fact, Snail can bind with strong affinity to the E-boxes in the promoter of the E-cadherin gene and repress its expression (Batlle *et al.*, 2000). It has also been shown that ectopic expression of Snail in different types of epithelial cells caused a mesenchymal-like phenotype and in these cells E-cadherin expression is significantly reduced. Furthermore, Snail expression is abundant in highly tumorigenic and invasive areas in both murine and human carcinomas, while it is very low or absent in non carcinogenic regions (Cano *et al.*, 2000). Additionally, when Snail levels were estimated by immunohistochemical analysis in human breast cancer tissue, it was found that there was a significant correlation of elevated expression levels with infiltrating ductal carcinomas (IDCs) with a poor grade of differentiation, but that do not develop lymph node metastases. Therefore, Snail was suggested as a prognostic marker for the metastatic potential in breast cancer (Blanco *et al.*, 2002). Microarray analysis of human breast cancer samples also revealed that Snail was overexpressed in patients who had decreased relapse-free survival (Moody *et al.*, 2005).

Slug has also been shown to directly repress E-cadherin in breast cancer cell lines. In fact, both Snail and Slug down-regulated the expression of wild-type E-cadherin genes, whereas they failed to do so when the E-cadherin gene contained mutated E-box elements (Hajra *et al.*, 2002). High expression of Snail and Slug were inversely correlated with E-cadherin expression in a large number of cancerous cell lines, but the same was not observed in breast cancer samples. Both increased Slug and Snail levels were detected in breast tumors associated with lymph node metastases, but Slug was

also overexpressed in semi-differentiated tubules of ductal carcinoma (Côme *et al.*, 2006).

Twist is a basic helix-loop-helix transcription factor expressed during embryonic development. It is also overexpressed in many cancers, including breast cancer. MCF-7 cells overexpressing Twist exhibited loss of E-cadherin and gain of vimentin expression. These cells also acquired increased motility and invasive potential (Mironchik *et al.*, 2005). Twist has been found to directly repress E-cadherin in a dose dependent manner. Increased expression of Twist and decreased expression of E-cadherin have been associated with grade III tumors in human breast cancer (Vesuna *et al.*, 2008).

1.14.4 Other transcription factors involved in EMT

The ZEB family of transcription factors consists of two members ZEB1 (or EF1) and ZEB2 (or SIP1). These are composed of two zinc finger domains located at their ends and a central homeodomain. The ZEB transcription factors bind to specific E-boxes in the E-cadherin promoter through the zinc-finger domains and they induce its down-regulation leading to EMT (Comijn *et al.*, 2001).

The human Forkhead box (FOX) gene family of transcription factors is a large family of proteins consisting of more than 43 members. These contain a 100 amino-acid long DNA binding domain, the forkhead box. Several members of the FOX family have been found to be involved in cancer progression, with FOXC2 first being reported to play a role in the induction of EMT and metastasis (Katoh and Katoh, 2004). Additionally, FOXC1 has been shown to drive EMT in the MCF-12A cell line and its action has also been correlated with basal-like breast cancer (Bloushtain-Qimron *et al.*, 2008). What is more, FOXQ1 also seems to promote, while FOXA2 has been found to inhibit the EMT process (Feuerborn *et al.*, 2011).

The Kruppel-like factors (KLF) also contain zinc-finger domains through which they regulate several cellular functions, such as proliferation, differentiation and apoptosis. For instance, transfection of 4T1 orthotopic mammary cancer cells with KLF4 led to the significant attenuation of primary tumor formation and micrometastases to the lungs and liver and it was accompanied with a decrease of the Snail levels (Yori *et al.*, 2010). KLF17 has also been suggested as a suppressor of EMT in breast cancer, whereas KLF8 has been shown to promote EMT by regulating MMP-9 and E-cadherin (Wang *et al.*,

2007b). KLF6 is also thought to take part in the TGF-β driven EMT of proximal tubule cells (Holian *et al.*, 2008).

1.14.5 Developmental EMT signalling pathways de-regulated in breast cancer

EMT that participates in normal development has been better characterized; however, the pathological EMT mechanisms have not been clearly defined. Overall, EMT has been divided into three categories; Type I: developmental EMT, Type II: fibrosis and wound healing-related EMT and Type III: cancer EMT (Kalluri and Weinberg, 2009). Interestingly, oncogenic EMT is thought to occur due to the abnormal activation of developmental EMT (Micalizzi *et al.*, 2010). In line with this hypothesis, several EMT regulators have been found to be inappropriately expressed in human cancers leading to less coordinated features than the ones seen in developmental EMT (Gavert and Ben-Ze'ev, 2008). As a consequence, EMT is believed to drive cancer progression, as it has been associated with poor patient outcomes and increased tumor aggressiveness (Prasad *et al.*, 2009; Logullo *et al.*, 2010). It has also been suggested that all the oncogenic effects of EMT are linked with its involvement in the signalling pathways that regulate the self-renewal of CSCs, as described in more detail below. Defining the molecular mechanisms that are involved in cancer EMT is challenging, since it can result from unpredictable genetic abnormalities or changes in the microenvironment and it can be limited to a certain number of cells at any one time (Micalizzi *et al.*, 2010).

1.14.6 The TGF-β signalling pathway

TGF-β is thought to play an essential role in the induction of EMT not only during embryogenesis, but also during cancer progression. TGF-β has a direct effect on EMT by down-regulating epithelial markers and by up-regulating mesenchymal markers (Micalizzi *et al.*, 2010). It has also been shown to regulate the differentiation and proliferation of both normal mammary and cancer stem cells (Mani *et al.*, 2008; Scheel *et al.*, 2011). TGF-β can also activate additional molecules, such as MAPK, PI3K or GTPases belonging to the Rho family of proteins (Moustakas and Heldin, 2007). The TGF-β action is mediated upon its binding to the TGF-βR I and TGF-βR II receptors which have a serine/threonine kinase activity. These can phospholyrate downstream cytoplasmic molecules e.g., Smad 2 and 3 and activate them. Phosphorylated Smad 2 and 3 (p-smad 2/3) can in turn bind to Smad 4 and enter the nucleus, where they form complexes with other factors and promote the expression of several target genes related to proliferation, differentiation, apoptosis and cell migration (Figure 1.7). The TGF-β

cascade can also be smad-independent, but its role in stem cell regulation has not yet been clarified (Sakaki-Yumoto *et al.*, 2013).

Although, TGF-β has tumor promoting effects in almost all types of cancer, in some types, including breast cancer, it seems to have a dual role. It acts as a tumor suppressor at early stages, whereas at later stages of the disease it drives invasion and metastasis. Tumor suppression activity can be seen in breast cancer due to the presence of particular mutations at genes encoding either TGF-β receptors or the three Smad molecules that participate in the TGF-β signalling pathway. For instance, abnormal signalling is found in advanced breast cancers because of point mutations in the kinase domain of TGF-β I or II receptor (Pardali and Moustakas, 2007). Additionally, several reports have associated the elevated expression of TGF-β isoforms with poor patient outcomes in breast cancer (Ghellal *et al.*, 2000; Mu *et al.*, 2008).

Despite the number of reports indicating that TGF-β signalling is responsible for the enhancement of the CSC phenotype and its role in the induction of oncogenesis (Mani *et al.*, 2008; Hollier *et al.*, 2013), TGF-β has also been shown to reduce the number of CSCs and inhibit tumor formation (Tang *et al.*, 2007; Yin *et al.*, 2008). In fact, the link of BCSCs to EMT and metastasis has been recently reviewed (Mallini *et al.*, 2014). First of all, Mani et al. induced EMT in a non tumorigenic, immortalized human mammary epithelial cell line (HMLE) by introducing either the Twist or Snail gene or by exposing these cells to TGF-β. All these approaches resulted in the generation of CD44+/CD24- cells with increased mammosphere forming efficiency and self-renewal capacity. Conversely, CD44-/CD24+ cells were not able to generate CD44+/CD24- cells, confirming that the latter have a stem cell-like phenotype (Mani *et al.*, 2008). Additionally, Shipitsin et al. examined the molecular profiles of CD44+ and CD24+ cells derived from normal and breast cancer tissue and reported that there was up-regulation of the TGF-β signalling pathway in CD44+ cells with a high expression of TGF-β1 and one of its receptors TGF-βR II compared to the CD24+ cells, while the TGF-βR II gene was hypermethylated in CD24+ cells, explaining its low levels of expression in these cells. Interestingly, these results were reversed upon the addition of a TGFβ-R inhibitor in CD44+ cells which became more epithelial-like (Shipitsin *et al.*, 2007). Genome-wide transcriptional profiling of breast cancer cell lines revealed that the 'Basal B'/mesenchymal cells have a more enhanced invasive potential, in comparison to 'Luminal' or mixed basal/luminal ('Basal A') features (Neve *et al.*, 2006). Additionally, Basal B cells are mostly correlated with a CD44+/CD24- phenotype while differences in expression of 299 genes were found between

CD44+/CD24- and CD44-/CD24+ cells. This means that the Basal B subtype might be stem-cell driven. Ideally, the molecular characterization of BCSCs could enable the identification of new genes that can be targeted in patients with this particular subtype (Blick *et al.*, 2010).

Furthermore, induced expression of Twist2 in the immortalized and naturally non-transformed mammary epithelial MCF-10A cell line led to a fibroblast-like morphology and changes in expression patterns of epithelial and mesenchymal markers (down-regulation of epithelial markers and up-regulation of mesenchymal markers). The migratory ability of both MCF-7/Twist2 and MCF-10A/Twist2 expressing cells was assessed by using wound healing assays and was significantly increased compared to controls (cells that were transfected only with the vector without the Twist2 gene). The tumorigenicity of the Twist-2-expressing cells was also enhanced compared to the control leading to the formation of large tumors after injection into nude mice. In addition, an increased number of CD44+/CD24- cells were identified and it was suggested that Twist2 not only promotes the EMT programme, but also generates cells with stem cell-like properties (Fang *et al.*, 2011).

It has also been reported that Twist directly regulates the transcription of CD24 in the breast cancer cell lines MCF-7 and MCF-10A. Following transfection with Twist, the cells were characterized as having a CD44+/CD24- profile, increased ALDH1 activity and increased export of Hoechst 33342 and Rhodamine 123 dyes due to high ABCC1 expression. Also, only 20 cells of this subpopulation were enough to promote new tumor formation in the mammary fat pads of immunodeficient mice. All these effects were reversed when Twist was silenced by the use of short hairpin RNA in MCF-7 and MCF-10A cells overexpressing Twist (Vesuna *et al.*, 2009).

Another study aimed at investigating the effect of silencing of FOXC2 in HMLE cells that were driven to EMT by overexpression of Snail, Twist or TGF-β1. This resulted in the production of cells with epithelial-like characteristics, reduction of CD44+/CD24- cells and a significant decrease in their mammosphere formation ability. Conversely, the opposite properties were seen in FOXC2 overexpressing cells that displayed increased drug resistance and tumor initiation ability, leading to the suggestion that FOXC2 is sufficient to induce BCSCs generation (Hollier *et al.*, 2013).

The gene expression patterns of CD44+/CD24- and CD44-/CD24+ cell populations of the basal cell phenotype MCF-10A cell line have been investigated. 32 genes were found to be differentially expressed in the two subpopulations and most importantly Slug overexpression was reported to increase the number of CD44+/CD24- cells.

However, overexpression of Slug in the luminal type breast cancer cell line MCF-7 could only give rise to CD44+/CD24+ cells, suggesting that Slug can only induce the generation of this phenotype in basal cell types and presumably basal types of breast cancer (Bhat-Nakshatri *et al.*, 2010). Moreover, treatment of H-Ras-V12 transfected HMLE cells with TGF-β resulted in the emergence of CD24- from CD24+ cells, while the opposite was not shown, suggesting that there is a cooperative effect of the TGF-β and the Ras-MAPK signalling pathways on promoting the generation of BCSCs (Morel *et al.*, 2008).

On the other hand, TGF-β treatment was found to decrease the percentage of the SP cells of the MCF10A cell line, by down-regulating Id1, which is involved in self-renewal and prevents differentiation in many types of tissue (Perk *et al.*, 2005). Similarly, Yin and colleagues also demonstrated the negative regulatory effect of TGF-β on the SP population of the MCF-7 breast cancer cell line (Yin *et al.*, 2008). Taken together, TGF-β seems to also inhibit tumor growth by inducing the differentiation of CSCs. These findings indicate the complex roles of TGF-β in the regulation of CSCs and contrasting effects of TGF-β on CSC populations depending on tumor type. The elucidation of the TGF-β mechanisms that are involved in the CSC function are of great scientific and clinical importance.

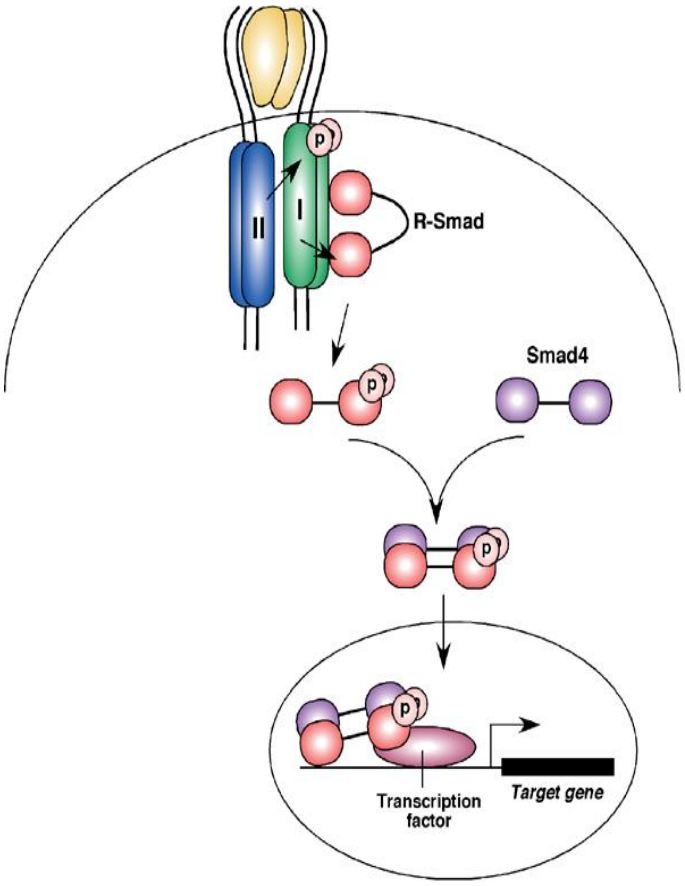


Figure 1.7: Schematic representation of the smad-dependent TGF-β signalling pathway. Upon binding of TGF-β to the TGF-βR I and TGF-βR II receptors, receptor-regulated smads (known as R-smads), including smad 2 and 3 are phosphorylated through the receptors' serine/threonine kinase activity. p-smad 2/3 can then bind to Smad 4 and enter the nucleus, where in association with other factors they promote the expression of several target genes related to EMT (Izzi and Attisano, 2004).

1.14.7 The Wnt signalling pathway

The Wnt signalling pathway plays a pivotal role at different stages of the normal mammary gland development including the mammary duct formation during embryogenesis and the alveolar differentiation and duct branching during pregnancy. It is also known to regulate cell fate determination and maintenance of the progenitor cell populations in their stem cell state (Brennan and Brown, 2004). The main molecule in the Wnt pathway is β-catenin whose cellular localization determines the effects of the pathway. In fact, when it is located in the cell membrane it is connected with E-cadherin and provides tight contacts between epithelial cells. Alternatively, when β-catenin accumulates in the cytoplasm it translocates into the nucleus where it activates the transcription of the Wnt target genes, such as c-Jun, c-Myc, fibronectin and Cyclin D (Kalluri and Weinberg, 2009).

When the Wnt ligand is absent, the cytoplasmic β-catenin protein is degraded by the Axin complex, consisting of the scaffolding protein Axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 are responsible for the phosphorylation of β-catenin, leading to its ubiquitination by β-Trcp and protein degradation. Therefore, β-catenin fails to reach the nucleus and induce the expression of the Wnt target genes, which are repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. Upon binding of the Wnt ligand to a seven-pass transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its homolog LRP5 promotes the recruitment of the scaffolding protein Dishevelled (Dvl) and leads to LRP6 phosphorylation and activation. These molecular events bring the Axin complex in close proximity to the receptors and block the Axin-driven β-catenin phosphorylation resulting in the stabilization of β-catenin and its nuclear translocation (MacDonald *et al.*, 2009).

Deficiency of any of the Wnt signalling components, including the inactivation of the Axin complex, mutations in β-catenin or in the tumor suppression gene Wnt5 might be involved in the abnormally regulated self-renewal of normal stem cells, leading to the generation of CSCs (Howe and Brown, 2003). Additionally, WNT1 has been identified as an oncogene whose activation leads to the transformation of mouse mammary cells (Harada *et al.*, 1999). Notably, no mutations have been found in breast cancer, but since active Wnt signalling has been detected in breast cancer specimens from patients with poor prognosis (Prasad *et al.*, 2009; Logullo *et al.*, 2010), research has focused on the

epigenetic alterations that might affect the normal pathway action (Gangopadhyay *et al.*, 2013).

Furthermore, activation of the Wnt pathway has led to the induction of EMT in various *in vitro* models, including mammary epithelial and carcinoma cells lines (Kim *et al.*, 2002a; Yook *et al.*, 2006). Wnt activity has also been shown to regulate the mammary stem cells at the basal layer of the mammary ducts. It has also been demonstrated that treatment of mammary stem cells with the Wnt3 protein enhanced their colony formation capacity *in vitro*, which exhibited high mammary gland formation efficiency (Zeng and Nusse, 2010)**.** Interestingly, MMTV-WNT1 mice were characterized with a significantly higher proportion of mammary stem cells, supporting the involvement of the Wnt pathway in the BCSC function (Shackleton *et al.*, 2006).

1.14.8 The Notch signalling pathway

Notch signalling is involved in cell fate determination and it is mediated through Notch receptors (Notch 1-4) and 5 ligands (Jagged-1 and 2 and Delta-like (Dll)-1, -3, -4). The pathway activation is initiated when cells expressing the receptor interact with neighbouring cells secreting the ligand and this in turn leads to the cleavage of the intracellular domain of the receptor by the γ-secretase complex. This domain then translocates into the nucleus where it promotes the transcriptional activation of the Notch target genes, belonging to the Hes and Hey families of transcription factors which are responsible for the prevention of cell differentiation and maintenance of a stem cell state (Miele, 2006).

Notch signalling is essential in mammary gland development where it acts as a regulator of stem cell self-renewal and differentiation. Interestingly, Notch activation has been shown to contribute to the regulation of asymmetric division in mammospheres at early developmental stages, while at later stages it promotes the generation of myoepithelial cells from progenitor cells, which facilitates branching morphogenesis. Additionally, it has been concluded that de-regulation of these processes might lead to aberrant self-renewal in normal breast stem cells, resulting in the acquisition of breast cancer stem cell properties (Dontu *et al.*, 2004). Indeed, loss of Numb expression, a negative regulator of the Notch pathway, has been found in more than 50% of cases, while elevated expression of Notch-1 and Jagged-1 has been associated with poor prognosis in breast cancer (Yu *et al.*, 2007; Fillmore and Kuperwasser, 2008).

Furthermore, Farnie and co-workers introduced a new primary cell culture method for DCIS and they demonstrated that these cells had a greater mammosphere forming efficiency (MFE) compared to cells derived from normal breast tissue. In fact, MFE increased with increased grade DCIS. Notch signalling activation was found to be more apparent in the DCIS mammospheres and the use of the γ-secretase inhibitor decreased MFE, but not to the same degree as treatment with a Notch-4 neutralizing antibody did. Therefore, the authors suggested that this technique might be useful for the investigation of the molecular events that regulate self-renewal in BCSCs from DCIS patients and the efficiency of potential inhibitors that disrupt Notch signalling could be examined (Farnie *et al.*, 2007). Intriguingly, in a more recent study it was reported that blocking signalling through the Notch-4 rather than the Notch-1 receptor more effectively reduces the percentage of the CD44+/CD24-/ESA+ cells isolated from both breast cancer cell lines and patients, suggesting that targeting BCSCs can be more specific (Harrison *et al.*, 2010). Similarly, it has also been shown that Hes-1 was highly expressed in all mammospheres derived from breast cancer cell lines and patients and it was shown that the MRK003 was the most effective γ-secretase inhibitor at eliminating the formation of mammospheres *in vitro*, while it retained its activity through the whole treatment period (Grudzien *et al.*, 2010). Finally, Qiu et al also reported that treatment with a Notch-1-specific antibody alone or in combination with docetaxel significantly reduced mammosphere formation in a SUM149 model by down-regulating Hes-1 and c-Myc, while it also decreased the metastatic potential and delayed tumor re-occurence in mice (Qiu *et al.*, 2013).

1.14.9 The Hedgehog signalling pathway

The Hedgehog signaling pathway also participates in normal mammary gland development. The actions of this signalling pathway are dependent on the presence or absence of the hedgehog ligands and they are mediated through the GLI zinger-finger transcription factors (GLI1, 2, 3). GLI1 is thought to act as a transcriptional activator only, whereas GLI2 and 3 have been reported to act either as activators or as repressors of gene expression. In mammary gland development the role of the Indian hedgehog ligand and of the *Patched* receptor (PTC1) seem to be important. When the ligand is present it binds to PTC1 and it inhibits the action of *Smoothened* (SMO). SMO is a seven-pass transmembrane protein which interacts with a multiprotein complex which is supported by its connection with the microtubules and it consists of the dynamin-related

protein Costal-2 (COS-2), the serine-threonine kinase Fused (FU), the suppressor of Fu, (SUFU) and one of the GLI transcription factors (Gangopadhyay *et al.*, 2013).

In the absence of the ligand the GLI proteins are phosphorylated by a cAMP-dependent protein kinase A (PKA) or GSK3 and CK1. This in turn promotes their cleavage into a repressor form that translocates into the nucleus and blocks the transcription of the Hedgehog target genes. However, in the presence of the ligand the GLI complex dissociates from the microtubules and its cleavage is inhibited by the action of PP2A-like phosphatase. As a result, the uncleaved form of the GLI proteins can then translocate into the nucleus and induce the expression of the target genes (Lewis and Veltmaat, 2004).

Dysfunction of any of the Hedgehog signalling components is thought to contribute to tumor progression in the mammary gland (Liu *et al.*, 2005). For instance, overexpression of GLI1 or Bmi-1, which are both downstream molecules of the Hedgehog pathway, has led to increased tumor growth in mice (Fiaschi *et al.*, 2009). O'Toole and co-workers also reported that high Hedgehog ligand expression is associated with increased risk of metastasis and death from breast cancer and it was particularly correlated with the basal-like breast cancer subtype. These findings were confirmed with the use of a mouse model representing this phenotype in which overexpression of the ligand was induced leading to the acceleration of metastasis and the decrease of survival. Accordingly, treatment with the monoclonal antibody against the ligand (5E1) reversed these effects (O'Toole *et al.*, 2011). Similarly, it has been shown that abnormal activation of this pathway can promote bone metastasis in breast cancer (Das *et al.*, 2012a; Das *et al.*, 2012b).
Moreover, the SP and CD44+/CD24-, which were found to partially overlap in the MCF-7 cell line, were characterized by the overexpression of Hedgehog components, including SHH and GLI1 on both mRNA and protein level. Silencing of GLI1 by siRNA resulted in the inhibition of proliferation and reduction of the percentages of both BCSC populations. Therefore, it was suggested that targeting the Hedgehog pathway might have a therapeutic potency for the prevention of metastasis (Tanaka *et al.*, 2009). It has also been found that Bmi-1 seems to be responsible for the self-renewal and differentiation of BCSCs. In fact, Bmi-1 and c-Myc levels were higher in the corresponding metastases than in the primary tumors and the highest expression of Bmi-1 was present in late relapse tumors. These findings were also correlated with the high expression of Snail in metastases (Joensuu *et al.*, 2011). Another study showed that

Bmi-1 was up-regulated in primary cancerous tissue compared to the non cancerous tissue. This was inversely correlated with E-cadherin expression providing evidence that Bmi-1 might also be involved in EMT. This was confirmed by the use of human mammary cell lines. In fact, MCF-10A cells overexpressing Bmi-1 exhibited increased cell motility and acquired invasive properties, while they also expressed mesenchymal markers. Conversely, silencing of Bmi-1 in the highly invasive MDA-MB-435S cell line resulted in the opposite effects (Guo *et al.*, 2011). What is more, Liu et al. demonstrated that there was a 6-fold increase in the levels of Bmi-1 when the Hedgehog pathway was active, but Bmi-1 was significantly down-regulated when this pathway was inhibited. Furthermore, Bmi-1 was up-regulated up to 5 times in the CD44+/CD24- human breast cancer stem cell subpopulation. Therefore, it is reasonable to suggest that Bmi-1 is an essential stem cell regulator in breast stem cells and that de-regulation of the Hedgehog pathway might result in the generation of BCSCs which can cause malignancies (Liu *et al.*, 2006).

1.14.10 EMT and BCSC features in breast cancer patients

Several studies have aimed to investigate the association of EMT with the presence of CSCs in breast cancer patients. The analysis of 226 blood samples derived from 39 metastatic breast cancer patients undergoing chemo-, antibody or hormonal therapy revealed a correlation of ALDH1 with the expression of EMT markers (Aktas *et al.*, 2009). In another study the evaluation of breast cancer samples from patients at all stages led to the identification of CSCs in 66% of the patients and these were further examined for the presence of ERα, HER2, ALDH1, vimentin and fibronectin. Interestingly, ALDH1 expression was significantly associated with the disease stage and the expression of vimentin and fibronectin, suggesting that EMT is involved in this CSC phenotype (Raimondi *et al.*, 2011). Vimentin was also found to be prevalent in CSCs in 77% and Twist was highly expressed in 73% of patients with primary disease, while all of the patients with the metastatic disease expressed both markers at high levels (Kallergi *et al.*, 2011).

What is more, the presence of breast cancer tumor initiating cells was associated with the claudin-low breast cancer subtype, which represents 5% of the breast cancer patients. It was also reported that these cells were characterized with the elevated expression levels of mesenchymal markers, including FN1, VIM, FOXC2, MMP2 and MMP3, while the expression of epithelial markers, such as CDH1 and DSP were almost

undetectable (Creighton *et al.*, 2010). Finally, it is worth mentioning that 50% of human breast cancers have a dysfunctional Notch signalling pathway, predominantly due to reduced expression of the Notch inhibitor, NUMB (Dontu *et al.*, 2004; Liu *et al.*, 2005). In conclusion, the correlation of EMT and CSC traits in certain subgroups of breast cancer patients increases the need for the development of more effective strategies to target these cells, therefore many research groups have focused on the determination of the EMT mechanisms that regulate CSC function in breast cancer metastasis and drug resistance.

1.14.11 The role of EMT in the induction of MDR

There is accumulating evidence to support the contribution of EMT to drug resistance as we also summarized in our recent review (Mallini *et al.*, 2014). In fact, when breast cancer cells were treated with doxorubicin for 1 week, the expression of many ABC transporters and EMT markers was up-regulated in invasive cell lines only. Interestingly, induction of EMT by TGF-β1 treatment or Twist1 overexpression in the non-invasive cell line MCF-7 led to elevated expression of ABC transporters, while knocking down of Twist and Zeb1 reversed dox-mediated EMT and drug resistance. It has also been shown that there are binding sites for several EMT transcription factors (Snail, Twist, Slug and FOXC2) in 16 ABC transporters and Chip analysis revealed that Twist directly binds to E-boxes in the promoter region of ABCC4 and ABCC5 in MCF-7 cells transfected with Twist (Saxena *et al.*, 2011).

Vesuna and co-workers demonstrated that induced expression of Twist in MCF-7 cells promoted the increased efflux of Hoechst 33342 and Rhodamine 123 dyes caused by the up-regulation of the ABCC1 transporter (Vesuna *et al.*, 2009). On the other hand, depletion of Slug/Snail2 in the basal/HER2+ cells resulted in the up-regulation of CD24, increased sensitivity to trastuzumab and led to the inhibition of tumor growth *in vivo*, providing further evidence for the role of EMT in drug resistance in BCSCs (Oliveras-Ferraros *et al.*, 2012). Similarly, FOXC2 knockdown in HMLE cells that were previously induced to undergo EMT through Snail or Twist transfection or TGF-β exposure led to the decrease of the CD44+/CD24- population, which were also characterized with reduced resistance to paclitaxel (Hollier *et al.*, 2013).

Additionally, tamoxifen resistant MCF-7 cells were found to have a more aggressive and invasive behavior that could be reversed upon the inhibition of the autocrine EGFR pathway, also known to be involved in EMT, by using an EGFR tyrosine kinase inhibitor (EGFR-TKI), gefitinib (Hiscox *et al.*, 2004).

Moreover, doxorubicin treatment of murine 4T1 cells led to an increase in Sca-1 cells with a high metastatic potential (Bandyopadhyay *et al.*, 2010). The same effect was shown after TGF-β mediated EMT in pancreatic cell lines and this was reversed upon the removal of TGF-β (Kabashima *et al.*, 2009). In contrast, TGF-β treatment in MCF-7 cells eliminated the SP population, decreased the levels of ABCG2 expression and reduced cell viability in the presence of mitoxantrone and these changes were also reversed when TGF-β was removed. E-cadherin knockout also reduced the SP numbers, but this was not as significant as the TGF-β driven EMT or the ABCG2 knockout and did not affect the mRNA or proteins levels of ABCG2. Consequently, the authors claimed that EMT controls the post-translational regulation of ABCG2 via E-cadherin (Yin *et al.*, 2008). In our laboratory we have also been able to demonstrate that treatment of the MCF-7 cell line with TGF-β1 results in the reduction of the SP phenotype (Mallini *et al.*, 2014).

1.15 Hypoxia in cancer

The rapid expansion of tumor cells creates large distances from the blood vessels which support their proliferation with oxygen and nutrients and leads to altered microenvironmental conditions, such as low oxygen concentration, acidosis and nutrient deprivation (Brown and Giaccia, 1998). Low concentration of oxygen in cells or tissues, referred to as hypoxia, is the most studied and best characterized of tumor microenvironment conditions and it seems to play a crucial role in carcinogenesis. Hypoxia has an effect on many aspects of tumor progression especially on cancer cell survival, resistance to apoptosis, invasion, metastasis, chemo-radiation resistance and angiogenesis, while recent evidence suggests that it is also responsible for CSC self-renewal and maintenance. It is usually caused due to insufficient blood supply (transient hypoxia) or increased oxygen diffusion due to tumor expansion (chronic hypoxia) (Bao *et al.*, 2012).

Notably, hypoxia or anoxia have been found to be prevalent in up to 60% of advanced solid tumors (Favaro *et al.*, 2011). In fact, the components of the hypoxia-regulated pathways have been suggested as clinical prognostic markers for patients with solid tumors (Jubb *et al.*, 2010). Additionally, adaptation to hypoxia has been correlated with a more aggressive phenotype and increased resistance to chemo- and radiotherapy (Moulder and Rockwell, 1987). For instance, hypoxia has been associated with poor clinical outcome and decreased survival in cervical cancer (Nordsmark *et al.*, 2005),

while management of hypoxia has been shown to improve the efficiency of radiation therapy in head and neck cancer patients (Overgaard, 2011).

1.15.1 Molecular regulation of hypoxia

The hypoxia-inducible factors (HIFs) are the fundamental molecular mediators of hypoxic adaptation. The HIF family of proteins (1, 2 and 3) belong to the per-aryl hydrocarbon receptor nuclear translocator (ARNT)-sim (PAS) basic helix-loop-helix (bHLH) heterodimeric transcription factors and they are composed of the α and the β subunits. The expression of the α subunit depends on oxygen availability and under low oxygen conditions both mRNA and protein levels elevate, while the β subunit is constitutively expressed (Wang *et al.*, 1995). Interestingly, hypoxia can regulate the expression of up to 1.5% of the genes in the human genome (Favaro *et al.*, 2011).

The most common heterodimer which is involved in hypoxic adaptation is HIF-1α/β. Although, HIF-1α and HIF-2α have very similar sequences and undergo similar regulation processes, HIF-1α seems to be more commonly expressed, whereas the expression of HIF-2α is more restricted (Wiesener *et al.*, 2002). HIF-3α has not been studied to the same extent as 1 and 2α (Rohwer and Cramer, 2011). During normal oxygen conditions the HIF-α subunits are hydroxylated by prolyl-hydroxylase (PHD) at two specific prolyl residues located in their oxygen-dependent degradation (ODD) domain. As a result, they interact with the von-Hippel-Lindau (VHL) complex, which recruits an E3 ubiquitin ligase complex and leads to their proteolytic degradation (Ivan *et al.*, 2001; Yu *et al.*, 2001).

Under hypoxic conditions, hydroxylation by PHD is inhibited resulting in the accumulation of the HIF-α subunits, which upon their translocation to the nucleus form heterodimers with the β subunits and associate with other co-activators, including p300 and CBP. Binding of these complexes to the hypoxia-responsive elements (HRE) within the promoter regions of target genes can in turn regulate their expression (Mahon *et al.*, 2001). Apart from low oxygen availability, hypoxia effects can also be induced by other factors, such as reactive oxygen species (ROS) (Dewhirst *et al.*, 2008) (Figure 1.8).

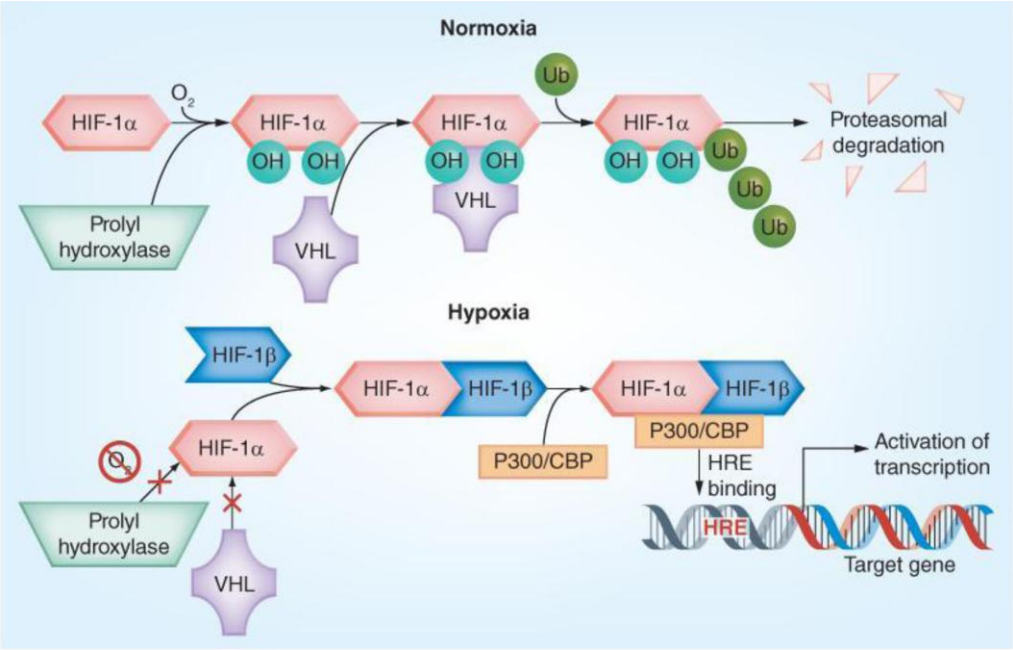


Figure 1.8: Schematic representation of the HIF signalling pathway after normoxic and hypoxic conditions. During normal oxygen conditions PHD hydroxylates HIF-1α leading to its targeting for protein degradation via its binding to the VHL complex. During low oxygen conditions PHD can no longer hydroxylate HIF-1α, which can then enter the nucleus and associate with HIF-1β and the P300/CBP complex. These in turn bind to the HRE and transcriptionally activate the expression of several hypoxia target genes (Burroughs *et al.*, 2013).

1.15.2 The role of hypoxia in the induction of EMT

There is evidence to suggest that hypoxia can also drive EMT in several *in vitro* systems, including cancer cells (Lee *et al.*, 2006; Chang *et al.*, 2011). Despite the fact that the exact contribution of hypoxia to the EMT process has not been fully described, it has been proposed that this is achieved via several mechanisms. First of all, it has been demonstrated that hypoxia-induced EMT results in the acquisition of mesenchymal properties and the up-regulation of the E-cadherin repressors, Twist, Snail, Slug, SIP1/ZEB2 (Sahlgren *et al.*, 2008; Klymkowsky and Savagner, 2009). Furthermore, hypoxia seems to stimulate the activation of known EMT-related pathways, including the TGF-β, the Notch, the Wnt and the Hedgehog cascades (Koong *et al.*, 1994; Eger *et al.*, 2000; Sahlgren *et al.*, 2008). It has also been shown to regulate the expression of cytokines that participate in EMT-related inflammatory response, such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) (Chuang *et al.*, 2008; St. John *et al.*, 2009). Next, hypoxia is also thought to have a direct or indirect impact on the mediation of cell-matrix interactions that are associated with migration and invasion through affecting the expression of LOX/LOX2 and urokinase-type plasminogen activator (uPA) (Chen *et al.*, 2010). It has also been reported that angiogenesis, which is induced by hypoxia, can also drive EMT through the VEGF pathway (Gonzalez-Moreno *et al.*, 2010).

In addition, hypoxia seems to regulate EMT through known stem cell self-renewal pathways. For instance, it has been shown that it can activate the Notch signalling pathway via direct binding of HIF-1 to the HES-1 promoter and thus resulting in EMT and increased cell invasion and migration in breast cancer (Chen *et al.*, 2010). There also seems to be an interaction of the HIF and the Wnt signalling pathways, although the exact mechanisms by which this occurs have not been fully elucidated. In fact, increased HIF-1α expression has been associated with increased activation of the Wnt pathway. Finally, there might be a crosstalk between the Hedgehog and the HIF pathways, as the former has been suggested to contribute to the angiogenic process (Bicknell and Harris, 2004). Taken together, hypoxia plays a role in the regulation of EMT via various pathways, indicating that targeting hypoxia might be an effective approach to also inhibit EMT and cancer progression. The involvement of hypoxia in the activation of stem cell self-renewal pathways might be the reason why it also regulates the function of CSCs, which will be described in more details in a following section.

1.15.3 The role of hypoxia in the induction of MDR

There is accumulating evidence to support the role of hypoxia in the induction of drug resistance in cancer, although there are also conflicting reports. However, the molecular events underlying this effect are not fully understood and they depend on tumor type. It should be noted that the HIF-1α– mediated effects on MDR have been studied more extensively than the HIF-2α ones (Rohwer and Cramer, 2011). Several mechanisms have been suggested with one of them being the HIF-1α driven changes in cell proliferation and survival, as HIF-1α has been shown to inhibit apoptosis. In fact, knocking down of HIF-1α has resulted in increased cell death following chemotherapeutic treatment in tumor cells with several origins (Ricker *et al.,* 2004; Peng *et al.,* 2006; Hao *et al.,* 2008). It has also been demonstrated that these effects are due to inhibition of p53-mediated apoptosis by HIF-1α (Bertout *et al.,* 2008; Rohwer *et al.,* 2010).

Additionally, Sullivan and colleagues reported that hypoxia can affect the irreversible cell cycle arrest, known as senescence, which is caused by the cellular DNA-damage response mechanisms in response to drug treatment. Further evidence supports that this depends on HIF-1α activity as shown by siRNA targeting of HIF-1α in breast and colon cancer cells, which led to the reversion of drug resistance (Sullivan *et al.,* 2008). Furthermore, it was reported that HIF-1α blocked the etoposide-induced DNA damage in breast and prostate cancer cells (Sullivan and Graham, 2009). It has also been suggested that hypoxia regulates various metabolic processes in cancer, including mitochondrial activity. Mitochondria are the main sources of intracellular ROS and ROS production in tumor cells by radiation or chemotherapy is a common way of targeting them (Fruehauf and Meyskens, 2007). However, HIF-1α has been shown to decrease ROS production and thus increase cancer cell survival in response to chemotherapy, while silencing HIF-1α led to the opposite effects (Rohwer *et al.,* 2010).

The involvement of hypoxia in the induction of MDR via the regulation of ABC transporter expression is becoming increasingly evident. Silencing of HIF-1α using si-RNA in T98G human glioma cells resulted in decreased mRNA and protein levels of both HIF-1α and ABCC1 and these cells became more sensitive to doxorubicin and etoposide (Chen *et al.,* 2009). In this context, Li and co-workers also demonstrated that sh-RNA knockdown of HIF-1α in MCF-7 cells led to the down-regulation of HIF-1α target genes, including VEGF, Glut-1, PGK and ABCB1 and these cells exhibited higher sensitivity to methotrexate (Li *et al.,* 2006). Further evidence suggests that

hypoxia can induce the increase in drug export, since knock-down of the HIF-1α gene in the gastric cancer cell line SGC7901 decreased their resistance to 5-FU, vincristine, cisplatin, etoposide and adriamycin and was accompanied with a decrease of both ABCB1 and ABCC1 mRNA and protein expression. Notably, co-injection of vincristine with HIF-1α si-RNA into nude mice reduced the tumor size by a half in comparison to vincristine alone (Liu *et al.*, 2008).

Finally, hypoxia also seems to promote MDR through the regulation of the already drug resistant CSCs. Prostate cancer SP numbers were enhanced in the prostate cancer cell lines PC-3 and DU145 when exposed to hypoxic conditions, while the percentage of CD44+ cells and the expression of OCT 3/4, NANOG and ABCG2 were also increased (Ma *et al.*, 2011). Cardiac SP cells treated with hydrogen peroxide were also increased due to ABCG2 up-regulation (Martin *et al.*, 2008). Of note, Liu and colleagues demonstrated that the up-regulation of ABCG2 in kidney SP cells due to hypoxia and re-oxygenation was mediated by the MEK/ERK pathway (Liu *et al.*, 2013). According to another study, the up-regulation of ABC transporters prevents the accumulation of porphyrins and heme under hypoxia, since the production of ROS and mitochondrial dysfunction can lead to cell death (Krishnamurthy *et al.*, 2004).

1.15.4 The role of hypoxia in the generation of BCSCs

Hypoxia seems to be involved in the development of breast cancer and it has been associated with more aggressive breast cancer phenotypes (Mimeault and Batra, 2013). For instance, HIF-2α expression has been significantly correlated with high ABCG2 expression, histology-grade and Ki67 expression in invasive breast cancer patients, indicating that targeting HIF-2α could serve as an effective therapeutic strategy for the management of drug resistance and metastasis in breast cancer (Xiang *et al.*, 2012). Hypoxia presumably induces all these effects by affecting BCSCs, which are characterized with improved survival mechanisms against oxygen deprivation (Mimeault and Batra, 2013). Accordingly, HIF-1α expression has been associated with the presence of the CD44+/CD24- phenotype in 253 specimens from patients with breast ductal carcinoma who had poor prognosis (Oliveira-Costa *et al.*, 2011).

Furthermore, HIF-1α expressing CD44+/CD24- cells were also found to express high levels of mesenchymal and low levels of epithelial markers and had increased mammosphere forming and tumorigenic ability under normal and hypoxic conditions compared to their differentiated progenies (Conley *et al.*, 2012; Han *et al.*, 2012). Louie

et al showed similar findings for the CD44+/CD24-/ESA+ cells of the MDA-MB-231 and BCM2 breast cancer cells lines when they exposed these to three cycles of hypoxia and re-oxygenation (Louie *et al.*, 2010). Finally, the up-regulation of both CD44 and VEGF in the MDA-MB-231 and SUM-149 breast cancer cell lines was also attributed to hypoxia (Krishnamachary *et al.*, 2012).

1.16 Hypothesis

Triple negative breast cancer patients fail to respond to current therapeutic options and it has been reported that they have an increased SP prevalence, which may be responsible for their poor clinical outcome (Britton *et al.*, 2012). What is more, ER expression has been found to be inversely correlated with TGFB-RII expression (Arteaga *et al.*, 1988) and the CD44+/CD24- cell phenotype has been associated with ER-/TGFB-RII+ patients (Shipitsin *et al.*, 2007). Therefore, I hypothesize that ER status and the presence of a functional TGF-β signalling pathway will be crucial for the regulation of putative BCSCs through EMT in different breast cancer patients. As a consequence, in this project I used the MDA-MB-231 (ER-/PR-/HER2-) and MCF-7 (ER+/PR+/HER2-) breast cancer cell lines representing triple negative and luminal epithelial breast cancer subtypes, respectively. Since MDA-MB-231 cells are characterized with an intact TGF-β signalling pathway and MCF-7 cells have been found to lack essential components of the pathway, including TGFB-RII expression, I hypothesize that the effect of EMT on the regulation of BCSCs contained in the former will be stronger than the one in the latter. Furthermore, based on the effect of hypoxia on several CSCs and its definite impact on EMT, I also hypothesize that there will be a potential response of BCSCs to hypoxic culture conditions. Finally, I hypothesize that there will be a relationship between hypoxia and the induction of drug resistance. It was expected that this project would provide insight to the mechanisms that are involved in the regulation of BCSCs depending on breast cancer subtype and that this could be the basis of promising and more effective therapeutic strategies for the prevention of metastasis and drug resistance in breast cancer.

1.17 General aims

- To determine the effects of EMT induced by TGF-β1 treatment of BCSCs in the MDA-MB-231 and MCF-7 cell lines.
- To investigate the properties of the TGF-β signalling pathway in both cell lines in order to explain the potentially different effect of EMT on the SP cells from these.
- To study the effects of hypoxia induced by $CoCl_2$ treatment on the MDA-MB-231 and MCF-7 BCSCs.
- To examine the possible impact of hypoxia on the transcriptional activation of EMT related genes, stem cell markers and ABC transporter genes.
- To determine the possible relationship of hypoxia with the induction of drug resistance in BCSCs of the MDA-MB-231 and MCF-7 cell lines.
- To test whether there is an interaction between the hypoxia and TGF-β signalling pathways by investigating the combined effect of hypoxic and TGF-β on BCSCs in the MDA-MB-231 and MCF-7 cell lines.

Chapter 2: Materials & Methods

2.1 General Laboratory Practice

All experimental procedures were carried out according to the Control of Substances Hazardous to Health (COSHH and BIOCOSHH) regulations. All work was conducted in accordance with Newcastle University Safety policies. Tissue culture was performed in compliance with the regulations related to containment of class II pathogens.

2.2 Tissue culture

2.2.1 Definition of cell lines used

MDA-MB-231

MDA-MB-231 is a breast cancer cell line isolated from pleural effusions of a 51 year old female. This cell line is ER-/PR-/HER2- (Cailleau *et al.*, 1978).

MCF-7

MCF-7 is a breast cancer cell line obtained from a pleural effusion of a 69 year old Caucasian woman with MBC. This cell line is ER+/PR+/HER2- (Levenson and Jordan, 1997).

Human Mammary Epithelial Cells (HMEpC)

HMEpC are derived from normal adult mammary glands. They are cryopreserved at fifth passage and can be propagated 16 population doublings.

2.2.2 Validation of cell lines

The MDA-MB-231 cell line from MD Anderson were validated by short tandem repeat (STR) DNA fingerprinting using the AmpF_STR Identifier kit according to manufacturer's instructions (Applied Biosystems). STR profiles were compared to known ATCC fingerprints, and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808. The STR profiles matched known DNA fingerprints or were unique. Cell lines from ECACC were validated using the Applied Biosciences Identifier Plus system for DNA profiling (Applied Biosystems). STR profiles were compared to those held on ECACC's database. The authentication of the MCF-7 cell line was performed by ECACC using the AmpFISTR®SGM Plus® PCR amplification kit and the ABI Prism 3730 genetic analyser (SOP ECACC/047).

2.2.3 Cell culture conditions and maintenance

Human breast cancer cell lines, MDA-MB-231 (provided by MD Anderson) and MCF-7 (purchased from ECACC) were cultured in the presence of complete media (cDMEM) which is DMEM (Sigma) without phenol red, supplemented with 10% FBS (Lonza), 2mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen). The cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. All procedures were carried out under aseptic conditions in a class II laminar flow hood (SAFE 2020, Thermo Scientific) and all working areas were decontaminated by using 70% ethanol. Medium was changed every 4-5 days and cells were split when ~80% confluent. For maintenance of cell lines; DMEM was discarded and the flasks were washed with 1X PBS. 1X trypsin EDTA (Sigma) (T/E) was added for approximately 3 min. T/E was neutralized with the addition of sufficient amount of cDMEM and the mixtures were centrifuged at 3000 rpm, 4^{o}C for 5 min. The supernatant was discarded and the pellet was re-suspended in cDMEM. The cell suspensions were transferred into new flasks containing cDMEM.

2.2.4 Cryopreservation of cells

1x10^{6} cells were re-suspended in 1ml aliquots of recovery cell culture freezing medium (Gibco). Cells were cryopreserved in 2 ml cryogenic vials which were placed in a cell freezing container [Nalgene] containing 250ml of 100% isopropyl alcohol [VWR BD Prolabo] and stored at -80^{o}C. This ensures successful cell cryopreservation and recovery by providing -1°C/minute cooling rate. Frozen stocks were rapidly thawed and the recovery cell culture freezing medium was washed with 1 x Phosphate Buffered Saline (PBS) and centrifuged at 3000 rpm, 4^{o}C for 5 min. Pellets were re-suspended in cDMEM and cells were cultured as described above.

2.2.5 Cell counting

Cells were counted prior to cryopreservation or experiments using a haemacytometer (Scientific Laboratory Supplies). 10 μl of cell suspension were diffused under a coverslip at both edges of the haemacytometer. The number of cells in the 25 squares of the grid were counted and multiplied by 1 x 10^{4}, in order to get the total cell number per 1ml of media.

2.2.6 Mycoplasma detection and treatment

Cells were routinely tested for mycoplasma contamination (every 4 months) using the MycoAlert^{TM} mycoplasma detection kit by Lonza. This is a selective biochemical test

that detects the activity of certain mycoplasmal enzymes that react with the MycoAlert™ substrate leading to the conversion of ADP to ATP. An increase of ATP is indicative of mycoplasma contamination and it can be assessed by measuring the increased light intensity emitted in the following bioilluminescent reaction catalyzed by luciferase: ATP + Luciferin + O_2 → Oxyluciferin + AMP + PPi + CO_2 + Light. 100 µl of the MycoAlert Reagent were added to 100 µl of cell culture supernatant followed by a 5 min incubation and then Reading A was taken. 100 µl of the MycoAlert™ substrate were added to the sample and followed by a 10 min incubation before taking Reading B. Measuring the emitted fluorescence before (Reading A) and after (Reading B) the addition of the MycoAlert™ substrate can allow the calculation of the ratio: Reading A/Reading B. A ratio of 1 was indicative of an uninfected culture, while a ratio higher than 1 identified the presence of mycoplasma contamination.

2.2.7 TGF-β treatment

1 x 10^5 cells were seeded in 100-mm dishes. After 24 h cells were treated with 5 ng/ml or 10 ng/ml TGF-β1 (R & D Systems) for 72 hours. 5 ng/ml and 10 ng/ml were chosen as the optimal concentrations in the MDA-MB-231 and MCF-7 cells, respectively (data presented in Chapter 3). Cells treated in parallel with the carrier only were used as controls. 1, 3 and 5 µM SB-505124 (Sigma-Aldrich), a selective inhibitor of the TGFB-RI receptor, was added 30 min prior to TGF-β1 treatment to separate cells cultured under the same conditions to confirm the inhibition of TGF-β1. 5 µM SB-505124 was chosen as the optimal concentration (data presented in Chapter 3). After 72 hours cells were harvested for further analysis.

2.2.8 Cobalt (II) chloride hexahydrate ($CoCl_2$) treatment

$CoCl_2$ has been shown to up-regulate the expression of HIF-1α by affecting the intracellular ascorbate concentration and thus it promotes iron oxidation and inactivation of prolyl hydroxylase (Salnikow *et al.*, 2004). 2 x 10^5 cells were plated in 100 mm dishes. After 24 hours cells were treated with 200, 400 and 600 µM $CoCl_2$ (Sigma-Aldrich), while the 400 µM was chosen as the optimal concentration for all experiments (data presented in Chapter 4). Untreated cells were used as controls. Cells were harvested after 24h for qPCR and after 48 h for protein or SP analysis.

2.2.9 Mitoxantrone treatment

Mitoxantrone is an anthracenedione antineoplastic agent, which is used for the treatment of certain types of cancer, including metastatic breast cancer, acute myeloid leukaemia, and non-Hodgkin's lymphoma (Katzung, 2006). 5 x 10^5 cells were plated in 100 mm dishes. After 24 hours cells were treated with 0.5, 1 and 2 μg/ml mitoxantrone, while 1 μg/ml was chosen as the optimal concentration (data presented in following section). Cells were harvested after 48h as previously optimized by Britton et al (Britton *et al.*, 2012) for SP analysis.

2.3 Cell viability assessment using the MTS assay

The CellTiter 96® AQ_{ueous} One Solution Reagent containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) was used for the assessment of cell viability in the presence of $CoCl_2$ and mitoxantrone. MTS is reduced by dehydrogenase enzymes of viable cells producing formazan, which is soluble in cell culture medium. The amount of the formazan product is directly proportional to the number of living cells in culture (Promega Website).

2,000 cells in 200 μl of complete media per well were plated in a 96-well plate and incubated for 24 h. The media was then removed and replaced with media containing 200, 400 and 600 μM $CoCl_2$ or 0.5, 1 and 2 μg/ml mitoxantrone. Untreated cells were used as controls. Cells were treated for 48 h and the media was removed and replaced with 100 μl of fresh media. 20 μl MTS was added to each well followed by a 2 hour incubation at 37^{o}C with 5% CO_2. The absorbance was read at 492 nm in a Thermo Multiskan Ascent ELISA plate reader. Media only controls were used in triplicate and the average of these was subtracted from all absorbance values. The percentage of living cells was calculated according to the following equation:

% Living cells = (Absorbance of treated cells/Absorbance of untreated cells) x 100

The concentration of 1 μg/ml mitoxantrone and 400 μM $CoCl_2$ were preferred for all future experiments (Figures 2.1 and 2.2).

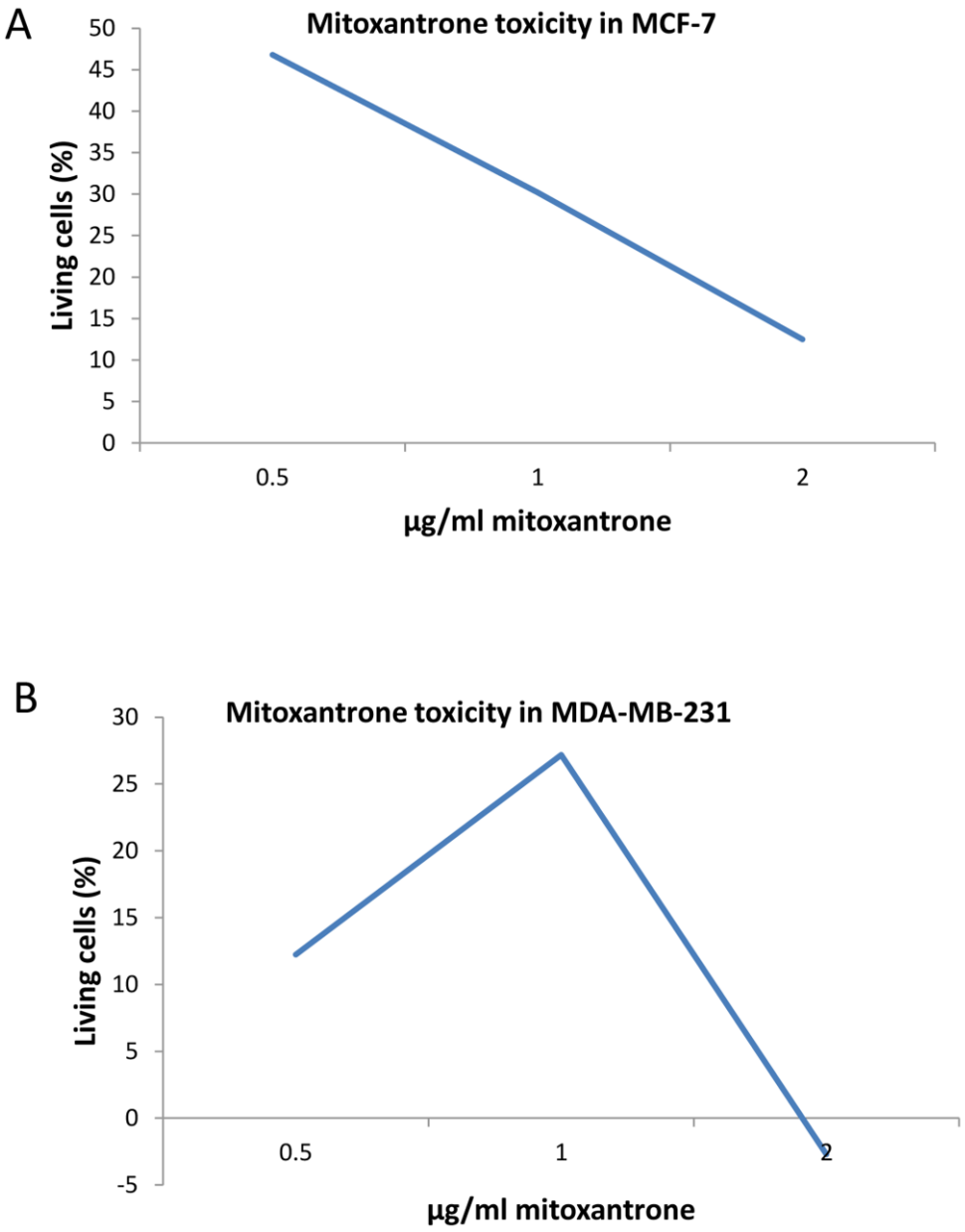


Figure 2.1: Percentages of living cells in the presence of 0.5, 1 and 2 µg/ml mitoxantrone in A) MCF-7 and B) MDA-MB-231 cells (n=1).

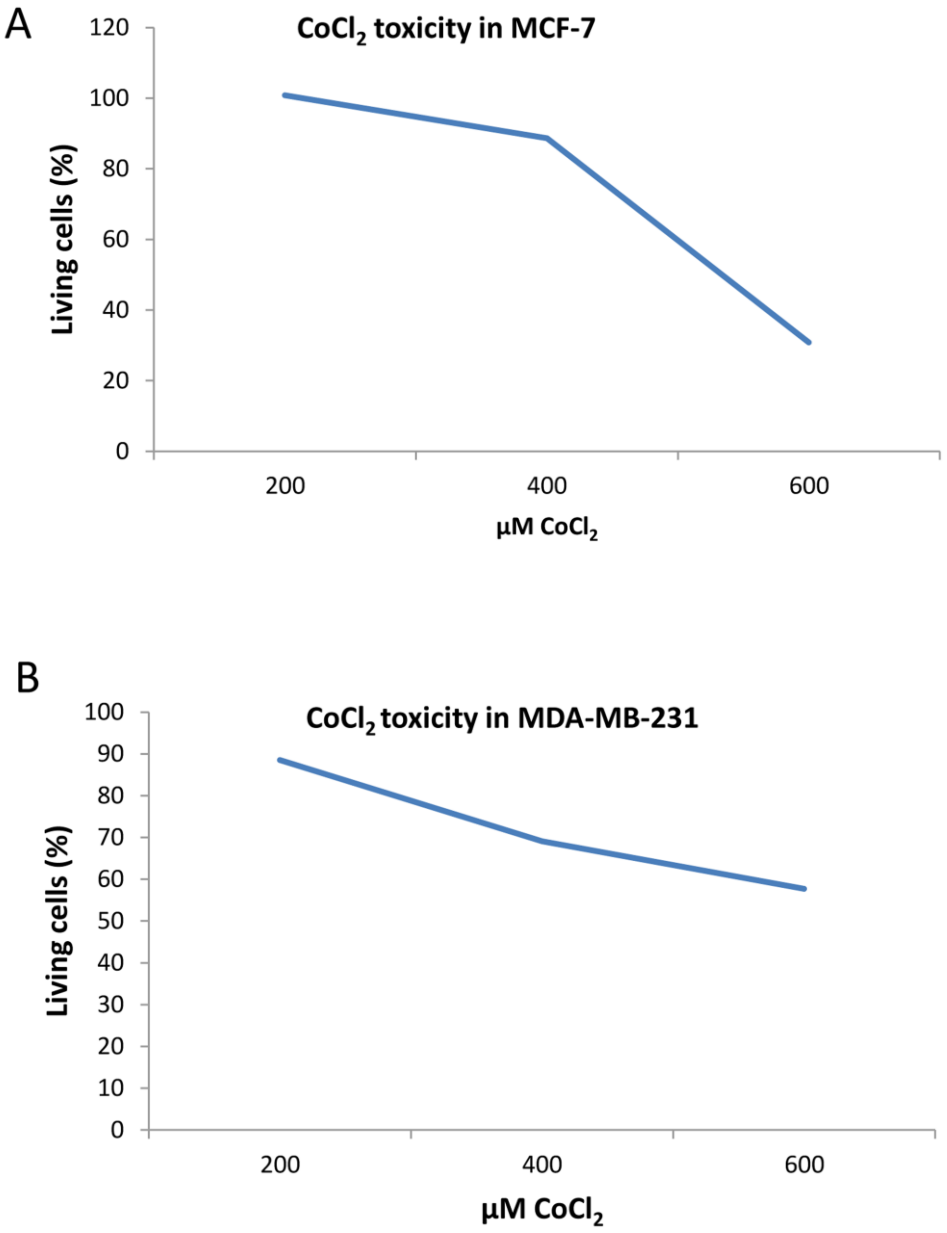


Figure 2.2: Percentages of living cells in the presence of 200, 400 and 600 µM $CoCl_2$ in A) MCF-7 and B) MDA-MB-231 cells (n=1).

2.4 RNA extraction

2.4.1 RNA isolation from cells

RNA extraction was performed using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. 1 x 10^6 cells were lysed in a total volume of 350 µl of RTL buffer containing 10% β-mercaptoethanol and vortexed for 30 sec. Lysate was added to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 sec at 11,000 rpm. The column was discarded. 350 µl of 70% ethanol were added and mixed by pipetting. The mixtures were transferred to an RNeasy Min Elute spin column placed in a 2 ml collection tube and centrifuged for 15 sec at 11,000 rpm. The flow through was discarded. 700 µl of RW1 buffer were added and centrifuged for 15 sec at 11,000 rpm. The flow through was discarded. 500 µl of RPE buffer were added and centrifuged for 2 min at 11,000 rpm. The flow through was discarded. 500 µl of 80% ethanol were added and centrifuged for 2 min at 11,000 rpm. The flow through was discarded. The spin column was transferred into a new 2 ml collection tube and centrifuged for 5 min at 11,000 rpm with open lid. The tube and contents were discarded. The spin column was placed in a 1.5 ml eppendorf, 14 µl of RNase free water were added and the columns stayed on ice for 10 min before centrifuging for 1 min at 13,000 rpm. The eluted RNA could be then stored at -80^{o}C.

2.4.2 Estimation of RNA concentration and purity using NanoDrop

The NanoDrop spectrophotometer 2000 (Thermo Scientific) was used for measurement of the RNA concentration. Absorbance at 260nm (A260) measures the RNA concentration and 280nm (A280) measures the protein concentration. The instrument was blanked using 1µl RNAase free water in which the extracted RNA was eluted. 1µl of each sample was applied on the lower arm of the machine and then the readings of the concentrations were recorded. The generally accepted reading for the RNA concentration is ~40 ng/µl and for the A260/A280 ratio is ~2.0.

2.4.3 Determination of the RNA integrity

The RNA integrity was determined by running a 1% agarose gel in 1X Tris/Borate/EDTA (TBE) buffer [Sigma], containing 2 µl ethidium bromide (BIOLINE). The gel run was run at voltage of 85 V for approximately 45 min and visualized under a UV gel documentation system [UVP Ltd, Cambridge, UK]. The total RNA that is isolated includes messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), but almost the 85% consists of rRNA. Therefore, the presence of two clear 18S and 28S rRNA bands that encode for the two ribosomal sub-units is

indicative of a good RNA integrity (Figure 2.3). The intensity of the 28S rRNA band should be approximately twice as higher as the 18S rRNA band. Degraded RNA is expected to appear as a smear.

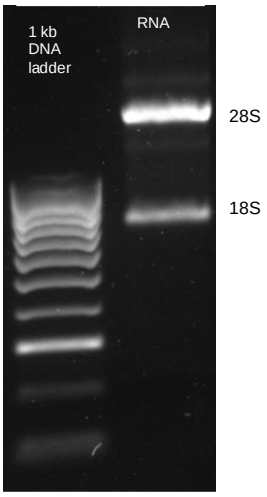


Figure 2.3: Determination of RNA integrity. Lane 1: DNA ladder. Lane 2: RNA from MDA-MB-231 cells.

2.5 cDNA synthesis by reverse transcription

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is used to generate complementary DNA sequences using RNA as a template. Small fragments consisting of T nucleotides (oligo $(dT)_{18}$) are used to ensure binding to the poly-A tale of the mRNA molecules. RT-PCR can be performed in one or two steps, depending on whether the reverse transcription and PCR are carried out in one or in separate reaction tubes, respectively. The BIOLINE cDNA synthesis kit was used for the preparation of cDNA. RNA aliquots were prepared by diluting the RNA samples to get a final concentration of 1000 ng/µl. 1µl of 10mM dNTP, 1µl of oligo$(dT)_{18}$ and RNAase/DNAase free water was added to make the volume up to 10 µl. The mixtures were then incubated at 65^{o}C for 10 min and placed on ice for 2 min. Reverse Transcription master mix was prepared, containing 4 µl 5 X RT buffer, 1µl RNase inhibitor, 0.25 µl reverse transcriptase (200 U/µl) and 4.5 µl DEPC- treated water per reaction. 10 µl of this was then added to each of the RNA samples and mixed by pipetting. The mixture was then incubated at 45^{o}C for 50 min and reactions were terminated by incubating at 70^{o}C for 15 min. The cDNA samples could be then stored at -20^{o}C.

2.6 Polymerase Chain Reaction (PCR)

PCR is a method developed by Kary Mullis in the 1980s. It utilizes the ability of the enzyme DNA polymerase to amplify specific DNA sequences by synthesizing their DNA complementary strand. As a result, at the end of PCR billions of copies of a particular target sequence can be produced. The most commonly used DNA polymerase is the *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus*, which is heat resistant and can perform at high temperatures. This enzyme acts by extending the 3' end of smaller DNA sequences, known as primers, with a 5'→ 3' direction. PCR involves the following 4 steps:

1) Denaturation: This step is achieved at a temperature of 94-96^{o}C for 30 sec and it results in the separation of the two complementary DNA strands by breaking their hydrogen bonds.
2) Annealing: This step consists of the binding of the forward and the reverse primer to complementary sequences on the single stranded RNA (50-60^{o}C for 30 sec) allowing DNA polymerase to bind to their 3' end and begin DNA synthesis.
3) Extension: This step is usually carried out at 72^{o}C and it involves the elongation of the primer sequences, resulting in the production of double PCR products at the end of each PCR cycle. Every synthesized DNA sequence of one PCR cycle becomes the template for the next cycle leading to a chain reaction and the exponential amplification of DNA.
4) Final elongation: After steps 2-3 have been repeated for 20-40 cycles. This step is carried out at 72 ^{o}C for 5 min to ensure that any remaining single stranded DNA will be extended.

2.6.1 Conventional (qualitative) PCR and primer design and optimization

Conventional PCR was carried out for 30, 35 and 40 cycles using a G-storm thermocycler. 25 μl reactions were prepared, containing 12.5 μl of 2X PCR Master Mix (Promega; *Taq* DNA polymerase (50 units/ml), dNTPs and 3mM $MgCl_2$), 10μM Forward primer, 10μM Reverse Primer, 2.5 μl of cDNA and Nuclease Free water (Promega) up to 25 μl. For each primer pair gradient annealing temperatures from 51 to 60^{o}C were used. The thermocycling programme was set at 94°C for 30 seconds for denaturation and at 72°C for 1 minute for elongation. For all reactions a non template control (NTC) and a reaction containing the corresponding RNA template were used to ensure that no genomic DNA was amplified.

All gene sequences and refseq accession numbers were obtained from the National Centre for Biotechnology Information (NCBI) and entered into the Primer-Blast website, in order to get specific primer sets. Only primer sets that spanned an exon-exon junction were selected to avoid genomic contamination. The optimal primer length was between 18 and 22 bp and the CG content was approximately 40-60%. The optimal primer melting temperature (Tm) was in the range of 52-58^{o}C, while the differences in the Tm between the forward and reverse primers of each set did not surpass 2. A maximum product length of 200 bp was preferred. All primers sequences and product lengths are listed in Table 2.1.

Primer optimization was carried out with conventional PCR for 30, 35 and 40 cycles using a G-storm thermocycler. 25 μl reactions were prepared, containing 12.5 μl of 2X PCR Master Mix (Promega; *Taq* DNA polymerase (50 units/ml), dNTPs and 3mM $MgCl_2$), 10μM Forward primer, 10μM Reverse Primer, 2.5 μl of cDNA and nuclease free water (Promega) up to 25 μl. For each primer pair gradient annealing temperatures from 51 to 60^{o}C were used. PCR for GAPDH was used to ensure good cDNA quality, while RNA and nuclease free water were used to ensure genomic contamination free PCR products. The thermocycling programme was set at 94°C for 30 seconds for denaturation and at 72°C for 1 minute for elongation.

Gene name	Primer	Sequence (5'-3')	Product Length (bp)
E-cadherin	Forward	TGCCATTTCCACTCGGGCTG	172
	Reverse	AGGTTCTGGTATGGGGGCGT	
ABCG2	Forward	GAGCGCACGCATCCTGAGAT	157
	Reverse	TCATTGGAAGCTGTCGCGGG	
FOXC2	Forward	TCGCACGCAAGAATCTTACCC	100
	Reverse	ACAGCAACCAAGGACAGGTTT	
Δp63α	Forward	GTGATGATGGTTCACGTTGG	143
	Reverse	ACATGACGTCGGGTGTTTTT	
ABCB1	Forward	CTGACGTCATCGCTGGTTTC	116
	Reverse	ATTTCCTGCTGTCTGCATTGTG A	
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC	150
	Reverse	GCCTTCTCCATGGTGGTGAA	

Table 2.1: List of primer sequences and product lengths.

2.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate a mixed population of DNA or RNA fragments depending on their size. An electric field is applied, so that the negatively charged molecules can move towards the positive electrode, with the smaller fragments migrating faster. 1% agarose gel in 1X Tris/Borate/EDTA (TBE) buffer [Sigma], containing 2 µl ethidium bromide was prepared (BIOLINE). 18 µl of PCR product were mixed with 2 µl of DNA loading dye (Biolabs) and loaded to each well of the agarose gel. The gel run was performed at voltage of 85 V for approximately 45-60

min and PCR products were visualized under a UV gel documentation system [UVP Ltd, Cambridge, UK] (Figure 2.4).

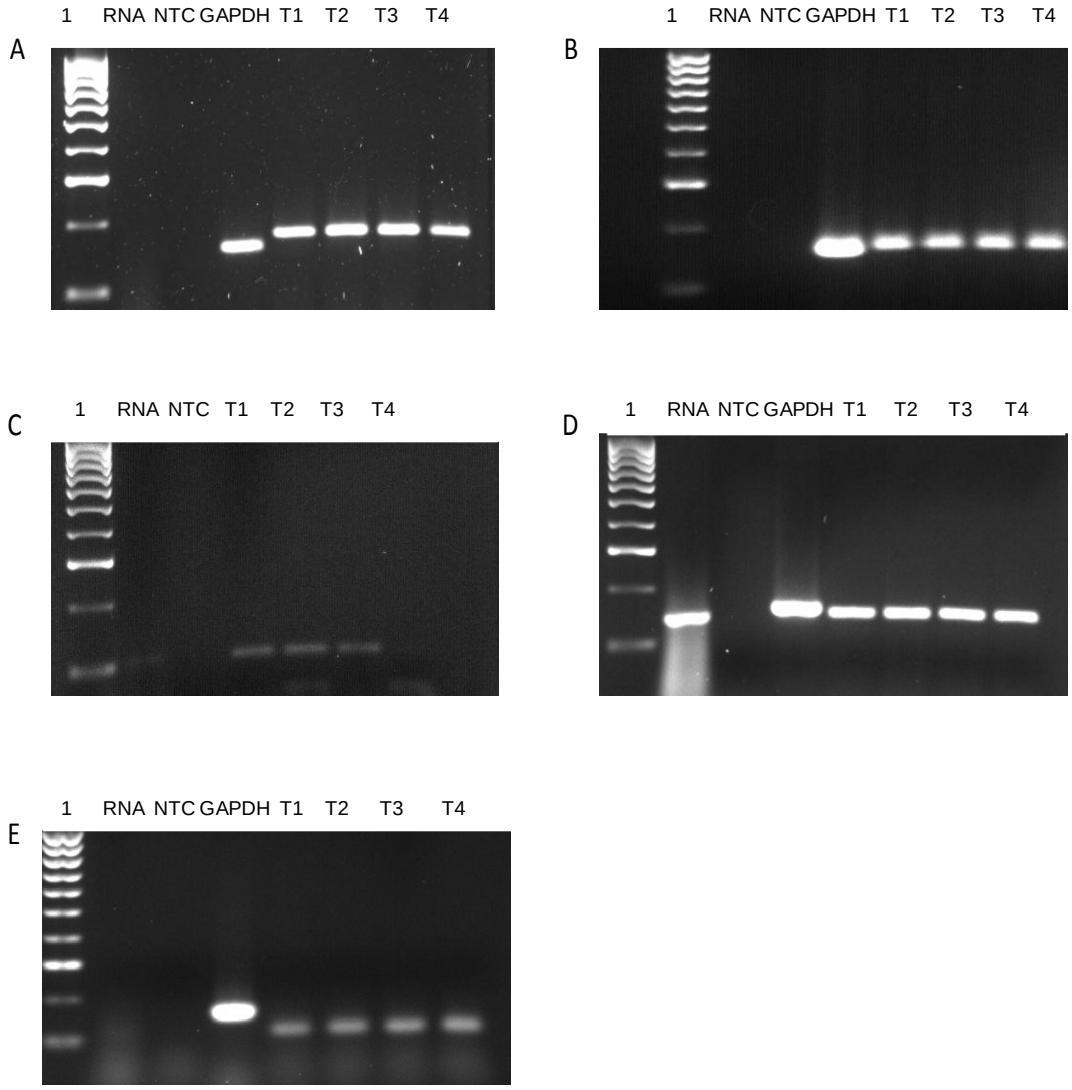


Figure 2.4: PCR products for primer optimization A: E-cadherin, B: ABCG2, C: FOXC2, D: Δp63α E: ABCB1

1: 1 kb DNA ladder, RNA (RNA template), NTC (Non Template Control), T1-T4: gradient annealing temperatures

T1: 51.8 ^{o}C, T2: 53.7 ^{o}C, T3: 56 ^{o}C, T4: 58.6 ^{o}C.

The cDNA template was not sufficient for GAPDH in C, while DNAase treatment needs to be performed prior to the preparation of the PCR reactions in D.

2.6.3 Quantitative Real-time PCR (qPCR)

qPCR has the advantage of measuring the amount of PCR product after each PCR cycle while it is produced. This is achieved by the use of several fluorescent dyes with the fluorescent signal being directly proportional to the amplified PCR products (amplicons). By plotting fluorescence against the cycle number (Ct value) an amplification plot is generated providing information about the accumulation of the product over the duration of the PCR reaction. Therefore, target sequences that are highly expressed can be detected at earlier cycles, while the ones that have a lower expression are detected at later cycles. Reactions are usually performed at 40 cycles with each cycle consisting of the same steps as conventional PCR. qPCR products are quantified in the beginning of the exponential phase as opposed to conventional PCR, in which the final amplicons can be visualized. This provides more accurate data, since all the reagents are still in abundance and DNA polymerase is highly active at this stage.

Two of the main fluorescent technologies commonly used in qPCR are: the SYBR Green dye and the TaqMan- based technology. The SYBR Green dye is a DNA binding dye which binds to double-stranded DNA and generates a stronger fluorescent signal than the unbound dye. However, the specificity of SYBR green is questionable, as it can bind to any double stranded DNA regardless of if it is target or non target. The Taqman-based technology utilizes two gene-specific PCR primers and a TaqMan probe that is attached to a reporter at its 5' end and a quencher at its 3' end. The reporter and the quencher are both fluorescent dyes with the reporter having a longer emission wavelength compared to the quencher. When these two dyes are in close proximity, excitation of the reporter causes its emission energy to be transferred to the quencher, suppressing its signal by a phenomenon called Fluorescent-Resonance Energy Transfer (FRET). During PCR, the 5' nuclease activity of Taq DNA polymerase cleaves the probe and releases the reporter from the quencher allowing it to emit its fluorescence (Life Technologies, 2014) (Figure 2.5).

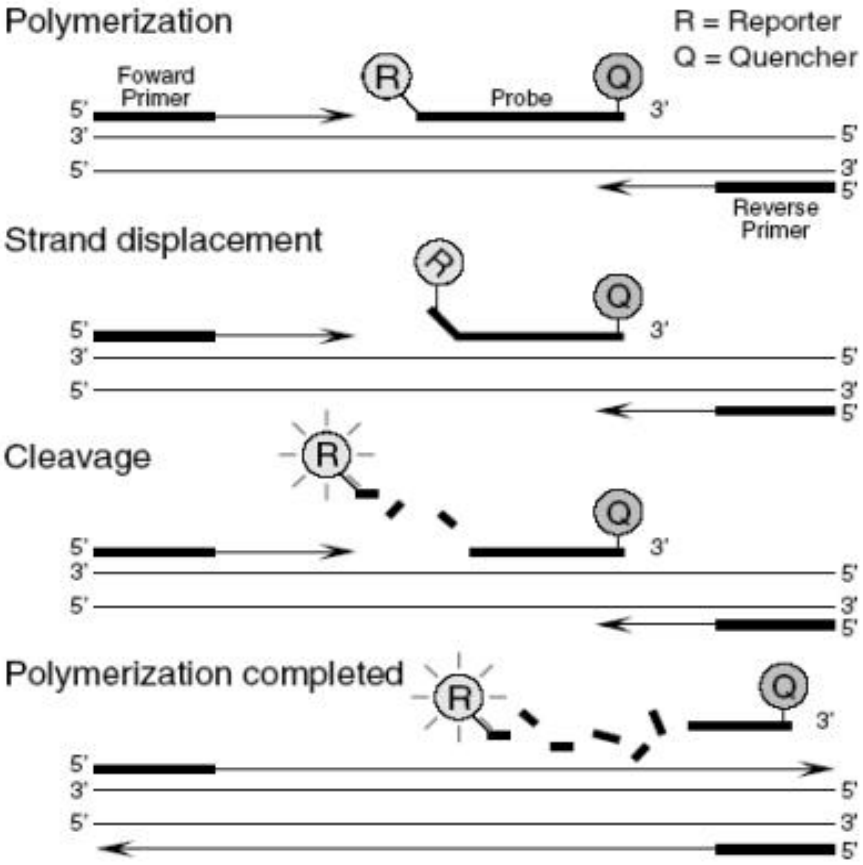


Figure 2.5: The principles of qPCR (German Cancer Research Centre Website, 2013)

2.6.4 Preparation of qPCR reactions and data analysis

20 µl qPCR reactions were prepared in triplicates for each sample, containing 2µl cDNA, 1µl primer probes (Applied Biosystems), 10µl Brilliant II QPCR master mix with High ROX (Agilent) and DNase-free water (Promega) to make up a total volume of 20µl. NTCs were used to ensure that all procedures were free of DNA contamination. All reactions were pipetted in triplicate into a 96-well plate (Applied Biosystems), covered with plastic seal and centrifuged briefly. The run was performed in an Applied Biosystems Step One thermocycler at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The comparative ΔΔCt method was used for the assessment of the expression levels of each gene of interest in comparison to the expression of the housekeeping gene (beta-actin) in both the controls and the samples. This method involves the following calculations:

ΔCt= Ct of the gene of interest – Ct of the housekeeping gene

ΔΔCt= ΔCt of sample – ΔCt of control

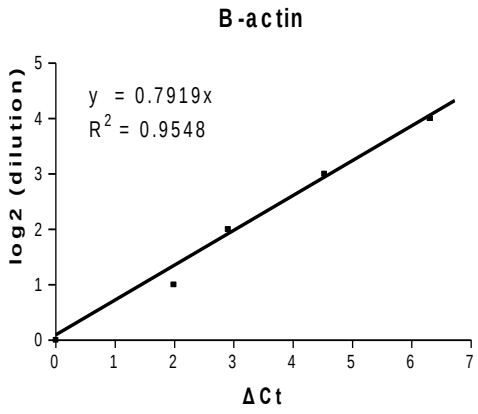
Fold change= $2^{\wedge -\Delta\Delta Ct}$

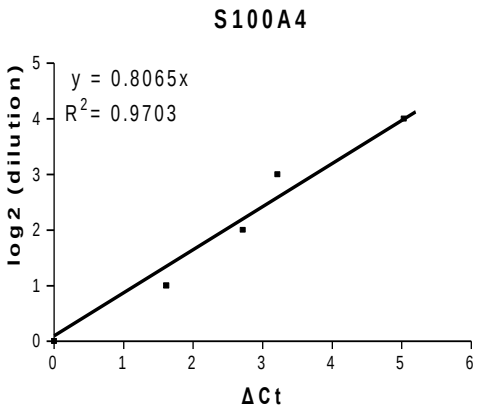
2.6.5 Validation of efficiency of primer probes for qPCR

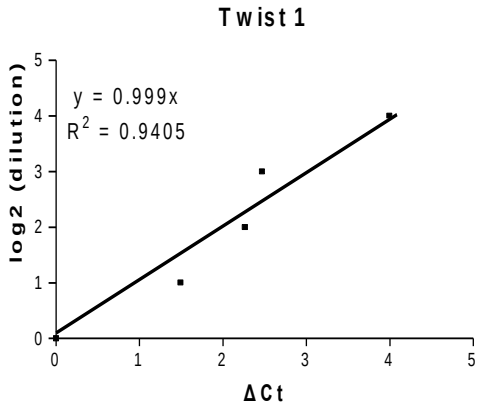
Taqman gene expression assays (Applied Biosystems) were used as primer probes (Table 2). The efficiency of each primer probe was validated by the use of serial dilutions of the cDNA (1:10, 1:20, 1:40, 1:80, 1:160), 1µl primer probes (Applied Biosystems), 10µl Brilliant II QPCR master mix with High ROX (Agilent) and DNase-free water (Promega) to make up a total volume of 20µl. All reactions were pipetted in triplicate into a 96-well plate (Applied Biosystems), covered with plastic seal and centrifuged briefly. The run was performed in an Applied Biosystems Step One thermocycler using the thermocycling parameters mentioned above. The ΔCt values were plotted against the logarithmic value of 2 of each dilution and analyzed for linear regression curve with 95% confidence interval (Figure 2.6). The x value (slope) of the equation was considered as the efficiency of each primer set. The acceptable value for the efficiency was ~90%. Cell lines that express ABCB5 and VEGF-A at high levels were not available, therefore the validation of the qPCR probes for these genes was not successful (Table 2.2).

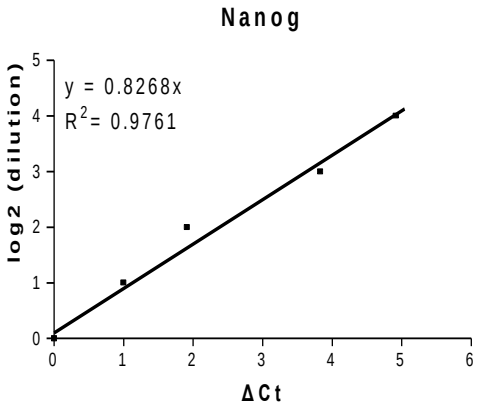
Assay ID	Gene name	Cells used	Efficiency (%)
Hs01060665_g1	ACTB (Beta Actin)	MCF-7	79.1
Hs01053049_s1	SOX2	MCF-7	84.5
Hs04260366_g1	NANOG	MCF-7	82.6
Hs00243202_m1	S100A4	MCF-7	80.6
Hs00270951_s1	FOXC2	Endothelial cells	84.8
Hs00195591_m1	SNAI1 (Snail)	HEYA8MDR	78.7
Hs00950344_m1	SNAI2(Slug)	HTCEC	96.7
Hs01675818_s1	TWIST1	MCF-7	99.9
Hs02379973_s1	TWIST2	HEYA8MDR	88
Hs00999632_g1	POU5F1 (Oct4)	MCF-7	89.9
Hs00900055_m1	VEGFA	*MDA-MB-231	*63.5
Hs00607978_s1	CXCR4	HEYA8MDR	91.7
Hs00153153_m1	HIF1A	MCF-7	85.1
Hs01053790_m1	ABCG2	MDA-MB-231	87.3
Hs00978473_m1	ABCC3	MCF-7	87.5
Hs02889060_m1	ABCB5	*	*
Hs00184500_m1	ABCB1	HEYA8MDR	84.9
Hs01075861_m1	CD44	MDA-MB-231	90.9
Hs00185584_m1	VIMENTIN	MDA-MB-231	78.3
Hs01023894_m1	E-CADHERIN	MCF-7	75.8

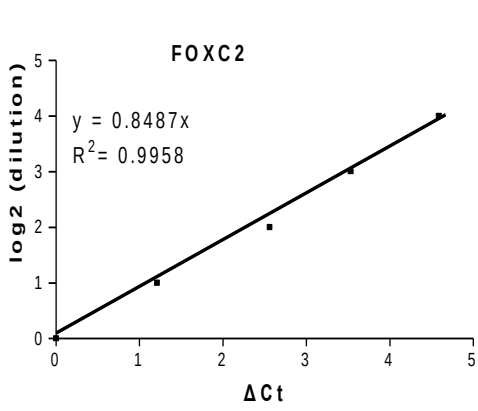
Table 2.2: Taqman Gene Expression assay IDs and efficiencies. * indicates no availability of the appropriate cDNA template.

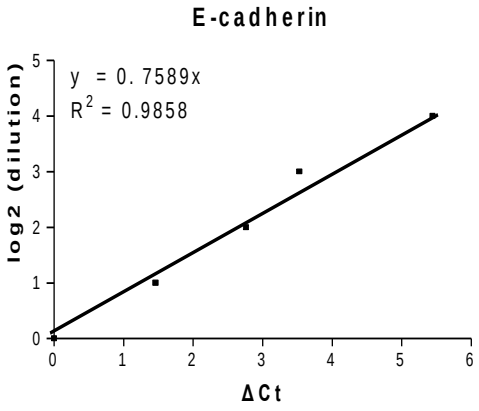

B-actin
y = 0.7919x
R2 = 0.9548
log2 (dilution)
ΔCt


S100A4
y = 0.8065x
R2= 0.9703
log2 (dilution)
ΔCt


Twist 1
y = 0.999x
R2 = 0.9405
log2 (dilution)
ΔCt


Nanog
y = 0.8268x
R2= 0.9761
log2 (dilution)
ΔCt


FOXC2
y = 0.8487x
R2= 0.9958
log2 (dilution)
ΔCt


E-cadherin
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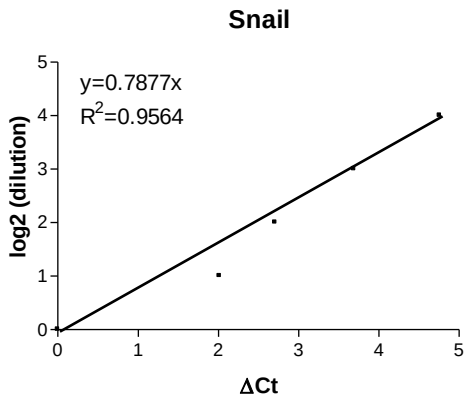
R2 = 0.9858
log2 (dilution)
ΔCt


Snail
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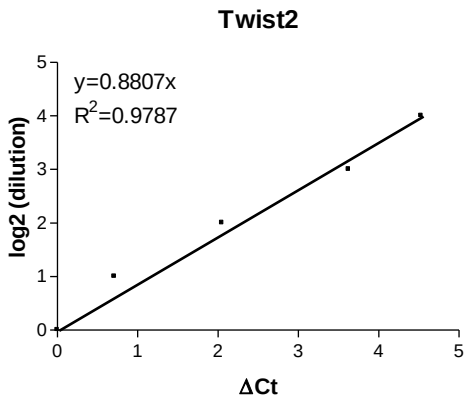
log2 (dilution)
ΔCt


Twist2
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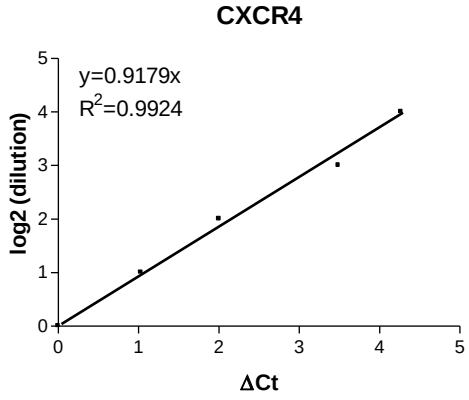
R2=0.9787
log2 (dilution)
ΔCt


CXCR4
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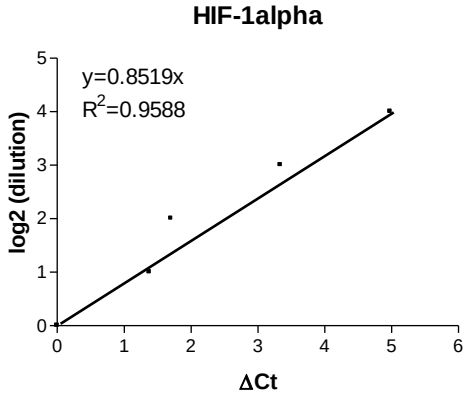
log2 (dilution)
ΔCt


HIF-1alpha
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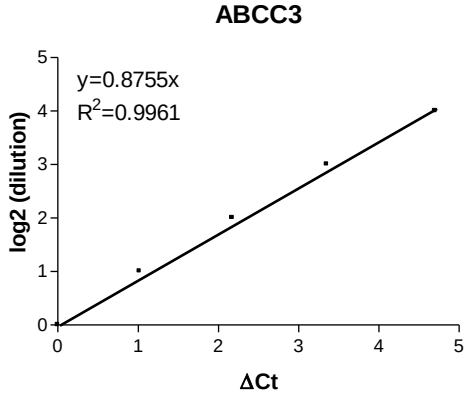
R2=0.9588
log2 (dilution)
ΔCt


ABCC3
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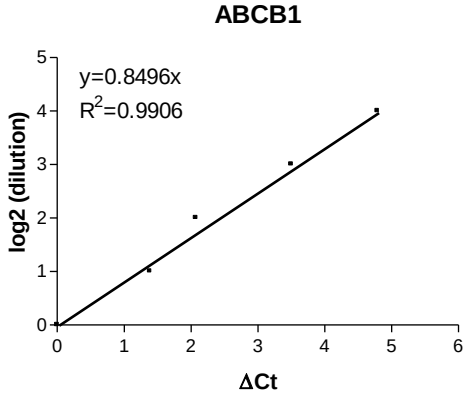
R2=0.9961
log2 (dilution)
ΔCt


ABCB1
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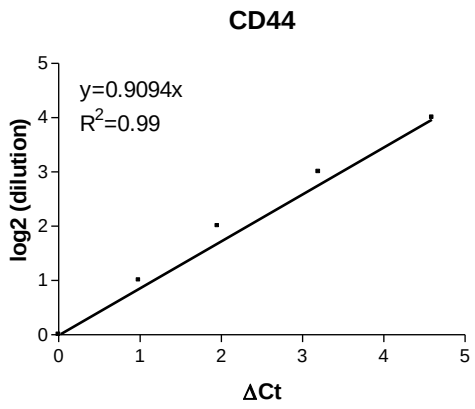
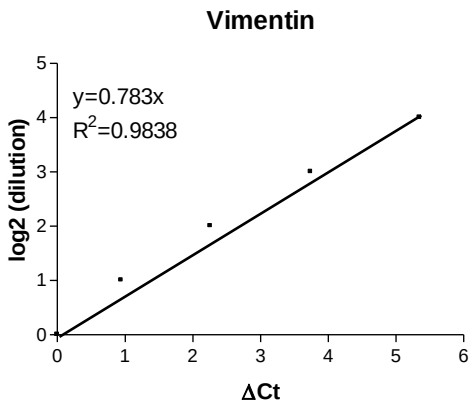
R2=0.9906
log2 (dilution)
ΔCt


CD44
y=0.9094x
R2=0.99
log2 (dilution)
ΔCt


Vimentin
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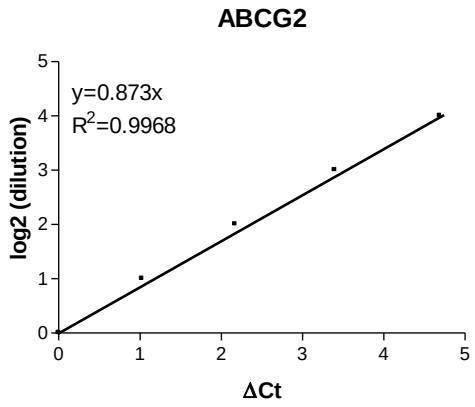
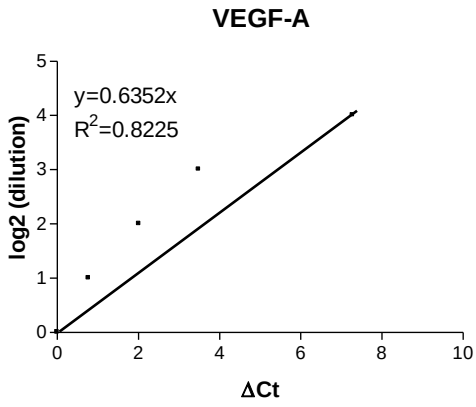
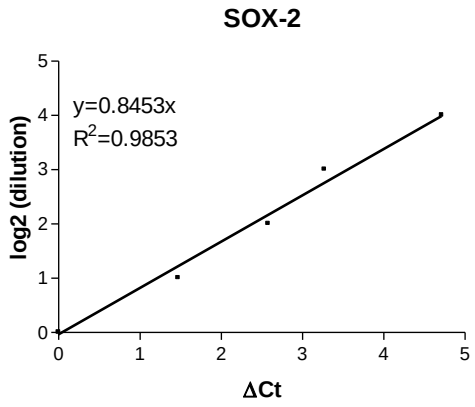
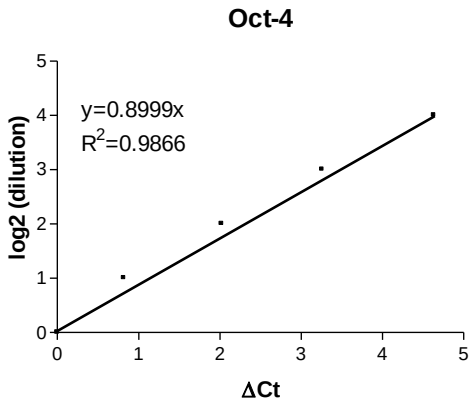
R2=0.9838
log2 (dilution)
ΔCt


ABCG2
y=0.873x
R2=0.9968
log2 (dilution)
ΔCt


VEGF-A
y=0.6352x
R2=0.8225
log2 (dilution)
ΔCt


SOX-2
y=0.8453x
R2=0.9853
log2 (dilution)
ΔCt


Oct-4
y=0.8999x
R2=0.9866
log2 (dilution)
ΔCt

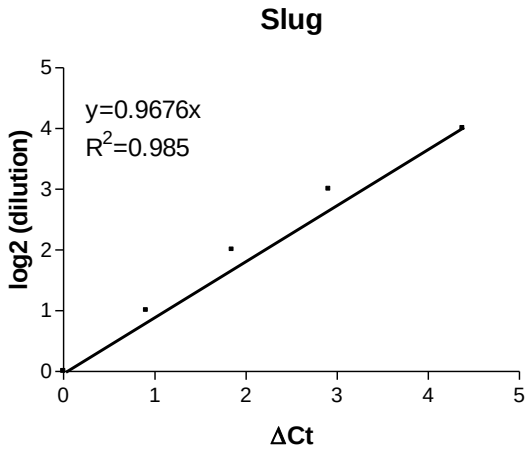


Figure 2.6 Graphical representation of efficiency curves for qPCR primer probes

2.7 Flow cytometry: Definitions and principles

Flow cytometry is a technology that analyzes multiple properties of single particles, most commonly cells, with a size of 0.2-150 μm as they flow through a beam of light. An optical-to electronic coupling system enables the measurement of the size, granularity or internal complexity and the relative fluorescence intensity of these particles. A flow cytometer consists of three main systems: 1) the fluidics system which transports particles to the laser beam. 2) the optics system in which the lasers illuminate the particles and the optical filters direct the scattered light to the detectors and 3) the electronics system which converts all the detected light signals into electronic signals that can be analyzed by a computer.

2.7.1 Forward Scatter (FSC) and Side Scatter (SSC)

When the light strikes the single-file particles it scatters depending on the particle's size and internal complexity. FSC is proportional to the cell surface area or size of the particle. FSC allows the identification of particles with a bigger size and it can be used in many applications, including immunophenotyping. SSC is proportional to the cell granularity and internal complexity. SSC is usually collected at 90^{o} to the laser beam. Combined FSC and SSC measurements can be used to distinguish different cell types within a heterogeneous cell population.

2.7.2 Fluorescence

Fluorescence is termed as the transition of energy from an excited electron of a fluorescent compound when this absorbs light from a light source with the appropriate wavelength. The range of emitted wavelengths for a particular compound is known as emission spectrum. Multiple fluorescent compounds can be analyzed as long as their emission wavelengths do not overlap. When a fluorescent dye is conjugated with a monoclonal antibody, it can be used to identify a particular cell type that expresses cell surface antigens specific to this antibody.

2.7.3 Principles of cell sorting

Cell sorting allows the collection of the cells of interest for further analysis. This process requires the identification of distinct cell populations within a heterogeneous cell population. Gates are used to indicate which cells will be sorted. Droplet sorting is an effective technique for distributing cells into collection containers. A single stream is vibrated to form uniform droplets downstream with each one of these containing a single particle of interest. The droplets pass through the laser beam one by one and an electrical charge is applied to each one of them as it approaches the break off point. As droplets pass between electrical plates, charged droplets are sorted into a collection tube, while uncharged droplets pass into the waste aspirator (Figure 2.7).

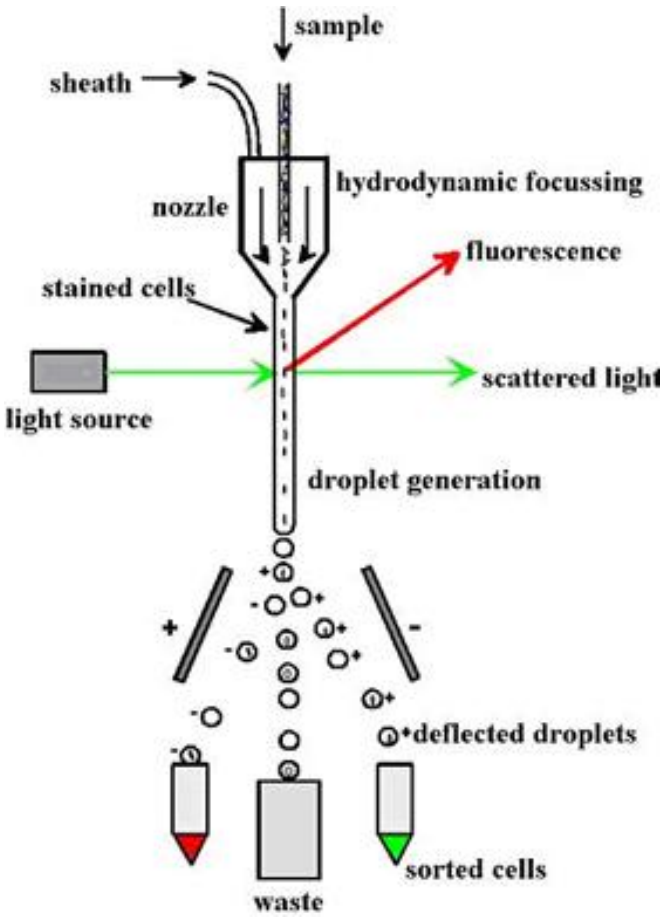


Figure 2.7: The principles of cell sorting (Applied Cytometry Website, 2013)

2.7.4 SP assay

The SP assay was first introduced by Goodell in 1996 who used it on murine bone marrow cells (Goodell *et al.*, 1996). This assay is based on the differential ability of cells to export the Hoechst 33342 dye through members of the ABC transporter family of proteins (Golebiewska *et al.*, 2011). The SP phenotype has mainly been associated with high expression of ABCB1, ABCG2 and ABCC1 (Schinkel, 1999; Zhou *et al.*, 2001; Sun *et al.*, 2003; Robey *et al.*, 2009). This subpopulation of cells is thought to be enriched in stem cells and the SP assay has been used for the identification of such cells in several types of tissue, including umbilical cord blood (Storms *et al.*, 2000) skeletal muscle (Asakura *et al.*, 2002), kidney (Iwatani *et al.*, 2004), mammary gland (Clayton *et al.*, 2004) and others. Furthermore, several ABC transporters are distributed in tissues all over the human body contributing to the protection of these tissue cells from xenobiotics. These transporters are normally expressed at essential pharmacological barriers, such as the brush border membrane of intestinal cells or the epithelium that contributes to the BBB (Gottesman, 2002). Of note, it has been suggested that the ability of SP cells to more actively efflux the Hoechst 33342 dye could be the mechanism by which chemotherapeutic drugs are exported, leading to drug resistant tumors. Therefore, SP cells could also represent a putative cancer stem cell population (Hirschmann-Jax C, 2005).

2.7.5 Hoechst 33342 dye and mechanism of action

The Hoechst 33342 dye is a DNA binding dye that specifically binds to AT-rich regions withing the minor groove of the DNA (Lalande and Miller, 1979). This dye can be passively diffused in all cells, but only the ones that express sufficient levels of ABC transporters can actively export it. In the presence of an ultraviolet (UV) laser Hoechst 33342 emits fluorescence that can be visualized at two distinct wavelengths: one at 450/50 nm (Hoechst blue) and one at 675/20 nm (Hoechst red), while a dichroic mirror is used to split these two wavelengths. As a result, SP cells appear as a tail near the G0/G1 cells, while several inhibitors (verapamil:specific to ABCB1 and ABCG2, Fumitremorgin C:specific to ABCG2) block the dye efflux by competing with the binding of the Hoechst dye and are used to confirm the SP phenotype, as they restore the intracellular accumulation of the dye (Golebiewska *et al.*, 2011) (Figure 2.8).

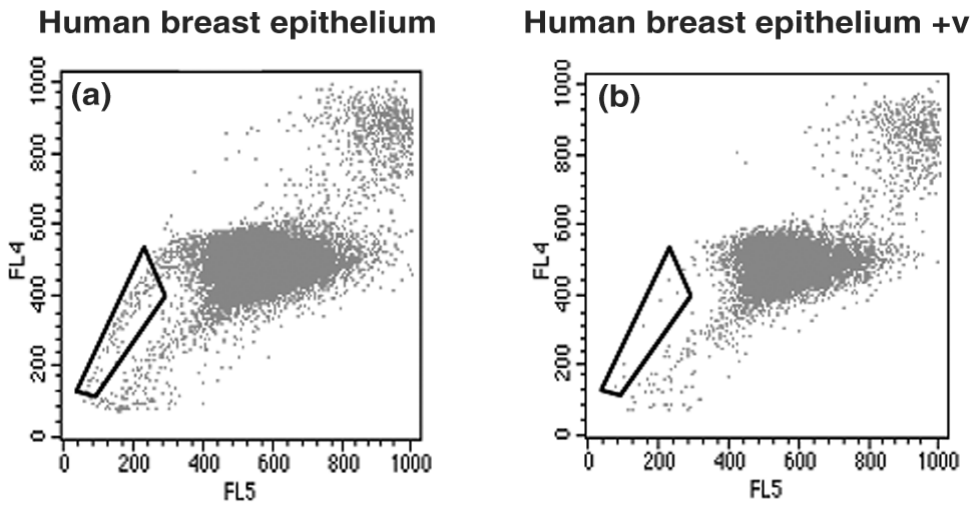


Figure 2.8: SP profile of human breast epithelial cells using Hoechst staining. **A.** Clear discrimination of the SP population, representing approximately 0.25% **B.** Note the 12-fold reduction of the SP population after the addition of verapamil (Alvi *et al.*, 2002).

2.7.6 SP identification in breast cancer cell lines; optimization of the Hoechst 33342 dye concentration

One of the most critical factors that affects the efficiency of the SP assay is the concentration of the Hoechst 33342 dye, as unsaturated Hoechst staining can result in a mistaken SP profile and oversaturated Hoechst staining can even cause increased cell death or loss of the SP phenotype (Montanaro *et al.*, 2004). Cells were re-suspended to give a final concentration of 1 x 10^6 cells/ ml in pre-warmed complete DMEM media. 5 µl of DNase (Ambion) was added to each tube to prevent cell clumping. 5 µl of FTC (10 mM; Axxora) was added to one tube containing 1 x 10^6 cells/ ml and incubated with all other tubes at 37^{o}C on a MACsMix rotor (Miltenyi Biotec) housed within a standard TC incubator for 15 min, after this 3µg/ml, 5µg/ml and 7µg/ml of Hoechst 33342 (1µg/ml; Sigma-Aldrich) were added into separate tubes. 5µg/ml of Hoechst 33342 was added to the tube containing the inhibitor, all samples were then incubated rotating at 37^{o}C in a TV incubator for 90 min and then washed in ice-cold 1X PBS and centrifuged at 2500 rpm for 5 min. Cells were re-suspended in 700 µl ice-cold 1X PBS and filtered through 70 µm cell strainers (BD Biosciences) into sterile FACs tubes (BD falcon). The cells were maintained on ice in the dark and prior to cell analysis, non-viable cells were excluded by the addition of 2µl propidium iodide (2µg/ml) (Sigma). LSRII flow cytometer (BD Biosciences) was used for SP assays and the FACS Diva software was used for the analysis of the data. 5 µg/ml and 7 µg/ml Hoechst were used for the MDA-

MB-231 and the MCF-7 cells, respectively for all future experiments (Figures 2.9 and 2.10).

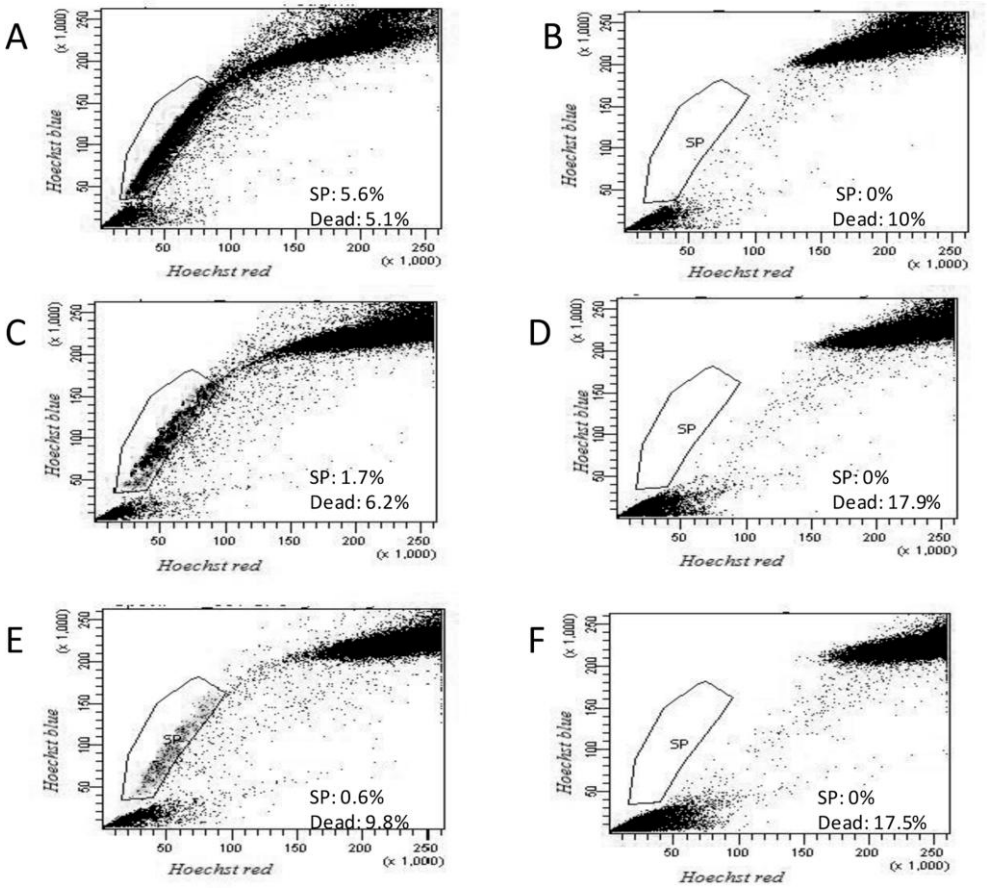


Figure 2.9: FACS profiles for the identification of SP cells in the MCF-7 breast cancer cell line using dual-wavelength flow cytometry combined with **A)** 3µg/ml Hoechst dye. **C)** 5µg/ml Hoechst dye. **E)** 7µg/ml Hoechst dye. **B)**, **D)** and **F)** represent the inhibited samples of A, C and E, respectively upon the addition of 10µm FTC. Note the absence of SP cells in the inhibited samples. Dead cells were excluded by the addition of propidium iodide prior to analysis (not shown).

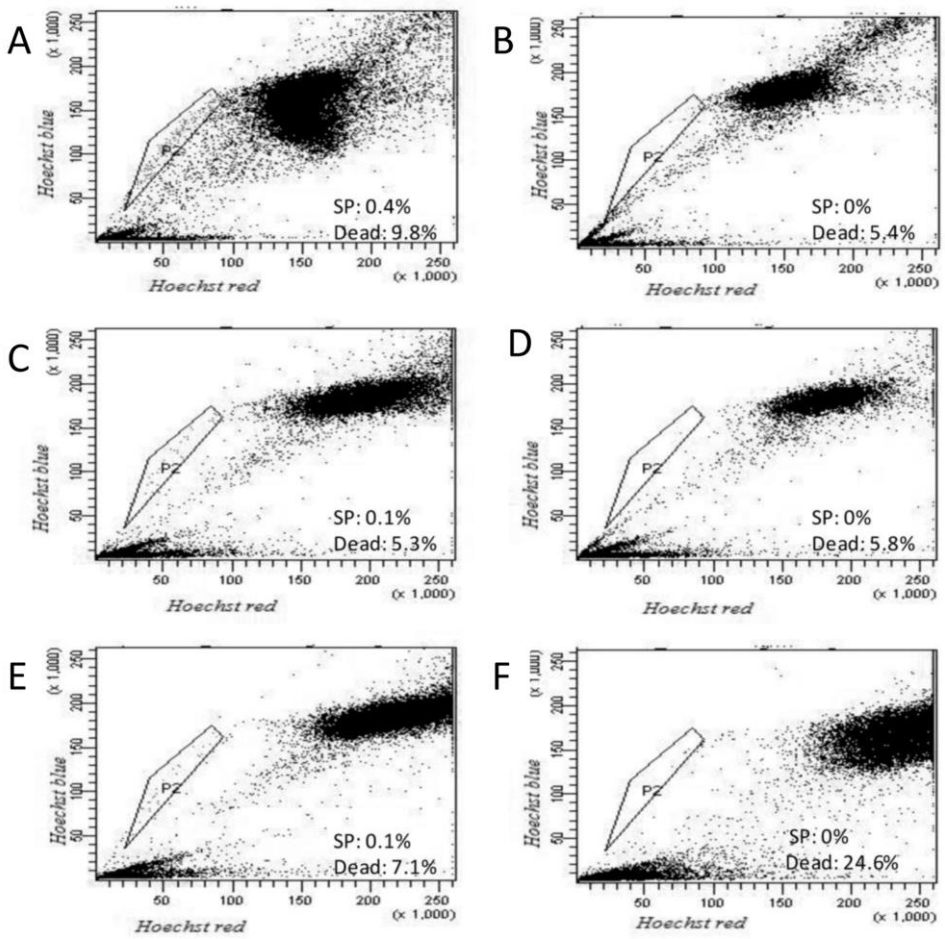


Figure 2.10: FACS profiles for the identification of SP cells in the MDA-MB-231 breast cancer cell line using dual-wavelength flow cytometry combined with **A)** 3µg/ml Hoechst dye. **C)** 5µg/ml Hoechst dye. **E)** 7µg/ml Hoechst dye.**B)**, **D)** and **F)** represent the inhibited samples of A, C and E, respectively upon the addition of 10µm FTC. Note the absence of SP cells in the inhibited samples. Dead cells were excluded by the addition of propidium iodide prior to analysis (not shown).

2.7.7 Determination of the effects of confluency on the SP numbers in breast cancer cell lines

Cell density is another important factor that requires optimization to ensure the successful identification of SP cells (Tavaluc *et al.*, 2007). For each cell line 1, 2, 4 and 6 X 10^6 cells were seeded into T75 flasks in cDMEM. 48 h later cells were harvested and stained with the optimal concentrations of Hoechst 33342 for SP isolation. Negative control samples containing 5 µl of FTC (10 mM) and Hoechst 33342 were also used. The cells were maintained on ice in the dark and prior to cell analysis, non-viable cells were excluded by the addition of 2µl propidium iodide (2µg/ml) (Sigma). LSRII flow cytometer (BD Biosciences) was used for SP assays and the FACS Diva software was used for the analysis of the data. The lowest cell numbers were used for future SP assays to ensure high SP percentages (Figures 2.11 and 2.12).

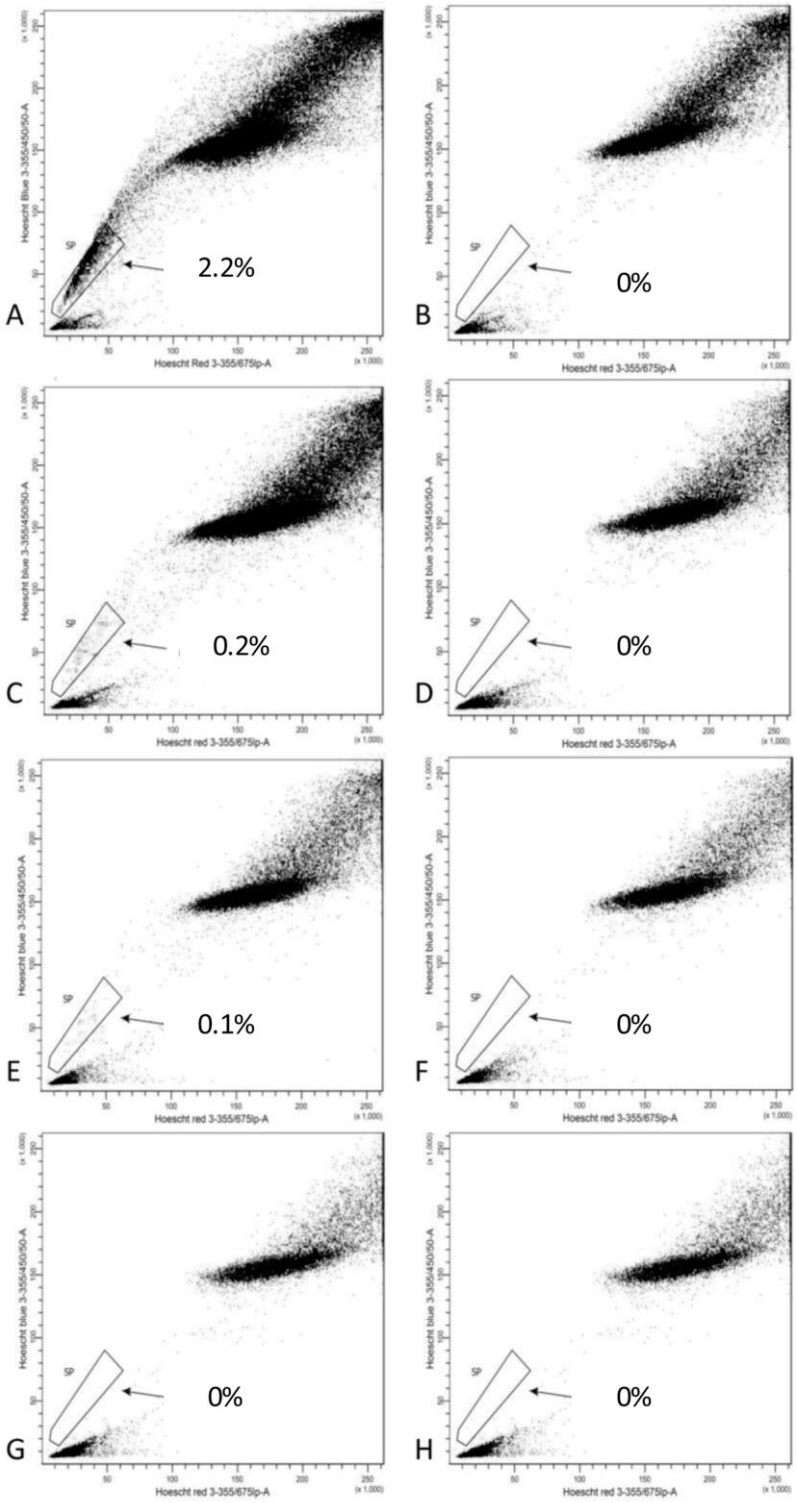


Figure 2.11: FACS profiles for the identification of SP cells in the MCF-7 breast cancer cell line at different confluencies. **A)** 10% confluency **C)** 20% confluency **E)** 40% confluency and **G)** 60% confluency. **B), D), F)** and **H)** represent the inhibited samples of A, C, E and G, respectively upon the addition of 10μm FTC. Note the absence of SP cells in the gated regions of the inhibited samples. Dead cells were excluded by the addition of propidium iodide prior to analysis (not shown).

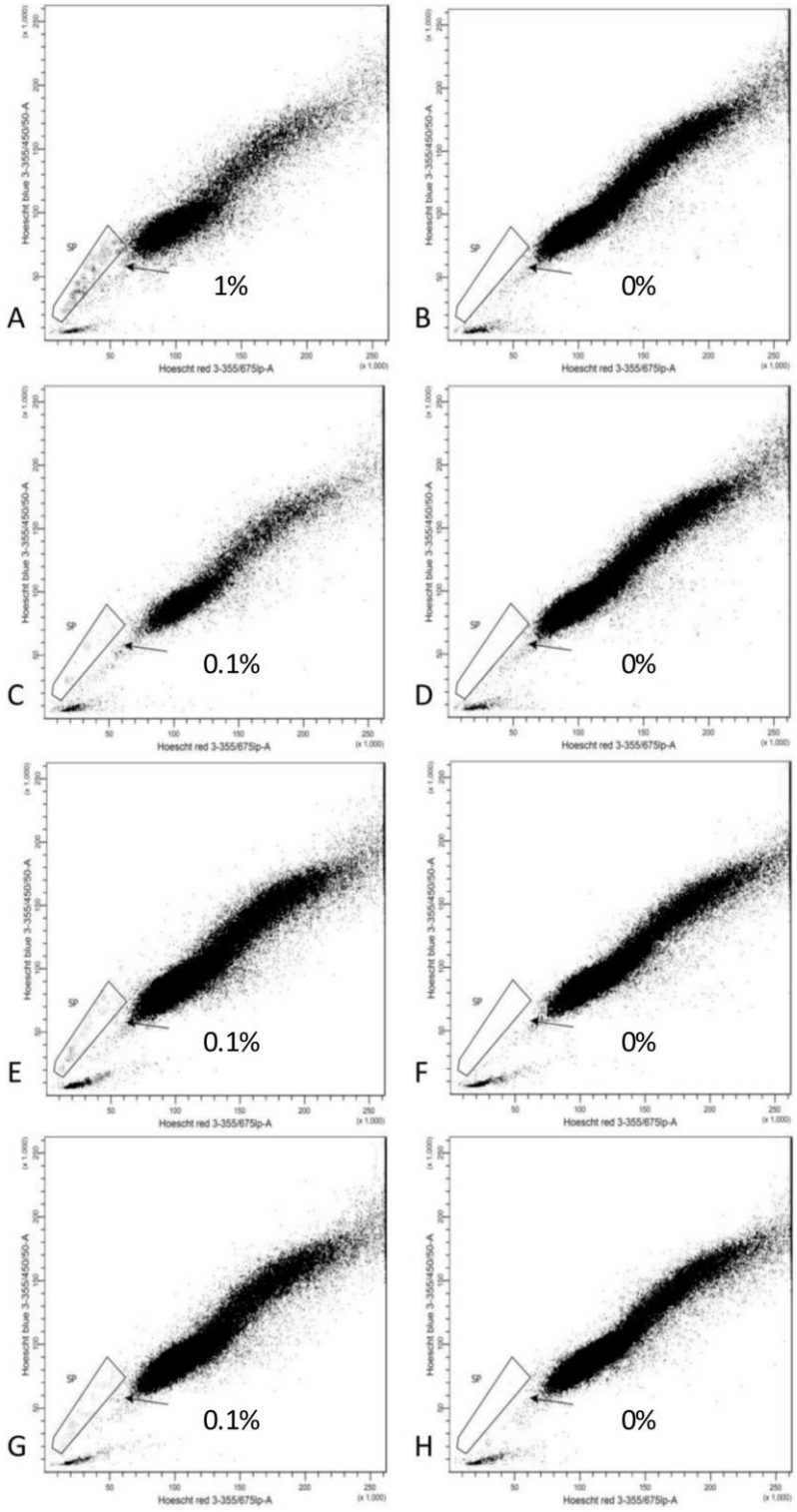


Figure 2.12: FACS profiles for the identification of SP cells in the MDA-MB-231 breast cancer cell line at different confluencies. **A)** 10% confluency **C)** 20% confluency **E)** 40% confluency and **G)** 60% confluency. **B), D), F)** and **H)** represent the inhibited samples of A, C, E and G, respectively upon the addition of 10μm FTC. Note the absence of SP cells in the gated region in the inhibited samples. Dead cells were excluded by the addition of propidium iodide prior to analysis (not shown).

2.7.8 SP isolation from Fine Needle Aspirates (FNAs)

Needles were flushed out with 1 ml of Tissue culture PBS into a 1.5 ml eppendorf which was then centrifuged at 3000 rpm at 4^{o}C for 5 minutes to pellet the cells. The pellet was then re-suspended in 1 ml of complete DMEM and added to a 50 ml tube containing 3 ml of complete DMEM and 21 ml of Red Blood Cell (RBC) lysis buffer (0.2% Tris Base, 0.75% ammonium chloride in distilled water, pH 7.5). This was followed by 5 min incubation at RT and centrifugation at 3000 rpm for 5 min. The lysis step was repeated and the pellet was re-suspended in 2ml of complete DMEM and incubated overnight in a 24 well plate. On the next day, cell suspensions or adhered cells were pelleted at 3000 rpm at 4^{o}C for 5 min and re-suspended in 1 ml of pre-warmed Hanks' balanced salt solution (Sigma; 2% FBS, 1% P/S) and counted. The protocol described in section 2.14 was used with the alterations of 2.5 μg/ml Hoechst 33342 (1μg/ml; Sigma-Aldrich) and 25 μM Verapamil (Sigma-Aldrich) for 1 x 10^{6} cells/ml. All tubes were incubated at 37^{o}C by using a MACsMix rotor in incubator for 20 min before the addition of Hoechst 33342 and for 45 min after the addition of it. The cells were maintained on ice in the dark and prior to cell analysis, non-viable cells were excluded by the addition of 2μl propidium iodide (2μg/ml) (Sigma). LSRII flow cytometer (BD Biosciences) was used for SP assays and the FACS Diva software was used for the analysis of the data (Figure 2.13).

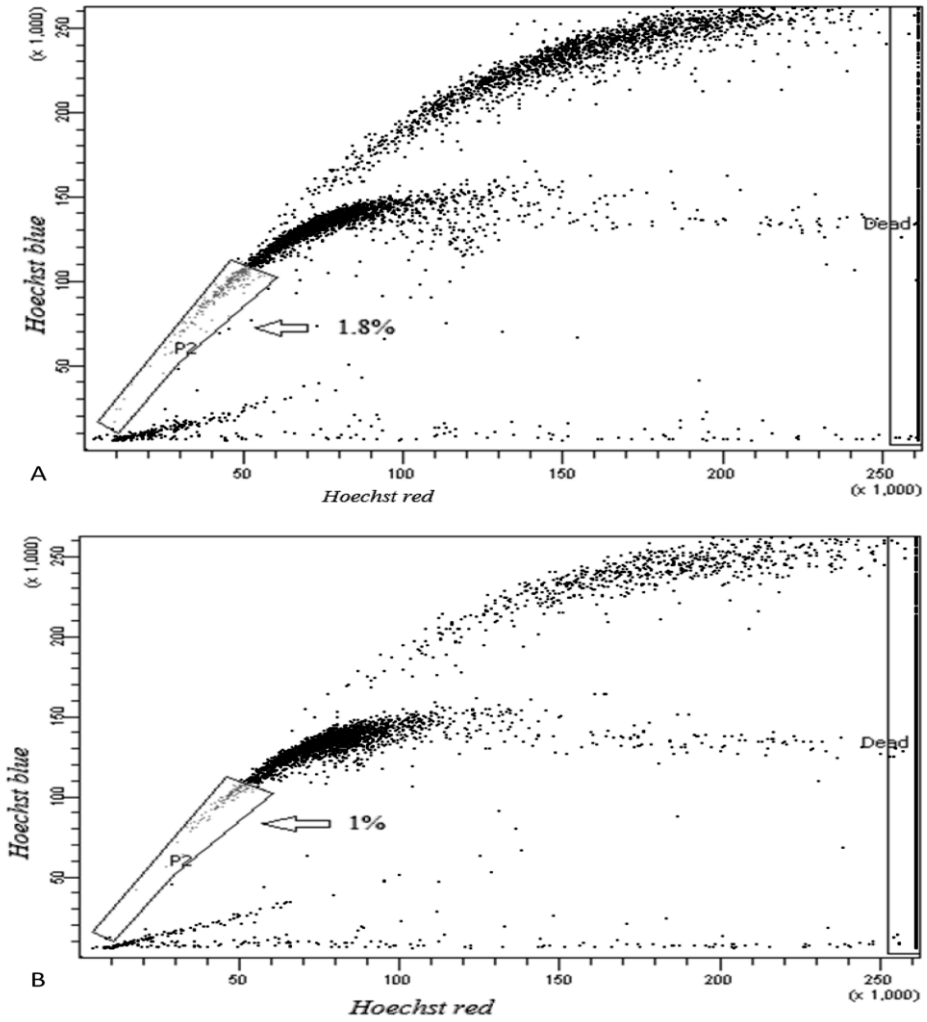


Figure 2.13: FACS profile of cells obtained from a breast cancer patient's FNA (Patient 3, see Appendix A). A) Identification of an SP population B) Note the partial inhibition on the addition of 25 μM Verapamil.

2.7.9 Staining cell surface proteins in suspended cells; staining breast cancer cell lines with anti-CD44 and anti-CD24 antibodies; optimization of antibody concentrations

Cells were re-suspended in 1X PBS and counted to get aa final number of 1x 10^6 cells per tube. MDA-MB-231 cells were used for the optimization of the CD44 antibody and MCF-7 cells were used for the optimization of the CD24 antibody. The following tubes were set up: 1 x 10^6 cells in 100 μl PBS and 1) 20 μl 2) 10 μl 3) 5 μl 4) 2 μl BD Pharmingen PE mouse anti human CD24, 1 x 10^6 cells in 100 μl PBS and 5) 20 μl 6) 10 μl 7) 5 μl 8) 2 μl BD Pharmingen APC mouse anti human CD44 9) 1 x 10^6 unstained MCF-7 and 10) unstained MDA-MB-231 cells . All mixtures were incubated on ice for 20 min in the dark and then washed x2 in excess cold PBS. The pellets were re-suspended in 500 μl PBS, filtered through 70 μm cell strainers (BD Biosciences) and they were then ready for FACS analysis. The mean values of fluorescence intensity were increased gradually with increasing concentrations of the antibodies and the highest concentrations were preferred for all future experiments to ensure antigen saturation (Figures 2.14 and 2.15).

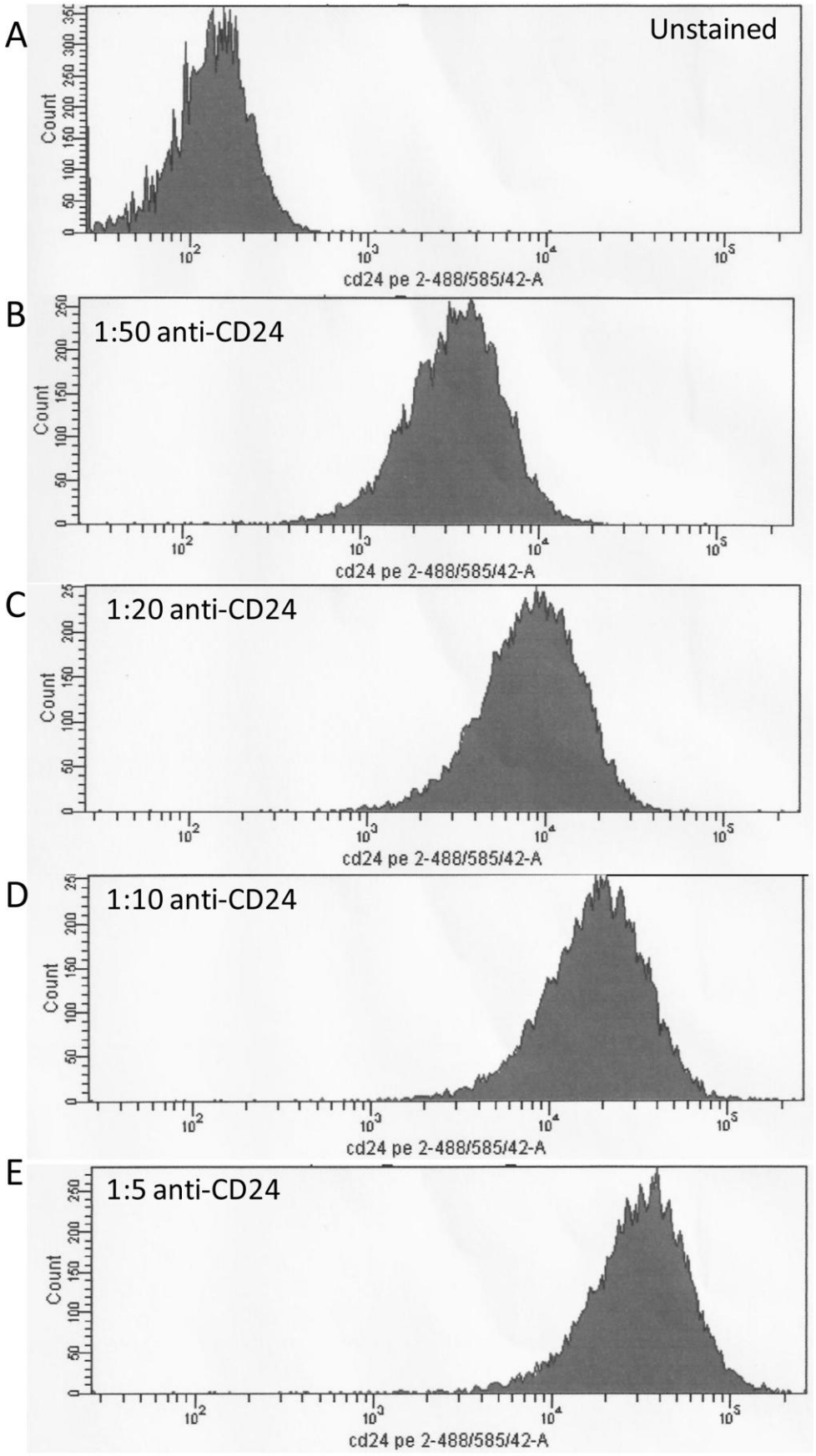


Figure 2.14: Optimization of the concentration of anti-CD24 in the MCF-7 cell line

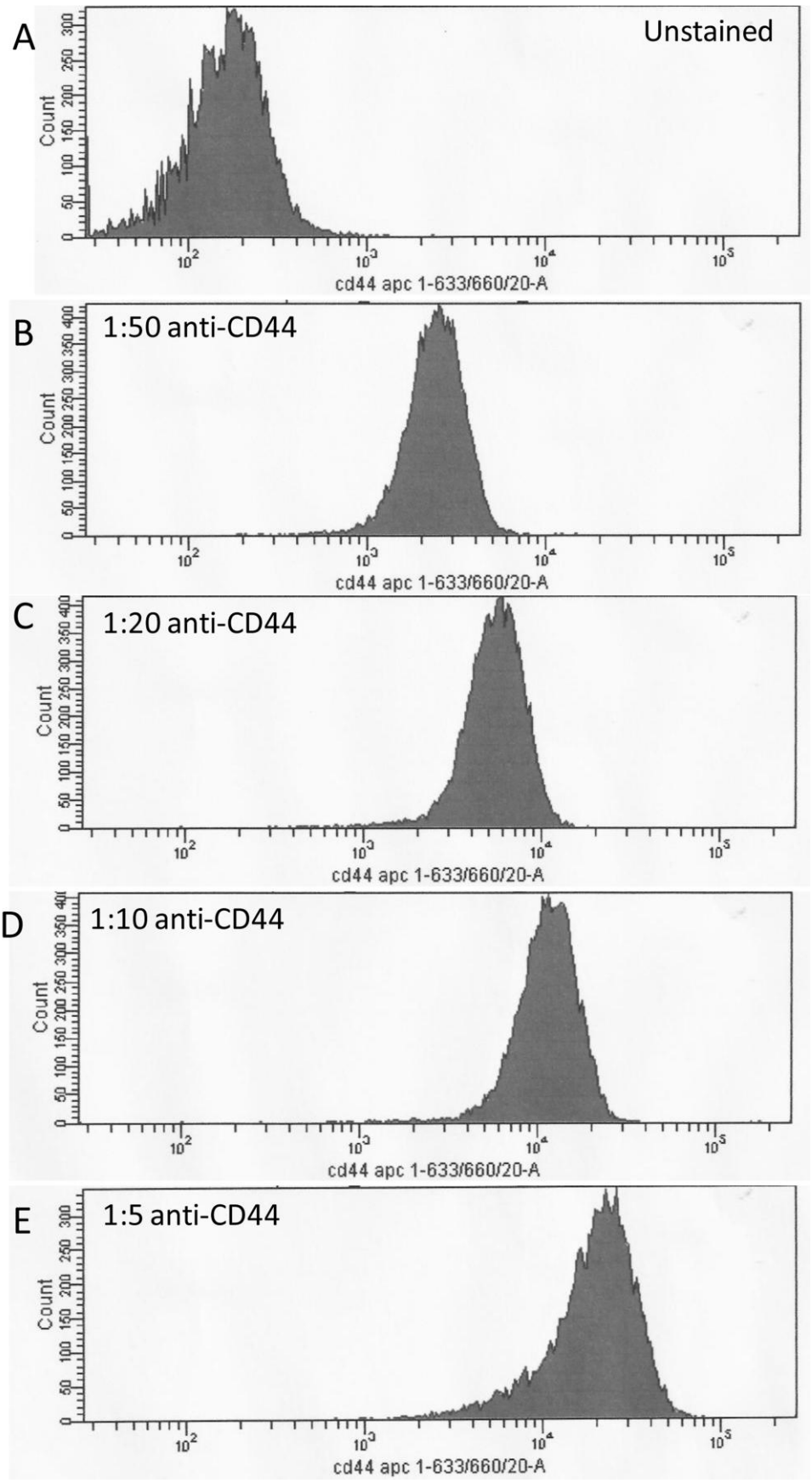


Figure 2.15: Optimization of the concentration of anti-CD44 in the MDA-MB-231 cell line

2.8 Immunocytochemistry (ICC)

ICC allows the detection of antigens or proteins and their cellular localization by the use of specific primary antibodies. Primary antibodies can be conjugated with a fluorochrome or another detectable tag (direct method) or alternatively these can be attached to secondary antibodies that bind to the Fc domain of the primary antibodies (indirect method). The use of fluorophores enables the localization of proteins in question when a light source with the appropriate wavelength is applied by a fluorescence or confocal microscope. Secondary antibodies can also be bound to enzymes which upon the addition of the appropriate substrate, also known as chromogens [such as AEC (3-Amino-9-EthylCarbazole) or DAB (3,3'-Diaminobenzidine Tetrahydrochloride)] generate a detectable product, such as color, in the areas where the protein of interest is localized.

2.8.1 Preparation of cells on chamber slides

10,000 cells were seeded into each chamber of an 8-chamber slide (BD Falcon) and cultured in the presence of complete DMEM until they become sufficiently confluent. The media was then removed and chambers were washed x3 with 1 X PBS. The cells were fixed by incubation in cold methanol (BDH Laboratory Supplies) for 20 min at -20^{o}C.

2.8.2 Protocol 1: Vectastain Elite ABC kit

The Vectastain Elite ABC kit (Vector Laboratories; mouse PK6102 and rabbit PK6101) was used for the ICC staining for the (anti-rabbit) TGFB-RI and (anti-mouse) II receptors in MDA-MB-231 and MCF-7 cells according to the manufacturer's instructions. Briefly, cells were fixed by incubation in cold methanol (BDH Laboratory Supplies) for 20 min at -20^{o}C. Slides and the inserts were washed x2 with Tris Buffered Saline (TBS) pH 7.6 for 5 min. Cells were covered with blocking buffer (15 μl normal horse serum in 1000 μl TBS) for 10 min and then incubated for 30 min with primary antibodies diluted in TBS (1:50). Negative controls for the primary (TBS instead of the primary antibody) were also used. The washing step was repeated as described above. Cells were covered with the appropriate biotinylated secondary antibody solutions (15 μl normal horse serum + 5 μl Biotinylated secondary antibody in 1000 μl TBS) for 30 min. The washing step was repeated as described above. The avidin peroxidase complex was added [20 μl of Avidin DH (Reagent A) and 20 μl of Biotinylated Horseradish Peroxidase H (Reagent B) in 1000 μl TBS] and incubated for 30 min. The washing step was repeated as described above. Chambers were removed and DAB was added as a

peroxidase substrate for 2-3 min [One DAB tablet was added to 10 ml TBS. To this 200 µl H_2O_2 in distilled water (5.8 ml distilled water + 200 µl H_2O_4) were added]. Slides were washed in tap water for 5 min and cells were counterstained with the nuclear counterstain haematoxylin. Slides were then dehydrated and cleared through alkaline solution and graded ethanol series and mounted in DPX. Slides were visualized under a microscope using bright-field illumination.

2.8.3 Protocol 2: Fluorescence

Slides and the inserts were washed x2 with 1 X PBS and permeabilized by soaking into 0.3% (v/v) Triton X-100 (Fisher Scientific) in PBS for 10 min when staining for intracellular proteins. For extracellular or cell surface proteins this step was omitted. Slides were then washed once with PBS and non-specific binding sites were blocked by the addition of 5% normal goat serum (NGS; Sigma)/PBS for 30 min. This was followed by a 1 hour incubation in humid chamber with the primary antibody, diluted in 0.5% NGS/PBS in a range of antibody concentrations was tested (see Table 2.3 Slides were then washed 3 times with PBS for 5 min followed by a 30 min incubation in the presence of the appropriate secondary antibody (diluted 1:25; Jackson Labs; Table 3) again in a humid chamber in the dark Slides and the inserts were washed x3 with PBS for 5 min and chambers were removed. Slides were mounted in vectashield anti-fading medium (Vector Laboratories) and coversliped. Negative controls for the primary (0.5% NGS/PBS instead of the primary antibody) and the secondary antibody (0.5% NGS/PBS instead of the secondary antibody) were also used. Slides could then be stored at 4^{o}C in the dark until microscope visualization or for longer term at -20^{o}C.

Antibody	Optimal concentration
Mouse monoclonal Nanog (2.6 µg/µl, ab62734)	7 µg/µl
Mouse anti-Oct 3/4 (250 µg/ml, BD Biosciences)	2.5 µg/ml
Rabbit polyclonal SOX-2 (1 mg/ml, ab97959)	5 µg/ml
Rabbit polyclonal S100A4 (0.72 mg/ml, ab27957)	1:100
Rabbit polyclonal p-smad 2/3 (Ser 423/425)(200 µg/ml, sc-11769)	1:100
Rabbit polyclonal Twist1 (1.100 mg/ml, ab50581)	1 µg/ml
Rabbit polyclonal TGF beta Receptor I (ab31013)	1:50
Mouse monoclonal TGF beta receptor II (ab78419)	1:50

Table 2.3: Antibody names, codes and optimized concentration for use.

2.9 Statistical analysis

Statistical analyses and graphical representation of results were carried out using GraphPad Prism version 3 (GraphPad, San Diego, USA) and included unpaired and paired two tailed student t-tests or column statistics or ANOVA statistical tests. Each figure displays one representative experiment, while the number of repeats is described in each figure legend. All data were considered statistically significant at $p<0.05$.

Chapter 3: The effect of TGF-β treatment on BCSCs

3.1 Introduction

CSCs are good candidates for tumor regrowth and drug resistance due to their self renewal capacity, resistance to apoptosis and ability to expel chemotherapeutic drugs through ABC transporters (Reya *et al.*, 2001). In fact, SP cells, a putative CSCs population, can be identified by their ability to efflux vital dyes through these transporters (Gottesman *et al.*, 2002). SP cells isolated from both mouse and human primary breast tissue were able to generate epithelial and luminal cells and structures *in vitro* and *in vivo*, respectively (Alvi *et al.*, 2002). Moreover, ABCG2 was found to be highly expressed in the SP compared to the NSP compartment suggesting that this is a distinctive property of SP cells in most cell types (Zhou *et al.*, 2002; Patrawala *et al.*, 2005; Steiniger *et al.*, 2008). Additionally, complete elimination of the SP population was achieved by the use of FTC, a specific inhibitor of ABCG2, and siRNA for ABCG2 in the MCF-7 breast cancer cell line (Rabindran *et al.*, 2000).

The expression of high levels of CD44 and low levels of CD24 in eight out of nine patients with breast cancer has also been suggested as a property of another BCSC population. The tumorigenic ability of CD44+/CD24- cells was demonstrated in immunocompromised mice, a few of these cells were enough to form new tumors, while a high number of cells with alternative profiles failed to do so. Al-Hajj and colleagues also showed that CD44+/CD24- cells were able to give rise to new tumorigenic and non tumorigenic cells (Al-Hajj *et al.*, 2003).

It is becoming increasingly evident that EMT is involved in the regulation of BCSCs. EMT is thought to contribute to the metastatic process during which tumor cells at the primary site acquire an increased migratory potential and colonize at distant sites where they proliferate and form new tumors (Evans, 1991; Britton *et al.*, 2011). Several growth factors and cytokines are involved in EMT. TGF-β binding to TGFB-RII, results in the heterodimerization of TGFB-RII and RI. TGFB-RI phosphorylates and activates downstream cytoplasmic molecules such as smad 2 and 3. p-smad 2/3 can then form complexes with Smad 4 and enter the nucleus, where it induces the expression of several target genes related to EMT in association with other transcription factors (Moustakas and Heldin, 2007). This pathway seems to play a dual role in carcinogenesis; at early stages it promotes inhibition of cell growth and apoptosis, while at more advanced stages cancer cells become resistant to the TGF-β induced suppressive

activity leading to excessive cell proliferation, motility and invasiveness (Pardali and Moustakas, 2007). Interestingly, higher mRNA (MacCallum *et al.*, 1994) and protein (Walker and Dearing, 1992; Auvinen *et al.*, 1995) levels of TGF-β1, 2 and 3 were found in primary breast cancer tissue specimens and the latter were significantly increased in more aggressive carcinomas compared to *in situ* carcinomas (Walker and Dearing, 1992). Elevated circulating TGF-β plasma levels were also detected in breast cancer patients with worse survival rates (Grau *et al.*, 2008).

Induction of EMT in the HMLE led to an increase of CD44+/CD24- cells which had a higher mammosphere forming and self-renewal ability compared to the CD44-/CD24+ cells (Mani *et al.*, 2008). Similarly, introduction of the Twist1 gene (Vesuna *et al.*, 2009) in MCF-7 and MCF-10A cells and of the Twist2 gene in the MCF-10A cells (Fang *et al.*, 2011) led to an increase of the CD44+/CD24- cells, which acquired enhanced migratory (Fang *et al.*, 2011) and invasive (Vesuna *et al.*, 2009) characteristics and had an increased export activity of the Hoechst 33342 and Rhodamine 123 dyes (Vesuna *et al.*, 2009). Conversely, FOXC2 silencing mediated by shRNA in HMLE cells that were induced to undergo EMT resulted in the generation of cells with epithelial-like properties, a reduction of CD44+/CD24- cells which demonstrated a decreased ability to form mammospheres (Hollier *et al.*, 2013).

Interestingly, microarray analysis of the gene expression patterns of the CD44+/CD24- and the CD44-/CD24+ cells in the MCF-7 and MCF-10A cell lines led to the identification of 32 genes that are differentially expressed in these two subpopulations with the Slug gene being able to promote the generation of CD44+/CD24- cells in the basal MCF-10A cell line only (Bhat-Nakshatri *et al.*, 2010) Additionally, genome-wide transcriptional profiling of the above mentioned subpopulations revealed that there is higher expression of TGF-β and TGF-BRII in the CD44+/CD24- compared to the CD44-/CD24+ cells, suggesting that BCSCs are more prone to undergo EMT (Shipitsin *et al.*, 2007).

TGF-β1 treatment of the MCF-7 cell line led to the loss of the SP phenotype, decreased ABCG2 expression and reduced cell viability in the presence of mitoxantrone (Yin *et al.*, 2008). We have also confirmed these findings (Mallini *et al.*, 2014). In most studies it was shown that EMT has a negative regulatory role in the SP from several cancer cell lines. TGF-β significantly reduced the SP percentage of human diffuse-type gastric

carcinoma cells by down-regulating ABCG2 through direct binding of p-smad2/3 to the ABCG2 promoter region (Ehata *et al.*, 2011). Similarly, SP cells isolated from pancreatic cell lines were more likely to gain and lose E-cadherin expression before and after TGF-β exposure *in vitro* and they had a greater *in vivo* metastatic potential compared to NSP cells (Kabashima *et al.*, 2009).

3.2 Chapter hypothesis

Autocrine production of TGF-β in the MDA-MB-231 cells is thought to be essential for their growth and maintenance, since inhibition of the TGF-β signalling pathway has led to apoptosis (Lei *et al.*, 2002). This cell line represents basal-like or hormone non responsive breast cancer subtypes, who have been found to have a high SP prevalence (Britton *et al.*, 2012), whereas the MCF-7 cell line represents hormone-dependent subtypes. Additionally, the TGF-β signalling pathway has been found to be less functional in ER+ patients due to low TGF-BRII expression (Arteaga *et al.*, 1988) and this phenotype has also been correlated with the presence of CD44-/CD24+ cells, which are not considered CSCs (Shipitsin *et al.*, 2007). Therefore, I hypothesize that there will be potential differences in the responsiveness of the SP cells to EMT in these cell lines, and that the BCSCs in MDA-MB-231 will be more prone to undergo EMT due to the presence of an intact TGF-β signalling pathway, and to their increased mesenchymal and migratory potential compared to the MCF-7 cell line. It was expected that this would also explain different clinical outcomes and response to current therapeutic options depending on the hormonal status of breast cancer patients. Ideally, the identification of the exact EMT mechanisms that regulate the SP population in these cell lines would enable the development of more efficient patient-specific therapeutic approaches for overcoming drug resistance and metastasis caused by the presence of BCSCs.

3.3 Aims

- To determine the effects of TGF-β1 treatment on the SP population in the MDA-MB-231 cell line.
- To determine the effects of TGF-β treatment on the CD44+/CD24- population in the MDA-MB-231 and MCF-7 cell lines
- To confirm the impact of EMT on the MCF-7 SP cells, (previously reported), and compare it to that of the MDA-MB-231 SP cells.

- To investigate the properties of the TGF-β signalling pathway in both cell lines in order to explain the potentially different effect of EMT on the SP cells from these.

3.4 Experimental design

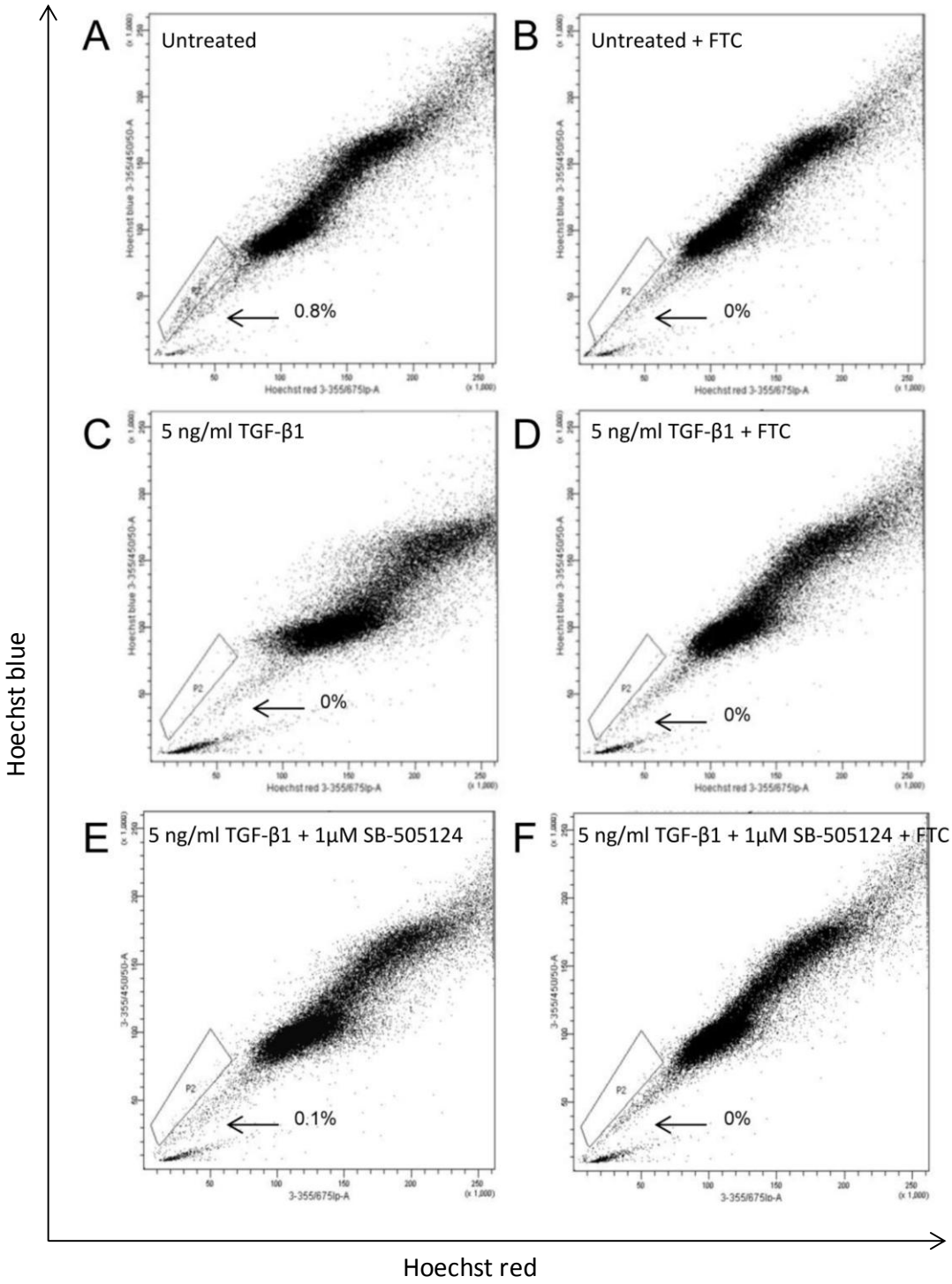
- EMT was induced in the MDA-MB-231 and MCF-7 cell lines by treatment with TGF-β1 for 3 days followed by SP analysis to examine the effect of EMT on these SP populations. The TGF-BRI inhibitor, SB-505124, was used to reverse these effects and therefore confirm that they are due to the TGF-β signalling pathway.
- The activation of the pathway was further confirmed with the detection of p-smad 2/3 by ICC in response to TGF-β treatment with or without the addition of SB-505124 or just SB-505124 to test whether the autocrine TGF-β signalling was blocked.
- The expression levels of TGF-β1 and its receptors, TGF-BRI and II, were assessed by qPCR and by qPCR and ICC, respectively.

3.5 Results

3.5.1 The effect of TGF-β1 treatment on SP numbers in breast cancer cell lines

To determine the role of EMT on the SP population of the MDA-MB-231 cell line unfractionated MDA-MB-231 cells were treated with 5 ng/ml TGF-β1 for 3 days and SP analysis was performed. TGF-β1 treatment resulted in the complete abrogation of the SP phenotype of the SP population (p= 0.04). The use of increasing doses of SB-505124 [1, 3 and 5 μM] in the presence of TGF-β1 led to a gradual increase in the SP percentage (from 0.1 to 0.8%) to that seen in the untreated cells (0.8%) confirming that the loss of the SP phenotype in this cell line was due to the activation of the TGF-β signalling pathway (Figure 3.1). In order to further confirm that the action of this pathway is responsible for reducing the SP numbers, SB-505124 was added to unfractionated MDA-MB-231 cells without the presence of any exogenous TGF-β1. This experiment showed that the SP percentage was further increased compared to the untreated cells (p=0.03) (Figures 3.2, 3.3).

Since the addition of 5 ng/ml did not effectively eliminate the SP population (Figure 3.4), unfractionated MCF-7 cells were treated with 10 ng/ml TGF-β1 for 3 days and SP analysis was performed. This induced a significant reduction (p=0.03), but not complete abrogation of the SP numbers (Figures 3.5 and 3.6). The use of SB-505124 was not possible in the MCF-7 cells, as it caused cell death (data not shown).


A
Untreated
0.8%
B
Untreated + FTC
0%
C
5 ng/ml TGF-β1
0%
D
5 ng/ml TGF-β1 + FTC
0%
E
5 ng/ml TGF-β1 + 1μM SB-505124
0.1%
F
5 ng/ml TGF-β1 + 1μM SB-505124 + FTC
0%
P2
Hoechst blue
Hoechst red

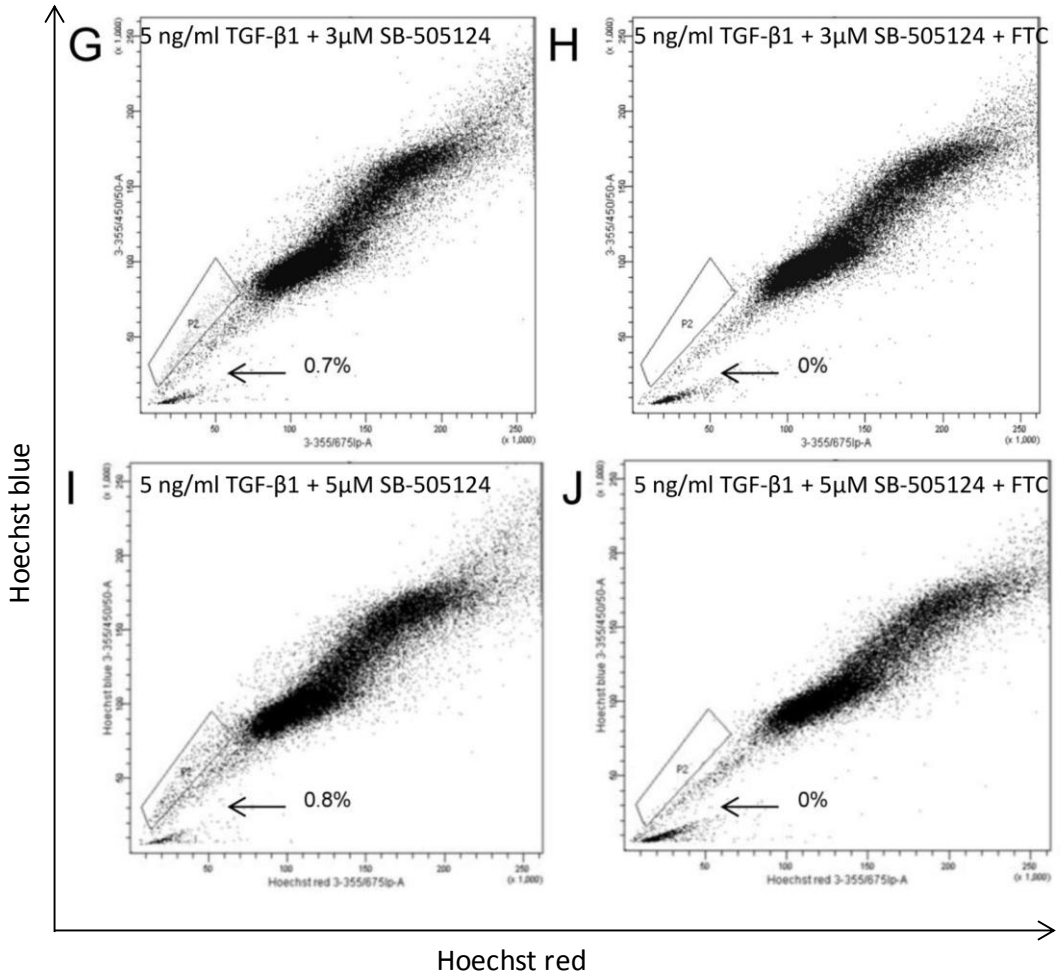


Figure 3.1: Representative FACS images of SP analysis in MDA-MB-231 cells. A) Untreated C) Treated with 5 ng/ml TGF-β1 E) Treated with 5 ng/ml TGF-β1 and 1 μM SB-505124 G) Treated with 5 ng/ml TGF-β1 and 3 μM SB-505124 I) Treated with 5 ng/ml TGF-β1 and 5 μM SB-505124. FTC, an ABCG2 inhibitor, was used to confirm the SP phenotype in B), D), F), H) and J). (n=8, p=0.04 for 5 μM SB-505124). **(student's paired t-test). Results were considered significant when p<0.05.**

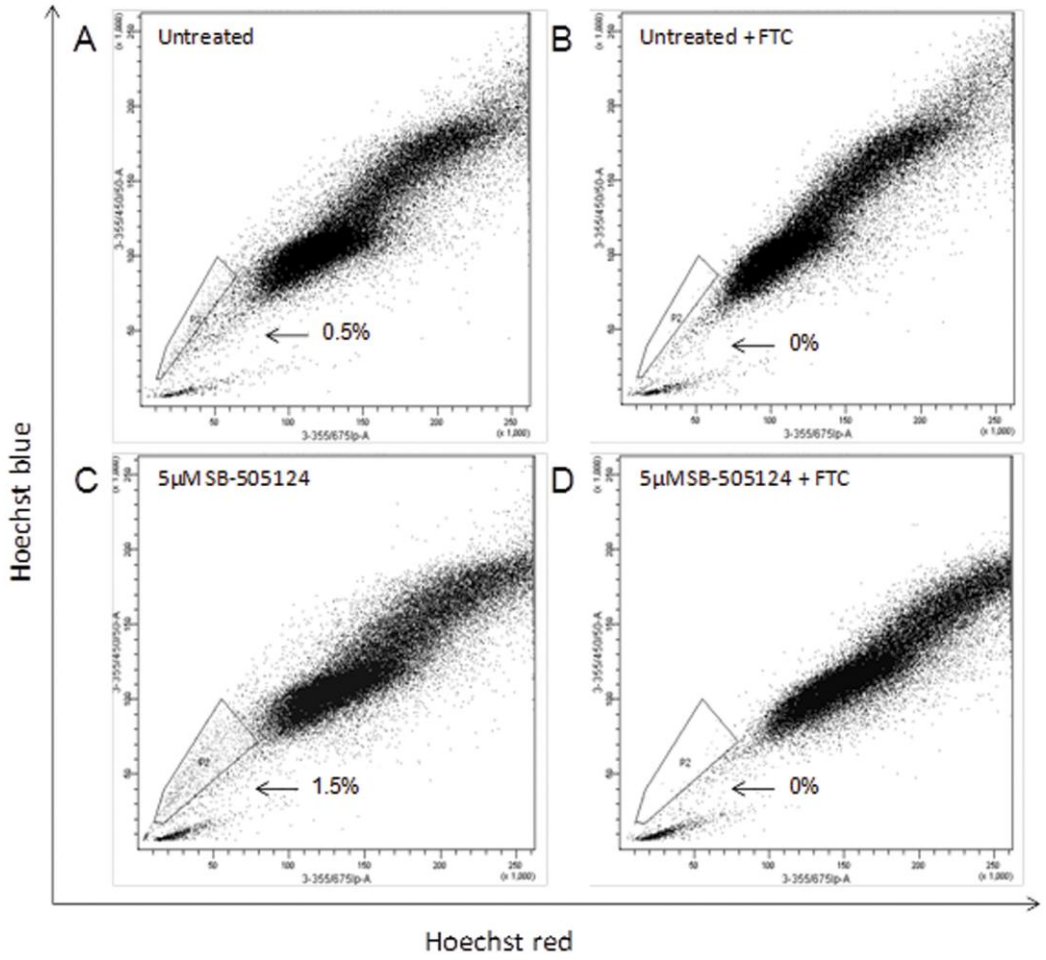


Figure 3.2: Representative FACS images of SP analysis in MDA-MB-231 cells. A) Untreated C) Treated with 5 µM SB-505124. FTC, an ABCG2 inhibitor, was used to confirm the SP phenotype in B) and D). n=6, p=0.03 **(student's paired t-test). Results were considered significant when p<0.05.**

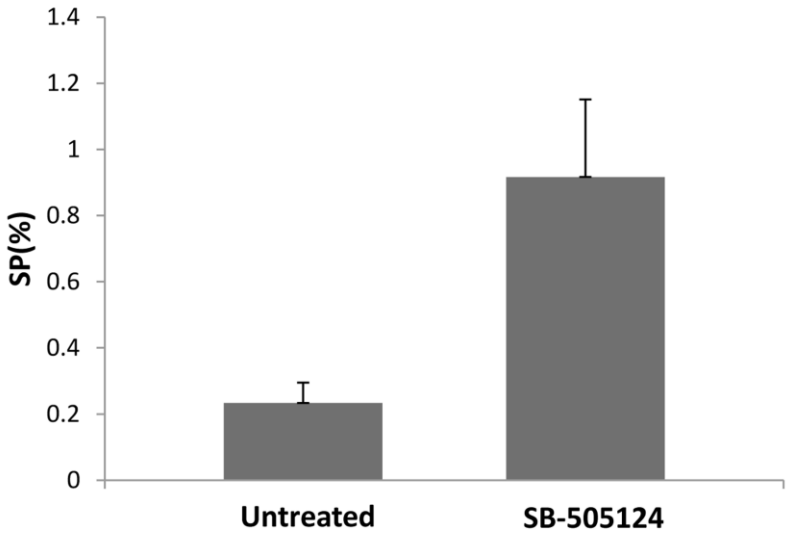


Figure 3.3: Graphical representation of flow cytometry data. Percentages of SP cells in untreated and treated with 5 μM SB-505124 unfractionated MDA-MB-231cells. Bars represent an average of n=6 individual experiments, p=0.03. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

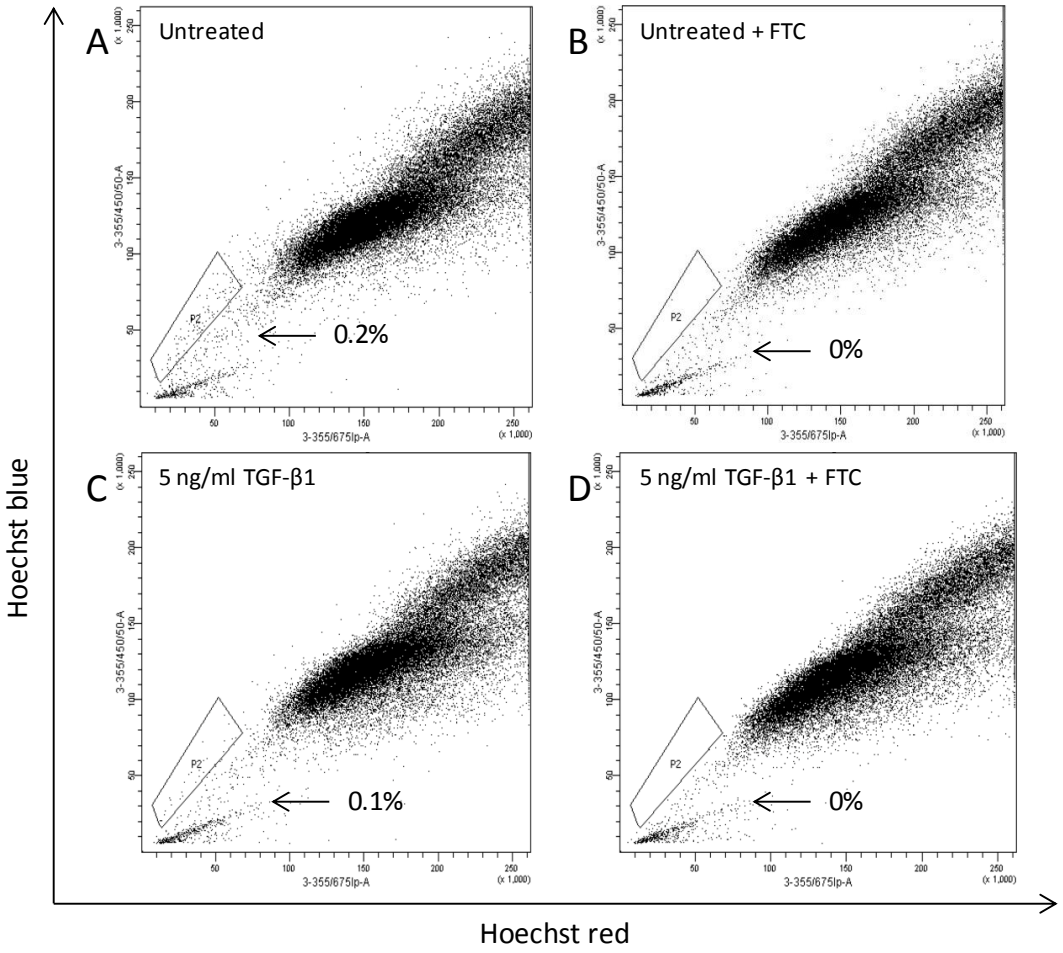


Figure 3.4: FACS images of SP analysis in MCF-7 cells. A) Untreated C) Treated with 5 ng/ml TGF-β1. FTC, an ABCG2 inhibitor, was used to confirm the SP phenotype in B) and D). n=1

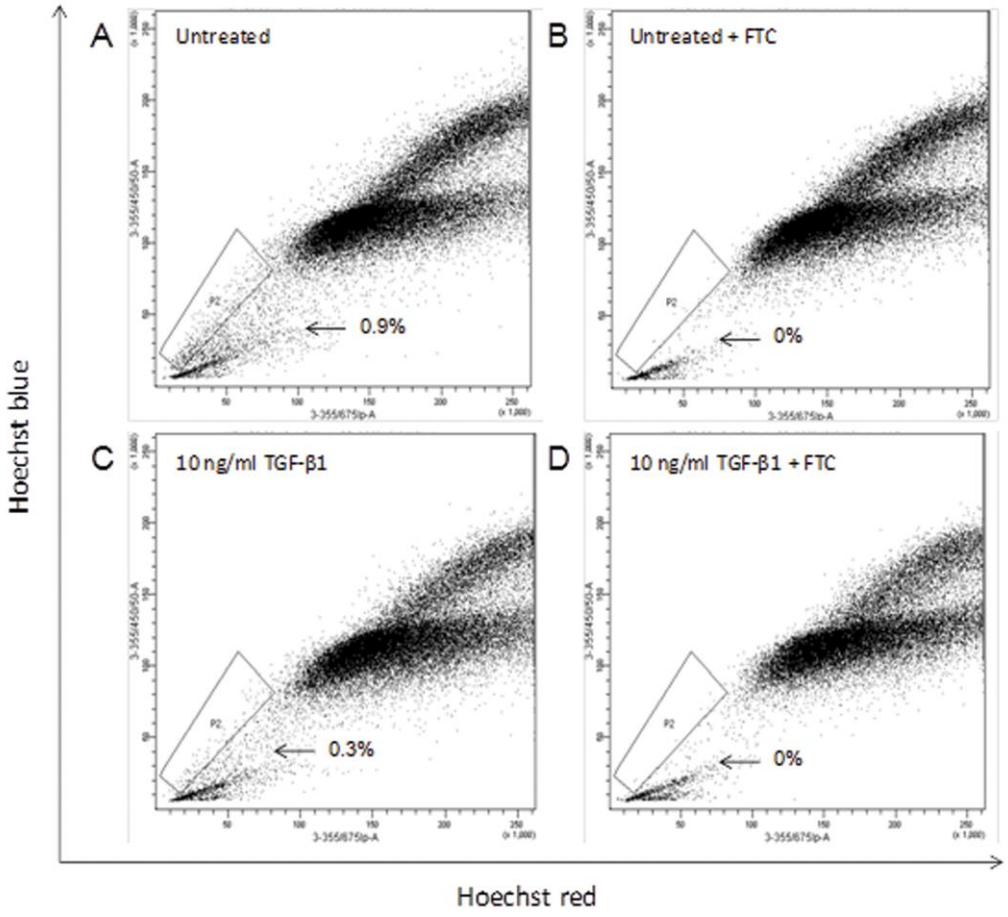


Figure 3.5: Representative FACS images of SP analysis in MCF-7 cells. A) Untreated C) Treated with 10 ng/ml TGF-β1. FTC, an ABCG2 inhibitor, was used to confirm the SP phenotype in B) and D). n=7, p=0.03 **(student's paired t-test). Results were considered significant when p<0.05.**

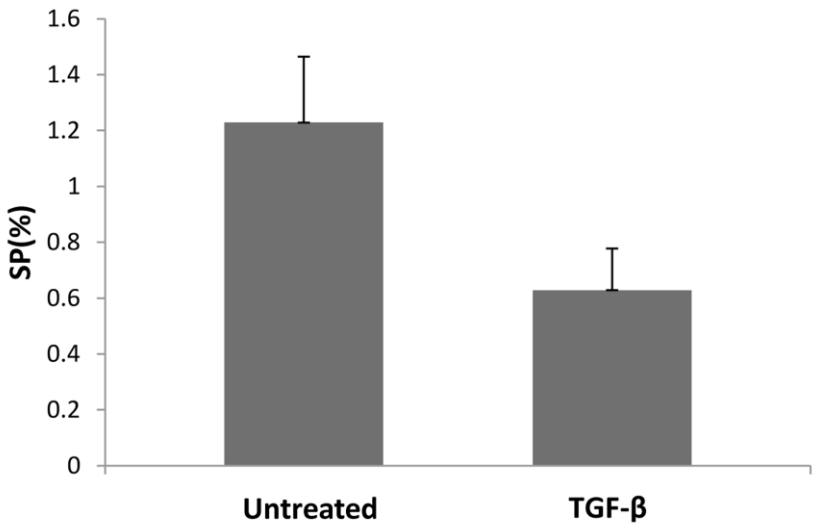


Figure 3.6: Graphical representation of flow cytometry data. Percentages of SP cells in untreated and treated with 10 ng/ml TGF-β1 unfractionated MCF-7 cells. Bars represent an average of n=7 individual experiments, p=0.03. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

3.5.2 The effect of TGF-β treatment on the CD44+ population in breast cancer cell lines.

To investigate the role of TGF-β treatment on the CD44+/CD24- cells in the MDA-MB-231 and MCF-7 cells, staining for CD44 and CD24 was performed in untreated and treated cells. CD24 staining gave double peaks in the MCF-7 cells, which made the interpretation of data impossible. For the MDA-MB-231 cells the decrease in CD24 expression was very low in the treated with TGF-β1 sample (Appendix B, Supplementary data, pages 217 and 218). Treatment with TGF-β1 resulted in a higher increase of the CD44 protein levels in the MDA-MB-231(Figure 3.7) compared to the MCF-7 (Figure 3.8) cells, yet non significant (Figures 3.9-3.10).

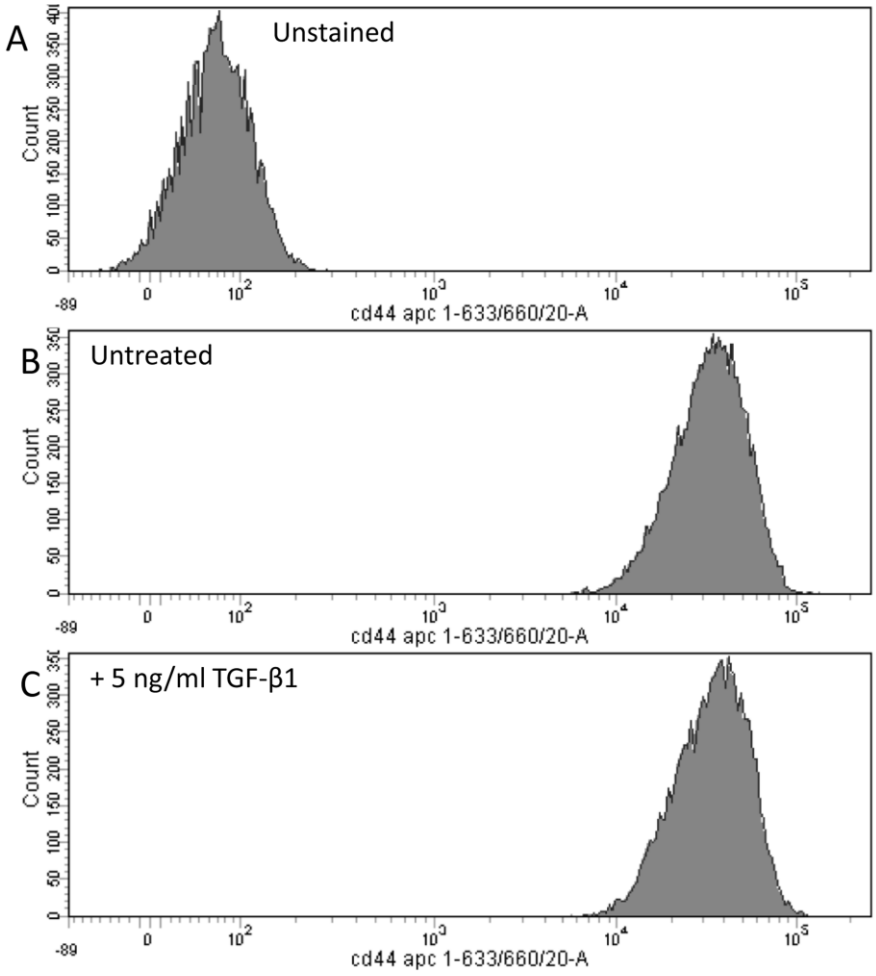


Figure 3.7: Representative FACS images from CD44 staining in MDA-MB-231 cells. A) Unstained B) Untreated C) Treated with 5 ng/ml TGF-β1 for 3 days. n=3, p=0.21 **(student's paired t-test). Results were considered significant when p<0.05.**

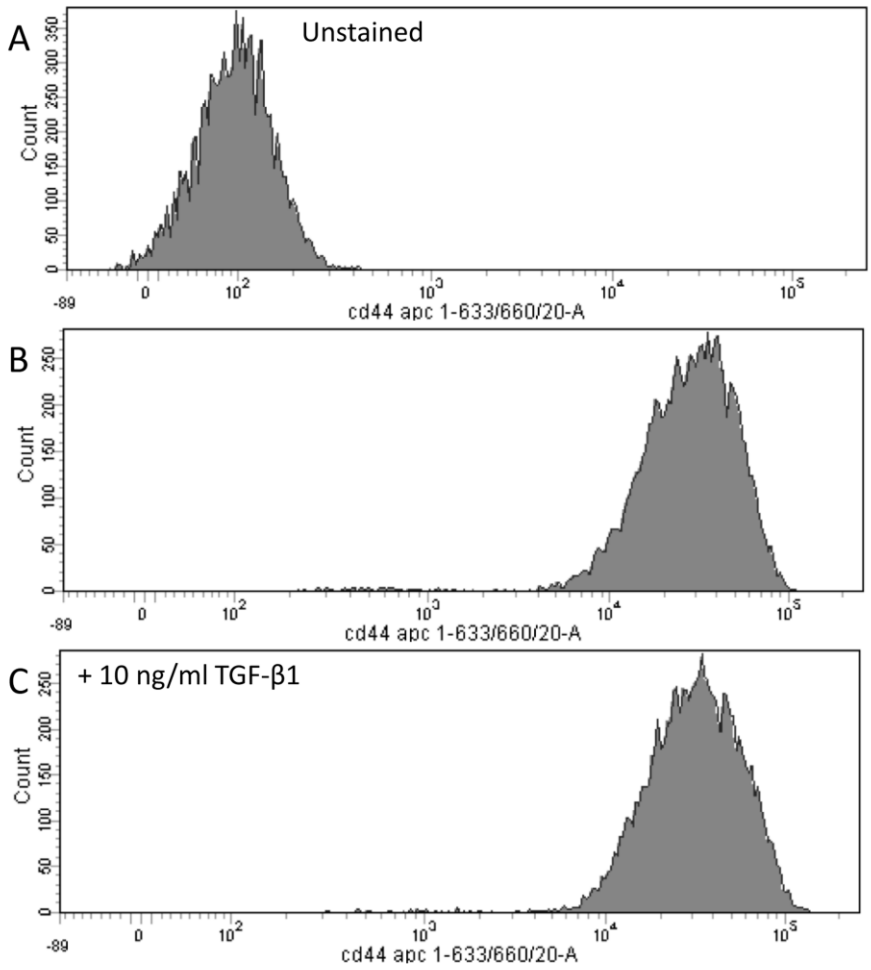


Figure 3.8: Representative FACS images from CD44 staining in MCF-7 cells. A) Unstained B) Untreated C) Treated with 10 ng/ml TGF-β1 for 3 days. n=3, p=0.19 **(student's paired t-test). Results were considered significant when p<0.05.**

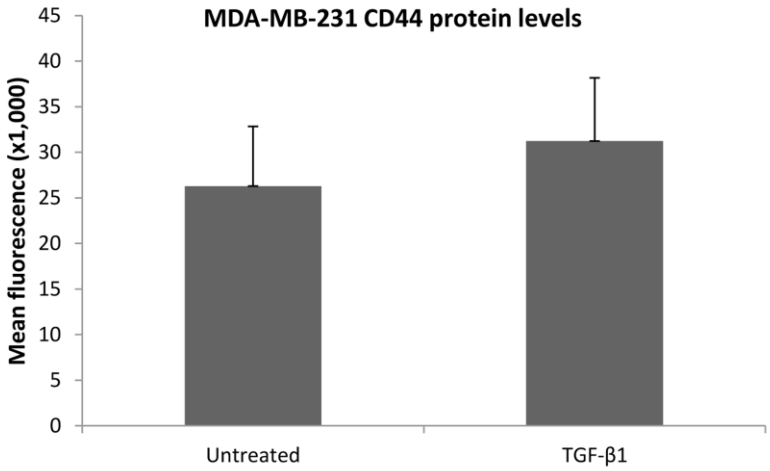


Figure 3.9: Graphical representation of flow cytometry data. Protein expression levels of CD44 in untreated and treated with 5 ng/ml TGF-β1MDA-MB-231 cells. Bars represent an average of n=3 individual experiments, p=0.21. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

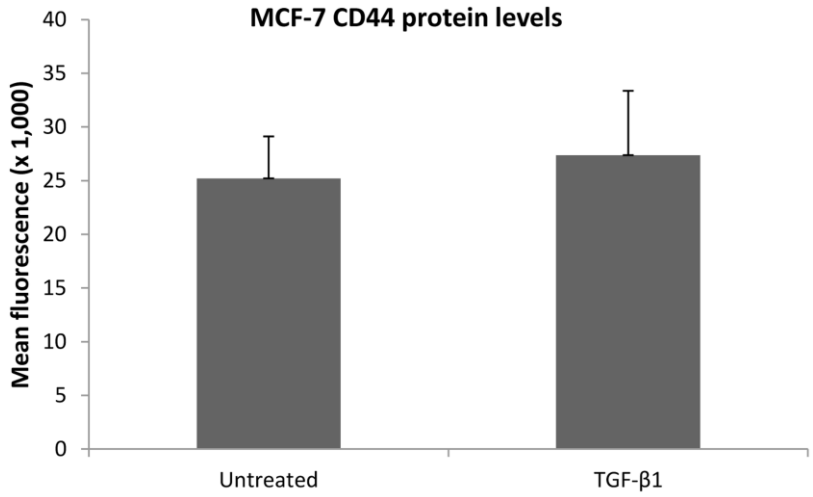
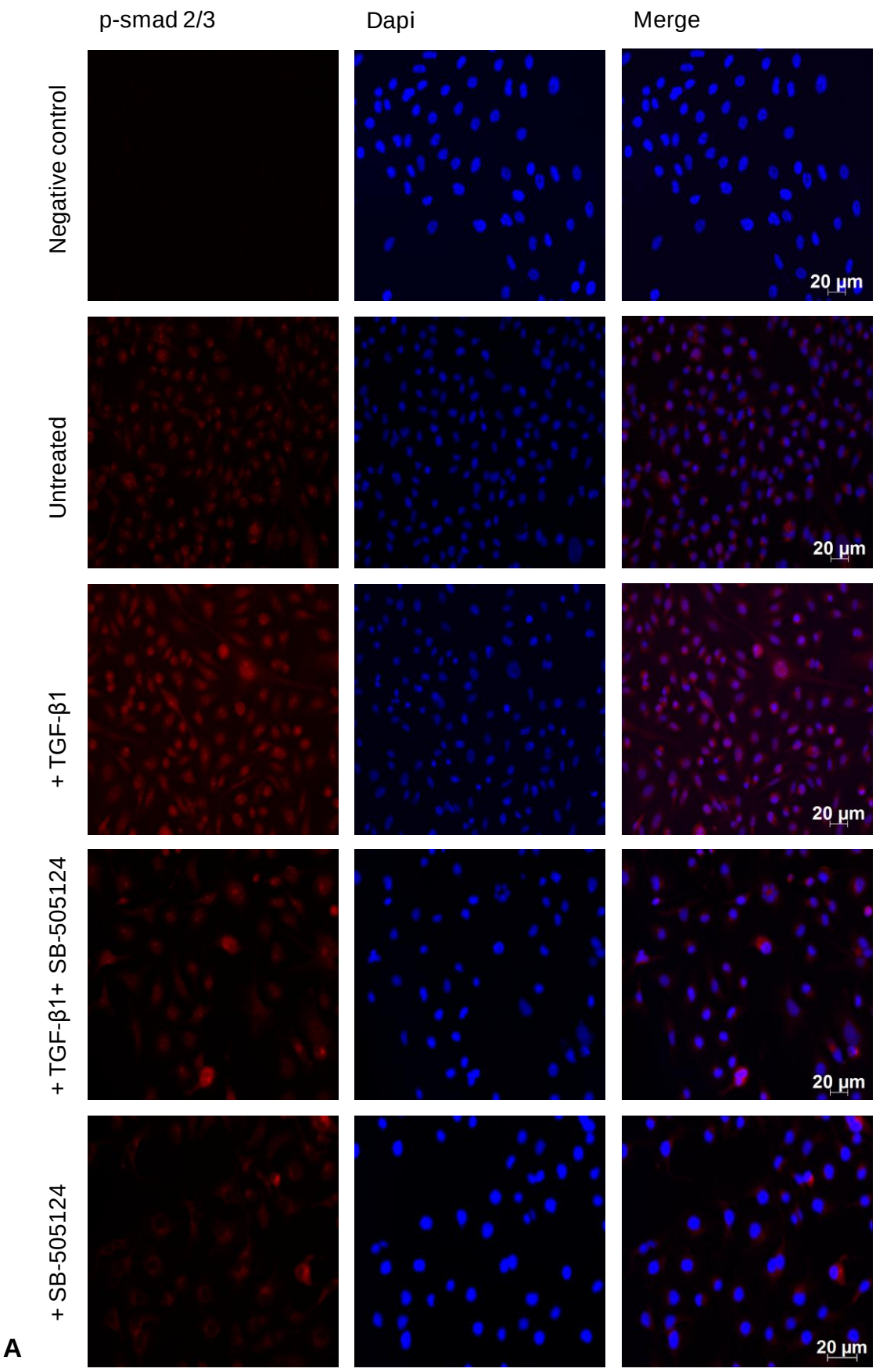


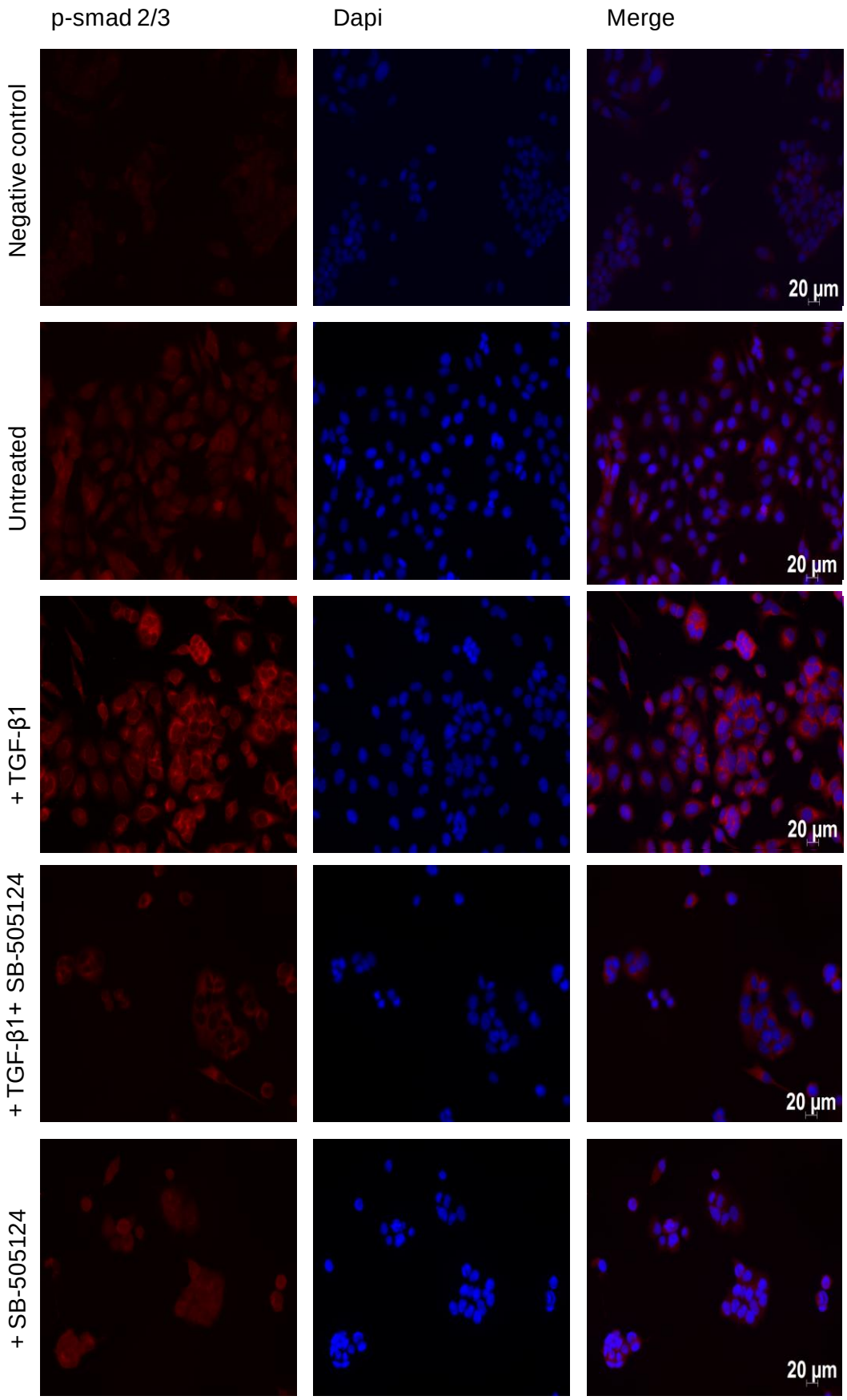
Figure 3.10: Graphical representation of flow cytometry data. Protein expression levels of CD44 in untreated and treated with 10 ng/ml TGF-β1MCF-7 cells. Bars represent an average of n=3 individual experiments, p=0.19. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

3.5.3 Confirmation of TGF-β pathway activation through TGF-β1 treatment

To confirm the activation of the TGF-β pathway by the addition of TGF-β unfractionated MDA-MB-231 cells were treated with 5 ng/ml TGF-β1 for 3 days and nuclear p-smad 2/3 levels were assessed by ICC. Untreated cells showed low p-smad 2/3 levels, suggesting that there is autocrine production of TGF-β1 in the MDA-MB-231 cells. Additionally, successful activation of the TGF-β signalling pathway was achieved by TGF-β1 exposure as seen by the significant increase of p-smad 2/3 ($n=3$, $p=0.001$) and these effects were reversed upon the addition of the TGF-B-RI antagonist, SB-505124 with or without the presence of TGF-β1 (Figures 3.11A and 3.12). Since treatment of the MCF-7 cells with 5 ng/ml TGF-β1 was not sufficient to activate the pathway (data not shown), 10 ng/ml were added and significantly increased p-smad 2/3 ($n=3$, $p=0.0008$), but not to the same degree as in the MDA-MB-231 cells. The use of SB-505124 also reduced the levels of nuclear p-smad2/3 in the MCF-7 cell line (Figures 3.11B and 3.13).


p-smad 2/3
Dapi
Merge
Negative control
Untreated
+ TGF-β1
+ TGF-β1+ SB-505124
+ SB-505124
20 µm
20 µm
20 µm
20 µm
20 µm

A


p-smad 2/3
Dapi
Merge
Negative control
Untreated
+ TGF-β1
+ TGF-β1+ SB-505124
+ SB-505124
20 µm
20 µm
20 µm
20 µm
20 µm

B

Figure 3.11: ICC images for nuclear p-smad 2/3 staining showing activation of the TGF-β signalling pathway. A) MDA-MB-231 B) MCF-7 cells upon exposure to 5 ng/ml TGF-β1 (MDA-MB-231) and 10 ng/ml (MCF-7) for 3 days (red). p-smad 2/3 is decreased or almost absent with the addition of 5 μM SB-505124 with or without TGF-β1, respectively. Nuclei were visualized by 4', 6-diamidino-2-phenylindole DAPI stain (blue). Negative controls for the primary antibody were used. All images were taken at 20X magnification and a 20 μm scale bar was used. Representative images from n=3 experiments.

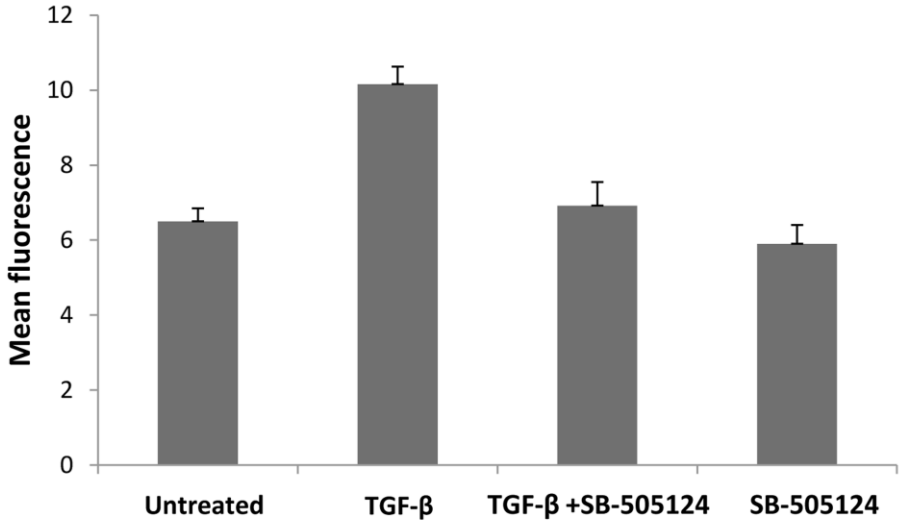


Figure 3.12: Graphical representation of ICC data. p-smad 2/3 levels in untreated, treated with 5 ng/ml TGF-β1, 5 ng/ml TGF-β1 + 5μM SB-505124 and 5μM SB-505124 MDA-MB-231 cells. Bars represent an average of n=3 individual experiments, p=0.001. Results are expressed as the mean SEM using a one way ANOVA test and were considered significant when $p<0.05$.

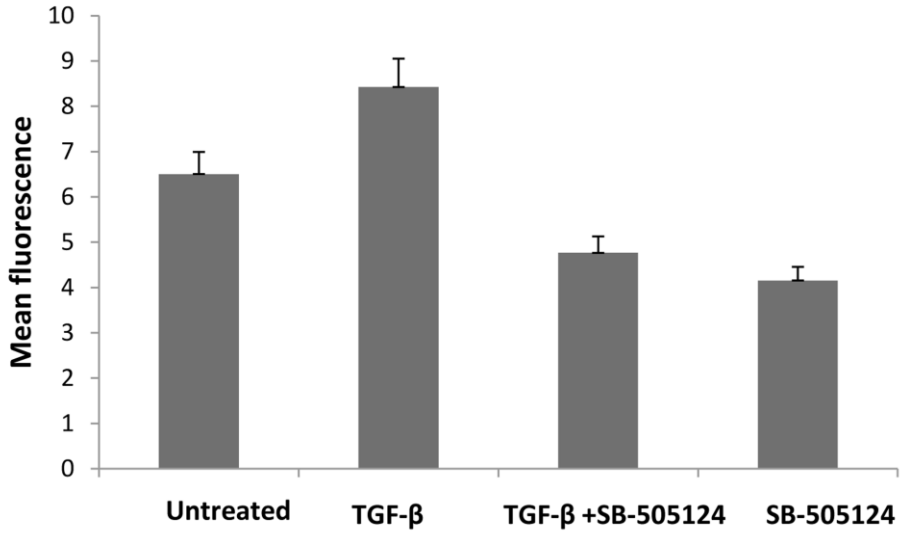


Figure 3.13: Graphical representation of ICC data. p-smad 2/3 levels in untreated, treated with 10 ng/ml TGF-β1, 10 ng/ml TGF-β1 + 5μM SB-505124 and 5μM SB-505124 MCF-7 cells. Bars represent an average of n=3 individual experiments, p=0.0008. Results are expressed as the mean SEM using a one way ANOVA test and were considered significant when $p<0.05$.

3.5.4 Assessment of endogenous TGF-β1 production and TGF-BRI and II expression in MDA-MB-231 and MCF-7 cells

To explain the different responsiveness of SP cells in the MDA-MB-231 and MCF-7 cells, I used q-PCR and ICC experimental approaches to characterize the properties of the TGF-β signalling pathway in the above mentioned unfractionated cell lines. In fact TGF-β1 mRNA and TGFB-RI and II mRNA and protein expression were assessed by qPCR and ICC, respectively. Although mRNA expression of TGFB-RI was not significantly different between the two cell lines, levels of TGFB-RII were significantly higher in the MDA-MB-231 than in the MCF-7 cells (p=0.04, n=6 (Figure 3.14A and B). The levels of all three isoforms of endogenous TGF-β (data only shown for TGF-β1) were also measured by qPCR in MDA-MB-231 and MCF-7 cells, with only TGF-β1 being produced at measurable levels by both cell lines. Interestingly, the mRNA expression of TGF-β1 was significantly higher in the MDA-MB-231 cells (p=0.04, n=3) (Figure 3.14C). ICC analysis for the TGFB-RI and II receptors confirmed that although TGFB-RI protein levels were similar in both cell lines, TGFB-RII protein expression was increased in the MDA-MB-231 compared to the MCF-7 cells (Figure 3.15).

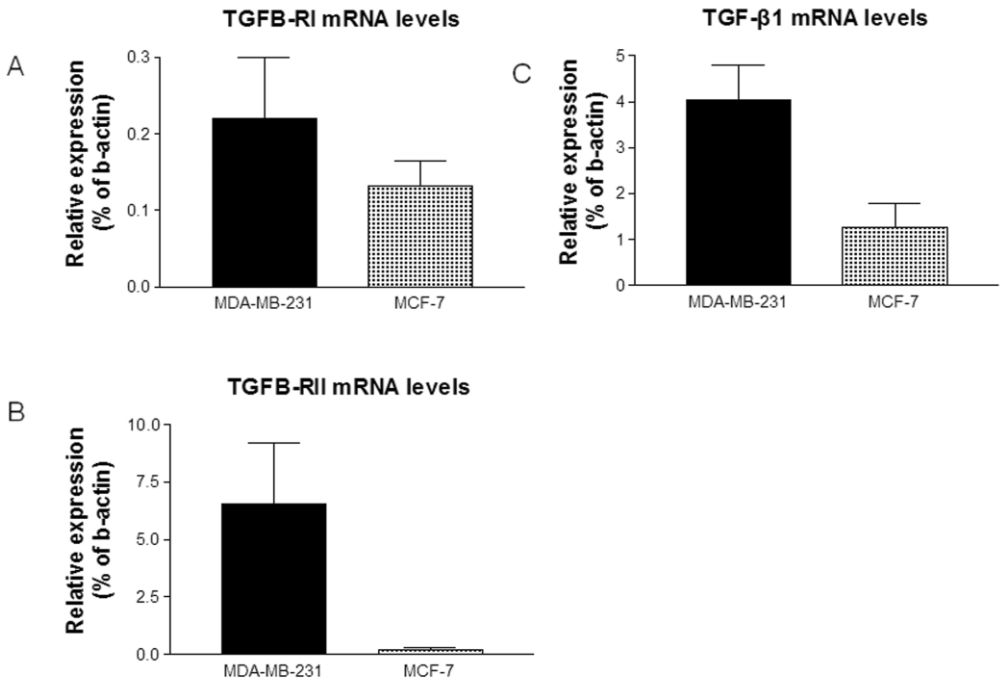
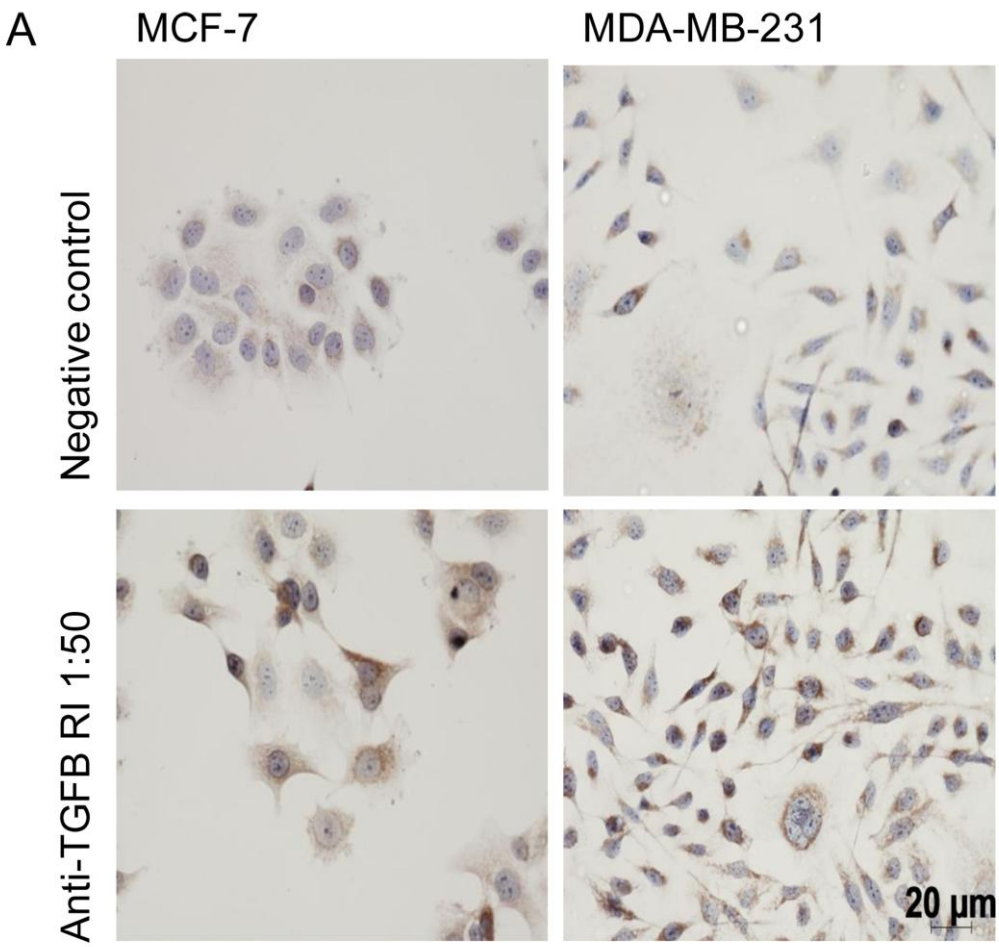


Figure 3.14: Graphical representation of qPCR data A) TGFB-RI B) TGFB-RII and C) TGF-β1 mRNA levels in MDA-MB-231 and MCF-7 cells. Results are expressed as the mean SEM for A) n=6, p=0.33 and B) n=6, p=0.04 and C) n=3, p=0.04 using an unpaired student's t-test.


A
MCF-7
MDA-MB-231
Negative control
Anti-TGFB RI 1:50
20 µm

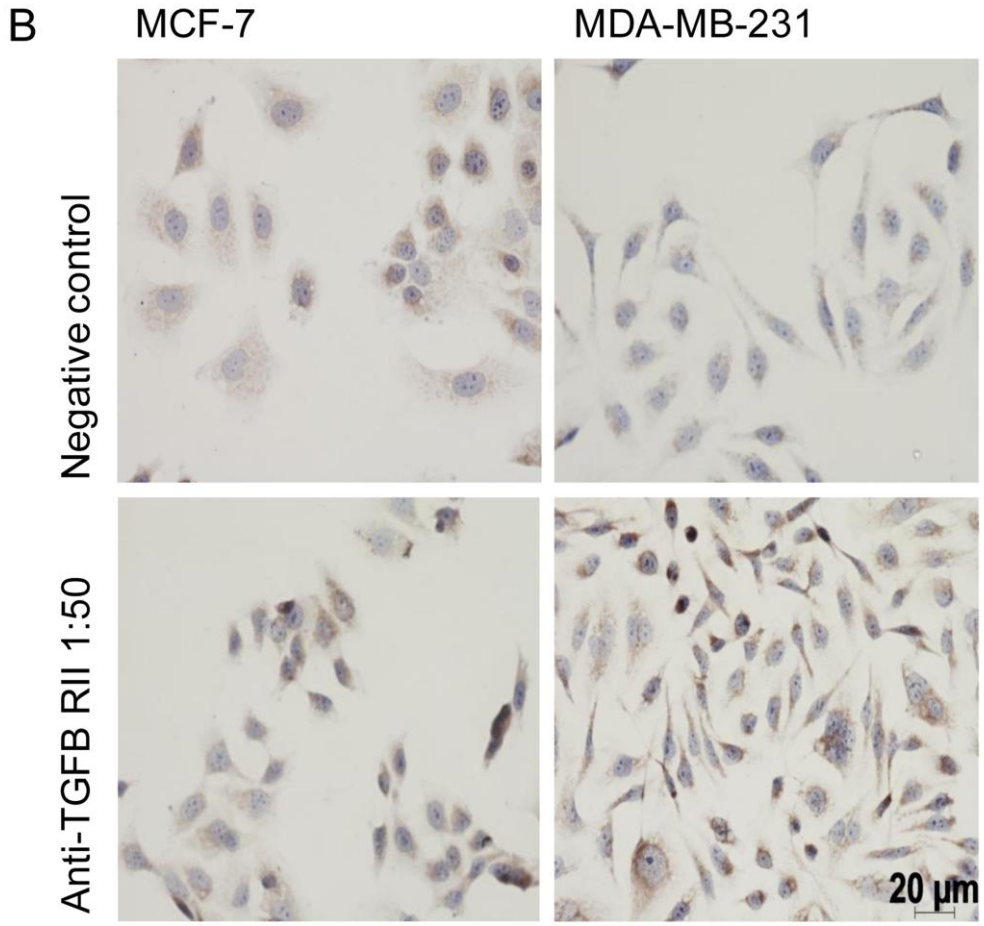


Figure 3.15: ICC staining for A) TGFB-RI and B) TGF-RII protein expression in MDA-MB-231 and MCF-7 cells. All images were taken at 40X magnification and a 20 µm scale bar was used. Representative images from n=2 experiments.

3.5.5 SP isolation from a breast cancer patient's Fine Needle Aspirate (FNA)

Obtaining a breast cancer patient's FNA containing a high number of cells allowed me to perform a series of experiments in order to detect the presence of SP cells and investigate the expression of ABCB1 and ABCG2 in this patient. SP analysis of this FNA revealed that there was a distinct SP population, which was only partially inhibited upon the addition of Verapamil (Figure 3.16). In addition, the use of the normal primary cells (HMEpC) isolated from normal adult mammary glands served as a negative control and allowed me to confirm that there is no SP population in these. In Figure 3.17 ABCG2 and ABCB1 are both expressed in the FNA sample, but ABCG2 expression seems higher than ABCB1, which could explain the incomplete inhibition by Verapamil, since it is known that it is more specific for ABCB1. The absence of an SP population in the HMEpC cells was validated by the very low ABCG2 and ABCB1 expression.

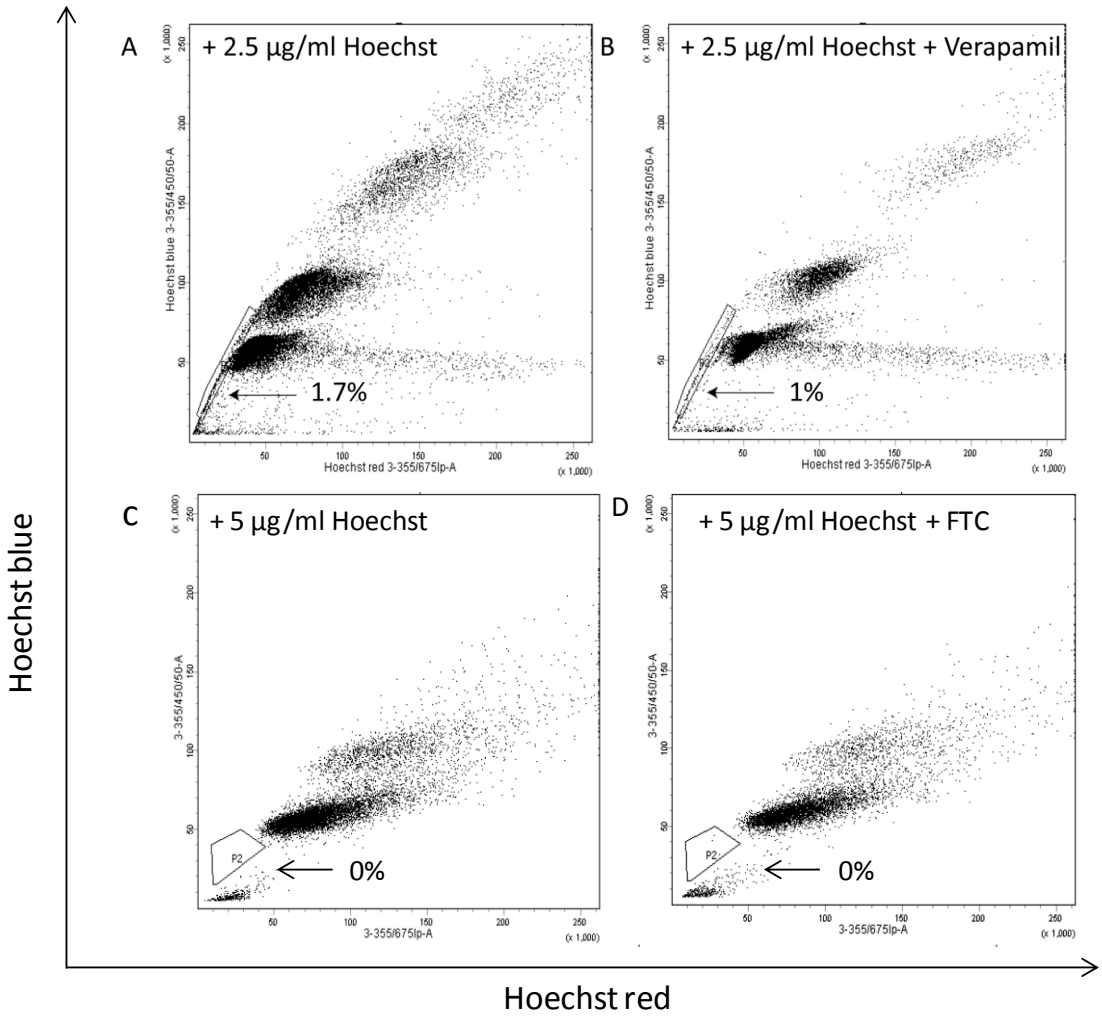


Figure 3.16: SP profile obtained from a breast cancer patient's FNA (Patient 5, see Appendix A). **A)** FACS profile showing an SP population **B)** Note the partial inhibition

of the SP phenotype as indicated by the reduced numbers of cells in the SP gate on the addition of 25 μM Verapamil. **C)** Negative control: HMEpC **D)** Note the absence of an SP population on the addition of 10 mM FTC.

3.5.6 Investigation of EMT marker expression in cells derived from a breast cancer patient's FNA

The expression of several EMT markers in this FNA was also assessed by PCR and it was shown that the patient's cells express mRNA for E-CADHERIN, TWIST, SLUG and FOXC2. HMEpC were used as a negative control and showed lower expression of E-CADHERIN, TWIST and SLUG and almost no expression of FOXC2 (Figure 3.17). The cells obtained from this FNA were cultured and ICC for EMT and stem cell markers was performed. High TWIST and S100A4 protein expression was seen (Figure 3.18) and there was also low expression of OCT 3/4 and SOX-2 and high expression of NANOG (Figure 3.19).

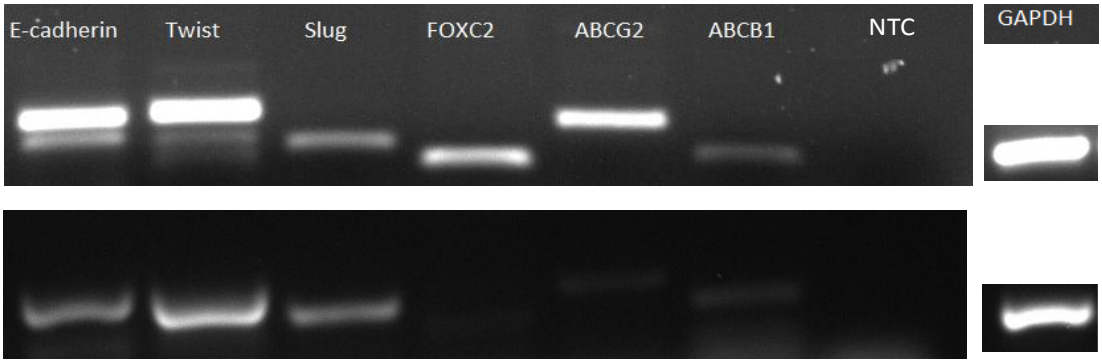


Figure 3.17: PCR products for EMT markers and ABC transporters gene expression obtained from a breast cancer patient's FNA (top panel) and from normal primary cells HMEpC (bottom panel). GAPDH was used as a housekeeping gene and a NTC ensured no genomic contamination.

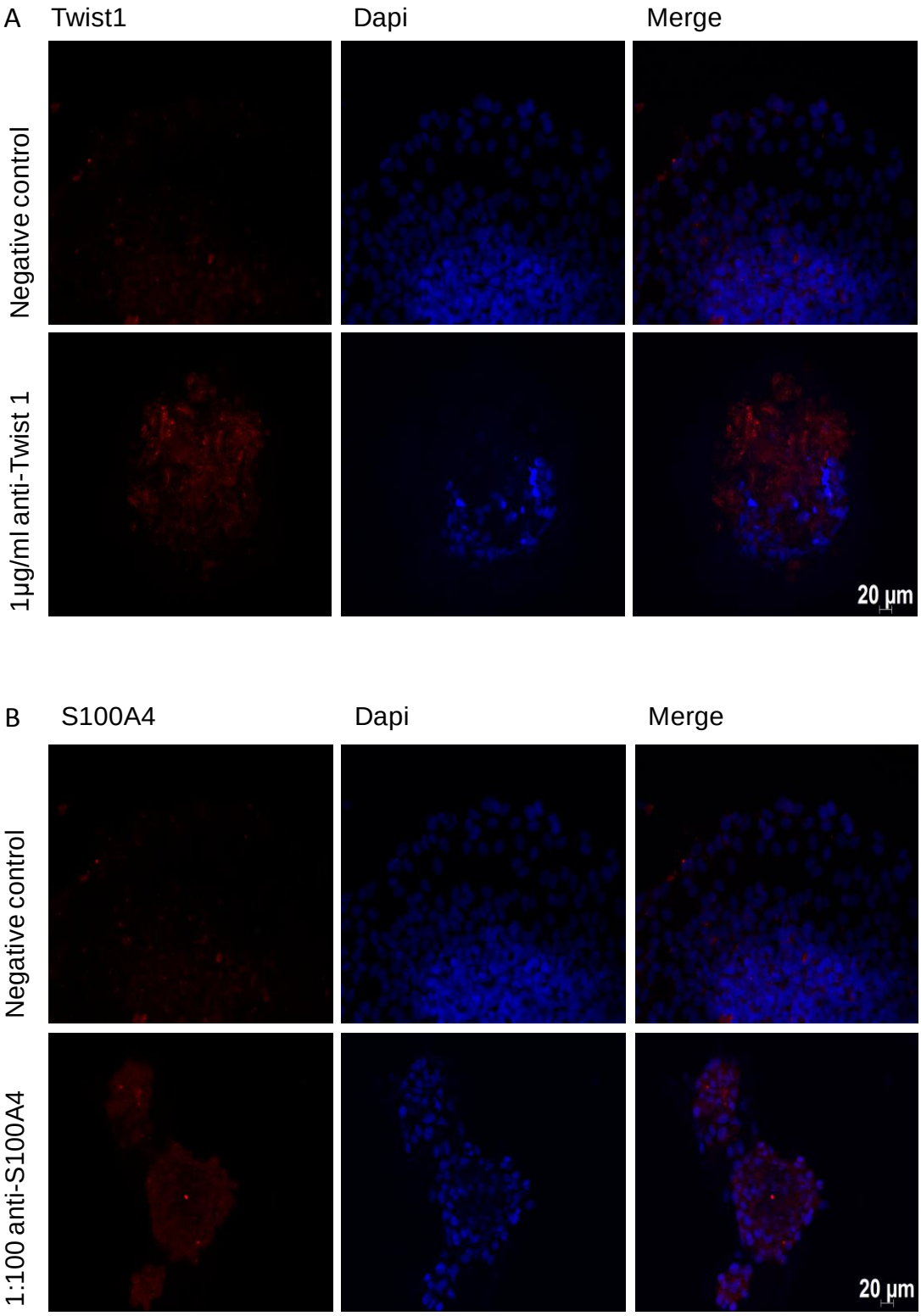
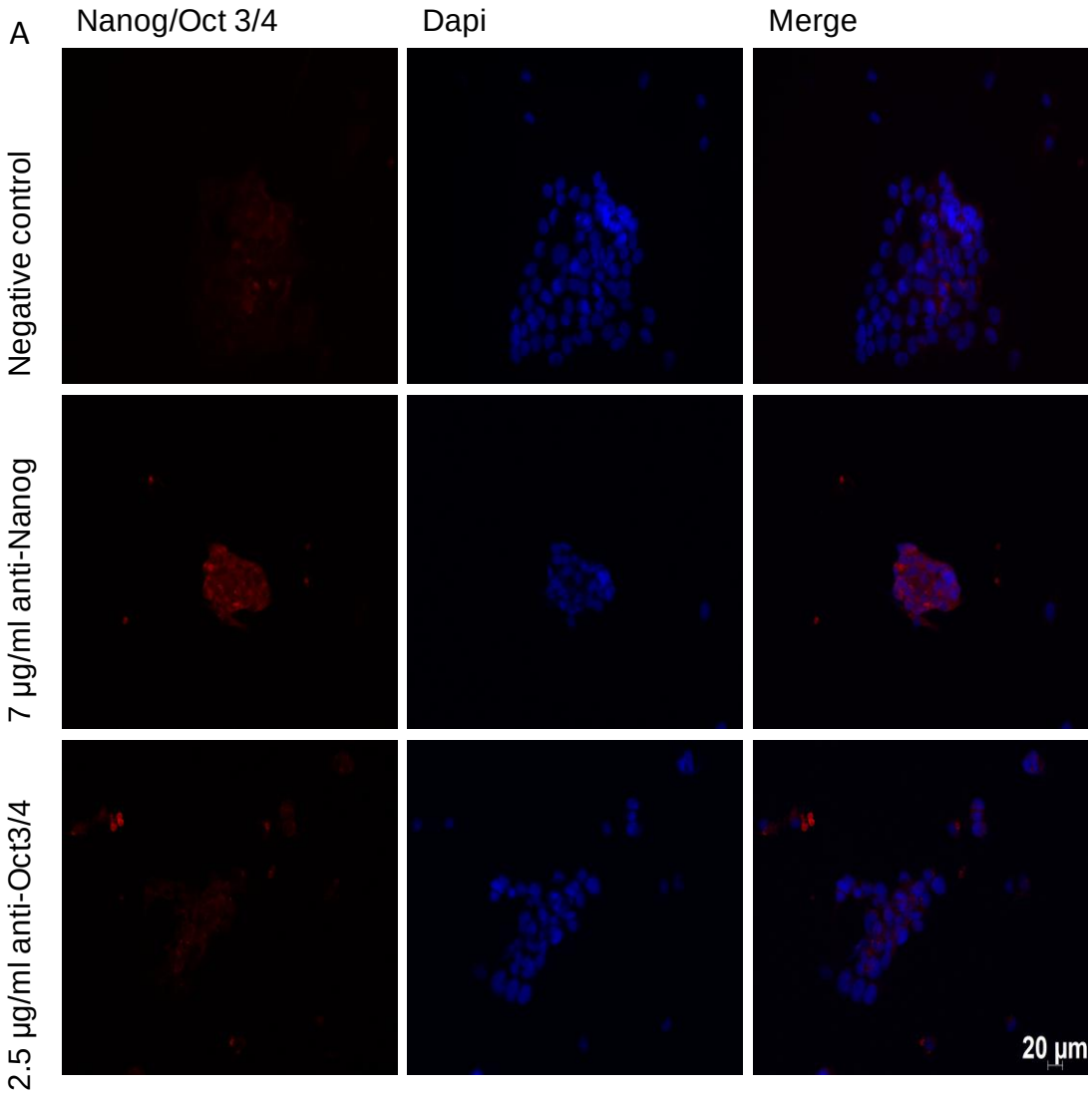


Figure 3.18: ICC images for EMT markers obtained from a breast cancer patient's FNA A) TWIST1 B) S100A4 protein expression. Images were taken at 20X magnification and a 20 µM scale bar was used.


A
Nanog/Oct 3/4
Dapi
Merge
Negative control
7 µg/ml anti-Nanog
2.5 µg/ml anti-Oct3/4
20 µm

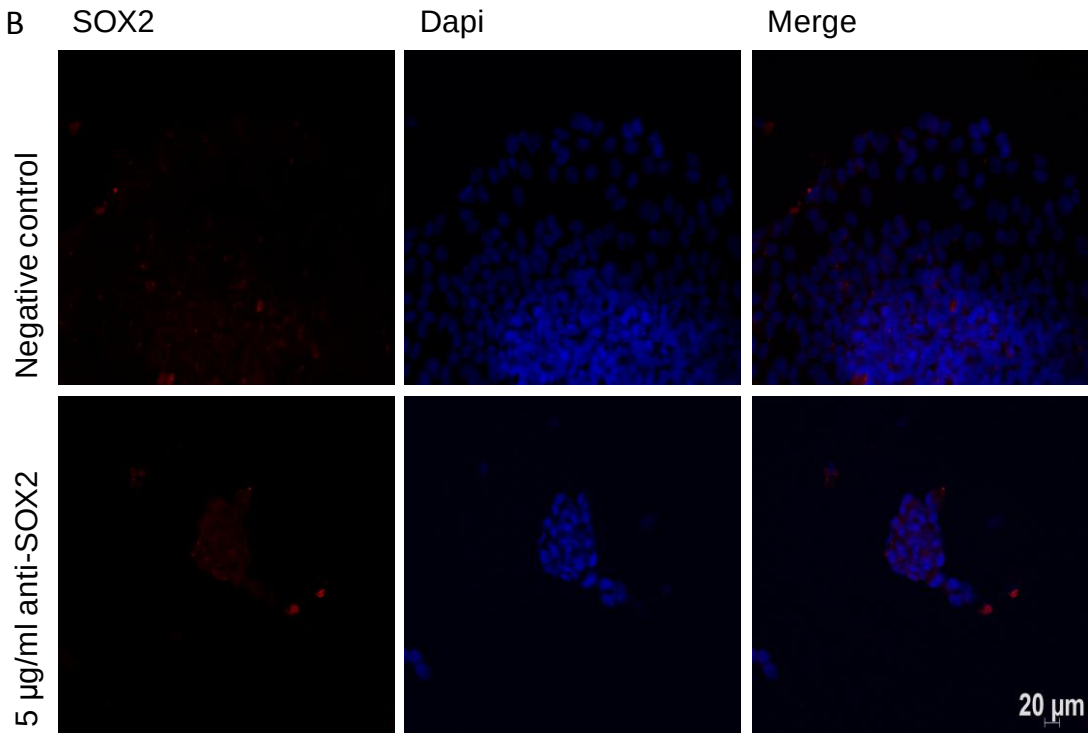


Figure 3.19: ICC images for stem cell markers obtained from a breast cancer patient's FNA. A) NANOG and OCT 3/4 B) SOX2. Images were taken at 20X magnification and a 20 µM scale bar was used.

3.6 Discussion

The relationship between EMT, BCSCs and multidrug resistance has become of increasing interest in recent years, with the TGF-β signalling pathway having a role in inducing EMT via down-regulation of E-cadherin (Moustakas and Heldin, 2007). The TGF-β signalling pathway has a key role in normal mammary gland development by maintaining the mammary epithelium in an undifferentiated state. It is also responsible for the induction of apoptosis in the alveolar department and for the inhibition of cell growth in the ductal epithelial compartment, indicating its multiple roles depending on cell and environmental context (Wakefield *et al.*, 2000). However, during tumorigenesis tumor epithelial cells acquire an altered responsiveness to TGF-β, they increase the secretion and/or activation of it, promoting invasion and metastasis (Wakefield and Roberts, 2002).

TGF-β signalling plays a role in the regulation of both normal and cancer stem cells in the breast. However, its impact differs depending on the cell type and physiological cell state and other microenvironmental factors. In normal mammary gland development, TGF-β is thought to highly contribute to the regulation of the stem cell properties of normal epithelial stem cells (Sakaki-Yumoto *et al.*, 2013). Additionally, it has been found that induced expression under the WAP promoter in mice, which is mostly active during late pregnancy and lactation, has led to the impairment of alveolar growth and development and to premature alveolar cell apoptosis. Mammary epithelial stem cells were also prematurely senescent and thus failed to form ductal mammary trees due to overexpression of TGF-β1 (Kordon *et al.*, 1995). Of note, in another study it has been reported that these effects prevented tumor formation caused by MMTV-induced tumorigenesis presumably by reducing the self-renewal capacity of mammary epithelial stem cells and by promoting their differentiation (Boulanger and Smith, 2001).

Although in some studies TGF-β has been shown to promote the generation of CSCs, there are contrasting reports which demonstrate an inhibitory role of TGF-β on CSCs (Sakaki-Yumoto *et al.*, 2013). Indeed, the effect of EMT through the TGF-β pathway on several BCSCs populations has been studied previously and it has been reported that it leads to the enhancement of the CD44+/CD24- phenotype (Mani *et al.*, 2008) or to significant reduction of the SP phenotype (Yin *et al.*, 2008). In my study SP analysis of TGF-β1 treated cells showed loss of the SP phenotype in the MDA-MB-231 and a significant reduction of cells with an SP phenotype in the MCF-7 cell line. A similar observation on the MDA-MB-231 SP has also been made by Tang and colleagues, who

demonstrated that SP fraction was decreased in response to TGF-β exposure (Tang *et al.*, 2007). Furthermore, my data regarding the MCF-7 SP are consistent with those of Yin et al., who demonstrated that the decrease of the SP percentage was due to ABCG2 down-regulation caused by TGF-β exposure (Yin *et al.*, 2008).

Additionally, it has been shown that treatment of the MCF10ACa1h subline, transformed with the activation of the Ha-ras oncogene, with TGF-β also resulted in significant decrease of the SP cell percentage and these effects were also observed in the mouse tumor cell line Wnt1. TGF-β treatment also reduced the mammosphere forming ability of the SP cells in the Ca1h cells and this was reversed by the loss of TGFB-RII expression in the Ca1h cells transfected with the dominant negative form of the TGFB-RII gene (Ca1h-DNR). Notably, the SP numbers were increased in tumors formed by injection of the Ca1h-DNR cells in mice, supporting the role of the TGF-β signalling pathway both *in vitro* and *in vivo*. It is worth mentioning that in the same study TGF-β was found to be responsible for also promoting the differentiation of a more committed, but highly proliferative progenitor cell population as shown by the elevation of the MUC1 and CK8 luminal differentiation markers, while in addition it reduced the proliferation rates of this progenitor cell population (Tang *et al.*, 2007).

I anticipate that there is a stronger impact on the MDA-MB-231 SP cells, since these have been shown to express no significantly different protein levels of ABCG2 compared to the MDA-MB-231 NSP cells (Britton *et al.*, 2012). However, there was a significantly higher ABCG2 protein expression in the MCF-7 SP cells than in the MCF-7 NSP cells (Britton *et al.*, 2012), which is presumably more difficult to down-regulate. I also observed differences in the responsiveness of the two cell lines to TGF-β1 treatment, the effects of which could be reversed by the use of the SB-505124 inhibitor in the MDA-MB-231 cells, as shown by the re-appearance of the SP population in cells treated with SB-505124 in the presence of TGF-β1. Most importantly, treatment of MDA-MB-231 cells with SB-505124 in the absence of exogenous TGF-β1 further enhanced the SP phenotype, indicating that the autocrine TGF-β1 production is responsible for reducing the SP numbers in this cell line. MCF-7 cells did not survive the addition of SB-505124, which presumably was due to the decreased internalization of TGF-β1 and weak TGF-β signalling.

Findings of this study are consistent with previous reports in which TGF-β treatment also decreased the SP fraction in other cancer cell lines. Ehata et al demonstrated that

ABCG2 was down-regulated upon the addition of TGF-β to the culture media of several cancer cell lines, including MDA-MB-231 cells. Accordingly, ABCG2 was among the most up-regulated genes in tumors formed after the injection with the diffuse-type gastric carcinoma cell line, OCUM-2MLN which had been transfected with the dominant negative form of TGFB-RII (2MLN-dnTGFB-RII) into nude mice. Most importantly, SP cells exhibited a higher tumorigenicity *in vivo* compared to the NSP cells from diffuse-type gastric carcinoma cell lines and this was reduced when cells were pre-treated with exogenous TGF-β before being injected into nude mice. Knock-down of the smad4 gene did not result in the repression of ABCG2 expression in response to TGF-β, indicating that these effects were due to the TGF-β/smad dependent pathway (Ehata *et al.*, 2011).

What is more, isolated SP cells from the pancreatic cell lines PANC-1 and Capan-2 re-gained E-cadherin expression after 3 days in culture and this was down-regulated with TGF-β treatment for 2 days, whereas E-cadherin expression levels were lower before and after treatment in the NSP cells. E-cadherin expression was also restored upon withdrawal of TGF-β from the culture media. These observations indicated that SP cells were more responsive to EMT changes induced by TGF-β in comparison to NSP cells. This was further confirmed with luciferase reporter assays in the PANC-1 SP and NSP cells which showed that SP cells had a higher luciferase transcription activity even without the addition of any exogenous TGF-β. Furthermore, p-smad2/3 was increased in the SP compared to the NSP, suggesting that the TGF-β pathway was more active in the former (Kabashima *et al.*, 2009). Additionally, TGF-β exposure led to the decrease of SP hepatic stellate LX2 cells and the increase of collagen type I expression which was reversed upon the addition of the SB431542 inhibitor. It was concluded that TGF-β signaling participates in Extracellular Matrix (ECM) accumulation causing fibrosis in addition to the regulation of the SP population (Kim *et al.*, 2014).

The only study that reported a positive regulation of the SP population by TGF-β was in the human gallbladder cancer cell line GBC-SD. The SP cells generated through EMT induced TGF-β exposure were characterized with the up-regulation of ABCG2 and decreased sensitivity to mitoxantrone. These effects were reversed by the use of a specific si-RNA for smad 3 which resulted in the reduction of the SP abundance. I anticipate that TGF-β may have different regulatory roles depending on cellular context. However, it should be noted that SP cells in this study also showed a higher colony

forming efficiency *in vitro* and a higher tumorigenic ability *in vivo* compared to the NSP cells, confirming the notion that they are a CSC population (Zhang *et al.*, 2011).

The autocrine TGF-β1, β2 and β3 mRNA production by MDA-MB-231 and MCF-7 cells was assessed using qPCR, but only TGF-β1 was produced by these cells. The levels of TGF-β1 were significantly higher in the MDA-MB-231 cells compared to the MCF-7 cells. qPCR and ICC analysis for the TGFB- receptors I and II revealed that although the levels of TGFB-RI were similar, there was very low expression of the TGFB-RII in the MCF-7 cells. These results are consistent with published findings that MCF-7 cells lack expression of RII and when this was restored the cells became less tumorigenic both *in vitro* and *in vivo* (Sun *et al.*, 1994). The effects of defective TGF-β signalling by loss of TGFB-RII expression on the SP population have been demonstrated in the MCF10ACalh1 subline by Tang et al. (Tang *et al.*, 2007).

RII expression is considered more important for the activation of the TGF-β signalling pathway, since TGF-β1 bound RII forms a heterodimer complex with RI, which promotes the subsequent molecular events through its kinase activity (Vivien *et al.*, 1995). Additionally, all TGF-β isoforms are mainly produced in a latent form, therefore the small amount that is activated requires sufficient receptors on the cell surface to internalize it (Chen *et al.*, 1997). Low RII expression in the MCF-7 cells is presumably responsible for defective binding of TGF-β1, internalization and signalling in contradiction with the MDA-MB-231 cells.

It has been reported that RIII binds to RII and causes conformational changes, which facilitate the binding of the ligand to RII and enhance the pathway activation (López-Casillas *et al.*, 1993). Interestingly, RIII expression is also very low in MCF-7 cells and transfection with the RIII gene led to increased TGF-β promoter activity measured by luciferase and decreased levels of activated TGF-β1 in the conditioned media. Additionally, there was significant inhibition of anchorage independent colony formation and cell growth (Chen *et al.*, 1997). Moreover, ectopic expression of the dominant negative form of RIII (sRIII) in the MDA-MB-231 cell line had the same effects, but it also caused apoptosis by increasing the levels of PTEN (Lei *et al.*, 2002), which has a pro-apoptotic role by competing with phosphatidylinositol 3-kinase mediated Akt activation (Simpson and Parsons, 2001). Treatment with TGF-β1 prevented apoptosis and reduced the PTEN levels, suggesting that autocrine TGF-β1

production is essential for the proliferation and maintenance of these cells (Lei *et al.*, 2002).

TGF-β exerts its tumor suppressive activity by inhibiting cyclin-dependent protein kinases (cdk) that regulate the G1 phase of the cell cycle (cdk2, cdk4, cdk6) (Massagué, 2000; Massagué *et al.*, 2000). It is known to induce the elevation of p15Ink4b, which is a cdk4/6 inhibitor (Sandhu *et al.*, 1997). TGF-β also prevents c-myc from blocking p15Ink4b and thus it promotes inhibition of cell growth (Warner *et al.*, 1999). However, in MDA-MB-231 cells there is loss of c-myc down-regulation, leading to excessive cell cycle arrest and uncontrolled proliferation (Chen *et al.*, 2001). It has been proposed that MCF-7 cells are also resistant to the inhibitory effects of TGF-β (Lei and Bandyopadhyay, 2002; Lei *et al.*, 2002) due to the fact that RII, whose expression is low, can be saturated by TGF-β1 and therefore reaches the cells' maximal autocrine TGF-β activity more easily (Chen *et al.*, 1997).

The oncogenic effects of the TGF-β signalling pathway have not been attributed to the inactivation of any of the pathway components, since genetic alterations are thought to be extremely rare in breast cancer (Wakefield *et al.*, 2001). In fact, no mutations have been found in TGFB-RII in primary breast cancer tumors or breast cancer cell lines (Vincent *et al.*, 1996; Anbazhagan *et al.*, 1999; Tomita *et al.*, 1999) and in TGF-BRI in 20 breast cancer specimens from metastatic cases (Anbazhagan *et al.*, 1999), while 90% of primary cell cultures expressed normal levels of TGF-BRI (Chakravarthy *et al.*, 1999). Additionally, none of the 22 breast cancer cell lines showed any gene alterations in Smad 1, 3, 5 and 6 (Riggins *et al.*, 1997).

Nevertheless, it has been reported that breast cancer development can be caused due to loss of TGFB-RII expression at both early and late stages of the disease (Wakefield *et al.*, 2001). In 17% of women with epithelial hyperplasia lacking atypia (EHLA) there was a reduction to less than 25% of cells expressing TGF-BRII, which was correlated with increased risk of invasive breast cancer (Gobbi *et al.*, 1999). Furthermore, immunohistochemical analysis of specimens derived from human DCIS and IBS patients showed low TGFB-RII expression compared to the normal tissue. Therefore, it was suggested that resistance to the TGF-β suppressive actions caused by low or absent TGF-BRII expression may lead to the progression of a more aggressive disease (Gobbi *et al.*, 2000).

Moreover, prolonged TGF-β exposure may also lead to the inhibition of the apoptotic role of the TGF-β signalling and to the induction of EMT and migration as demonstrated in the murine mammary epithelial cell line namru murine mammary gland (NmuMG) by Gal and co-workers. The EMT related changes were found to be caused through the activation of the PI3K-Akt and the MAPK cascades, which were reversible upon TGF-β removal and the addition of an TGFB-RI inhibitor (Gal *et al.*, 2007).

Nuclear expression of p-smad 2/3 in treated MDA-MB-231 and MCF-7 cells was increased by exogenous TGF-β1 treatment. However, even treatment with higher concentration of TGF-β1 did not augment activation of the p-smad 2/3 pathway in MCF-7 cells to the same extent as in the MDA-MB-231 cells. Surprisingly, MCF-7 cells survived following addition of the antagonist SB-505124 probably due to the higher number of cells used in this experiment. Normally, a limited number of cells are plated for the SP assay in order to ensure low confluency and high SP percentage. Although the total cell population is responsive to the TGF-β exposure there seems to be a visible impact only on the SP phenotype. Interestingly, it has previously been shown that p-smad 2/3 directly binds to the ABCG2 promoter and down-regulates its expression in response to TGF-β (Ehata *et al.*, 2011). As a result, it is reasonable to suggest that effects on the SP population are caused through the TGF-β smad-dependent pathway.

There is accumulating evidence to support the cross-talk between the TGF-β and estrogen-signalling pathways. ER- breast cancer cell lines have been found to express receptors for TGF-β, while ER+ cell lines are characterized by undetectable levels of these. Furthermore, TGF-β treatment led to the inhibition of proliferation of almost all ER- breast cancer cell lines in a dose dependent manner, while all ER+ cell lines were unaffected. As a result, it was concluded that ER- cells produce and secrete large amounts of TGF-β into the cell culture medium, express sufficient receptors for it and respond to even low concentrations of exogenous TGF-β (Arteaga *et al.*, 1988).

Another study also showed that ER+ breast cancer cell lines were TGFB-RII- and resistant to TGF-β treatment, while the opposite was found in ER- cell lines. However, early passage MCF-7 cells (MCF-7E) were found to express TGF-BRII which was lost in late passage cells (MCF-7L). Using 5-aza-2'-deoxycytidine (5-aza-2'-dC), a DNA methyltransferase inhibitor, the authors were able to reverse methylation of the TGFB-RII gene and restore its expression in MCF-7L cells, which became sensitive to TGF-β

exposure. Interestingly, the TGFB-RII promoter region was found to contain 2 binding sites for the SP1 transcription factor and cells expressing a mutant form of SP1 failed to express TGFB-RII and respond to TGF-β treatment (Ammanamanchi *et al.*, 1998). Liu and colleagues also demonstrated that low TGFB-RII expression is partially due to insufficient amounts of SP1 protein, since co-transfection of MCF-7L with an SP1 plasmid and a TGFB-RII promoter construct resulted in the increase of TGFB-RII protein levels (Liu *et al.*, 2000)

In addition, Fujita and colleagues demonstrated that both ER-α and estradiol are essential for the inhibition of EMT through SNAIL repression and maintain E-cadherin expression and therefore the epithelial morphology of breast cancer cells (Fujita *et al.*, 2003). In another study, they further showed that ER-α transcriptionally activates the metastasis-associated protein 3 (MTA3) in MCF-7 cells by directly binding to both an SP1 and an ERE site, which are in close proximity in the MTA3 promoter region (Fujita *et al.*, 2004). It should be noted that MTA3 is an important subunit of the Mi-2/NuRD complex, which has histone deacetylase and chromatin remodeling ATPase activities and thus it contributes to the transcriptional repression of several genes (Fujita *et al.*, 2003; Bowen *et al.*, 2004).

Conversely, SNAIL and SLUG overexpression in MCF-7 cells promoted the acquisition of a more claudin-low- like phenotype caused by the induction of EMT and the up-regulation of genes that are involved in the TGF-β family, including Transgelin, SPARC and CTGF. The transfected MCF-7 cells also had an increased migratory potential, which was reversed by the use of TGF-β inhibitors. Most importantly, histone H3K9 was acetylated in the promoter of the TGFB-RII gene followed by SNAIL and SLUG overexpression in the MCF-7 and resulted in a pattern of this promoter in the MDA-MB-231 cells. Therefore, the authors suggested a model which explains the negative regulatory role of ER-α and MTA3 on the expression of SNAIL, which leads to the reduced activity of the TGFB-RII promoter and thus to weak TGF-β signalling in luminal type breast cancer cells (e.g MCF-7) (Dhasarathy *et al.*, 2011).

Intriguingly, two distinctive stem cell populations were identified in primary human breast cancer cells: CD44-, CD24+, ER+, TGFB-RII- or CD44+, CD24-, ER-, TGFB-RII+ with the latter only being able to undergo EMT due to TGF-β treatment (Shipitsin *et al.*, 2007). I also observed a higher increase of CD44+ cells in the MDA-MB-231

cells in response to TGF-β treatment in comparison to the MCF-7 cells. These findings further support the TGF-β signalling involvement in the regulation of the stem cell counterpart as well as ER signalling in breast cancer. Indeed, in MCF-7 cells the estrogen receptor α (ERα) induces p-smad 2/3 degradation by forming a complex with it and ubiquitin ligase Smurf (Ito *et al.*, 2010). Similarly, when the ERα gene was introduced in MDA-MB-231 cells it resulted in decreased tumor formation both *in vitro* and *in vivo*. These effects were reversed in cells overexpressing ERα and a constitutively active form of Smad 2. Consequently, ERα was proposed as negative regulator of the TGF-β/Smad dependent signalling pathway in later stages of breast cancer, although estrogen exposure drives tumor progression in early stages (Goto *et al.*, 2011).

Finally, I was able to identify a distinct SP population in the cells obtained from an FNA of a breast cancer patient. The use of verapamil did not completely inhibit the SP cells and this effect could be explained by PCR analysis, which showed that this patient's cells were characterized with a higher expression of ABCG2 than ABCB1. It is known that verapamil binds to ABCB1 with a higher affinity than ABCG2 (Britton *et al.*, 2012). The use of the normal primary mammary cells HMEpC for the SP analysis showed that there is no SP population in these, which indicates that the presence of this population in the breast cancer patient may be responsible for her clinical condition. These data can be supported by the PCR analysis which showed almost undetectable expression of ABCG2 and ABCB1 in HMEpC. It should be mentioned that other cell types are also present in the FNA sample, including blood cells, fat cells and fibroblasts, therefore, the use of a biological negative control, such as the HMEpC cells is extremely important. PCR analysis showed expression of several EMT markers, including E-CADHERIN, TWIST, SLUG and FOXC2, while E-CADHERIN, TWIST, SLUG had had lower expression and FOXC2 expression was absent in the negative control. In addition, protein levels of TWIST and S100A4 were also increased. OCT 3/4 and SOX-2 protein levels were slightly increased, while NANOG showed a higher expression. This suggests that there is a population of cells which express stem cell and/or EMT markers. However, it would be interesting to determine whether the stem cell population also expresses EMT markers, which would be indicative of the presence of a CSC population in this patient. This particular breast cancer patient was ER+/PR+/HER-, Grade 2 with 3 positive lymph nodes. The limited number of FNAs that I obtained during this project and the difficulties of the SP analysis due to the low

cell number in these did not allow me to associate the presence of SP cells and EMT/stem cell marker expression with any particular breast cancer patient subtypes. Any possible conclusions from this study would provide insight to the mechanisms that are involved in the regulation of SP cells by EMT and they could enable the identification of new target molecules for the prevention of metastasis. It should be noted that a recent study which analyzed 2,000 breast cancer tumors classified breast cancer into 10 different subtypes (Curtis *et al.*, 2012), therefore the specificity of future strategies that target SP cells based on the exact patient subgroup is of great importance.

Taken together, I have demonstrated that there is a clear impact of EMT on SP cells. Based on the literature (Yin *et al.*, 2008; Kabashima *et al.*, 2009; Ehata *et al.*, 2011) and my study I assume that TGF-β1 induces differentiation in the SP population. SP cells have been reported to be more capable of up-regulating E-cadherin and undergoing EMT when treated with exogenous TGF-β, suggesting that they are more prone to the phenotypic alterations caused by this process and thus they can acquire a higher metastatic and invasive potential compared to the NSP cells (Kabashima *et al.*, 2009). Yin et al. suggested that SP cells might re-emerge after the establishment of secondary tumors at metastatic sites (Yin *et al.*, 2008). MCF-7 cells have an epithelial origin and they could be more reliable models for defining the EMT-related changes caused by TGF-β exposure. However, the MDA-MB-231 cell line represents triple negative breast cancer subtypes, which have been significantly correlated with the presence of SP cells (Britton *et al.*, 2012).

Chapter 4: The effect of hypoxia on BCSCs

4.1 Introduction

Low concentration of oxygen in cells or tissues, known as hypoxia, is one of the conditions in the tumor microenvironment that is considered an important step in tumor progression, since it has a great impact on invasion, metastasis, chemo-radiation resistance and angiogenesis. It can result from insufficient blood supply (transient hypoxia) or increased oxygen diffusion due to tumor expansion (chronic hypoxia) (Bao *et al.*, 2012). Most importantly, hypoxia or anoxia have been found to be prevalent in up to 60% of advanced solid tumors (Favaro *et al.*, 2011). The effects of hypoxia are regulated by the HIF family of proteins (1, 2 and 3), which belong to the per-aryl hydrocarbon receptor nuclear translocator (ARNT)-sim (PAS) basic helix-loop-helix (bHLH) heterodimeric transcription factors and consist of the α and the β subunits. The most common heterodimer which is responsible for hypoxic response is HIF-1α/β. The expression of the α subunit is dependent on oxygen levels and under hypoxic conditions its mRNA and protein expression increases while the protein translocates into the nucleus (Wang *et al.*, 1995), where it can induce the expression of up to 1.5% of the genes in the human genome (Favaro *et al.*, 2011). During normoxia HIF-1α is hydroxylated by prolyl-hydroxylase and it is targeted for protein degradation by binding to the VHL complex (Yu *et al.*, 2001).

Hypoxia has been recognized as a critical regulator of self-renewal capacity and it is believed to be responsible for the maintenance of the undifferentiated state of stem cells during embryonic and adult development (Chandel and Simon, 2008; Simon and Keith, 2008). There is also accumulating evidence to support the role of hypoxia in the regulation and function of CSCs (Bao *et al.*, 2012), which are thought to result in the development of drug and chemo-resistant tumors and reduced patient survival (Reya, 2001). The mechanism by which this is mediated is not completely elucidated, but it has been suggested that necrotic or hypoxic tumor regions are considered as a niche for CSCs. In addition, it has been hypothesized that hypoxia or inappropriate expression of HIF can promote the expansion of CSCs by maintaining their undifferentiated state (Gustafsson *et al.*, 2005; Barnhart and Simon, 2007; Bao *et al.*, 2012) and this is reversible when oxygen levels are restored (Zeng *et al.*, 2011). Of note, the signalling pathways driven by HIF-1α and HIF-2 have been reported to be involved in the transcriptional activation of stem cells markers, such as OCT 3/4 (Covello *et al.*, 2006; Heddleston *et al.*, 2010). In fact, it has been demonstrated that hypoxia induces the

expression of genes that are responsible for stem cell maintenance (SOX2, OCT 3/4) in neuroblastomas through the activation of Notch signalling (McCord *et al.*, 2009).

What is more, recent evidence suggests that hypoxic regions or poorly vascularised tumors are enriched with BCSCs that express high levels of HIFs and EMT markers leading to the enhancement of angiogenesis and the formation of more metastatic phenotypes during breast cancer development (Louie *et al.*, 2010; Oliveira-Costa *et al.*, 2011; Wang *et al.*, 2011). Immunohistochemical analysis of 253 breast cancer specimens revealed an association of HIF-1α expression with the presence of CD44+/CD24- cells (Oliveira-Costa *et al.*, 2011). Notably, CD44+/CD24- cells overexpressing HIF-1α were found to also express mesenchymal markers, while they acquired an increased mammosphere and colony forming efficiency and tumorigenic capacity (Conley *et al.*, 2012; Han *et al.*, 2012). Similarly, repetitive cycles of hypoxia and re-oxygenation resulted in the generation of CD44+/CD24-/ESA+ in the MDA-MB-231 and BCM2 cell lines, and these cells had a higher metastatic potential to the lungs (Louie *et al.*, 2010). Moreover, Krishnamachary and colleagues demonstrated that hypoxia is responsible for the up-regulation of both CD44 and VEGF in the MDA-MB-231 and SUM-149 breast cancer cell lines (Krishnamachary *et al.*, 2012).

In addition, the contribution of hypoxia in the regulation of SP cells is becoming increasingly evident. Hypoxia increased the stem cell-like properties of cells of the prostate cancer cell lines PC-3 and DU145 by enhancing the number of SP cells. It also led to the increase of CD44+ cells which were characterized as having a higher expression of OCT 3/4, NANOG and ABCG2 and had a greater sphere and colony formation potential *in vitro* (Ma *et al.*, 2011). Liu et al also reported that hypoxia and re-oxygenation induced an increase in the expression of ABCG2 in SP cells of kidney cells from C57BL/6 mice and protected them against hypoxic damage through the MEK/ERK pathway (Liu *et al.*, 2013). The induction of ABCG2 expression has been found to prevent the accumulation of porphyrins and heme within a cell which increase under hypoxic conditions and lead to the production of reactive oxygen species and mitochondrial dysfunction (Krishnamurthy *et al.*, 2004). HIF-2α has been shown to directly bind to and up-regulate ABCG2 thus increasing the percentage of cardiac SP cells 3-fold, these SP then exhibited improved survival mechanisms when exposed to hydrogen peroxide (Martin *et al.*, 2008). Most importantly, HIF-2α expression was significantly correlated with high ABCG2 expression, histology-grade and Ki67 expression in a patient cohort consisting of 196 invasive breast cancer patients.

Therefore, it was proposed that HIF-2α could serve as a reliable prognostic marker for the prediction of drug resistance and metastasis in breast cancer (Xiang *et al.*, 2012).

4.2 Chapter hypothesis

Given the evidence regarding the effect of hypoxia on several CSCs and the lack of evidence regarding the regulation of SP cells by hypoxia particularly in breast cancer cell lines, I sought to determine the response of BCSCs populations (SP and CD44+ cells) to hypoxic culture conditions in the MDA-MB-231 and MCF-7 cell lines. I also aimed to study the possible transcriptional effect of hypoxia on stem cell and EMT markers in unfractionated cells, which would further support its potential role in the regulation of CSCs. Additionally, I was interested in determining whether there is an interaction between the pathways mediated by HIFs and TGF-β, since they have both been reported to induce EMT and I also addressed the question of how the BCSCs will respond to the concomitant stimulation of these. Finally, I examined the possibility of up-regulation of ABCG2 and other ABC transporters by hypoxia and the impact of this on the induction of drug resistance in the SP and NSP populations.

4.3 Aims

- To determine the effects of hypoxia on the MDA-MB-231 and MCF-7 SP cells
- To determine the effect on mRNA and protein expression of CD44 in MDA-MB-231 and MCF-7 cells as a result of exposure to hypoxia.
- To investigate the impact of hypoxia on the transcriptional activation of EMT related genes and stem cell markers.
- To examine whether there is a crosstalk between the hypoxia and TGF-β signalling pathways.
- To test the combined effect of hypoxic and TGF-β treatment on the above mentioned BCSCs in the MDA-MB-231 and MCF-7 cell lines.
- To study the impact of hypoxia on the transcriptional activation of members of the ABC transporter family.

- To investigate how hypoxia affects the drug resistance of SP cells of the above mentioned breast cancer cell lines.

4.4 Experimental Design

- I used the hypoxia mimetic $CoCl_2$ to chemically induce hypoxia, since it has been reported to interfere with the activity of prolyl-hydroxylase and prevent the hydroxylation of HIF-1α (Salnikow *et al.*, 2004). I then optimized the treatment conditions by assessing the mRNA levels of known hypoxia-responsive genes, including VEGF-A, CXCR4 and HIF-1α after treatment with $CoCl_2$ for 24 h.

- Following a 24h incubation with $CoCl_2$ I performed qPCR analysis for CD44, EMT markers (TWIST1, TWIST2, SNAIL, SLUG, E-CADHERIN, VIMENTIN and S100A4), stem cell markers (NANOG, SOX-2 and OCT 3/4) and ABC transporters (ABCB5, ABCB1, ABCG2 and ABCC3).

- After treatment of MCF-7 and MDA-MB-231 cells with $CoCl_2$ for 48 h I performed the SP assay or CD44 staining and examined the effect of hypoxia on BCSCs by flow cytometry.

- Following a 48 h treatment with $CoCl_2$ I used ICC to determine what happens to p-smad 2/3 expression and test whether hypoxia leads to the activation of the TGF-β signalling pathway.

- SP analysis and CD44 protein expression analysis by flow cytometry allowed the determination of treatment with TGF-β and $CoCl_2$ together or alone in cells treated for 3 and 2 days, respectively.

- SP analysis was performed on cells pre-treated with $CoCl_2$, mitoxantrone and both for 48 h to investigate the effect of hypoxia on the drug resistance of the SP and NSP cells.

4.5 Results

4.5.1 Optimization of $CoCl_2$-induced hypoxia conditions in breast cancer cell lines

To optimize the conditions for the induction of hypoxia I assessed the mRNA levels of three important hypoxia-responsive genes, including HIF-1α, VEGF-A and CXCR4 after treatment of the MDA-MB-231 and MCF-7 cells with 200, 400 and 600 μM $CoCl_2$ for 24 h. I found that HIF-1α levels exhibited the highest increase with 400 μM $CoCl_2$ in the MDA-MB-231 cells, while the increase in the MCF-7 cells was negligible even with the highest concentration of $CoCl_2$ (Figure 4.1). VEGF-A mRNA expression levels were increased approximately 40 times when treated with 400 μM $CoCl_2$ in the MDA-MB-231 and almost 3.5 and 5-fold with 400 and 600 μM $CoCl_2$, respectively in the MCF-7 cells (Figure 4.2). Finally, the highest CXCR4 expression was induced with 400 μM $CoCl_2$ in the MDA-MB-231, while no significant increase was seen in the MCF-7 cells (Figure 4.3). The treatment with 400 μM $CoCl_2$ was repeated 3 more times to confirm that the concentration of $CoCl_2$ was optimal and there was a significant increase of VEGF-A ($p=0.02$) and CXCR4 ($p=0.04$) levels in the MDA-MB-231 cells (Figure 4.4).

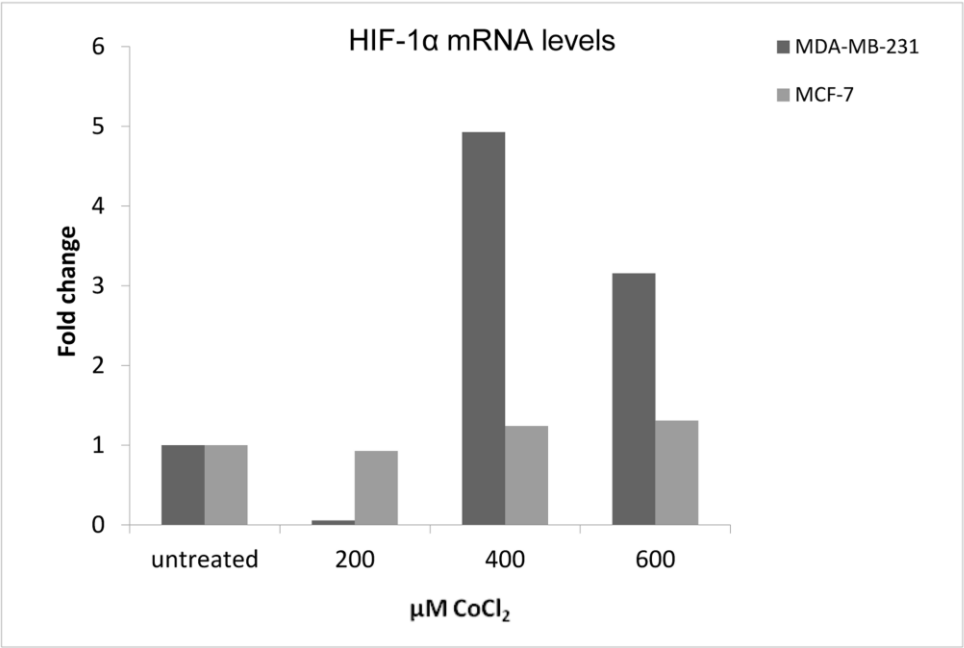


Figure 4.1: Graphical representation of qPCR data. mRNA expression levels of HIF-1α in MDA-MB-231 and MCF-7 cells treated with 200, 400 and 600 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of triplicates (n=1).

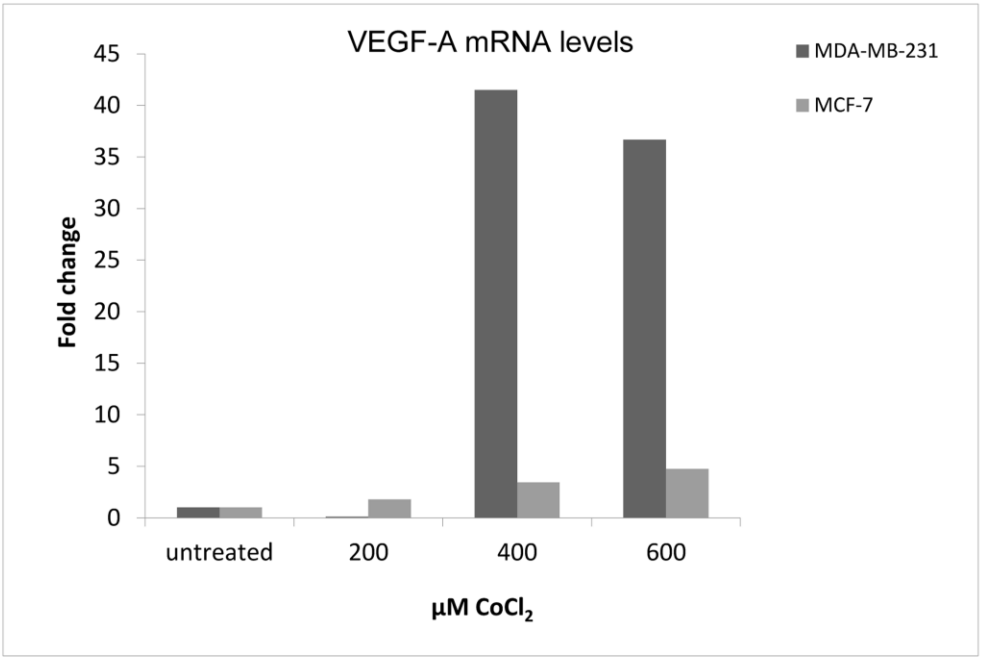


Figure 4.2: Graphical representation of qPCR data. mRNA expression levels of VEGF-A in MDA-MB-231 and MCF-7 cells treated with 200, 400 and 600 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of triplicates (n=1).

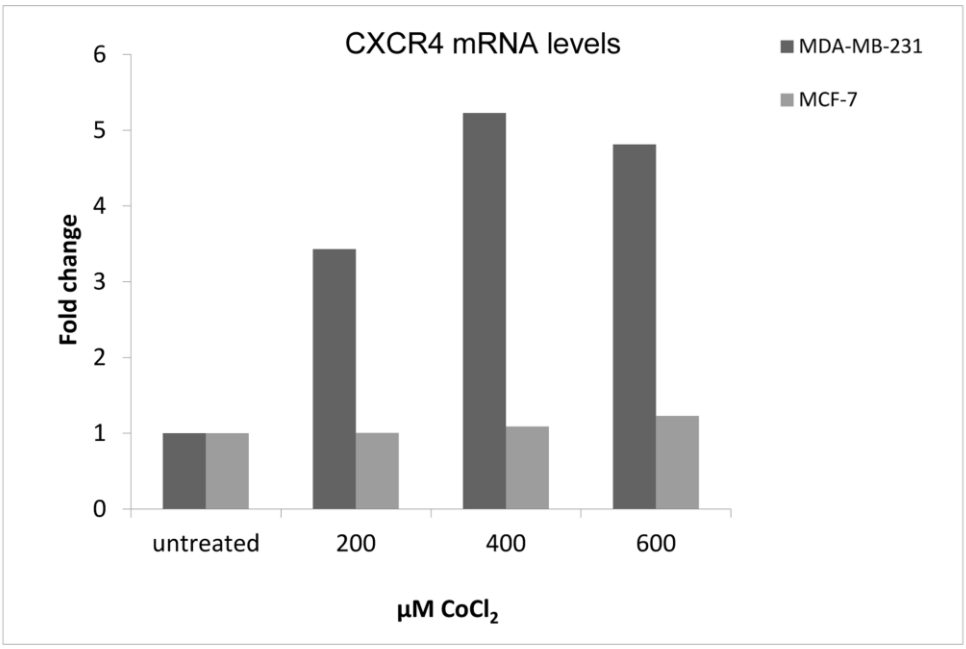


Figure 4.3: Graphical representation of qPCR data. mRNA expression levels of CXCR4 in MDA-MB-231 and MCF7 cells treated with 200, 400 and 600 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of triplicates (n=1).

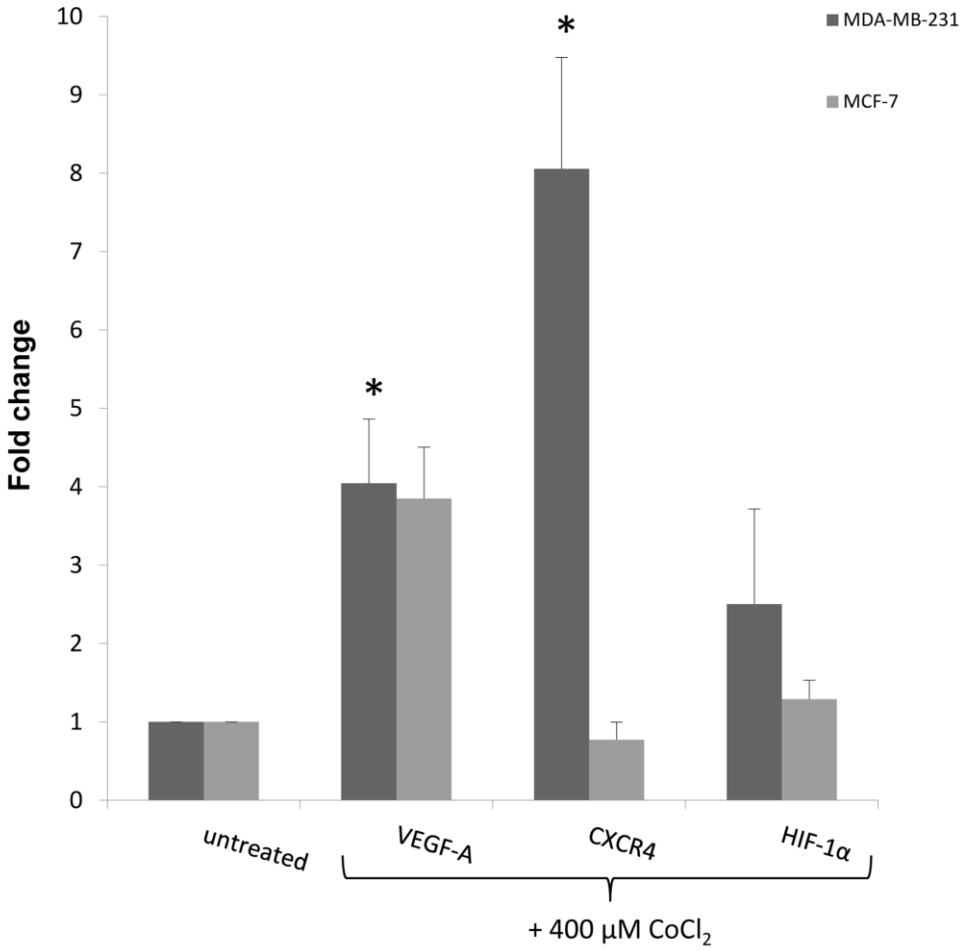


Figure 4.4: Graphical representation of qPCR data. mRNA expression levels of VEGF-A, CXCR4 and HIF-1α in MDA-MB-231 and MCF-7 cells treated with 400 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of n=3 individual experiments. Results are expressed as the mean SEM using a paired student's t-test. * $p<0.05$

4.5.2 The effect of $CoCl_2$-induced hypoxia on the transcriptional activation of stem cell marker genes.

To determine the effect of hypoxia on the potential induction of expression of stem cell marker genes I used qPCR analysis to determine the expression of these in unfractionated MDA-MB-231 and MCF-7 cells when these were exposed to 400 μM $CoCl_2$ for 24 h. The expression of NANOG (n=3, p=0.03) and OCT 3/4, (n=3 p=0.02) mRNA levels were significantly increased in the MDA-MB-231. SOX-2 was also increased in both cell lines and all genes were increased in the MCF-7, but not significantly (Figure 4.5).

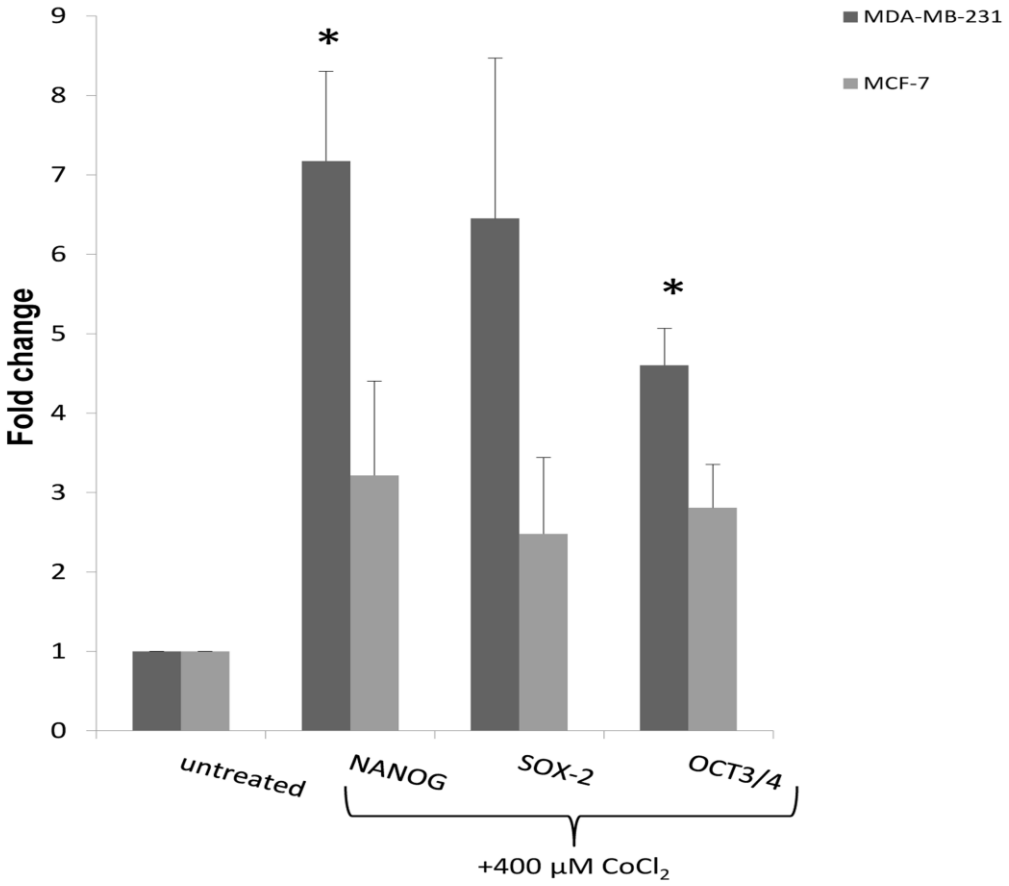


Figure 4.5: Graphical representation of qPCR data. mRNA expression levels of NANOG, SOX-2 and Oct 3/4 in MDA-MB-231 and MCF-7 cells treated with 400 μM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of n=3 individual experiments. Results are expressed as the mean SEM using a paired student's t-test. * $p<0.05$

4.5.3 The effect of $CoCl_2$-induced hypoxia on the transcriptional activation of EMT marker genes.

To confirm the role of hypoxia in the induction of EMT I assessed the expression of several EMT markers in unfractionated MDA-MB-231 and MCF-7 cells after exposure to 400 μM $CoCl_2$ for 24 h. qPCR analysis revealed that TWIST1 was significantly up-regulated in the MDA-MB-231 (n=7, p=0.03) and MCF-7 (n=7, p=0.03), while TWIST2 mRNA were only significantly increased in the MCF-7 (n=6, p=0.03) cells. SNAIL and SLUG mRNA levels were also significantly elevated in both cell lines (n=6, p=0.04 for SNAIL in both/n=4, p=0.01 in MDA-MB-231 and n=3, p=0.01 in MCF-7 for SLUG). S100A4 was slightly decreased in the MDA-MB-231 cells and increased in the MCF-7 and VIMENTIN increase was not significant in any of these. Surprisingly, E-CADHERIN levels were elevated in the MCF-7, but not significantly (Figure 4.6). The changes in E-CADHERIN expression in response to $CoCl_2$ treatment were not studied in the MDA-MB-231 cell line, since this is a mesenchymal cell line with undetectable E-CADHERIN expression (Figure 4.7).

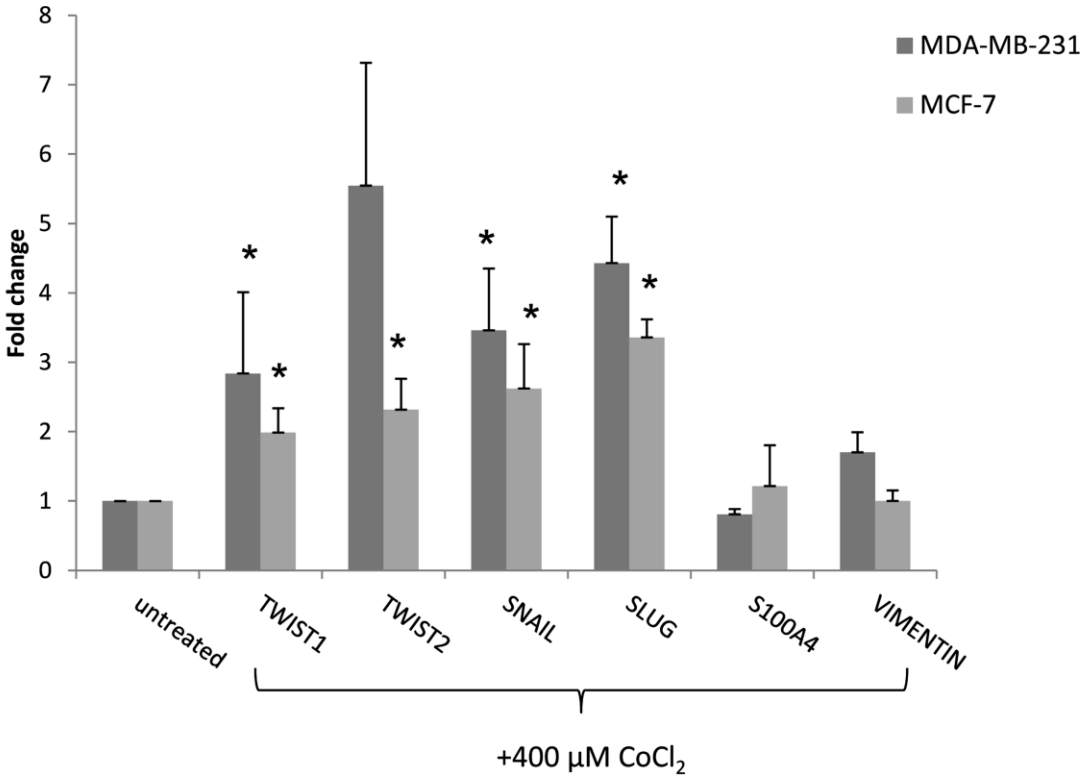


Figure 4.6: Graphical representation of qPCR data. mRNA expression levels of TWIST1, TWIST2, SNAIL, SLUG, S100A4 and VIMENTIN in MDA-MB-231 and MCF-7 cells treated with 400 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of n=7 (TWIST1), n=6 (TWIST2 SNAIL) n=4 (SLUG in MDA-MB-231, VIMENTIN), n=3 (SLUG in MCF-7, S100A4) individual experiments. Results are expressed as the mean SEM using a paired student's t-test. * $p<0.05$

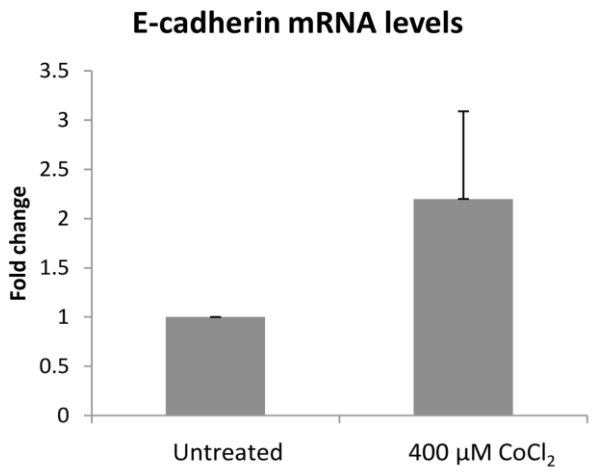


Figure 4.7: Graphical representation of qPCR data. mRNA expression levels of E-CADHERIN in MCF-7 cells treated with 400 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of n=4 individual experiments. Results are expressed as the mean SEM using a paired student's t-test.

4.5.4 The effect of $CoCl_2$ and low oxygen induced hypoxia on the SP cells of breast cancer cell lines.

Since I saw an up-regulation of both EMT and stem cell marker genes in the MCF-7 and MDA-MB-231 cell lines I investigated the impact of hypoxia on the SP cells in these. Treatment with 400 μM $CoCl_2$ for 48 h resulted in a significant increase of the SP percentage in the MCF-7 cells (n=4, p=0.03), while exposure to 1% O_2 for the same time only slightly increased the SP percentage, suggesting that $CoCl_2$ treatment is a more efficient method to induce hypoxic culture conditions for my study (Figures 4.8, 4.9). To determine whether exposure to 1% O_2 for a longer period of time would enhance the SP numbers to the same degree as the $CoCl_2$ I performed SP analysis after 2, 5 and 8 days of culturing the MCF-7 cells at low oxygen conditions. Interestingly, I observed high sensitivity of the cells to staining with the Hoechst 33342 dye, as the cell viability of the total cell population and of the SP population was reduced in a time-dependent manner (Figure 4.10). Additionally, SP analysis was carried out for MDA-MB-231 cells untreated and treated with 400 μM $CoCl_2$ for 48h and the SP population was eliminated (n=4, p=0.03) (Figure 4.11).

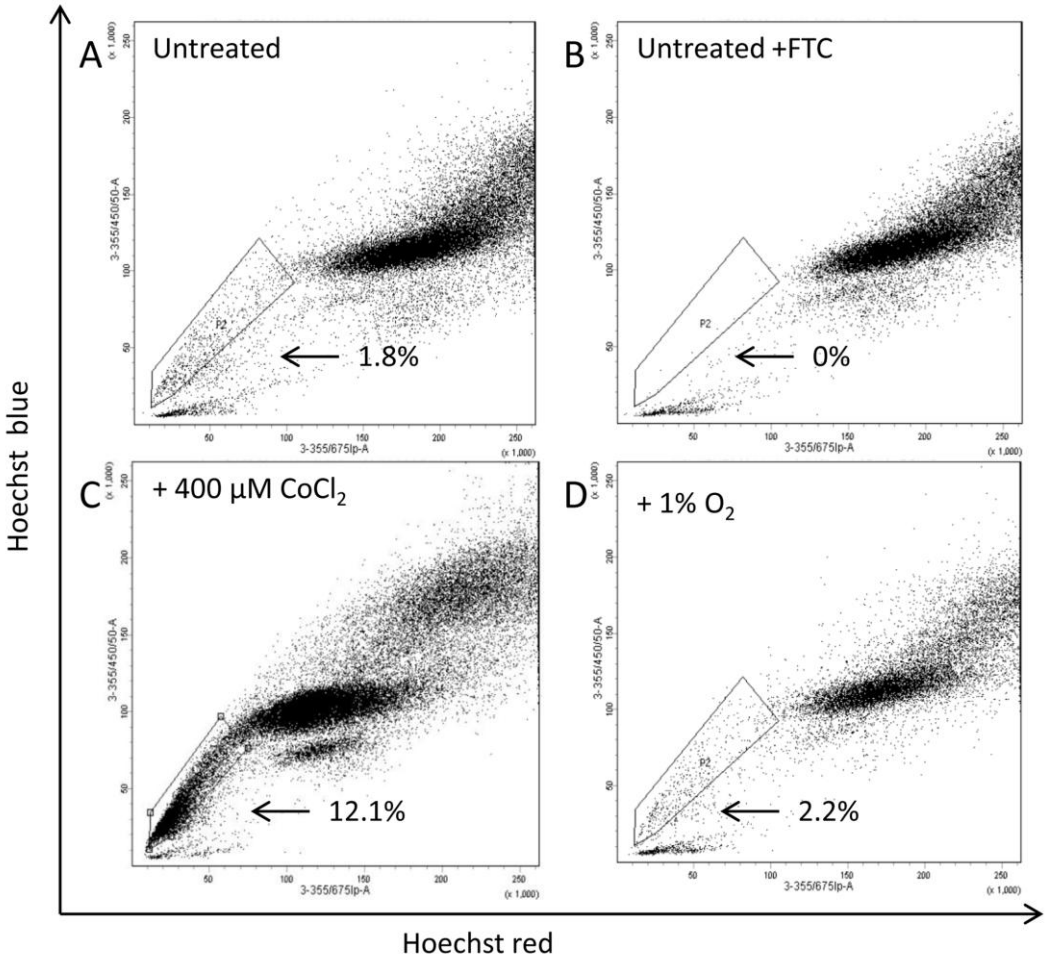


Figure 4.8: Representative FACS images of SP analysis in MCF-7 cells. A) Untreated B) With the addition of FTC to confirm the SP phenotype. C) Treated with 400 µM $CoCl_2$ for 48 h alone. n=4, p=0.03 D) Exposed to 1% O_2 for 48 h n=1 **(student's paired t-test). Results were considered significant when p<0.05.**

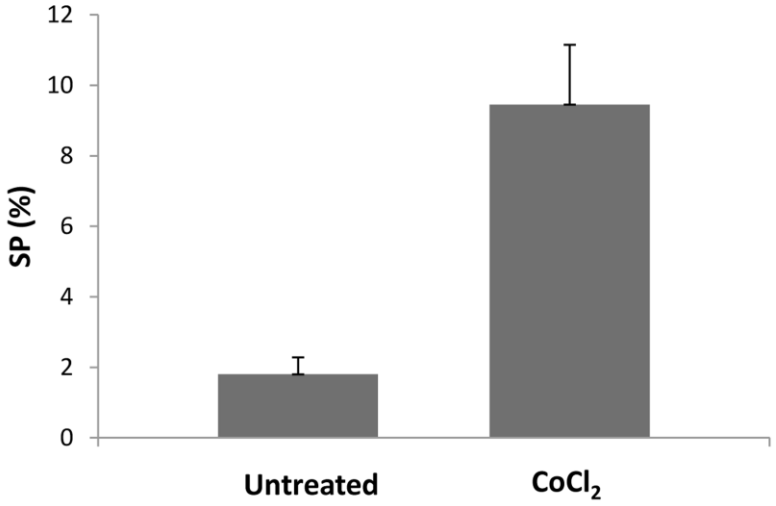


Figure 4.9: Graphical representation of flow cytometry data. Percentages of SP cells in untreated and treated with 400 μM $CoCl_2$ unfractionated MCF-7 cells. Bars represent an average of n=4 individual experiments, p=0.03. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

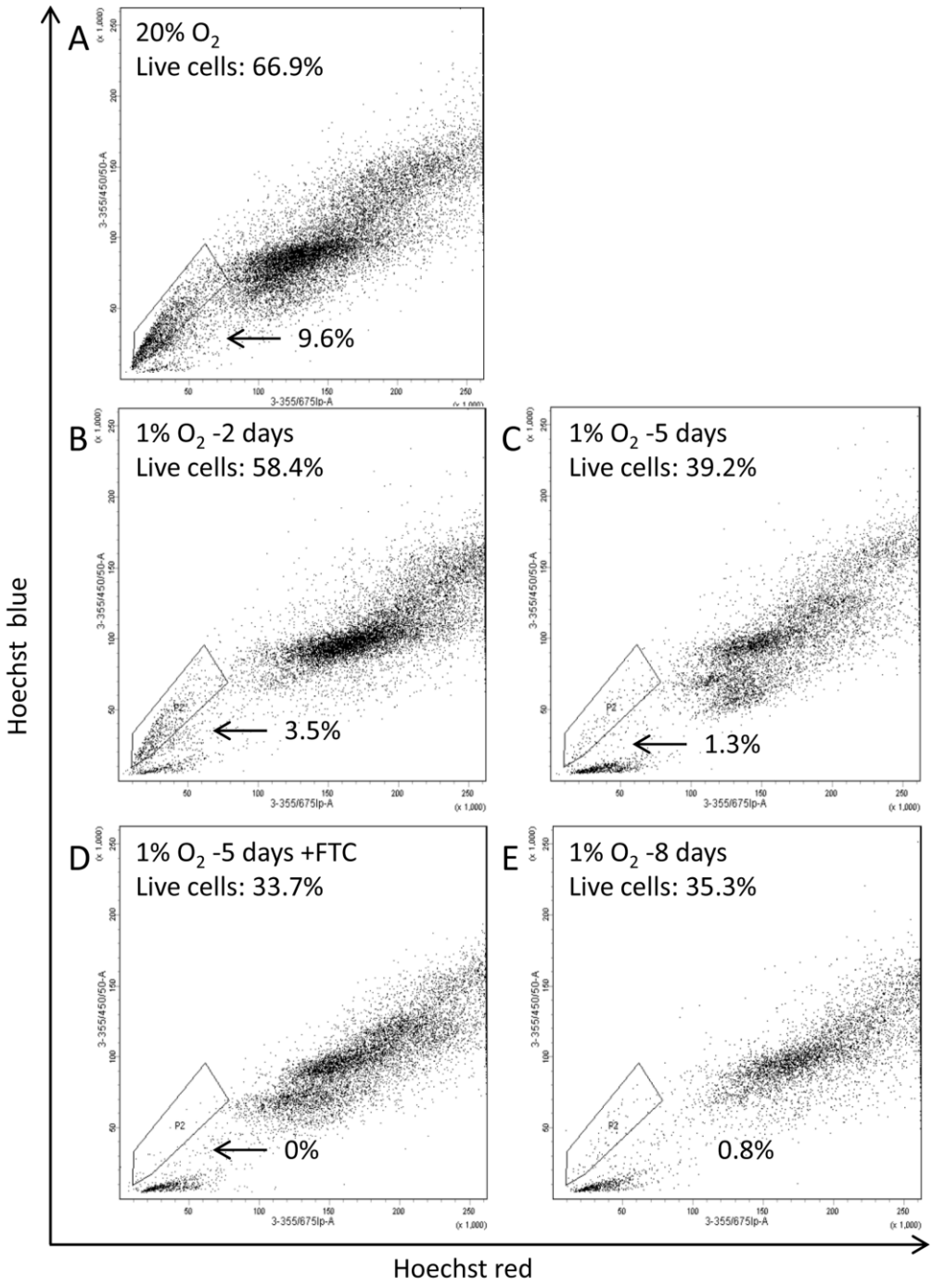


Figure 4.10: FACS images of SP analysis in MCF-7 cells. A) Exposed to 20% O_2 B) Exposed to 1% O_2 for 2 days D) Exposed to 1% O_2 for 5 days. E) Exposed to 1% O_2 for 8 days. FTC, an ABCG2 inhibitor, was used to confirm the SP phenotype in D). n=1.

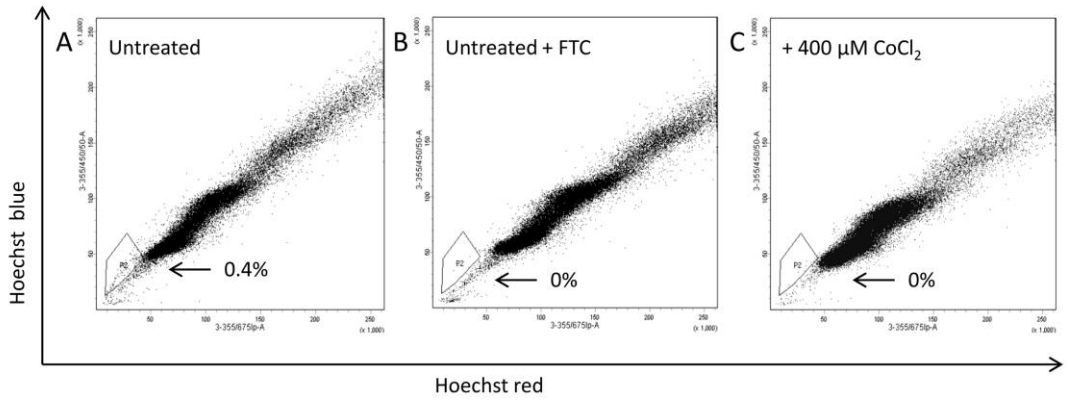
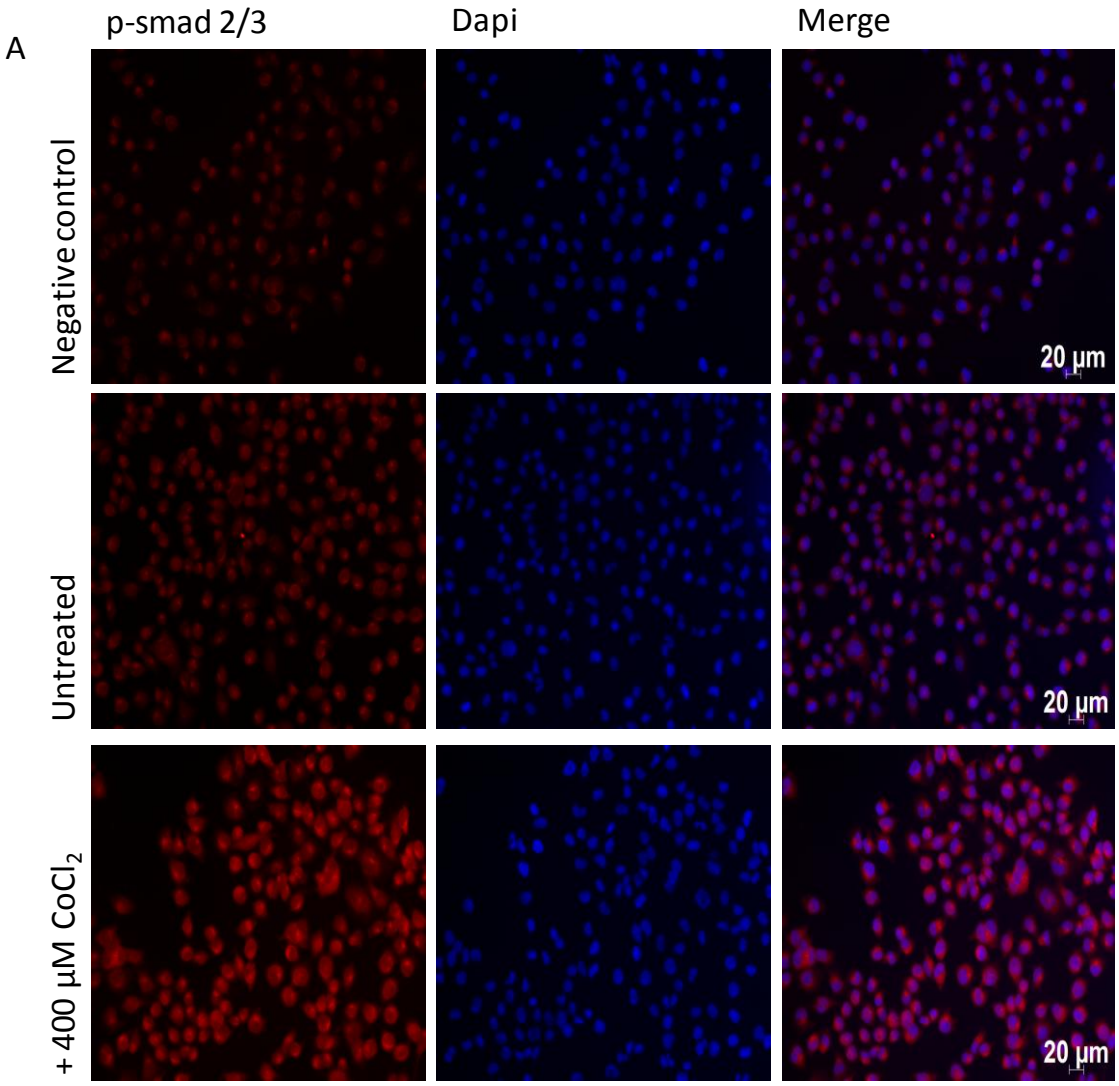


Figure 4.11: Representative FACS images of SP analysis in MDA-MB-231 cells. A) Untreated B) With the addition of FTC to confirm the SP phenotype. C) Treated with 400 µM $CoCl_2$ for 48 h alone. n=4, p=0.03 **(student's paired t-test). Results were considered significant when p<0.05.**

4.5.5 Investigation of TGF-β signalling pathway activation by $CoCl_2$ treatment in breast cancer cell lines.

To address the question of whether $CoCl_2$-induced hypoxia is responsible for activating the TGF-β signalling pathway I performed ICC for p-smad 2/3 in unfractionated MDA-MB-231 and MCF-7 cells untreated and treated with 400 μM $CoCl_2$ for 48 h $CoCl_2$ treatment led to a significant increase of the nuclear p-smad 2/3 levels (p=0.0003, p=0.005, respectively), indicating that hypoxia also activates this pathway. However, the increase was higher in the treated MDA-MB-231 compared to the treated MCF-7 cells (Figures 4.12, 4.13, 4.14).



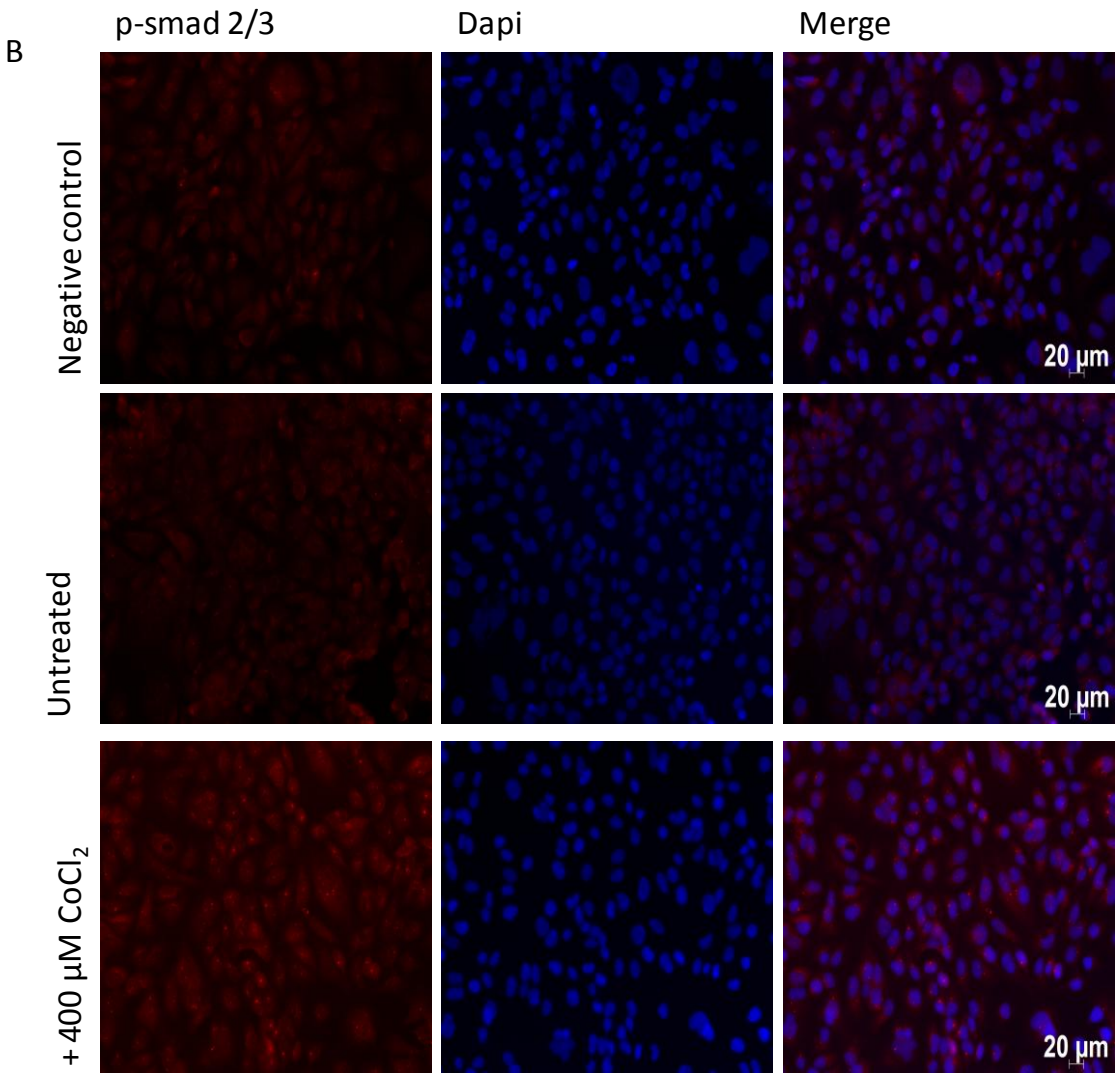


Figure 4.12: ICC images for p-smad 2/3 staining showing activation of the TGF-β signalling pathway. A) MDA-MB-231 B) MCF-7 cells upon exposure to 400 µM $CoCl_2$ for 48 h. p-smad 2/3 is increased after $CoCl_2$ treatment in both cell lines. Nuclei were visualized by 4', 6-diamidino-2-phenylindole DAPI stain (blue). Negative controls for the primary antibody were used. All images were taken at 20X magnification and a 20 µm scale bar was used. Representative images from n=3 experiments.

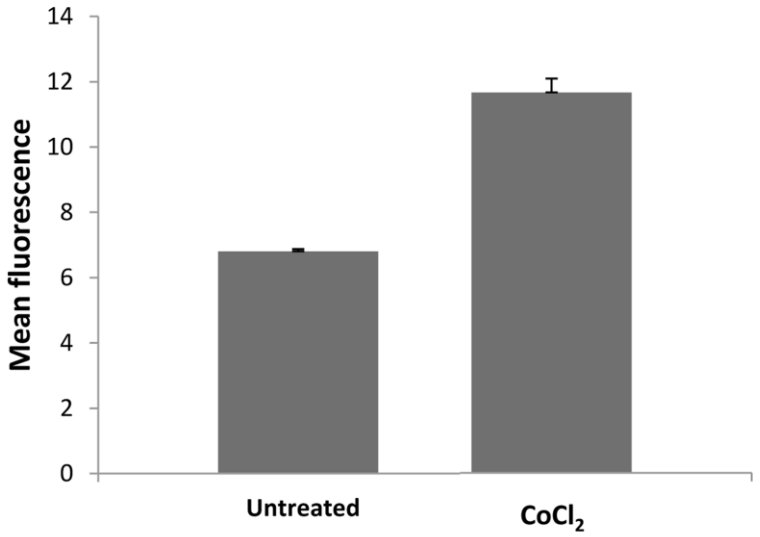


Figure 4.13: Graphical representation of ICC data. p-smad 2/3 levels in untreated and treated with 400 μM $CoCl_2$ MDA-MB-231 cells. Bars represent an average of n=3 individual experiments, p=0.0003. Results are expressed as the mean SEM using a paired student t- test and were considered significant when $p<0.05$.

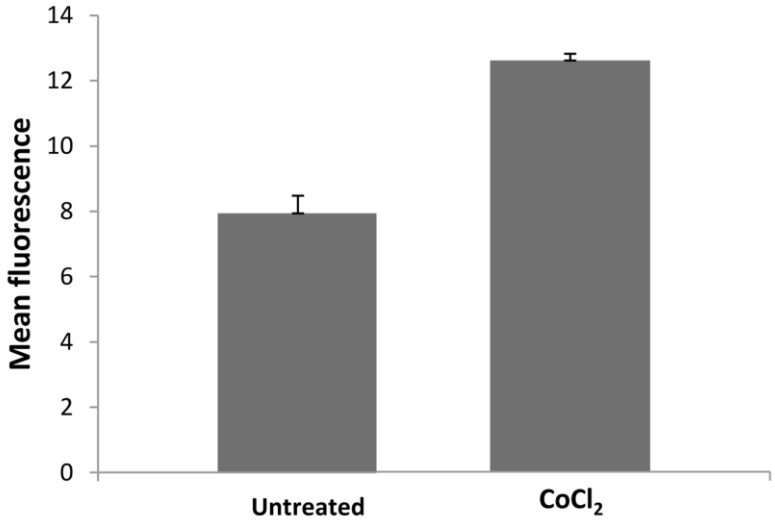


Figure 4.14: Graphical representation of ICC data. p-smad 2/3 levels in untreated and treated with 400 μM $CoCl_2$ MCF-7 cells. Bars represent an average of n=3 individual experiments, p=0.005. Results are expressed as the mean SEM using a paired student t-test and were considered significant when $p<0.05$.

4.5.6 The effect of $CoCl_2$ and TGF-β1 treatment on the SP population of the MCF-7 cell line.

Following the observation that $CoCl_2$ treatment also activates the TGF-β signalling pathway and since I observed a significant increase of the MCF-7 SP following $CoCl_2$ treatment as opposed to the results observed following TGF-β treatment (as described in Chapter 3), I wanted to explore the effect of combining the two treatments on the MCF-7 SP cells. Interestingly, treatment with both $CoCl_2$ (48 h) and TGF-β1 (72 h) simultaneously led to a significant decrease of the SP cells (n=3, p=0.02), suggesting that the impact of TGF-β is stronger than $CoCl_2$, although they both seem to activate the TGF-β signalling pathway (Figures 4.15, 4.16). The fact that the MDA-MB-231 SP cells were abrogated with both $CoCl_2$ and TGF-β1 (as described Chapter 3) when treated separately did not allow me to perform the combined treatment on this cell line, as no conclusions could have been drawn.

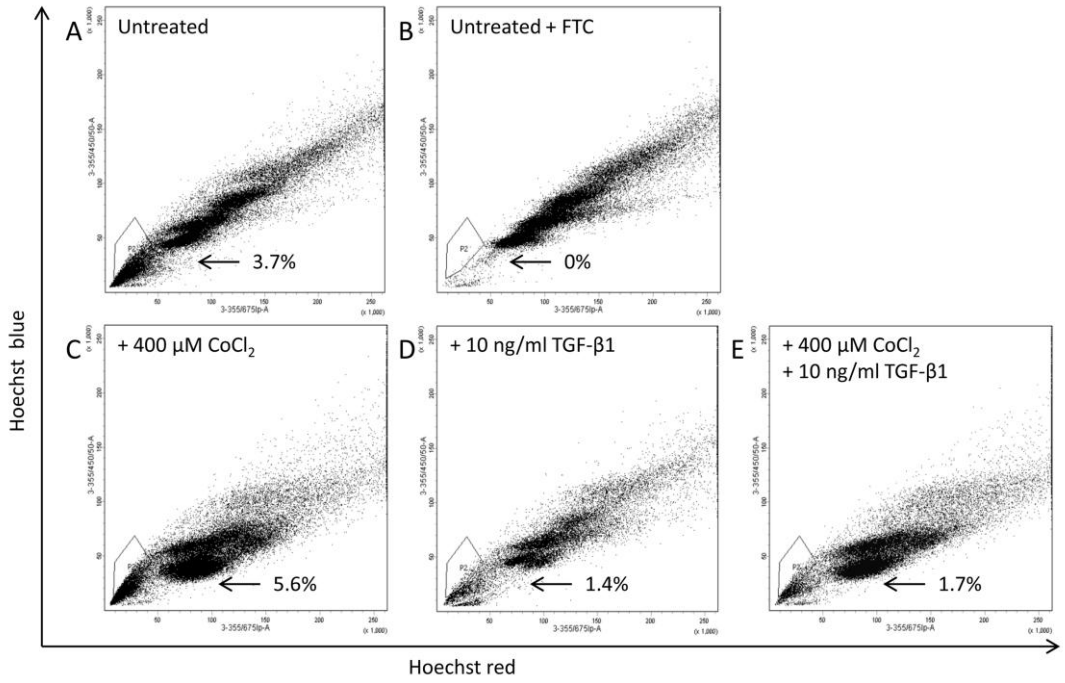


Figure 4.15: Representative FACS images of SP analysis in MCF-7 cells. A) Untreated B) With the addition of FTC to confirm the SP phenotype. C) Treated with 400 µM $CoCl_2$ for 48 h alone. D) Treated with 10 ng/ml TGF-β1 for 72 h alone E) Treated with both 400 µM $CoCl_2$ and 10 ng/ml TGF-β1for 48 and 72 h, respectively, n=3, p=0.02 **(student's paired t-test). Results were considered significant when p<0.05.**

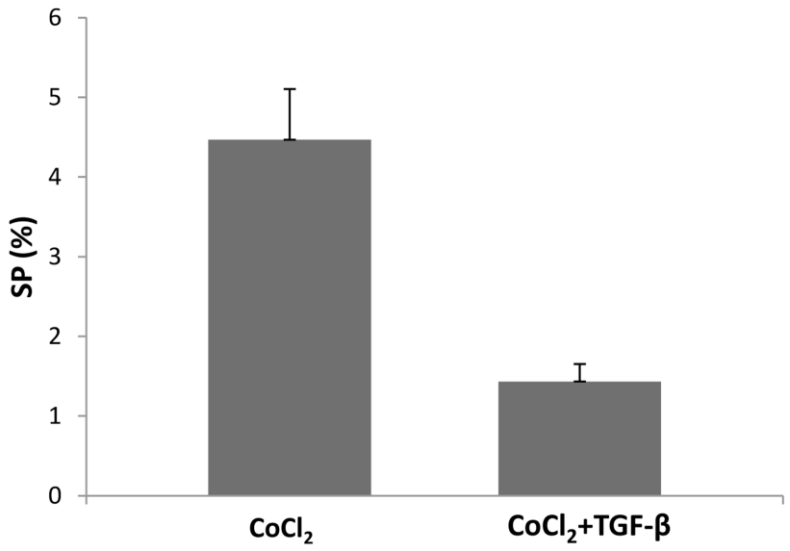


Figure 4.16: Graphical representation of flow cytometry data. Percentages of SP cells resulting from treatment of unfractionated MCF-7 cells with 400 μM $CoCl_2$ and treated with 400 μM $CoCl_2$ and 10 ng/ml TGF-β1. Bars represent an average of n=3 individual experiments, p=0.02. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

4.5.7 The effect of $CoCl_2$ and TGF-β1 treatment on the CD44+ population of breast cancer cell lines

I also investigated the role of $CoCl_2$, TGF-β1 and combined treatment on the CD44+ cells in both breast cancer cell lines. Addition of $CoCl_2$ led to a significant increase of the CD44 mRNA levels in the MDA-MB-231 cells (n=3, p=0.003). An up-regulation of CD44 was also induced by $CoCl_2$ in the MCF-7 cells, but it was non-significant (Figure 4.17). CD44 staining in combination with flow cytometry was also carried out to assess the levels of CD44 protein expression before and after TGF-β treatment, $CoCl_2$ treatment or combined treatment and none of the changes were significant (MDA-MB-231:p=0.72, MCF-7:p=0.5). Treatment with TGF-β alone resulted in an increase of the CD44 protein levels in both cell lines, but not to the same degree as $CoCl_2$ treatment. Combined treatment also induced a higher increase of CD44 above that of cells treated with TGF-β alone, but slightly lower than that of cells treated with $CoCl_2$ alone (Figures 4.18, 4.19 and 4.20, 4.21). CD24 protein levels were also assessed in the untreated and treated with $CoCl_2$ samples, but staining did not work in the MCF-7 cells, as it gave double peaks and in the MDA-MB-231 cells the expected changes were not seen with CD24 expression being slightly increased instead of decreased (Appendix B, Supplementary data, pages 217 and 218).

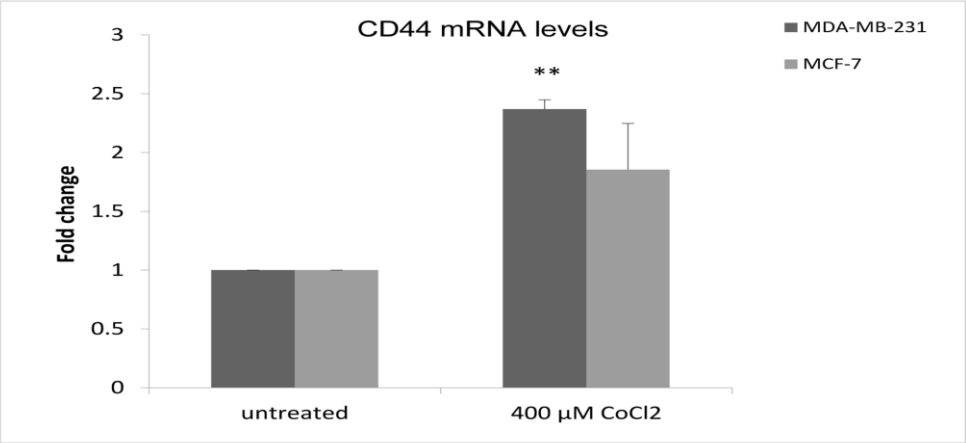


Figure 4.17: Graphical representation of qPCR data. mRNA expression levels of CD44 in untreated and treated with 400 µM $CoCl_2$ MDA-MB-231 and MCF-7 cells. Bars represent an average of n=3 individual experiments. Results are expressed as the mean SEM using a paired student's t-test. **** p<0.01**

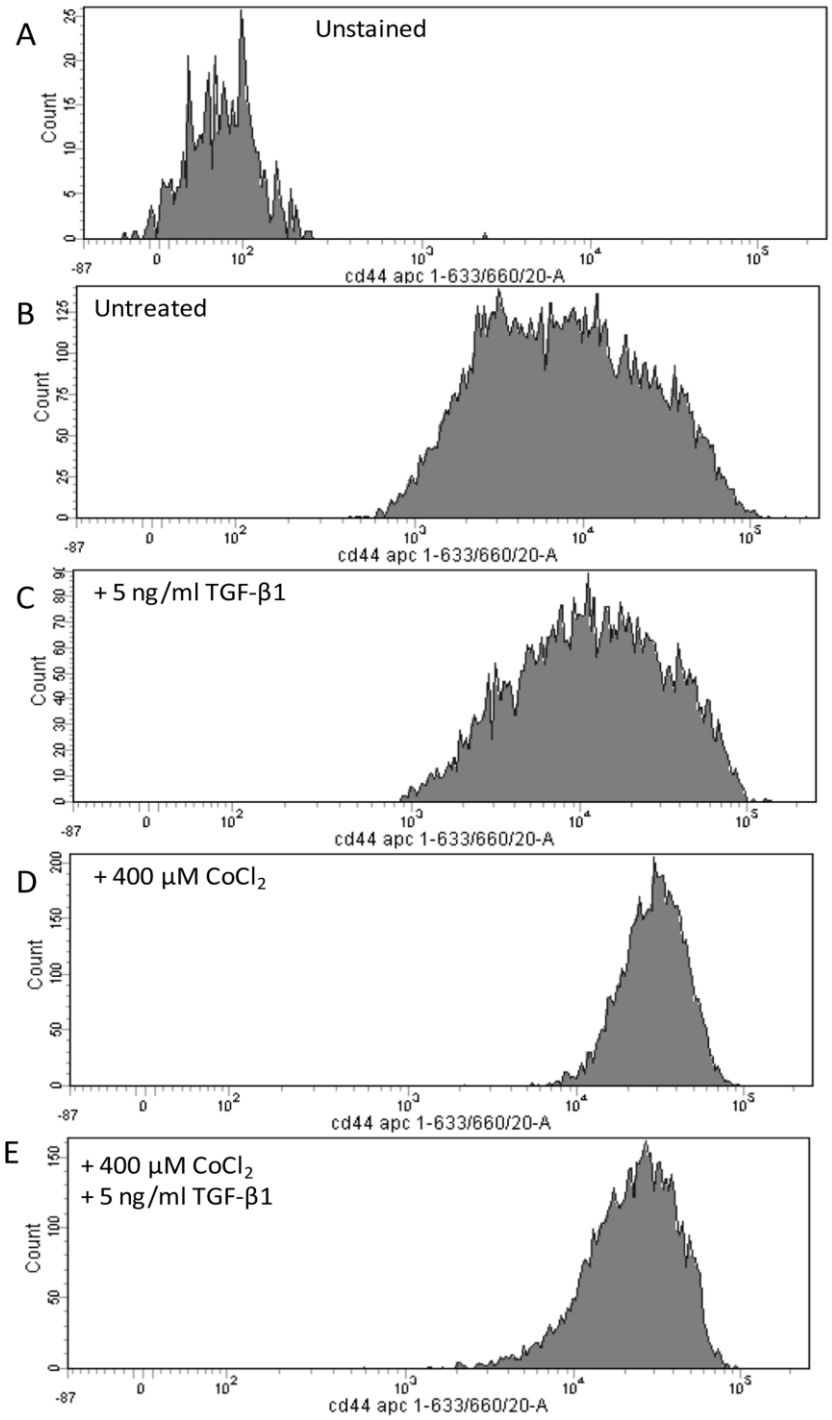


Figure 4.18: Representative FACS images from CD44 staining in MDA-MB-231 cells. A) Unstained B) Untreated C) Treated with 5 ng/ml TGF-β1 for 3 days D) Treated with 400 µM $CoCl_2$ for 2 days E) Treated with 5 ng/ml TGF-β1 and 400 µM $CoCl_2$ for 3 and 2 days, respectively.

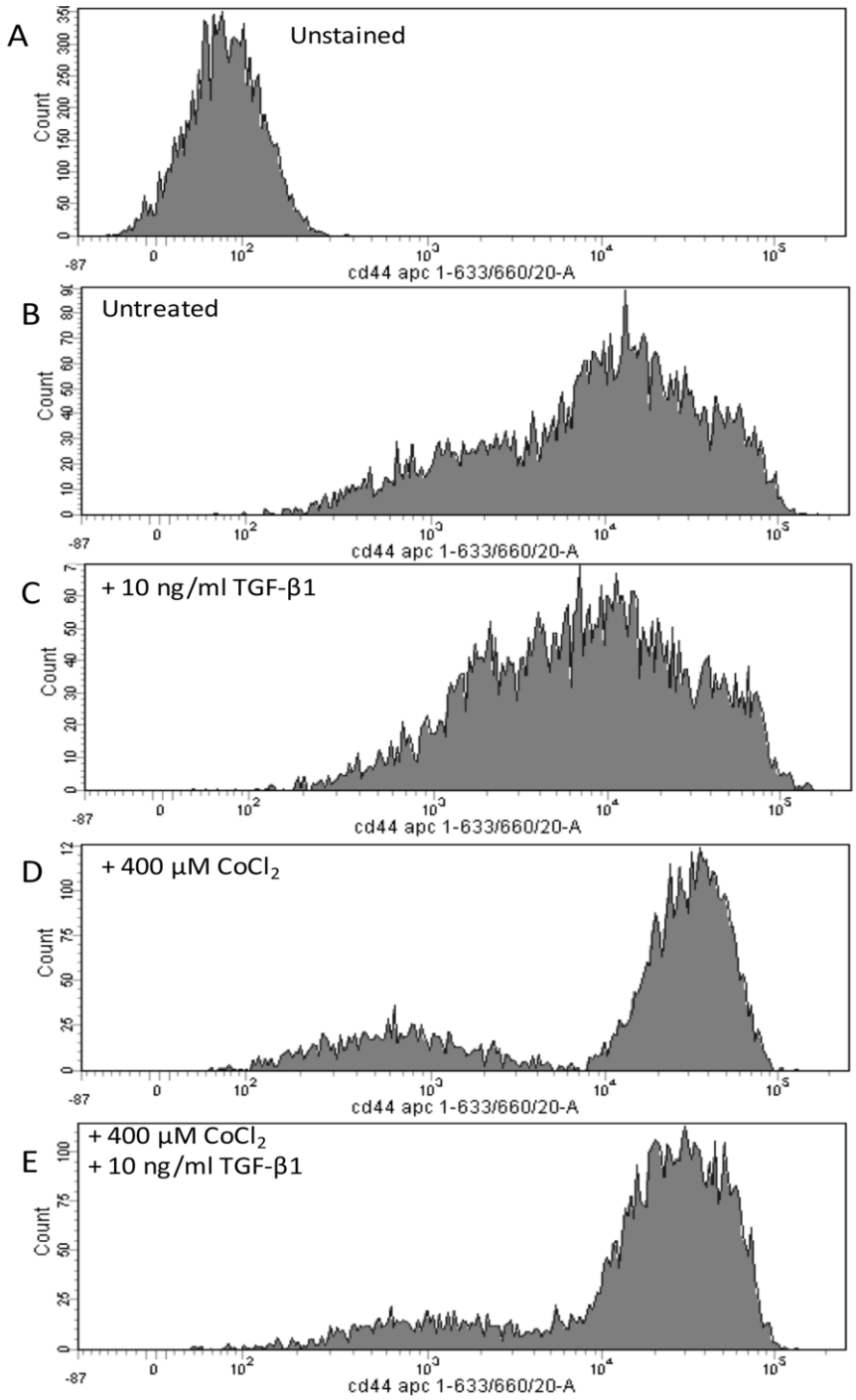


Figure 4.19: Representative FACS images from CD44 staining in MCF-7 cells. A) Unstained B) Untreated C) Treated with 10 ng/ml TGF-β1 for 3 days D) Treated with 400 µM $CoCl_2$ for 2 days E) Treated with 10 ng/ml TGF-β1 and 400 µM $CoCl_2$ for 3 and 2 days, respectively.

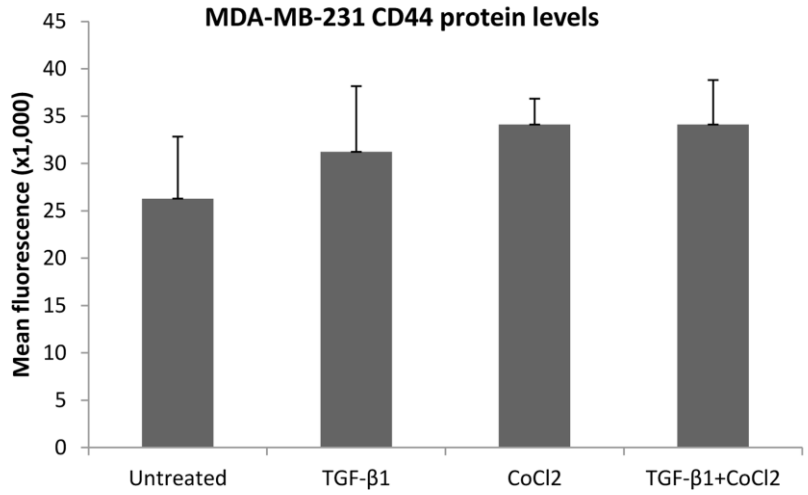


Figure 4.20: Graphical representation of flow cytometry data. Protein expression levels of CD44 in untreated and treated with 5 ng/ml TGF-β1, 400 μM $CoCl_2$ and both 5 ng/ml TGF-β1 and 400 μM $CoCl_2$ in MDA-MB-231 cells. Bars represent an average of n=3 individual experiments, p=0.72. Results are expressed as the mean SEM using a one way ANOVA test and were considered significant when $p<0.05$.

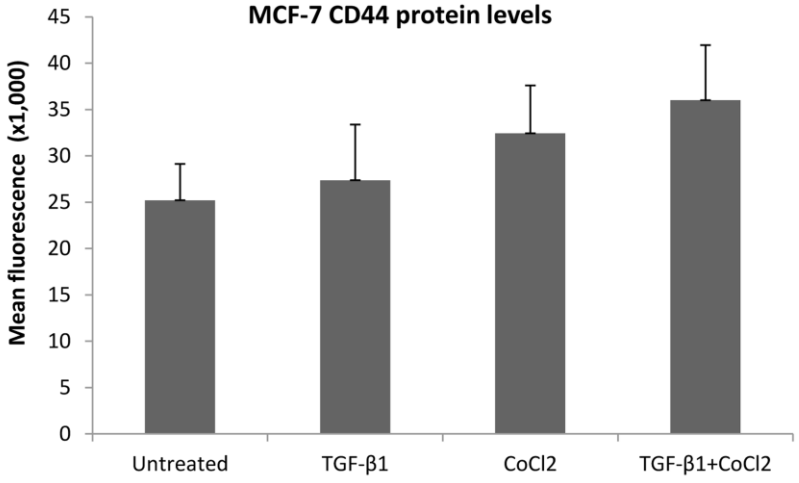


Figure 4.21: Graphical representation of flow cytometry data. Protein expression levels of CD44 in untreated and treated with 10 ng/ml TGF-β1, 400 μM $CoCl_2$ and both 10 ng/ml TGF-β1 and 400 μM $CoCl_2$ in MCF-7 cells. Bars represent an average of n=3 individual experiments, p=0.5. Results are expressed as the mean SEM using a one way ANOVA test and were considered significant when $p<0.05$.

4.5.8 The effect of $CoCl_2$-induced hypoxia on drug resistance in the MCF-7 breast cancer cell line.

To determine if hypoxia increases the drug resistance in the MDA-MB-231 and MCF-7 cell lines I first investigated the effect of $CoCl_2$ treatment on the expression of several ABC transporter genes, including ABCB1, ABCG2, ABCB5 and ABCC3 in unsorted cells. Analysis of qPCR results demonstrated a significant increase in ABCG2 (n=5, p=0.01: MDA-MB-231, n=5, p= 0.02: MCF-7). ABCC3 mRNA expression was also significantly elevated in the MCF-7 cells (n=4, p= 0.0003) and in the MDA-MB-231 (n=4, p=0.03). No increase was seen for ABCB1 in any of the cell lines and the increase in ABCB5 mRNA expression was non-significant. It should be noted that ABCB5 expression was barely detectable in the untreated cells of both cell lines and although this tended to increase with $CoCl_2$ treatment, it was still too low to determine if it was significant (Figure 4.22). I then treated unfractionated MDA-MB-231 and MCF-7 cells with 1 μg/ml mitoxantrone alone and in combination with $CoCl_2$ and I saw that hypoxia reduced the sensitivity of the cells to mitoxantrone and increased their viability (Figures 4.23 and 4.24). Furthermore, I performed SP analysis to examine whether hypoxia is responsible for the enhancement of the SP phenotype and thus for the induction of drug resistance to mitoxantrone in these cells. Mitoxantrone treatment led to the cell death of all NSP cells, while some of the SP cells still survived, confirming that these are more drug resistant compared to the NSP cells. $CoCl_2$ treatment alone led to an increase of the SP percentage and surprisingly it induced a significant increase of the SP numbers when combined with mitoxantrone (n=3, p=0.04). Notably, both SP and NSP cells were protected from the mitoxantrone cytotoxic effects when treated with $CoCl_2$ at the same time (Figures 4.25, 4.26). I only performed this experiment in the MCF-7 cell line, as $CoCl_2$ treatment resulted in the complete abrogation of the SP population in the MDA-MB-231 cell line as shown in Figure 4.11.

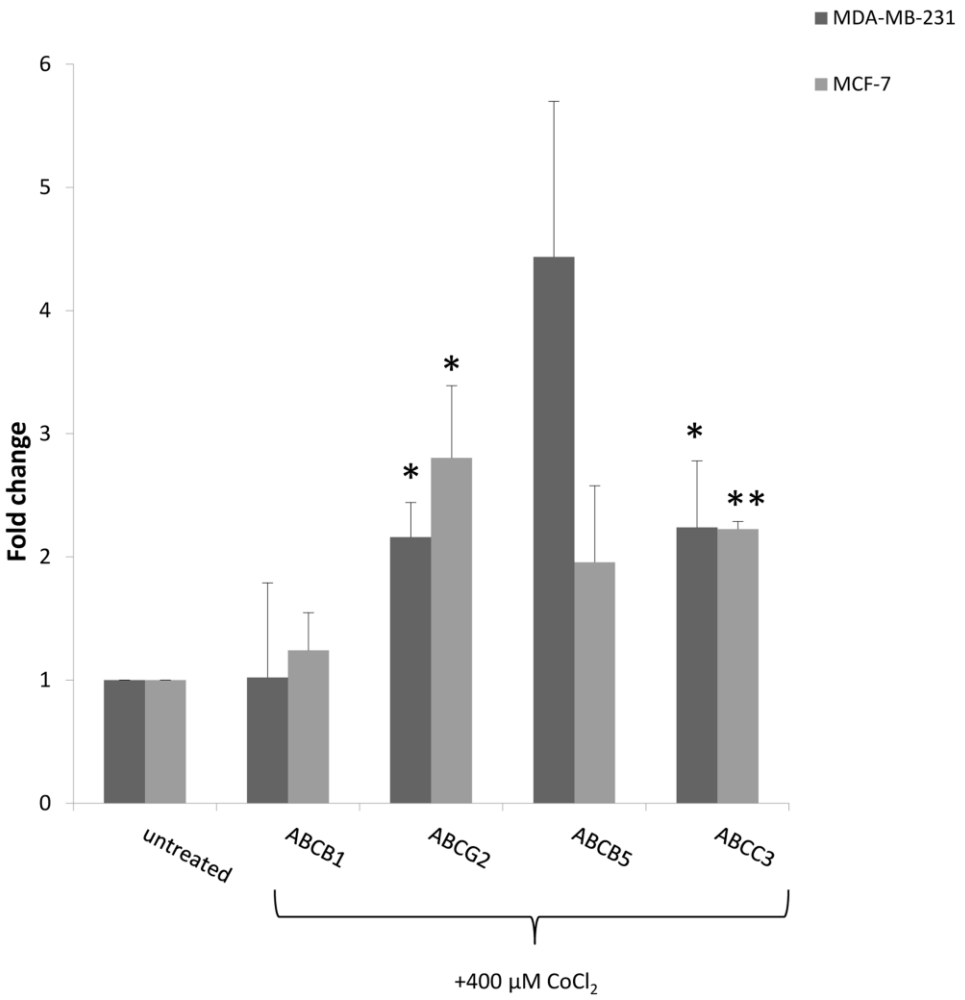


Figure 4.22: Graphical representation of qPCR data. mRNA expression levels of ABCB1, ABCG2, ABCB5 and ABCC3 in MDA-MB-231 and MCF-7 cells treated with 400 µM $CoCl_2$ for 24 h. Untreated cells are included as control. Bars represent an average of n=5 (ABCB5 in MDA-MB-231, ABCG2), n=4 (ABCB5 in MCF-7, ABCB1, ABCC3) individual experiments. Results are expressed as the mean SEM using a paired student's t-test. ***p<0.05 ** p<0.01**

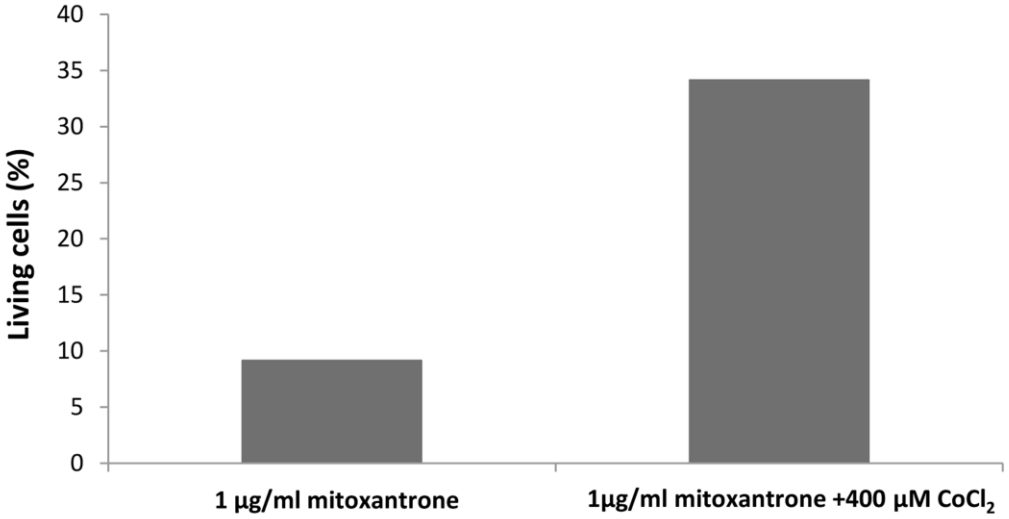


Figure 4.23: MTS assay for treated with 1µg/ml mitoxantrone alone and in combination with 400 µM $CoCl_2$ in the MDA-MB-231 cells (n=1)

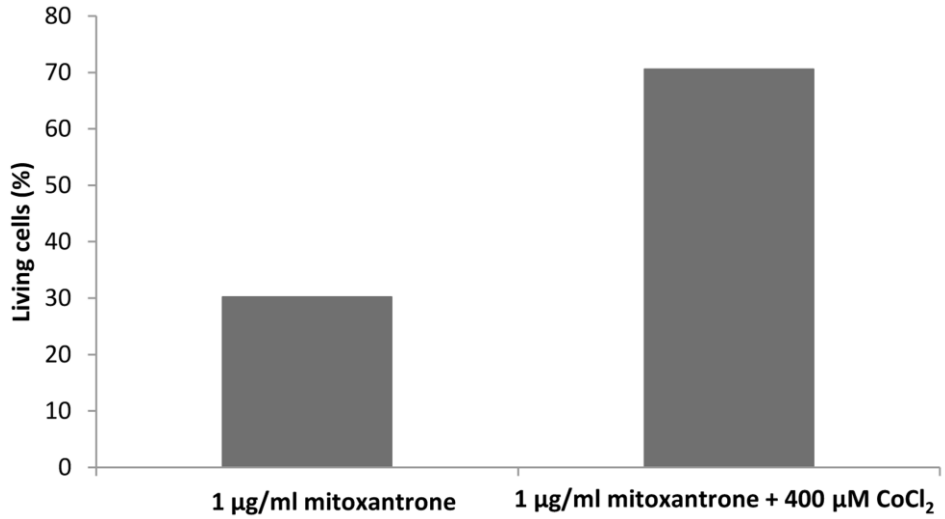


Figure 4.24: MTS assay for treated with 1µg/ml mitoxantrone alone and in combination with 400 µM $CoCl_2$ in the MCF-7 cells (n=1)

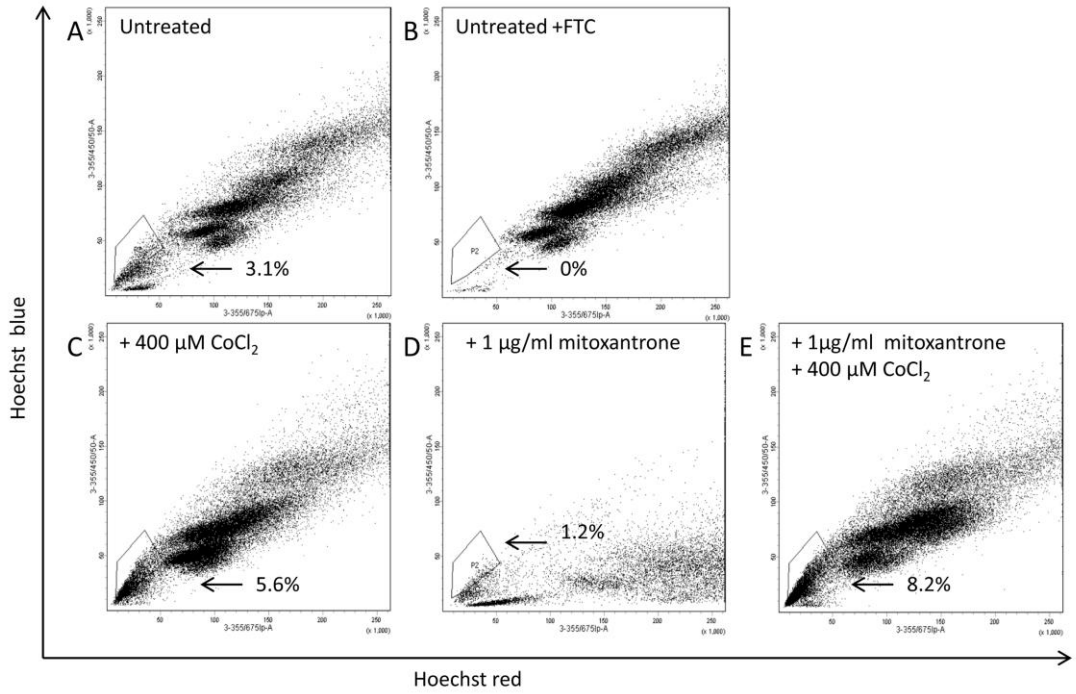


Figure 4.25: Representative FACS images of SP analysis in MCF-7 cells. A) Untreated B) With the addition of FTC to confirm the SP phenotype. C) Treated with 400 µM $CoCl_2$ for 48 h. D) Treated with 1µg/ml mitoxantrone for 48 h E) Treated with both 400 µM $CoCl_2$ and 1µg/ml mitoxantrone for 48, n=3, p=0.04 **(student's paired t-test). Results were considered significant when p<0.05.**

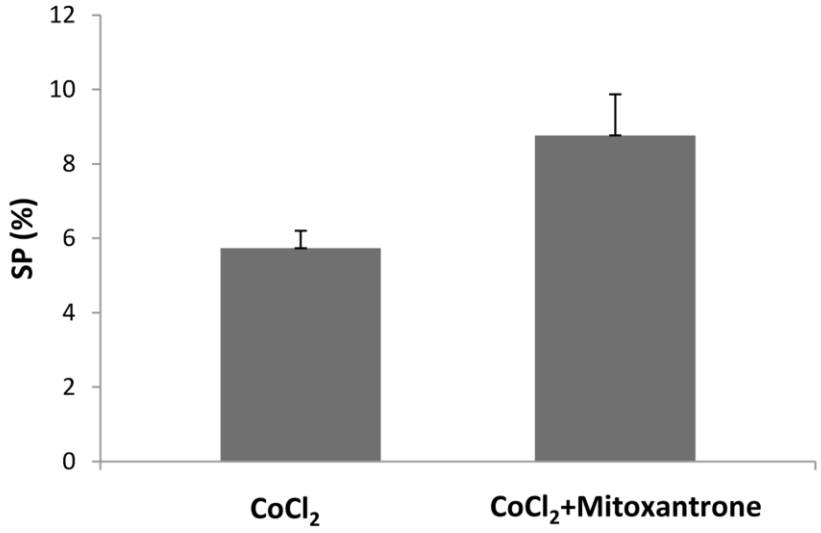


Figure 4.26: Graphical representation of flow cytometry data. Percentages of SP cells resulting from treatment of unfractionated MCF-7 cells with 400 μM $CoCl_2$ and treated with 400 μM $CoCl_2$ and 1 μg/ml mitoxantrone. Bars represent an average of n=3 individual experiments, p=0.04. Results are expressed as the mean SEM using a paired student's t-test and were considered significant when $p<0.05$.

4.6 Discussion

The role of CSCs in tumor re-occurrence has gained increasing attention over the last years due to the notion that CSCs are responsible for tumor re-growth, chemo- and radioresistance (Reya, 2001). EMT has also been reported to contribute to the regulation of several CSCs populations (Mani, 2008; Yin *et al.*, 2008). Hypoxia is thought to promote the EMT process (Marie-Egyptienne *et al.*, 2013) and thus many research groups have aimed to investigate its impact on particular CSC populations (Das *et al.*, 2008; Louie *et al.*, 2010; Ma *et al.*, 2011; Krishnamachary *et al.*, 2012).

In the present study, I sought to determine the effect of $CoCl_2$-induced hypoxia on the SP and CD44+ populations of the breast cancer cell lines MDA-MB-231 and MCF-7. Additionally, I assessed the mRNA expression levels of known stem cell and EMT markers, which would support the potential role of hypoxia in the above mentioned cell populations. I also examined whether hypoxia can drive the TGF-β signalling pathway and what the effect of combined $CoCl_2$ and TGF-β1 exposure would be on these cell populations. Finally, I studied the possible induction of drug resistance in cells treated with $CoCl_2$ by estimating the mRNA levels of several ABC transporters in both cell lines and I tested the resistance of the MCF-7 SP and NSP cells to mitoxantrone in response to hypoxia.

First of all, I optimized the hypoxic cell culture conditions to ensure the best efficiency at inducing hypoxia in all experiments. Exposure to 1% O_2 only slightly up-regulated HIF-1α levels after 30 days (data not shown), therefore I decided that the use of a hypoxia mimetic would be faster and more effective. $CoCl_2$ is a known chemical inducer of hypoxia and it acts by affecting the intracellular ascorbate concentration and thus by promoting the iron oxidation and inactivation of the enzyme prolyl hydroxylase, which is responsible for the hydroxylation of HIF-1α leading to its protein degradation (Salnikow *et al.*, 2004). $CoCl_2$ has been used for this purpose in many studies (Fu *et al.*, 2009; Krishnamachary *et al.*, 2012; Befani *et al.*, 2013; Huang *et al.*, 2014; Jeon *et al.*, 2014). To assess the efficiency of $CoCl_2$ I measured the mRNA levels of some important genes whose up-regulation is fundamental during hypoxia, including HIF-1α, CXCR4 and VEGF-A.

Interestingly, all the above mentioned genes were up-regulated in both cell lines apart from CXCR4 in the MCF-7 cells. HIF-1α, which is the up-stream molecule in the hypoxic pathway (Wang *et al.*, 1995), showed an increased expression in response to hypoxia, but this was not significant in any of the cell lines. Other HIF-α members also

contribute to this process (Wang *et al.*, 1995) with HIF-2α being important for the CSC maintenance, while HIF-1α has been reported to be present only under more severe hypoxic conditions and in both CSC and non-CSC populations (Li *et al.*, 2009).

However, both HIF-1α and HIF-2α are thought to regulate stem cell pathways, but HIF-1α seems to also activate metastasis-related pathways as reviewed by Barnhart and Simon (Barnhart and Simon, 2007). Indeed, HIF-1α has been suggested to be responsible for the stabilization of Notch1, leading to the maintenance of cells in an undifferentiated state (Gustafsson *et al.*, 2005), while it also promotes the expression of genes that are involved in invasion and metastasis, such as lysil oxidase (LOX) (Erler *et al.*, 2006) and matrix metalloproteases (MMPs) (Pouyssegur *et al.*, 2006). What is more, Pennacchietti et al. have described the role of HIF-1α in the up-regulation of MET, which is a proto-oncogene conferring self-renewal and metastatic properties to CSCs (Pennacchietti *et al.*, 2003). On the other hand, HIF-2α has been shown to promote the expression of OCT-3/4 (Covello *et al.*, 2006) and c-myc (Gordan *et al.*, 2007), two important stem cell markers.

Furthermore, $CoCl_2$ treatment resulted in the significant elevation of CXCR4 mRNA levels only in the MDA-MB-231 cells. I anticipate that the increase of CXCR4 levels was only apparent in the MDA-MB-231 cell line, since this is highly metastatic as opposed to the MCF-7 cell line. CXCR4 is known to be regulated by hypoxia in many ways and in many different cell types that participate in tumor progression and metastasis. In fact, CXCR4 was found to be highly expressed in tumor-associated macrophages (TAMs), which are involved in the inflammatory response during tumorigenesis in hypoxic tumor regions. CXCR4 levels were also increased under hypoxia and even after several hours of re-oxygenation in cancer cells, indicating that this is an essential property for them in order to be able to be ‘’attracted’’ by target organs secreting CXCL12 and metastasize (Schioppa *et al.*, 2003). Increased CXCR4 expression in response to hypoxia has been found in various hematologic malignancies (Kim *et al.*, 2009) and solid tumors (Phillips *et al.*, 2005; Sun *et al.*, 2010). Notably, luciferase reporter assays revealed that HIF-1α directly binds to the promoter of CXCR4 and it leads to a higher transcriptional activation under hypoxic conditions compared to normoxic conditions, while it has been suggested that this regulation occurs at the post-transcriptional level as well (Schioppa *et al.*, 2003).

VEGF-A mRNA levels showed a significant up-regulation in MDA-MB-231 after treatment with $CoCl_2$. VEGF-A is one of the main molecules that are involved in angiogenesis not only during early tumor development (Hanahan and Folkman, 1996), but also at more advanced stages and during hypoxia (Crowther *et al.*, 2001). Surprisingly, it has been demonstrated that chemotaxis between endothelial cells expressing CXCL2 and cancer cells characterized by high CXCR4 expression is mediated through the VEGF pathway (Nagasawa, 2001). As a consequence, it has been proposed that angiogenesis during hypoxia can be driven by high CXCR4 expression in endothelial cells, cancer cells and TAMs comprising the tumor microenvironment (Schioppa *et al.*, 2003). In addition, CXCR4 has been found to regulate VEGF expression via the PI3K/Akt pathway in the MDA-MB-231 cells, which was confirmed by the observation that VEGF levels were reduced in the presence of a known inhibitor of this pathway, LY294002. High CXCR4 mRNA levels were also associated with high VEGF mRNA levels in a breast cancer specimens from primary tumors (Liang *et al.*, 2007).

qPCR analysis of stem cell marker expression after $CoCl_2$ treatment revealed an increase in all three genes in both cell lines with NANOG and OCT 3/4 being significantly increased in the MDA-MB-231 cell line. These findings indicate the role of hypoxia in the induction of stem cell properties by potentially promoting the de-differentiation of differentiated cancer cells and leading to an increased CSC phenotype. Barnhart and Simon proposed a model to explain the potential contribution of hypoxia to the regulation of CSCs. They suggested that low oxygen conditions can a) induce de-differentiation of differentiated cells by activating stem cell related pathways, such as the Notch pathway or by causing the up-regulation of stem cell genes, such as OCT 3/4 and c-myc b) by maintaining the already existing CSCs into their undifferentiated state and c) by increasing their invasive and metastatic potential through EMT pathways (Barnhart and Simon, 2007). I hypothesize that hypoxia might also increase self-renewal and proliferation rate of CSCs resulting in the generation of higher CSC cell numbers.

I also investigated the effect of hypoxia on the expression of several EMT markers and I found that there was a significant elevation in mRNA levels of almost all genes in both cell lines, excluding TWIST2 in the MDA-MB-231 and S100A4 in both cell lines. These data support the role of hypoxia in the induction of EMT, but surprisingly I did not observe the expected changes in the mesenchymal marker VIMENTIN in neither

cell line nor changes in the epithelial marker E-CADHERIN in the MCF-7 cell line. In fact, VIMENTIN expression was increased, but not highly and E-CADHERIN expression was also increased in the MCF-7 cells. There is evidence to suggest that down-regulation of E-CADHERIN is not necessarily the hallmark of the EMT process, since SNAIL over-expression has led to the increased migratory potential of T-47D and MCF-7 cells despite the fact that E-CADHERIN expression remained unaffected. Accordingly, silencing of the SNAIL gene caused a significant decrease of the migratory capacity of the MCF-7, MDA-MB-231 and MDA-MB-468 without changing the expression of VIMENTIN or E-CADHERIN. Likewise, DCIS cells in necrotic tumor areas were found to be positive for nuclear SNAIL staining, while VIMENTIN and E-CADHERIN expression did not show any changes. As a result, the authors suggested that hypoxia only partially promotes the EMT process, although this is sufficient for the enhancement of cell movement (Lundgren *et al.*, 2009). In line with these findings, Barrallo-Gimeno et al. also claimed that the role of SNAIL is to increase the cells' migratory potential, rather than to induce complete EMT (Barrallo-Gimeno and Nieto, 2005). Intriguingly, the majority of the specimens from invasive breast carcinomas exhibited E-CADHERIN expression (Hashizume *et al.*, 1996) and no association between its expression and metastatic status has been found, while it has been concluded that complete EMT is not an essential factor for tumor invasiveness and aggressiveness (Parker *et al.*, 2001; Kovács *et al.*, 2003).

$CoCl_2$ treatment for 48 h resulted in a significant increase of the SP percentage in the MCF-7 cells, while exposure to 1% O_2 for even 8 days failed to do so and the cells became sensitive to the staining with Hoechst 33342 dye. In prostate cancer SP numbers were increased in the prostate cancer cell lines PC-3 and DU145 when cultured under hypoxic conditions this was accompanied by an increase of the CD44+ cells and higher expression of OCT 3/4, NANOG and ABCG2 (Ma *et al.*, 2011). Interestingly, the induction of ABCG2 expression in kidney SP cells caused by hypoxia and re-oxygenation was attributed to the MEK/ERK pathway (Liu *et al.*, 2013). This has been considered as a protective mechanism against the accumulation of porphyrins and heme, which leads to detrimental effects for the cells under hypoxia due to the production of reactive oxygen species and mitochondrial dysfunction (Krishnamurthy *et al.*, 2004). Indeed, in cardiac SP cells exposed to hydrogen peroxide HIF-2α was able to directly bind to and up-regulate ABCG2 leading to a significant increase of the SP percentage (Martin *et al.*, 2008). Notably, HIF-2α expression was significantly associated with high

ABCG2 expression, histology-grade and Ki67 expression in invasive breast cancer patients, suggesting that targeting HIF-2α could enable the prevention of drug resistance and metastasis in breast cancer (Xiang *et al.*, 2012).

The opposite effect was seen on the MDA-MB-231 SP cells, as they were completely diminished in the presence of $CoCl_2$. I anticipate that these differences in the responsiveness of the SP populations in these two cell lines could be explained by the fact that the MDA-MB-231 cells are characterized by the presence of more active TGFB-RII receptors, which are thought to be more essential for the activation of the TGF-β signalling pathway (Vivien *et al.*, 1995). However, there are almost undetectable levels of TGFB-RII in the MCF-7 cells as described in Chapter 3. As a consequence, it is reasonable to assume that hypoxia is more likely to lead to the activation of the EMT pathway in the MDA-MB-231 and have the same effects as exogenous TGF-β treatment as shown in Chapter 3. Indeed, I have demonstrated that hypoxia can lead to the phosphorylation of smad 2/3 and to its nuclear localization, which is indicative of the activation of the TGF-β signalling pathway, and this occurred to a greater degree in the MDA-MB-231 in comparison with the MCF-7 cells.

In a recent study, ERα was found to affect the responsiveness of CSCs to hypoxia both *in vitro* and *in vivo*. Firstly, the authors demonstrated that ERα is essential for the induction of hypoxia-related changes, since mammosphere forming efficiency was increased in ERα-positive primary samples and cell lines and it was decreased in ERα-negative primary samples and cell lines in response to hypoxia. These effects were reversed upon the addition of 4-hydroxytamoxifen (Tam), confirming that they were due to the activation of the ERα pathway. Additionally, hypoxic culture of ERα-positive primary samples and cell lines resulted in the transcriptional activation of known target genes of the ERα pathway (AREG, PIP, TFF1) and the significant up-regulation of ABCG2, CD44 and ALDH1 (Harrison *et al.*, 2013). The authors further examined the effects of hypoxia on the activation of the Notch signalling pathway in the above mentioned cells given that this pathway has been reported to play a pivotal role in the maintenance of cells into their stem cell state under hypoxia (Gustafsson *et al.*, 2005) and that Notch1 is a downstream target of ERα-positive primary samples and cell lines (Soares *et al.*, 2004). Indeed, both JAG1, which is a ligand and downstream target of the Notch signalling pathway, and HES1 and HEY2 were up-regulated in ERα-positive cell lines followed by hypoxic culture. Interestingly, a decrease in mammosphere formation was observed in the presence of a γ-secretase inhibitor (GSI) of the pathway,

dibenzazepine, in ERα-positive cell lines and accordingly over-expression of ERα in MDA-MB-231 and MDA-MB-468 cells led to an increase of mammosphere forming capacity. Furthermore, a xenograft model using cell lines and patient-derived cells with a different ER-α status further confirmed the contrasting hypoxic responsiveness of CSCs depending on this. Therefore, it was suggested that the breast cancer patient subtype should be considered in antiangiogenic therapies, as it could be combined with blocking both the ERα and the Notch signalling pathways in ERα positive patients to prevent the hypoxia-induced increase of CSCs, while increasing tumor hypoxia might be an effective therapeutic approach to eliminate the CSC population in ERα negative patients (Harrison *et al.*, 2013).

It should be noted that Clarke et al. demonstrated that breast SP cells had a 6-fold higher expression of ERα compared to NSP cells and they were also positive for $p21^{CIP1}$, CK19 and Msi1, which are putative stem cells markers. These were considered as the intermediate population during the development of the breast epithelium which could potentially give rise to a CSC population if they accumulate mutations (Clarke *et al.*, 2005). There are several reports that highlighted the regulation of the Notch signalling pathway by ERα. Soares and colleagues showed that co-culture of MCF-7 cells transfected with Jagged1 and MCF-7 cells expressing the Notch reporter gene resulted in a 45-fold increase in the reporter expression when 17 β-estradiol was added to the culture media, suggesting that Notch pathway activation is mediated through the ERα pathway. Intriguingly, HIF-1α was found to be one of the Notch1 target genes, this was confirmed by the decrease of HIF-1α expression when MCF-7 cells were transfected with the dominant negative form of Notch1. These findings indicated that Notch signalling can also promote tumor angiogenesis (Soares *et al.*, 2004). However, another study suggested that induced Notch signalling is only prevalent in ERα negative cells and that ERα leads to the accumulation of inactive Notch1 in the cell surface (Rizzo *et al.*, 2008).

The presence of both Notch1 and Notch4 have been associated with ductal carcinoma *in situ* in the breast (Brennan and Brown, 2003) and it was detected in the majority of ductal and lobular infiltrating carcinomas (Rizzo *et al.*, 2008). Abnormal Notch signalling has also been found in invasive breast cancer cases (Stylianou *et al.*, 2006). However, it has been reported that Notch4 and not Notch1 is responsible for the regulation of BCSCs as inhibition of the former only led to the reduction in the percentages of ESA+/CD44+/CD24- cells. Similarly, injection of MCF-7 cells in which

Notch1 was knocked down in mice promoted the formation of tumors whose volume was not significantly different in comparison to the control group, but no tumors were formed in Notch4 knockdown mice. The authors implied that Notch4 may have a role on the BCSC self-renewal potential and that Notch1 is presumably involved in the progenitor proliferation and luminal differentiation (Harrison *et al.*, 2010). Therefore, targeting Notch4 could be a more effective therapeutic strategy as BCSCs have been resistant to GSIs used in clinical trials (Li *et al.*, 2008).

In addition, my results show that CXCR4 expression is only significantly increased in the MDA-MB-231 cells in response to hypoxia, supporting the notion that the CSC population within this cell line is more prone to acquire an enhanced migratory capacity and metastasize. It is also known that CXCR4 expression is also regulated via the TGF-β signalling pathway (Javelaud *et al.*, 2007), which according to our study is also activated by hypoxia. Since this pathway is intact and more functional in the MDA-MB-231 cells, SP cells in this cell line can presumably become more migratory during hypoxia partially due to the elevation of CXCR4 expression levels.

I then investigated the impact of both $CoCl_2$ and TGF-β treatment on the MCF-7 SP cells, since I saw contrasting effects with each one of these alone in this particular cell line despite the fact that $CoCl_2$ also induced the activation of the TGF-β signalling pathway. Surprisingly, the TGF-β effect was dominant when this was combined with $CoCl_2$ and it led to the reduction of the SP numbers. I anticipate that these effects may vary *in vivo* depending on the concentration of TGF-β and the severity of hypoxic conditions in certain tumor areas. Alternatively, I assume that MCF-7 cells, which are characterized by a dysfunctional TGF-β pathway, require the co-operation of $CoCl_2$ in order to drive the EMT pathway, whereas $CoCl_2$ itself is enough and sufficient to cause the same effect in the MDA-MB-231 cells. In line with these findings, Dunn and colleagues found that there was an additive increase in the expression levels of VEGF and CXCR4 when both the TGF-β and HIF-1α pathways were activated simultaneously in the MDA-MB-231 cell line, suggesting that there is a small interaction between these two pathways (Dunn *et al.*, 2009). Therefore, combined treatment could also enhance the migratory and invasive potential of the MCF-7 SP cells in my study. Of note, the authors also showed that there is no direct regulation of the TGF-β signalling pathway by hypoxia. However, blocking both pathways might be a more promising therapeutic strategy to prevent metastasis, as this further decreased bone metastases *in vivo* (Dunn *et al.*, 2009). Additionally, SP cells that can be augmented in hypoxic tumor regions could

be targeted more effectively through ABCG2 down-regulation due to TGF-β treatment as targeting the HIF-1α pathway might have contrasting effects on the CSC population depending on the patient's ERα status as mentioned above.

Furthermore, I tested the impact of $CoCl_2$ alone and in combination with TGF-β treatment on the CD44 mRNA and protein expression in MCF-7 and MDA-MB-231 cells. Although there was an elevation in the CD44 mRNA levels in both cell lines, the changes were only significant in the MDA-MB-231 cell line. However, none of the cell lines showed a significant increase in CD44 protein expression. The MCF-7 cells exhibited a gradual tendency to respond to the treatment with TGF-β1, $CoCl_2$ and combined treatment, while the MDA-MB-231 cells did not show a further increase of the CD44+ population in response to the combined treatment than with the $CoCl_2$ alone.

Several reports have suggested the correlation of hypoxia with the CD44+/CD24- phenotype in breast cancer (Oliveira-Costa *et al.*, 2011; Conley *et al.*, 2012; Han *et al.*, 2012) and the same relationship was seen in the MDA-MB-231 cell line (Louie *et al.*, 2010). I also investigated the possible effect of hypoxia on CD24 expression, but the staining did not work in the MCF-7 cells and I could not observe the expected changes in the MDA-MB-231 cells. Several groups have only focused on the effect of hypoxia on CD44 expression; in fact, CD44+ cells have been shown to acquire higher ABCG2 expression due to exposure to hypoxic culture conditions (Ma *et al.*, 2011) and the up-regulation of ABCG2, CD44 and ALDH1 in response to hypoxia has also been documented (Harrison *et al.*, 2013). Therefore, given the fact that the data regarding CD24 expression were not satisfying following $CoCl_2$ treatment and the possible association of the CD44 up-regulation with the SP phenotype, I also focused on the analysis of the CD44 marker.

Additionally, Krishnamachary and colleagues demonstrated that hypoxia is responsible for the up-regulation of both CD44 and VEGF in the MDA-MB-231 and SUM-149 breast cancer cell lines (Krishnamachary *et al.*, 2012), but I was only able to detect this effect at the mRNA level. Consequently, based on my findings hypoxia affects the SP population in the MCF-7 and MDA-MB-231 cell lines. These variable results are presumably due to the different methodologies used in all these studies. I also assume that the CD44+ population might overlap with the SP phenotype in the MCF-7 cells, since the effect of hypoxia was similar. Nevertheless, the effects of the combined $CoCl_2$ and TGF-β1 treatment were different.

Finally, I examined the potential involvement of hypoxia in the induction of drug resistance by assessing the mRNA levels of some ABC transporters in response to $CoCl_2$ treatment. Interestingly, I observed a significant up-regulation of ABCG2 and ABCC3 in both cell lines, while ABCB1 expression was almost unaffected. Of note, ABCB5 expression was hardly detectable in both cell lines when untreated, however, there was a tendency for an increase in its mRNA levels after $CoCl_2$ treatment, but this would probably not relate to detectable or meaningful expression of protein. The increase of ABCG2 expression levels that I saw is in accordance with the effect that hypoxia has on the MCF-7 SP cells, but this fails to lead to the increase of the MDA-MB-231 numbers. Notably, previously published findings in my research group indicate that there is no significant difference of ABCG2 protein expression between SP and NSP cells in the MDA-MB-231 cell line (Britton *et al.*, 2012). Consequently, other ABC transporters apart from ABCG2 and ABCC3 could be responsible for the SP phenotype in this cell line, but since no increase of the SP percentage is seen, these are presumably unaffected by hypoxia.

Intriguingly, an ERE was discovered in the ABCG2 promoter and estrogen treatment promoted the up-regulation of ABCG2 mRNA levels in both ER+ cells (T-47D: A18) or cells induced to express ERα (PA-1). Furthermore, PA-1 cells transfected with the full-length ABCG2 promoter-luciferase construct as well as the ERα plasmid showed a higher promoter activity in response to estrogen treatment and these effects were reversed upon the addition of the ICI 182,780 inhibitor of the ER pathway. Additionally, site directed mutagenesis revealed that the ERE is located between the -243 and -155 positions in the ABCG2 promoter, due to the fact that luciferase activity was reduced when this region was mutated. Finally, electrophoretic mobility shift analysis showed that ERα directly binds to the ABCG2 promoter (Ee *et al.*, 2004). Therefore, I anticipate that the MCF-7 cells, which are ER+, have a higher potential to up-regulate ABCG2 than the MDA-MB-231 cells when exposed to hypoxia. What is more, the ER signalling pathway has also been reported to promote the degradation of p-smad 2/3 and thus it prevents the activation of the TGF-β signalling pathway (Ito *et al.*, 2010). As a result, I assume that the SP cells within this cell line are less prone to undergo EMT-related changes through this pathway as opposed to the MDA-MB-231 SP cells.

According to these findings I expect that preventing the hypoxia-induced up-regulation of certain ABC transporters would provide a beneficial method for overcoming drug

resistance to particular chemotherapeutic drugs. To further support this hypothesis, I aimed to determine the effect of $CoCl_2$ on the SP population of the MCF-7 cell line when combined with mitoxantrone. Interestingly, I saw a significant increase of the SP percentage in MCF-7 cells exposed to both $CoCl_2$ and mitoxantrone at the same time, while both the SP and the NSP populations were protected by $CoCl_2$ against mitoxantrone. This observation could be explained by the up-regulation of ABCG2 and ABCC3 in unfractionated cells, but the increase of the SP numbers is remarkable and indicative of the induction of resistance to mitoxantrone.

Hypoxia has been reported to be responsible for promoting resistance to certain chemotherapeutic drugs in cancer by several research groups. In fact, Chen t al. demonstrated that T98G human glioma cells in which HIF-1α was silenced by si-RNA exhibited lower mRNA and protein levels of both HIF-1α and ABCC1 and they were also characterized with increased sensitivity to doxorubicin and etoposide (Chen *et al.*, 2009). Similarly, sh-RNA knockdown of HIF-1α in MCF-7 cells was accompanied with suppression of expression of HIF-1α target genes, including VEGF, Glut-1, PGK and ABCB1 and these cells showed increased sensitivity to methotrexate (Li *et al.*, 2006). The gastric cancer cell line SGC7901 also had a reduced resistance to 5-FU, vincristine, cisplatin, etoposide and adriamycin when the HIF-1α gene was silenced. Additionally, vincristine treated SGC7901 cells which were exposed to hypoxic culture conditions have a reduced apoptotic rate as shown by Annexin V/PI staining with the anti-apoptotic Bcl-2 being up-regulated and the pro-apoptotic Bax being down-regulated. HIF-1α led to the increase of both ABCB1 and ABCC1 mRNA and protein expression and the intracellular adriamycin accumulation was decreased, suggesting that hypoxia can drive mechanisms to promote drug efflux. Of note, when vincristine was co-injected with HIF-1α si-RNA into nude mice the tumor size was decreased by a half compared to vincristine alone (Liu *et al.*, 2008). Finally, Sasabe and colleagues reported that HIF-1α targeting in OSCC cell lines established from patients with oral cancer resulted in the inhibition of cell growth and the induction of apoptosis, which were reversed in HIF-1α overexpressing cells. The increase of CDDP and 5-FU efflux was attributed to the increase of ABCB1 and not ABCC1 expression caused by HIF-1α overexpression (Sasabe *et al.*, 2007).

Interestingly, chemotherapy has also been reported to induce the activation of the HIF signalling pathway even under normoxic conditions. Cao et al. were the first to demonstrate that HIF-1α expression increased in response to doxorubicin treatment in

the 4T1ODD-luc and MCF-7 breast cancer cell lines, while it also promoted tumor angiogenesis via increasing VEGF expression (Cao *et al.*, 2013). More recently, Samanta and co-workers demonstrated that paclitaxel treatment can also promote an increase in the percentages of ALDH+ cells in the triple negative breast cancer cell lines SUM-159 SUM-149, MDA-MB-231, while it also increased their secondary mammosphere forming efficiency and these effects were reversed upon the addition of digoxin. It was also shown that the increase of the BCSC cells was due to the elevation of IL-6 and IL-8 in response to paclitaxel treatment, since the use of antibodies for these resulted in the abrogation of ALDH+ and mammosphere increase in the MDA-MB-231 and SUM-159 cell lines. The authors further reported that paclitaxel led to the elevation of ROS levels and of the histone demethylase JMJD1A and JMJD3 mRNA levels, which are both HIF target genes that bind to the promoters of IL-8 and IL-6, respectively to up-regulate their expression. Therefore, it was concluded that the paclitaxel indirectly triggers the stimulation of HIF signalling and induces the enrichment of BCSCs. Intriguingly, ABCB1 mRNA levels were also increased following paclitaxel exposure especially in the ALDH+ cells, which was inhibited when either HIF-1α or HIF-1β were silenced. Furthermore, paclitaxel treated cells led to increased tumor growth and increase of ALDH+ cells, which were characterized by high IL-6, IL-8 and ABCB1 expression and all these effects were inhibited when paclitaxel was combined with digoxin. It is also worth mentioning that the expansion of ALDH+ cells due to paclitaxel treatment was not restricted to triple negative cell lines, since MCF-7 cells exhibited the same pattern, while no significant increase of ALDH+ cells was observed in the HCC-1954 (HER2+) cell line. Finally, the HIF-1 signature was significantly associated with the triple negative breast cancer subtype, but not with the HER2+ subtype in 1,160 breast cancer specimens (Samanta *et al.*, 2014).

In summary, I have demonstrated that there is a definite impact of hypoxia in the regulation of SP cells in the breast cancer cell lines MCF-7 and MDA-MB-231 for the first time, whereas no significant effect was observed in the CD44+ population in these cell lines. All these effects were supported by alterations in mRNA levels of stem cell and EMT marker genes, which were more significant in the MDA-MB-231 cells. Strikingly, I was able to show contrasting effects of hypoxia on the MCF-7 and MDA-MB-231 SP cells, which I assume may mainly depend on their ERα status. Consequently, different therapeutic approaches should be used in the corresponding breast cancer patient subtypes, since hypoxia seems to have a positive and a negative

regulatory role in ERα positive and ERα negative cell lines, respectively. Targeting stem cell pathways, such as the Notch signalling pathway, might be a more effective strategy to eliminate the SP cells in ERα positive, while blocking EMT pathways could be promising for the prevention of metastasis in ERα negative breast cancer patients. Lastly, it is reasonable to suggest that targeting hypoxia might have a therapeutic potency to favour the reversion of drug resistance to chemotherapeutic drugs. It seems that chemotherapy further promotes the enrichment of BCSCs as demonstrated by our findings on the MCF-7 SP cells and as supported by recent research findings (Samanta *et al.*, 2014). Since chemotherapy has been found to stimulate the activation of HIF signalling (Cao *et al.*, 2013), it presumably affects the CSC population indirectly under normal oxygen conditions. Unlike the effects of hypoxia, the increase of ALDH+ cells in response to chemotherapy has not been found to depend on the ERα status (Samanta *et al.*, 2014). It is possible that different BCSCs populations respond differently to chemotherapy. Therefore, the identification of the exact ABC transporters whose expression and function is affected by hypoxia is crucial for each cancer type or even each patient subgroup with the same cancer type to ensure the efficiency of possible therapeutic strategies.

Chapter 5: General discussion

5.1 Summary of findings

- To determine the effects of EMT induced by TGF-β1 treatment of BCSCs in the MDA-MB-231 and MCF-7 cell lines.

The SP phenotype in MDA-MB-231 cells was lost while the MCF-7 SP cells were significantly decreased after TGF-β treatment. The use of the SB-505124 inhibitor allowed me to confirm that these effects were due to the exogenous addition of TGF-β. However, no significant effect was seen on the CD44+ cells in any of these cell lines. The negative regulatory role of EMT in the SP population has also been reported by other research groups (Tang *et al.*, 2007; Yin *et al.*, 2008; Ehata *et al.*, 2011).

- To investigate the properties of the TGF-β signalling pathway in both cell lines in order to explain the potentially different effect of EMT on the SP cells from these.

The impact of TGF-β treatment on the SP population was further confirmed by the observation that nuclear p-smad 2/3 levels increased when both cell lines were exposed to TGF-β and this was reversed when the SB-505124 inhibitor was added. In fact, this can be supported by the findings of Ehata and co-workers who showed that p-smad 2/3 is able to directly bind and down-regulate ABCG2 and therefore reduce the SP percentage (Ehata *et al.*, 2011). Additionally, MCF-7 cells, which are ER+, have a lower potential to exhibit this effect, since it has been shown that ERα is responsible for the degradation of p-smad 2/3 (Ito *et al.*, 2010). Most importantly, I demonstrated that there is very low mRNA and protein expression of TGFB-RII in the MCF-7 cells, while TGFB-RI expression was not different between MDA-MB-231 and MCF-7 cells. This is in accordance with previous reports suggesting that TGFB-RII expression is inversely correlated with ER expression (Arteaga *et al.*, 1988; Ammanamanchi *et al.*, 1998). TGFB-RII is thought to be more essential for the activation of the TGF-β signalling pathway (Vivien *et al.*, 1995) and suppression of its expression has resulted in the enhancement of the SP phenotype (Tang *et al.*, 2007).

- To study the effects of hypoxia induced by $CoCl_2$ treatment on the MDA-MB-231 and MCF-7 BCSCs.

The induction of hypoxia through $CoCl_2$ treatment led to the abrogation of the SP population in the MDA-MB-231, whereas it resulted in the significant expansion of these cells in the MCF-7 cell line. Nevertheless, there was an increase of the CD44+

cells in both cell lines, but it was not significant. The contrasting effects of hypoxia have been shown to depend on ERα status (Harrison *et al.*, 2013), while it has also been reported that the Notch signalling pathway is activated by ERα (Soares *et al.*, 2004). Therefore, it is reasonable to assume that hypoxia stimulates the self-renewal of BCSCs in the MCF-7, but it promotes their depletion in the MDA-MB-231 cell line. It is also possible that the more aggressive MDA-MB-231 cell line is more prone to undergo EMT through hypoxia.

- To examine the possible impact of hypoxia on the transcriptional activation of EMT related genes, stem cell markers and ABC transporter genes.

$CoCl_2$ treatment promoted the up-regulation of most EMT markers in both cell lines apart from TWIST2 in the MDA-MB-231 and S100A4 in both cell lines. Notably, E-CADHERIN expression was not decreased and VIMENTIN was not increased, but it has been suggested that complete EMT is not essential for the induction of invasion and metastasis (Parker *et al.*, 2001; Kovács *et al.*, 2003). Regarding the effect of hypoxia on stem cell marker expression I found that all markers were up-regulated in both cell lines, with the NANOG and OCT 3/4 elevation being significant in the MDA-MB-231 cells. Finally, the expression of both ABCG2 and ABCC3 was significantly increased in both cell lines, while ABCB1 expression was unaffected and ABCC5 expression was almost undetectable even in the treated cells.

- To determine possible relationship of hypoxia with the induction of drug resistance in BCSCs of the MDA-MB-231 and MCF-7 cell lines.

I performed the SP assay for the MCF-7 cells to test whether treatment with mitoxantrone would further enhance the SP numbers in the presence of $CoCl_2$. This experiment was carried out only in the MCF-7 cells, as the MDA-MB-231 SP cells were eliminated under hypoxic conditions. While treatment with mitoxantrone alone led to cell death of most of the cells in both the SP and the NSP compartment, treatment with $CoCl_2$ not only protected both populations from the cytotoxic effects of mitoxantrone, but it also significantly increased the SP numbers in comparison to the treatment with $CoCl_2$ alone. I assume that this effect could be due to the up-regulation of some members of the ABC transporter family of proteins, such as ABCG2 and ABCC3 in unfractionated cells. Chemotherapy also seems to induce the activation of the HIF signalling pathway (Cao *et al.*, 2013; Samanta *et al.*, 2014) and this presumably acts as a feedback loop mechanism by further increasing the SP percentage.

- To test whether there is an interaction between the hypoxia and TGF-β signalling pathways by investigating the combined effect of hypoxic and TGF-β on BCSCs in the MDA-MB-231 and MCF-7 cell lines.

SP analysis of MCF-7 cells treated with both $CoCl_2$ and TGF-β showed that there is a dominant effect of TGF-β leading to the significant reduction of the SP percentage compared to $CoCl_2$ alone. MCF-7 SP cells might need the co-operative action of both $CoCl_2$ and TGF-β for the induction of EMT, since I have already demonstrated that there is defective TGF-β signalling in the MCF-7 cell line. However, $CoCl_2$ or TGF-β alone are sufficient to promote the same effects on the MDA-MB-231 SP cells. The combined treatment only induced a slightly higher CD44 protein expression in the MCF-7 and had no additive effect in the MDA-MB-231 cells compared to $CoCl_2$ or TGF-β alone.

5.2 Limitations of study

One of the main limitations of the present study was the fact that there was a limited number of FNAs provided, so no significant conclusions could be drawn about the association of EMT marker expression and the presence of SP cells in particular breast cancer patient subgroups. What is more, in most cases the number of cells that were available was very low and this did not allow me to perform all the required experiments. Secondly, although I feel confident about our results on the TGF-β and hypoxia induced effects on the two different breast cancer cell lines, I was hoping to carry out the same treatment experiments on cells derived from actual FNAs from breast cancer patients. Furthermore, I anticipate that the ICC data could be better supported by the use of a more quantitative method for protein expression analysis, such as western blotting and it could also provide more reliable information about the localization of the proteins of interest. Therefore, this experimental approach could be performed in the future to enhance the reliability of the protein analysis. Finally, due to limited time I did not examine the possible functional differences of the SP and the NSP populations in response to EMT, including migration and invasion or clonal ability, but these could be some of the future directions of this study.

5.3 Clinical implications

Breast cancer is a complex disease, consisting of 10 different subtypes (Curtis *et al.*, 2012). In addition, the mammary gland is thought to contain undifferentiated cells that give rise to its myoepithelial and luminal compartment during puberty and pregnancy (Villadsen *et al.*, 2007). The elucidation of the mechanisms underlying normal

mammary development would reveal the actual source of CSCs. Furthering our understanding of the gene expression patterns that characterize normal and CSCs would enable the specific targeting of CSCs without having any negative effects on the normal stem cell population. Induction of the slow cycling and long-lived CSCs to undergo differentiation and incorporate with the bulk of the tumor cells might have a therapeutic potency. Thus, CSCs could become less resistant to conventional chemotherapy and they could ideally be detected at early stages of the primary disease favouring the prevention of metastasis. Understanding the molecular signature of breast cancer is challenging and the benefits of potential CSCs therapies need to be determined for each particular patient subgroup.

Blocking the ABC transporters expressed in most CSCs, including SP combined with current therapies might be more efficient. Strategies based on this approach need to be designed depending on the ABC transporter which is responsible for the SP phenotype in particular breast cancer patient subtypes, so that more specific ABC inhibitors can be developed. The use of general ABC inhibitors, such as verapamil for the reversion of drug resistance in patients with refractory myeloma in clinical trials has been unsuccessful due to toxicity issues (Dalton *et al.*, 1995). Nevertheless, the use of tyrosine kinase inhibitors (TKIs) which act by binding to ATP and preventing it from binding to the ATP binding site of several oncogenic tyrosine kinases seems more promising. For instance, it has been reported that some TKIs, such as nilotinib (Tasigna) can efficiently reduce the activity of ABCB1 and ABCG2 transporters (Tiwari *et al.*, 2009). Apatinib (YN968D1) is another TKI, which is at Phase III clinical trial in China for the treatment of gastric carcinoma and non-small cell lung cancer. Mi and colleagues explored the ability of apatinib to reverse MDR in breast cancer cell lines and in xenograft models of breast cancers overexpressing ABCG2 and/or ABCB1. Intriguingly, they found that apatinib remarkably enhanced the accumulation of doxorubicin and Rhodamine 123 dye in these cells by affecting the transporters' efflux function. Additionally, apatinib in combination with paclitaxel significantly increased the activity of paclitaxel in the animal models (Mi *et al.*, 2010).

Moreover, given the increasingly evident role of hypoxia in the induction of drug resistance (Sasabe *et al.*, 2007; Liu *et al.*, 2008; Chen *et al.*, 2009), molecular targeting of the HIF signalling pathway might be more effective for the prevention of all the downstream effects including ABC up-regulation caused by hypoxia. For example, the use of the HSP90 inhibitor in mice with triple negative breast cancer tumors resulted in

the degradation of HIF-1α and led to the reduction of tumor size and the decrease of the ALDH+ cell percentage in the residual tumor (Xiang *et al.*, 2014). What is more, Samanta and co-workers provided evidence that targeting HIFs may also reverse the increased hypoxic response due to chemotherapeutic treatment, therefore, they suggested that several potential HIF-inhibitors (Chintala *et al.*, 2010; Semenza, 2012; Xiang *et al.*, 2014) should be used in combination with chemotherapy in clinical trials.

Findings obtained from this study clearly demonstrate that TGF-β driven EMT has an impact on breast cancer SP cells, while this is stronger on the ER-/PR-/HER2- MDA-MB-231 than the ER+/PR+/HER2- MCF-7 cell line. The diversity of these effects depending on the hormonal status might also reflect the differences in the responsiveness of SP populations contained in patients with different breast cancer subtypes. TGF-β1 presumably promotes the differentiation of the SP cells, therefore, I expect that these could be targeted more efficiently in hormonal non responsive breast cancer patients, who are characterized with a more active TGF-β signalling pathway. I believe that this observation is of great clinical significance, since breast cancer patients belonging to this subgroup have limited therapeutic options and anti-hormonal treatment cannot be used in these cases. Most importantly, these patients' clinical condition and worse prognosis may mainly be due to the higher prevalence of the SP phenotype, which has been significantly associated with this particular breast cancer subtype (Britton *et al.*, 2012). Therefore, triple negative breast cancer patients can be benefited from the TGF-β tumor suppressive actions and the inhibitory role of this pathway on the SP population as opposed to patients with low or absent TGF-BRII expression.

Furthermore, inhibiting angiogenesis, which is one of the main effects of hypoxia, has been another therapeutic method for reducing oxygen and nutrient tumor supply and VEGF targeting is currently used in a number of clinical trials (Miller, 2003; Semenza, 2007). Although the initial results in most of these seem promising, cancer recurrence is very common. Indeed, the use of bevacizumab, a monoclonal antibody against VEGF-A, showed satisfactory results in terms of pathological response in triple negative breast cancer patients, whereas no improvement was seen in hormone receptor positive patients (von Minckwitz *et al.*, 2012). A recent study demonstrated that the adverse effects of such therapies could be because of differences in the responsiveness of BCSCs from ER-α positive and negative breast cancer cell lines and patients (Harrison *et al.*, 2013). In line with these observations, my results also support the contrasting

effects of hypoxia in different breast cancer cell lines depending on their ERα status and I suggest that these could be applied therapeutically.

Anti-angiogenic therapies, that are used to target tumor vasculature, may eventually lead to the induction of more severe hypoxic conditions (Conley *et al.*, 2012). These in turn can promote the expansion of BCSCs in ERα-positive patients and result in more aggressive and drug resistant tumors. However, hypoxia seems to have the opposite effect on the BCSC populations of ERα negative breast cancer patients. Consequently, immunohistochemical analysis of ERα expression can not only be carried out for anti-hormonal therapies in breast cancer, but it is also essential for the selection or not of anti-angiogenic therapies. I suggest that taking into account the patient's breast cancer subtype could facilitate the decision of whether these could be used in combination with approaches which aim to inhibit both the ERα and the Notch signalling pathways in ERα positive patients. Ideally the hypoxia-induced increase of CSCs could be prevented in these patients, whereas increasing tumor hypoxia might be a more efficient method to deplete the CSC population in ERα negative patients.

In addition, my findings on the combined effect of TGF-β and hypoxic treatment in the MCF-7 cell line indicate that blocking both pathways could more effectively target the SP population in patients represented by this cell line. Higher numbers of SP cells in hypoxic tumor regions of these patients could be eliminated through ABCG2 down-regulation induced by TGF-β treatment given that targeting hypoxia might promote diverse effects on the CSC population based on the patient's ERα status.

Finally, I was able to successfully demonstrate that mitoxantrone treatment further induces the enhancement of the SP phenotype under hypoxic conditions in the MCF-7 cell line, while it has also been shown by others that these effects can occur even under normal oxygen conditions (Samanta *et al.*, 2014). I anticipate that targeting hypoxia could prevent the enrichment of SP cells in estrogen responsive breast cancer patients, however, the chemotherapy-induced hypoxia and the subsequent increase of the ALDH1+ cells does not seem to depend on the ERα status. As a result, preventing hypoxia could potentially be used to target other BCSCs except SP cells in hormonal non responsive patients, but the exact mechanisms by which hypoxia regulates different types of BCSCs require further investigation.

5.4 Future directions

The results of the present study give rise to several research questions. First of all, HIF-1α was not significantly up-regulated with $CoCl_2$ treatment, so it might be worth assessing the levels of HIF-2α in response to hypoxia in the MDA-MB-231 and MCF-7 breast cancer cell lines. Given the role of HIF-2α in CSC maintenance (Li *et al.*, 2009) and the fact that HIF-2α has been shown to directly bind to and up-regulate ABCG2 (Martin *et al.*, 2008), I assume that it may play a more important role in the regulation of BCSCs. Additionally, HIF-2α expression has been significantly associated with high ABCG2 expression, histology-grade and Ki67 expression in patients with invasive breast cancer (Xiang *et al.*, 2012). I could also further investigate the role of both HIF-1α and HIF-2α, since HIF-2α is thought to act in both normoxic and hypoxic conditions, while HIF-1α seems to be essential only during hypoxia (Shaw *et al.*, 2012). Knocking down both HIF-1α and 2α would result in the reversion of the effects that I have observed and this would confirm that they are due to the activation of the HIF signalling pathway.

Furthermore, another experimental approach in order to confirm the potential involvement of the Notch signalling in the expansion of the SP population would be to block this pathway using the appropriate inhibitors, such as γ-secretase inhibitors, in the MCF-7 cells. Inhibiting the ER pathway by either silencing ERα or by using specific inhibitors for this, such as 4-hydroxytamoxifen, would lead to the same effects as the blocking the Notch pathway, since this has been reported to be activated by ERα (Soares *et al.*, 2004). In addition, Notch or ERα overexpression in the MDA-MB-231 cells might result in the increase instead of the depletion of the SP cells and this would provide further evidence that the Notch pathway, which is regulated by ERα is responsible for the enrichment of the SP cells in the MCF-7 cell line. It would also be important to determine which Notch is important for the regulation of SP cells, as targeting Notch4 rather than Notch1 has been suggested to be more effective for ESA+/CD44+/CD24- cells (Harrison *et al.*, 2010).

Moreover, transfection of MDA-MB-231 cells with ERα might also promote the increase of the SP cells in response to hypoxia considering the direct role of ERα in the up-regulation of ABCG2 (Ee *et al.*, 2004). This experiment would address the question why the SP percentage does not elevate although there is increase of the ABCG2 mRNA levels under hypoxic conditions. The protein expression changes in both the SP and NSP cells of this cell line could also be examined in comparison to the MCF-7 cell

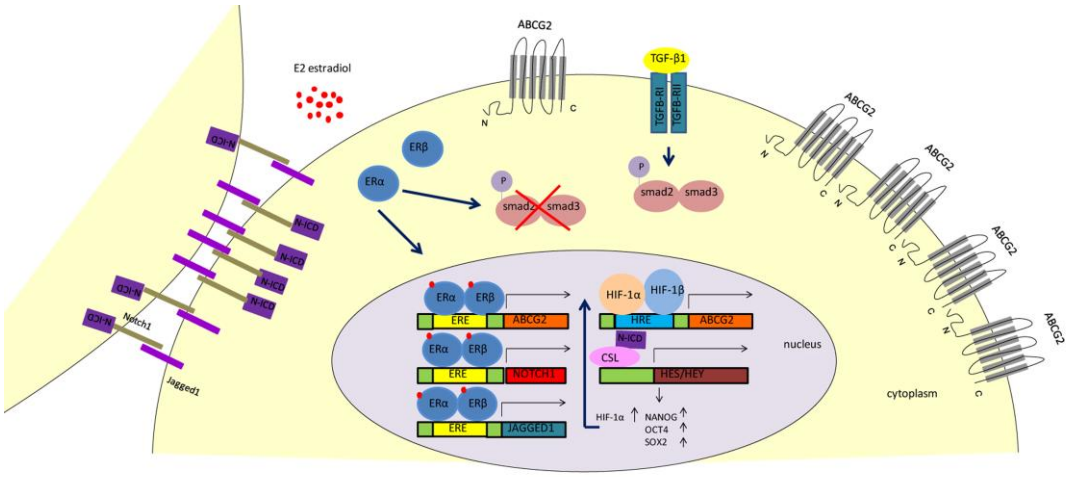
line, as it has been previously reported that the levels of ABCG2 protein expression are not different between the MDA-MB-231 SP and NSP cells (Britton *et al.*, 2012).

In addition to the role of hypoxia in the up-regulation of the ABCG2 protein levels, I could test if the ABCC3 protein levels are also increased by hypoxia as the mRNA levels. Further functional assays, such as invasion and migration assays as well as drug resistance tests to certain chemotherapeutic drugs (such as taxane due to ABCC3 up-regulation (O'Brien *et al.*, 2008) and mitoxantrone due to ABCG2 up-regulation (Mao and Unadkat, 2015), would confirm the role of hypoxia in the induction of a more metastatic and drug resistant phenotype in the SP cells in comparison to the NSP cells. Knocking down ABCC3 and ABCG2 individually or jointly under hypoxic conditions and performing the above mentioned experiments would reveal their involvement in the regulation of the SP population.

5.5 Conclusions

In this project I aimed to characterize the role of EMT induced by the activation of the TGF-β and the HIF-signalling pathways in the regulation of BCSCs in the breast cancer cell lines MDA-MB-231 and MCF-7. Hormonal status seems to play a role in the prevalence of BCSCs in breast cancer patients and thus I was interested in determining the possibly different effects in the above mentioned cell lines. Based on evidence about the effect of hypoxia on the stimulation of EMT and stem cell self-renewal pathways I investigated its potential to transcriptionally activate known EMT and stem cell markers. I was able to clearly demonstrate differences in the effects of EMT driven by TGF-β treatment and hypoxia on the SP populations of the MDA-MB-231 and MCF-7 cell lines, which I concluded that depend on ERα and TGFB-RII expression. Finally, given the emerging relationship between hypoxia and the induction of drug resistance, I aimed to study the impact of hypoxia on the expression of several ABC transporters and determine what the actual effect on the BCSCs is. I have shown that hypoxia can promote the up-regulation of several ABC transporters and enhance the SP phenotype, while combined chemotherapeutic and hypoxic treatment can further enrich the SP population in the MCF-7 cell line.

A



B

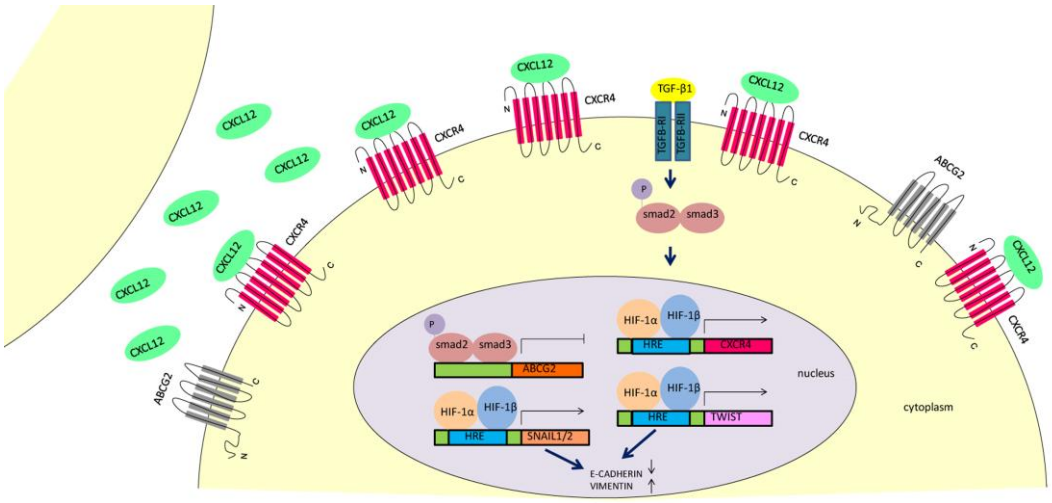


Figure 5.1: Proposed model of hypoxia response in A) ERα-positive breast cancer patients and B) ERα-negative breast cancer patients.

In A the TGF-β signalling pathway is less active due to the low levels of TGFB-RII receptor and the ERα-induced degradation of p-smad 2/3. Therefore, ABCG2 expression is not repressed by p-smad 2/3, but it is directly up-regulated by ERα leading to the enhancement of the SP phenotype. HIF-1α seems to positively regulate the expression of ABCG2 as well. ERα also promotes the activation of the Notch signalling pathway by activating the expression of Notch1 and Jagged1 resulting in the expression of stem cells markers and HIF-1α, which can further increase ABCG2 expression.

In B the TGF-β signalling pathway is intact and thus ABCG2 expression is repressed. However, during hypoxic conditions the elevation of the CXCR4, SNAIL1/2 and TWIST expression levels may be responsible for driving cell EMT and cell migration.

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Appendix A

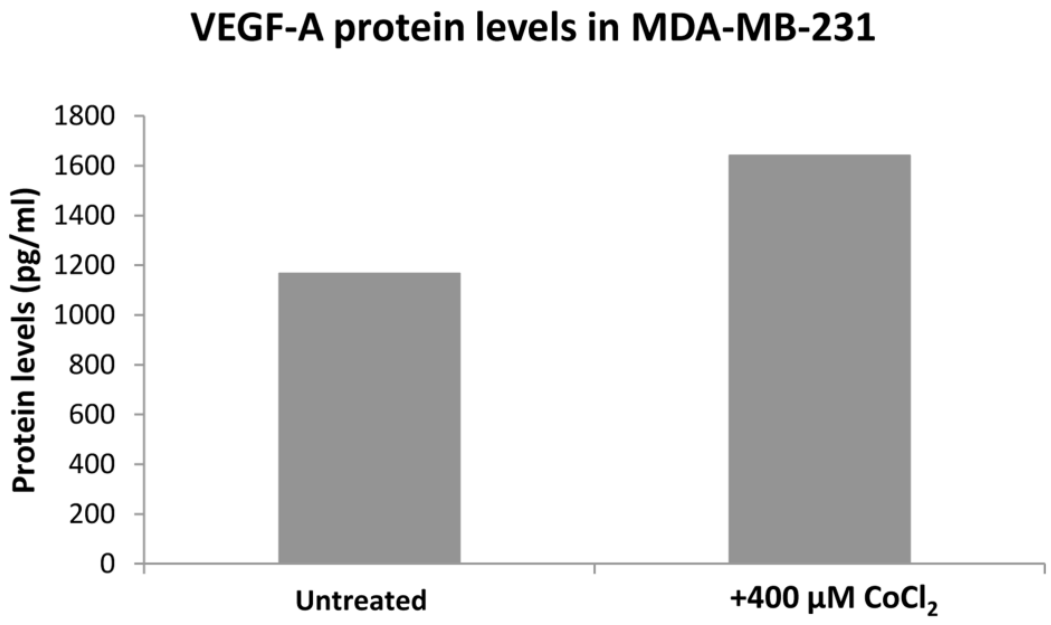
Patient No	Presence of an SP population	Clinical assessment	Hormonal status
1	Inadequate cell number	Ductal carcinoma in situ	N/A
2	Inadequate cell number	Invasive ductal carcinoma	ER+/HER2-
3	YES	benign/no evidence of malignancy	N/A
4	Inadequate cell number	Invasive ductal carcinoma	ER+/HER2+
5	YES	Invasive ductal carcinoma	ER+/PR+/HER2-

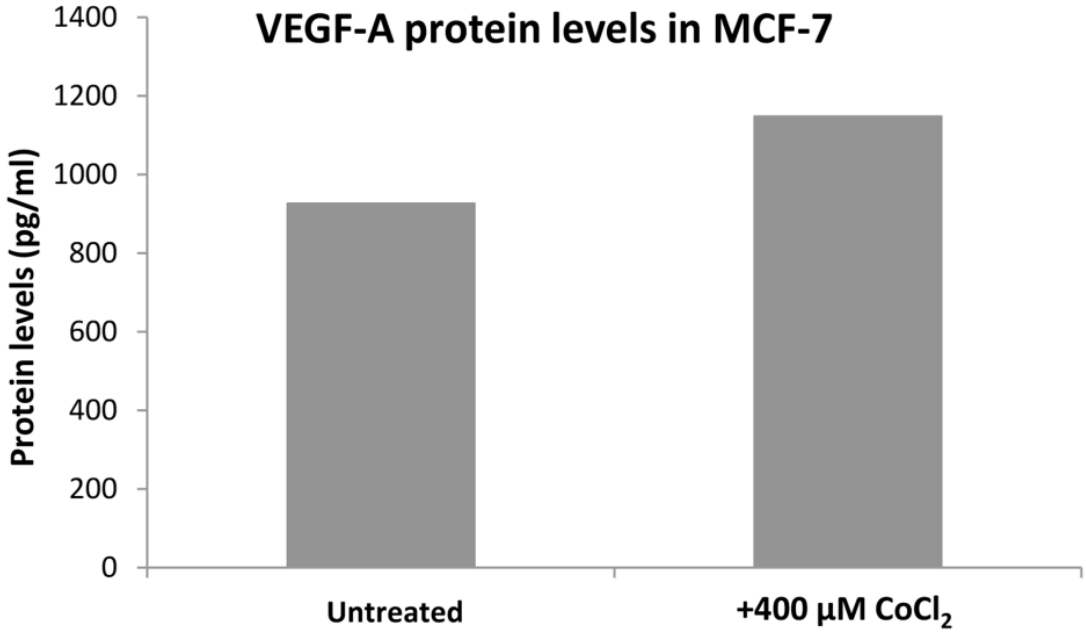
Comparison of FNA SP status to pathology of breast tumor

Appendix B

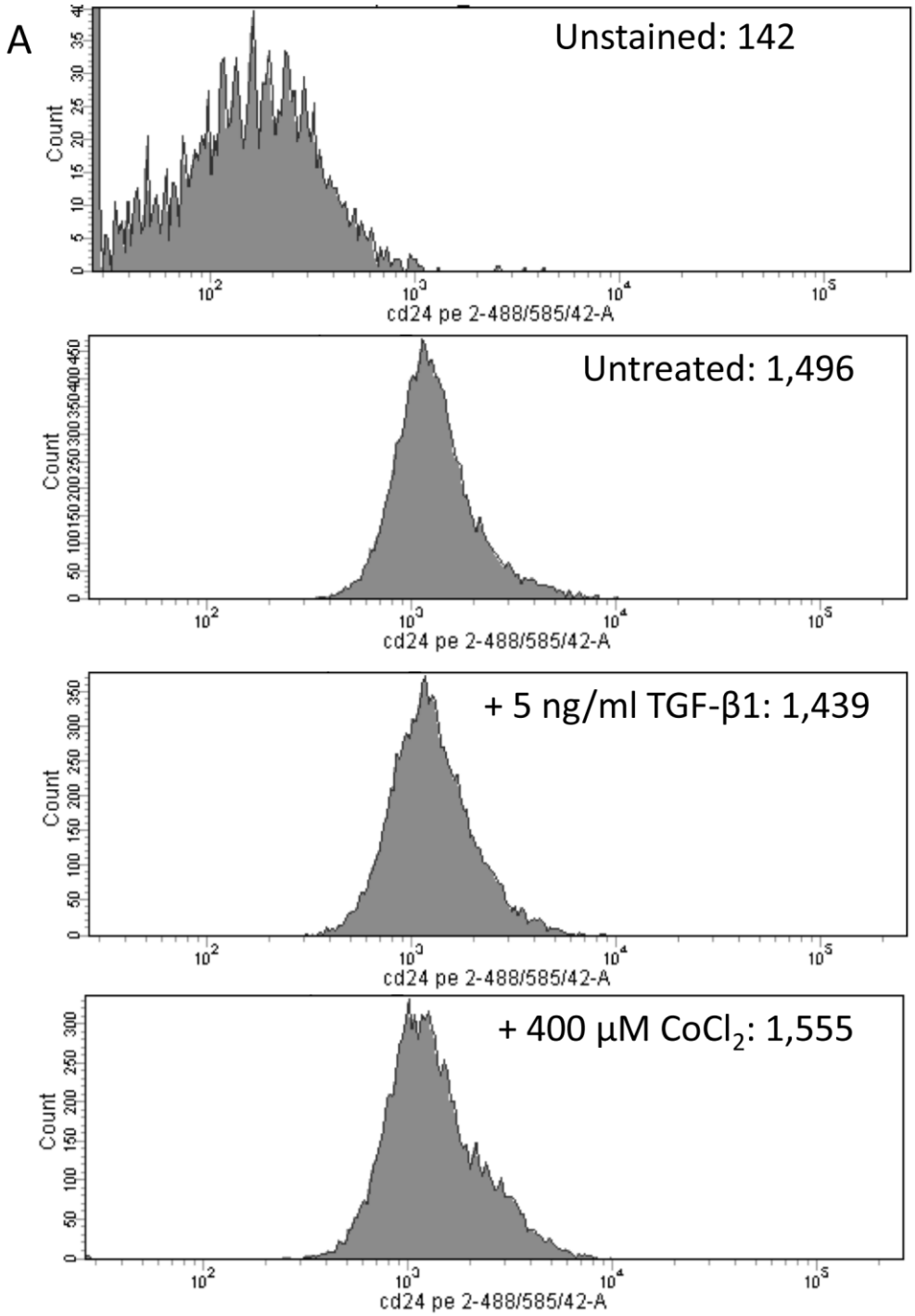
Supplementary data

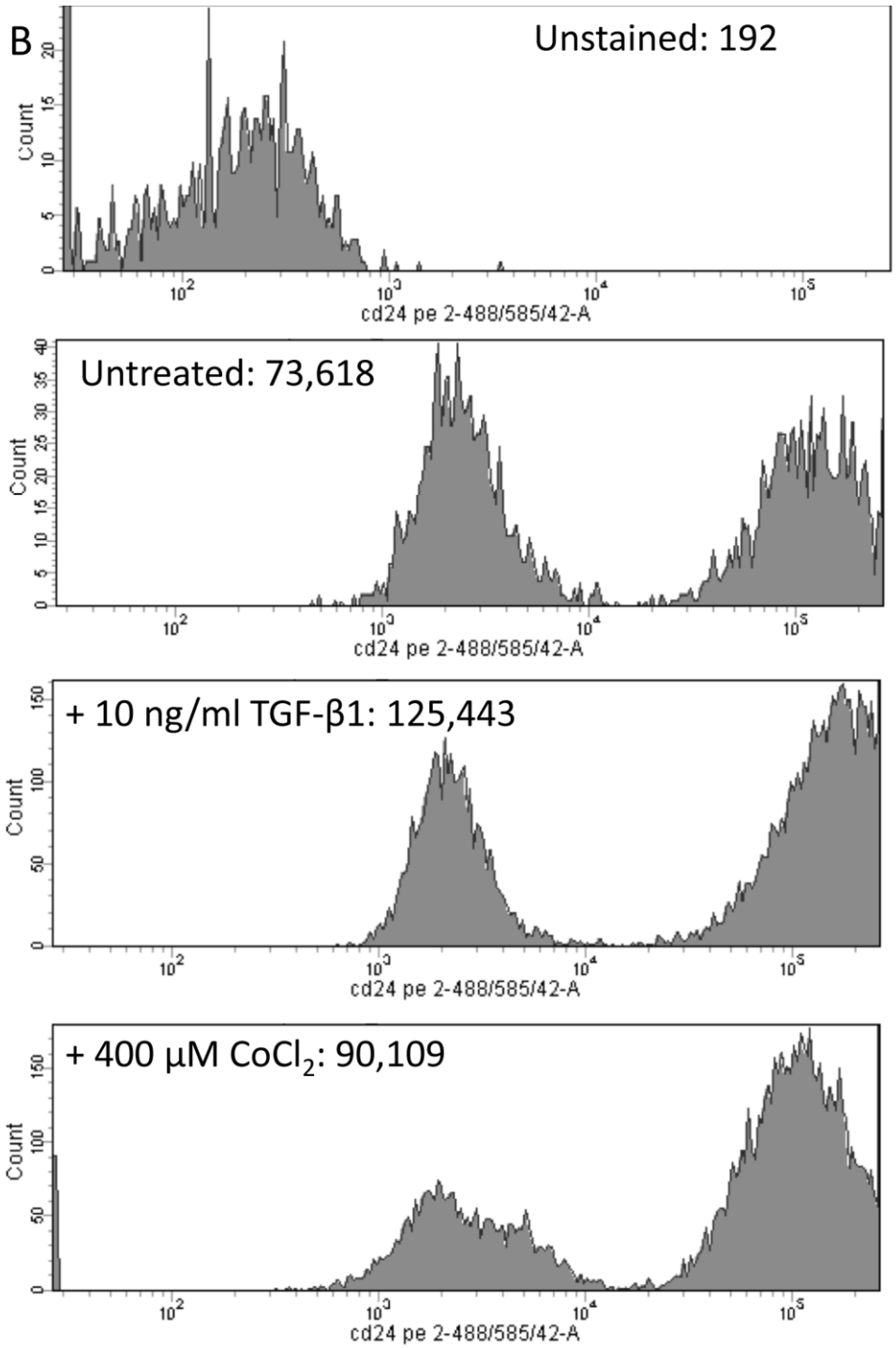
The induction of hypoxia was confirmed by Dr Gendie Lash who performed a protein array for angiogenic factors according to the manufacturer's instructions (Fast Quant Human Angiogenesis Kit Cat# 10486063). Briefly, this kit allows the simultaneous fluorescent detection of cytokines from any biological fluids, including cell culture supernatants, since it contains anti-cytokine monoclonal antibodies. The quantitative analysis is based on a seven-pont standard curve.





CD24 staining data in A) MDA-MB-231 cells and B) MCF-7 cells. In A) CD24 expression was only slightly decreased with TGF-β treatment, while it increased instead of decreasing with $CoCl_2$ treatment. In B) the staining was unsuccessful as double peaks were obtained. Values represent mean fluorescence.




B
Unstained: 192
Count
cd24 pe 2-488/585/42-A
Untreated: 73,618
Count
cd24 pe 2-488/585/42-A
+ 10 ng/ml TGF-β1: 125,443
Count
cd24 pe 2-488/585/42-A
+ 400 µM CoCl2: 90,109
Count
cd24 pe 2-488/585/42-A

Appendix C

Presentations and publications arising from this project

Mallini, P., Lennard, T., Kirby, J. and Meeson, A. (2014) 'Epithelial-to-mesenchymal transition: What is the impact on breast cancer stem cells and drug resistance', *Cancer Treat Rev* 40(3), pp. 341-348.

December 2013 Poster presentation at the Breast Cancer Meeting 2013 in San Antonio, Texas, USA

January 2013 Poster presentation on IGM Postgraduate research Day, Newcastle, UK.

May 2012 Poster presentation on NESCI Research Day, Newcastle, UK