FOXP3 regulates metastatic spread of breast cancer via control of expression of CXCR4 chemokine receptor

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Abstract

The FOXP3 transcription factor can regulate T cell migration by inhibiting expression of CXCR4, the receptor for the chemokine CXCL12. The increased expression of CXCR4 by breast cancer cells can drive metastatic migration towards sites that express CXCL12. Intracellular trafficking of FOXP3 to the nucleus is required in order for this factor to function. The presence of alternative splicing forms, mutations and post-translationally modified forms may disrupt FOXP3 nuclear localization. We hypothesised that FOXP3 tumour suppressor is inactive in breast cancer causing an increase in CXCR4 expression and the development of metastasis. The expression of FOXP3 and CXCR4 were measured at mRNA and protein (immunohistochemistry, immunofluorescence, FACS) levels. FOXP3 DNA sequences of normal and cancer cells were analysed. Stable FOXP3 overexpressing breast cancer transfectants were used to investigate the potential of FOXP3 to regulate chemotaxis. Normal breast epithelial cells (both patient-derived tissues and laboratory cultured cell lines) expressed FOXP3 in their nuclei but did not express CXCR4. Breast cancer cells overexpressed CXCR4 ($p<0.05$), whereas FOXP3 expression was decreased ($p<0.05$) and confined to the cytoplasm with negligible nuclear expression. Metastases expressed less FOXP3 and more CXCR4 than primary cancers ($p<0.05$). FOXP3 sequencing in breast cancer cell lines did not reveal mutations. However, there were at least three bands on the PCR electrophoreses gel. The predominant form in breast cancer cells contained an insertion of 120bp within the forkhead domain. Transfection of breast cancer cells with wild-type FOXP3 restored its nuclear expression, reduced CXCR4 expression and inhibited cell migration. This study demonstrated failure of nuclear localisation of FOXP3 in breast cancer cells and an inverse correlation between this failure and CXCR4 expression. Disruption of FOXP3 nuclear localisation may be due to abnormal co-expression of FOXP3 splice variants leading to increased CXCR4 expression and acquisition of the capacity to metastasize.

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Abbreviations

ANOVA	Analysis of variance
bp	Base pairs
BRCA 1/2	Breast cancer 1/2
BSA	Bovine serum albumin
cpm	Counts per minute
C_T	Threshold cycle
Da	Dalton
DAG	Diacylglycerol
DAPI	4', 6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMMB	Dimethyl-methylene blue
DMSO	Dimethyl sulphoxide
dNTP	Di-nucleotide triphosphate
dp	Degrees of polymerisation
DPX	Dibutylphthalate xylene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
ER	Oestrogen receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GlyCAM-1	Glycosylation-dependent cell adhesion molecule-1
GTP	Guanosine 5'-triphosphate
H&E	Haematoxylin and eosin
HBSS	Hanks' balanced salt solution
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSPG	Heparan sulphate proteoglycan
ICAM-1/2	Intercellular adhesion molecules-1/2
IDC	Infiltrating ductal carcinoma
IFN-g	Interferon-g
ILC	Infiltrating lobular carcinoma
IP3	Inositol 1,4,5-trisphophate
IPEX	Immunodysregulation, polyendocrinopathy, and enteropathy, x-linked
JAK	Janus kinase
JAM	Junctional adhesion molecule
LCIS	Lobular carcinoma in situ
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MT	Mutant
NDST	Heparan sulphate-N-deacetylase-N-sulphotransferase
NiDAB	Nickel-enhanced diaminobenzadine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDK1/2	Phosophoinositide-dependent protein kinase 1/2
PI	Propidium iodide
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma
RPMI	Roswell park memorial institute
SEM	Standard error of the mean
TAM	Tumour associated macrophage
Taq	Thermus aquaticus
TBS	Tris buffered saline
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNM	Tumour node metastasis staging system
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WT	Wild type

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"I thank everyone that has caused me to suffer, without you I would have no reason to express myself."
Author Unknown

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Chapter

1 Introduction

1.1 Breast cancer epidemiology

Breast cancer is the most common cancer in women worldwide, comprising 16% of all female cancers (WHO, 2009), causing 470,000 deaths annually mainly as a result of metastatic spread of the disease (WHO, 2002). The lifetime probability of developing breast cancer in the next 10 years is evaluated as 13.2% worldwide (2005-2006). Mortality rates rose in the years 1951 to 1990 but have since fallen in most European countries including the U.K. The reasons for improvement in mortality rates in Europe, Australia and the Americas include screening (Eisner et al., 2002), accurate and precise diagnosis and improved treatment; most notably hormonal therapy, including tamoxifen (Peto et al., 2000). Breast cancer metastasizes to specific organs: lymph nodes, bone marrow, lung and liver. There is increasing evidence that immune modulation is involved in breast cancer progression and metastatic spread (Fulton et al., 2006).

1.2 Risk factors associated with breast cancer

Non-modifiable risk factors of breast cancer development are: increasing age, female gender, genetic factors, family history of breast cancer, personal history of breast cancer, and race. Modifiable risk factors for breast cancers include smoking, alcohol consumption, use of hormonal replacement therapy, decreased physical activity, obesity and increased intake of animal fat (McPherson et al., 2000).

1.3 Structure of the human breast

The breast is a milk-producing, modified sweat gland composed of glandular tissue, fat and connective tissue. It is subdivided into multiple lobes. Lobes consist of lobules of glandular secretory tissue from which secretions pass to the nipple via ducts. Breast tissue is present on the anterior chest wall and extends towards the axilla as an axillary tail. Lymph nodes within the axilla collect the majority of the lymphatic drainage from the breast (Figure 1-1).

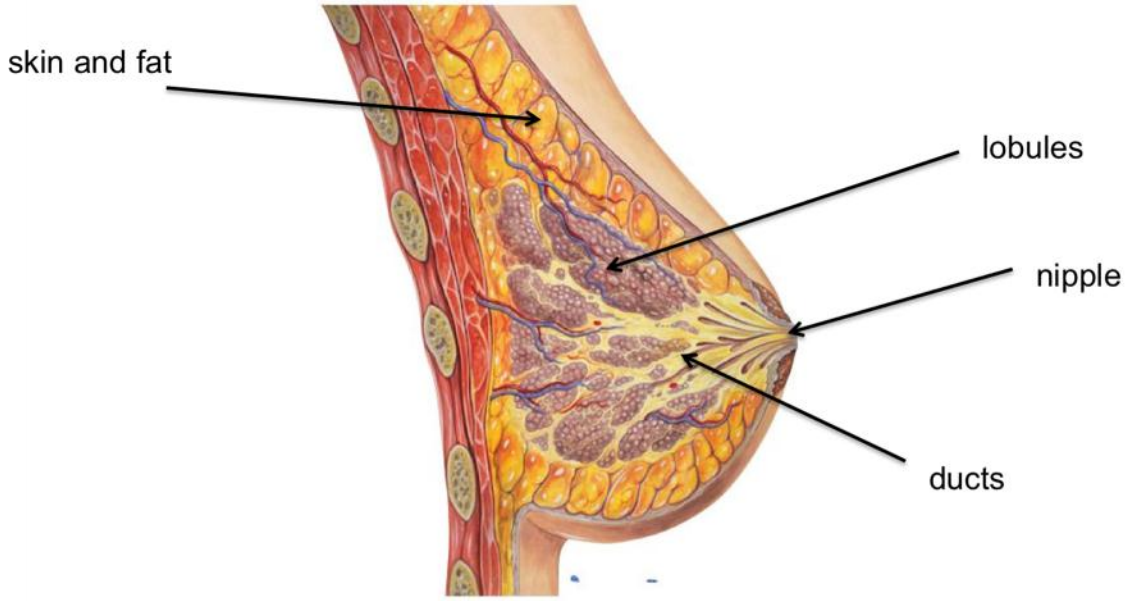


Figure 1-1: Structure of the human breast

The figure demonstrates structure of the human breast showing lobules, ducts and stromal elements. Lymph from the breast drains mainly into the ipsilateral axilla, axillary lymph nodes being a common site of breast cancer metastasis.

Modified from Medical illustration by Patrick J. Lynch (http://www.flickr.com/photos/patrlynch/).

1.4 Pathogenesis and pathology

Breast cancer tumorigenesis is a multi-step process progressing in the following manner: normal breast tissue, *in situ* carcinoma, invasive carcinoma and metastasis.

Initial changes of neoplastic transformation identified morphologically are known as *in-situ* carcinomas or (lobular/ductal) intraepithelial neoplasia.

The majority of breast cancers arise from the epithelial cells of the ducts or glands and are termed adenocarcinomas. Both ductal and lobular carcinomas arise from the terminal duct lobular unit. The most common invasive breast cancers are infiltrating ductal carcinoma (IDC) (80% cases) and infiltrating lobular carcinoma (ILC) (10% of cases).

Some tumours have distinct patterns of growth and cellular morphology, and are termed carcinomas of special type (tubular, cribriform, medullary, mucoid, papillary and classic lobular), the rest are considered to be of non-special type.

1.5 Oncogenes and Tumour Suppressor Genes

The multistep development of breast cancer is dependent on one or more regulatory gene mutations occurring at each step (Beckmann et al., 1997). The mutations involved in the development of hereditary and sporadic breast cancer occur in different genes. Hereditary breast cancers are linked with one or more germline mutations, whereas sporadic breast cancers develop from the accumulation of gene mutation that are somatic in nature (Kenemans et al., 2004). These mutations occur most commonly in regulatory genes collectively known as oncogenes and tumour suppressor genes (see Table 1-1).

Mutations in tumour suppressor genes can happen in both sporadic and hereditary breast cancer. Tumour suppressor genes are involved in the restraint of normal cell cycle progression especially when DNA damage is detected (Kenemans et al., 2004) and mutations in these genes are essential for breast cancer development.

The cell cycle is divided into four stages (Figure 1-2):

- Post-mitotic phase (interphase) when the cell prepares itself to cycle (G0 phase)
- Synthesis of new organelles and DNA checkpoint (G1 phase)
- DNA synthesis or replication (S phase)
- Preparation for mitosis and DNA checkpoint (G2 phase)
- Mitosis (M).

Mutations in the genes involved at any stage of the cell cycle allow cells harbouring other mutations to escape cell death by apoptosis and to proliferate without restraint.

There are a number of inherited breast cancer genes identified, of which mutations in the tumour suppressor genes breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) account for about 30-40% of cases (Venkitaraman, 2001). The BRCA genes are extremely penetrant genes that present an autosomal predisposition to the disease and have a strong relationship with cancer arising at a young age. The presence of the wild type BRCA genes is associated with protection against the development of breast cancer and therefore they play a role in tumour suppression (Clarke, 2002). In the majority of hereditary cases mutations in the BRCA genes are present on one allele with the wild-type protein present on the other allele. For breast cancer to develop the loss of the wild type must occur, possibly through errors in DNA replication. Mutations in BRCA1 or BRCA2 increase the lifetime risk of developing breast cancer to 60-80% in women. Less common gene mutations in hereditary breast cancer include the cell cycle check point kinase gene (CHEK) 2 truncating mutation 1100delC which accounts for 5% of cases and PTEN which is only responsible for 1% of cases (Sansal and Sellers, 2004) (Friedrichsen et al., 2004).

Recently, the x-linked breast cancer suppressor gene FOXP3 was identified (Zuo et al., 2007c). Following x-inactivation only one copy of FOXP3 is functional, allowing "one-hit" inactivation (FOXP3 will be extensively discussed in later chapters).

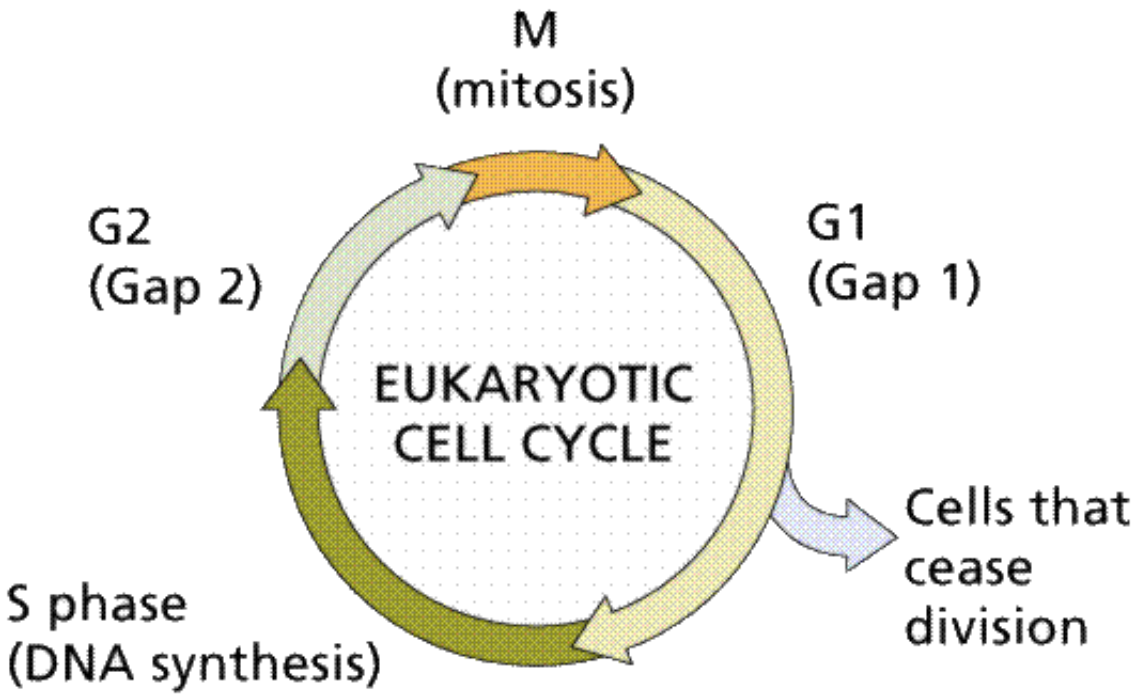


Figure 1-2: Cell cycle

The figure demonstrates eukaryotic cell cycle divided into phases: G1 phase, S phase, G2 phase and mitosis. Go phase (interphase) not shown on the picture.

Adapted from http://www.estrellamountain.edu/.

A.

Tumour suppressor gene	Normal function
p53	Triggers/facilitates apoptosis
p27	Inhibit cyclin-dependent protein kinases; arrests cell-cycle in G_1 phase
BRCA-1	Regulates DNA transcription; acts to repair damaged DNA
BRCA-2	Repairs damaged DNA
FOXP3	Master regulator in the development and function of regulatory T cells
CHK2	Cell-cycle checkpoint kinase, activates p53 after DNA damage
ATM	Checkpoint kinase, activates CHK2
PTEN	Phosphatase, negative regulator of Akt kinase
Rb	Retinoblastoma gene, repressor of cell cycle and protein translation

B.

Oncogene	Normal function
HER-2	Tyrosine kinase receptor
Ras	G-protein
PI3K	Cell signalling kinase
Akt	Cell signalling kinase
ElF-4E	Initiator of protein translation
Cyclin D1	Cell-cycle mediator
Cyclin E	Cell-cycle mediator
c-myc	Transcription factor
cerbB1-4	Gene encoding EGF receptor
Wnt1	Cell signalling

Table 1-1. Tumour suppressor genes and oncogenes in breast cancer

A list of tumour suppressor genes (A) and oncogenes (B) commonly associated with breast cancer, and their function in the absence of mutations (Osborne et al., 2004).

Generally mutations that affect the normal function of tumour suppressor genes differ from those seen with oncogenes. Unlike oncogenes, mutations must take place on both alleles for a change in the function of the majority of tumour suppressor genes, but this is not true for all tumour suppressor genes. Mutations in the tumour suppressor BRCA must occur on both alleles, whereas mutation of only one allele is sufficient to cause a pro-tumourigenic change with p53. The p53 gene is the most commonly mutated gene in sporadic cancers with mutations in p53 occurring late in sporadic breast cancer (Elledge and Allred, 1998). Following stress signals p53 becomes active and binds to DNA leading to the transcription of a multitude of proteins including p21 (Elledge and Allred, 1998). p21, a G_1 CDK inhibitor, complexes with cyclic dependent kinase (CDK) 2 causing G_1/S arrest in the presence of DNA damage.

Most of mutations in p53 are point mutations which prevent the binding of p53 to DNA and therefore the production of p21 (Liu and Kulesz-Martin, 2001). This allows cells containing mutations to progress through the cell cycle and proliferate. The retinoblastoma (Rb) tumour suppressor gene functions normally to prevent G_1/S transition in the presence of DNA damage. Under normal circumstances Rb is phosphorylated by CDKs preventing its interaction with the transcription factor E2F which promotes transcription of genes required for cell cycle progression (Cox and Lane, 1995). In the presence of DNA damage Rb is not phosphorylated and binds E2F preventing the transcription of cell cycle proteins. Mutations in Rb are required on both alleles and results in permanent phosphorylation of Rb preventing its interaction with E2F and unchecked cell cycle progression (Gewirtz et al., 2001).

Oncogenes develop as a consequence of a mutation or overexpression of proto-oncogenes that function under normal conditions to regulate proliferation and differentiation. An example of an oncogene linked with breast cancer progression is cyclin D1. Cyclin D1 is essential for progression from G_1/S phase in the cell-cycle. It binds to and activates CDK4 and CDK6 which then phosphorylate Rb. In the presence of DNA damage p53 inhibits cyclin D1 through p21, preventing

continuation of the cell-cycle (Harbour et al., 1999). Cyclin D1 expression is amplified up to 10-fold in 13-20% of breast cancers and functions to activate CDK4 and CDK6 leading to progression to the S phase, independent of p53-mediated cell-cycle arrest (Arnold and Papanikolaou, 2005).

1.6 Hormone and Growth Factor Receptors

A common feature of breast cancer is the upregulation of hormone receptor expression on the surface of the cells. Two hormone receptors upregulated in breast cancers are the oestrogen and progesterone receptors, which are present either individually or in combination in about 80% of all breast cancers.

Oestrogen is considered a major risk factor in the development of breast cancer. Oestrogen applies its effects on cell proliferation by two mechanisms. Firstly oestrogen can directly stimulate receptors on the plasma membrane resulting in increased levels of Ca^{2+} or nitric oxide, and activation of kinases (Song and Santen, 2006). The second mechanism of oestrogen action involves the diffusion of oestrogen into the cell and binding to the estrogene receptor (ER) located in the nucleus. Activation of the ER on the plasma membrane induces rapid responses to oestrogen whereas activation of the nuclear ER can be measured over the course of hours (Deroo and Korach, 2006). The ER exists in two forms the ER-a and the ER-b; as cancer progresses the expression levels of ER-b decrease and the levels of ER-a increase, which has led to the theory that ER-b may play a protective role in breast cancer through regulation of the ER-a, although most studies have focused on the ER-a (Bardin et al., 2004). About 50-70% of all breast cancers express the ER-a and are referred to as ER-positive tumours. These tumours have a lower proliferative rate and are better differentiated than ER-negative tumours. ER-positive tumours have a better prognosis and can be treated with anti-oestrogen therapies such as tamoxifen and raloxifene (Putti et al., 2005) (Platet et al., 2004).
Progesterone signals through the progesterone receptor (PR), which exists in two isoforms, PRA and PRB. The PR is induced by oestrogen through activation of the ER (Soyal et al., 2002). Unlike the ER the PR is only present intracellularly and not

on the plasma membrane. Following binding of progesterone, the nuclear receptor/ligand complex translocates to the nucleus interacting with the genome leading to the transcription of downstream gene targets (Ismail et al., 2003). In the case of breast cancer, progesterone is thought to function in a parallel manner to oestrogen by increasing cell proliferation. About 55% of tumours demonstrate increased expression of the PRB receptor with 50% tumours being both ER-positive and PR-positive and only 5% being ER-negative and PR-positive (Keen and Davidson, 2003). The tumours expressing both hormone receptors respond most favourably to hormone therapy, whereas those tumours that are either ER-negative or PR-negative do not respond to the same level.

The expression of growth factors receptors is frequently upregulated in breast cancer. One of these receptors is coded for by the oncogene HER2 or human epidermal growth factor receptor 2, also known as c-erbB2/neu, which participates in cell growth and proliferation (Brand et al., 2006). HER2 has no specific ligand but can form heterodimers with other members of the HER family, which increases their signalling potential and upon ligand binding activate a number of intracellular protein kinases leading to cell signalling. HER2 is overexpressed in 15-30% of breast cancers and is associated with poor prognosis and survival (Brand et al., 2006). HER2 amplification is associated with resistance to hormonal therapy and a decreased responsiveness to chemotherapy (De Placido et al., 1998, Shou et al., 2004, Giai et al., 1994, Stal et al., 1995, Muss et al., 1994). The growth of tumours and human breast cancer cell-lines overexpressing the HER2 receptor has been shown to be inhibited by the use of anti-HER2 monoclonal antibodies (mAbs), offering a potential target for cancer therapy.

1.7 Tumour grading

Prognostic information can be established by grading breast cancers according to their degree of differentiation. Nuclear pleomorphism, frequency of mitoses and the degree of glandular formation are scored from 1 to 3. The combine scores produce 3 grades: grade I (3-5), grade II (6-7) and grade III (8-9) which are an

important predictor of disease free survival and overall survival (Hatteville et al., 2002).

1.8 Tumour staging

The tumour node metastasis (TNM) staging system assesses the extent of local and systemic disease. It correlates with the UICC (International Union Against Cancer) system (Table 1-2). Increased sensitivity of techniques identifying metastases allow the identification of isolated tumour cells (<0.2mm), micrometastases (0.2-2mm) and metastases (>2mm). However, the clinical importance of the smaller metastases remains unclear. There is evidence that the number of patients developing axillary progression of breast cancer is far lower than expected, given the prevalence of lymph node metastases identified on histology (Zurrida et al., 2002). This suggests that the majority of tumour cells present in metastatic sites are not able to maintain tumour development and is consistent with a hypothesis that metastatic growth depends on the activation of tumourigenic stem cells. It is also consistent with a hypothesis suggesting that both the tumour and the metastatic site must have a compatible gene expression signature in order to achieve a tissue-specific metastasis (Kang et al., 2003b, Kang et al., 2003a).

1.9 Mutagenesis

Metastasis requires close collaboration between cancer cells, immune and inflammatory cells, and stromal elements. The process of metastasis consists of four major steps.

The first step is represented by epithelial-mesenchymal transition, in which cancer cells acquire fibroblastoid characteristics that increase their motility and allow them to invade epithelial and basal membranes and achieve efferent blood vessels or lymphatics (Kalluri and Weinberg, 2009). Loss of E-cadherin expression is considered as a key event in the epithelial-mesenchymal transition.

In the second step, cancer cells intravasate into blood vessels and lymphatics. The vascular permeability is increased by production of inflammatory mediators.

In the third step, metastasis-initiating cells survive and travel throughout the circulation. It has been estimated that only about 0.01% of cancer cells that enter the circulation will eventually survive and give rise to micrometastases (Joyce and Pollard, 2009). Next, integrin-mediated arrest allows the extravasation of circulating cancer cells.

Finally, single metastatic progenitors interact with immune, inflammatory, and stromal cells and start to proliferate (Polyak and Weinberg, 2009). Some of these cells may already be targeted to become premetastatic in response to tumor-generated inflammatory signals prior to the arrival of metastasis-initiating cancer cells (Kaplan et al., 2005). One of these inflammatory signals is the extracellular matrix component versican, which leads to macrophage activation and production of the metastasis-promoting cytokine TNF-α (Kim et al., 2009b).

Primary tumour (T)		
Tx		Primary tumour cannot be assessed
T0		No evidence of primary tumour
Tis		Carcinoma in-situ, or Paget's disease of nipple with no associated mass
T1		Tumour <2cm
T2		Tumour 2-5cm
T3		Tumour >5cm
T4		Tumour involving skin, chest wall or an inflammatory cancer
Regional lymph nodes (N)		
Nx		Lymph nodes cannot be assessed
N0		No regional lymph node metastases greater than 0.2mm
N1		1-3 lymph nodes metastatic
N2		4-9 lymph nodes metastatic
N3		>10 lymph nodes metastatic or supraclavicular/ infraclavicular
Metastasis (M)		
Mx		Presence of distant metastases not evaluated
M0		No distant metastases
M1		Distant metastases present
Stage		**Definition**
Stage 0		Tis, N0, M0
Stage I		T1, N0, M0
Stage II	Stage IIA	T0, N1, M0 T1, N1, M0 T2, N0, M0
	Stage IIB	T2, N1, M0 T3, N0, M0
Stage III	Stage IIIA	T0, N2, M0 T1, N2, M0 T2, N2, M0 T3, N1, MO T3, N2, M0
	Stage IIIB	T4, N0, M0 T4, N1, M0 T4, N2, M0
	Stage IIIC	Any T, N3, M0
Stage IV		Any T, any N, M1

Table 1-2: The Tumour, Lymph nodes and Metastasis system for classifying and staging breast cancer.

Adapted from Singletary at al, (2003). "Revision of breast cancer staging: the 6th edition of the TNM Classification." Semin Surg Oncol 21(1): 53-9.

1.10 Management of Breast Cancer

Management of breast cancer depends on the stage of the tumour, its size, the expression of hormone and growth factor receptors on the tumour cells and patients factors.

1.10.1 Surgery

In majority of cases surgery initiates breast cancer treatment by removing the tumour. The majority (75-83%) of tumours are small and undergo "conservative therapy" which includes lumpectomy known as "wide local excision"(Sarrazin et al., 1989). Large primary tumours can be reduced in size by primary chemotherapy prior to the surgery (Veronesi et al., 1995). Mastectomy is indicated if the tumours are too large for conservative therapy and cannot be reduced in size by primary chemotherapy. During surgery lymph nodes are removed to determine metastatic spread. Resection of lymph nodes may involve sentinel node biopsy, lymph node sampling or axillary clearance.

1.10.2 Radiotherapy

Radiotherapy is administered to the remaining breast tissue following either conservative surgery or mastectomy. If sampled lymph nodes are shown to contain cancer then radiotherapy is applied to the remaining nodes. Locally advanced tumours are also successfully treated with breast conserving surgery followed by radiotherapy if the tumours respond to induction chemotherapy (Fisher et al., 1997).

1.10.3 Chemotherapy

Chemotherapy reduces a patient's risk of recurrence and attempts to eradicate micrometastatic process. It can also be administered before surgery to reduce the size of a tumour as neoadjuvant therapy (Colleoni et al., 1998).

1.10.4 Hormone Therapy

Oestrogen (ER) and progesterone (PR) positive breast cancer are responsive to treatments targeting either the hormones or the hormone receptors on the breast

cancer cells. The ER receptor antagonist tamoxifen is recommended for 5 years following surgery in early breast cancers to prevent relapse of the cancer (Fisher et al., 1996). It has been proven that tamoxifen reduced the recurrence in 30-50% of women with ER-positive tumours (Fisher et al., 1998). Aromatase inhibitors block aromatase activity and prevent the production of oestrogen, which subsequently inhibits the development of ER-positive breast cancers. These medications are only effective in post-menopausal women since they cannot inhibit the ovaries of premenopausal women from producing oestrogen. Aromatase inhibitors can be used for treating both early and advanced breast cancer. These drugs are letrozole, anastrozole, and exemestane (Jelovac et al., 2004, Ingle et al., 2006, Steele et al., 2006). They are given to post-menopausal patients instead of tamoxifen for 5 years following surgery.

1.10.5 Molecular Therapies

Molecular therapies target a wide range of molecules such as growth factors, angiogenic factors and their receptors. The most popular is Trastuzumab (Herceptin), what is a monoclonal antibody that binds and blocks the HER2 receptor. HER2 is overexpressed in 15-30% of breast cancers and is associated with a poor prognosis. Herceptin is only effective in women with tumours overexpressing HER2.

Other targets of breast cancer cells include tyrosine kinases (Gefitinib), insulin-like growth factor and vascular endothelial growth factor (VEGF) (Bevacizumab) (Fountzilas et al., 2005b, Fountzilas et al., 2005a). Increased research into the molecular based therapies of cancer has led to extensive interest in the role of chemokines (chemo-tactic cyto-kines) and their receptors in cancer evolution and their potential as therapeutic targets.

1.11 Tumour inflammatory microenvironment

A link between inflammation and cancer was observed over 150 years ago when Rudolf Virchow noted that cancers tend to occur at sites of chronic inflammation (Balkwill and Mantovani, 2001). Inflammatory and infectious diseases are often associated with an increased risk of cancer (Coussens and Werb, 2002). In many ways, the microenvironment of tumours mimics that of tissues during an inflammatory response to injury (Coussens and Werb, 2002). However, unlike the ultimate resolution of the inflammation that occurs during healing of healthy tissue, tumours exist in a state of chronic inflammation. The tumour microenvironment is a vastly complex entity with multiple components affecting a tumour's growth, invasion and metastasis. Its components include endothelial cells, pericytes, fibroblasts, inflammatory cells, leucocytes, elements of the extracellular matrix (ECM) and network of angiogenic and angiostatic chemokines (Ali and Lazennec, 2007, Harvey, 2005, Harvey et al., 2007, Mellor et al., 2007). Since alternative environments such as bone marrow and lymph node are not naturally compatible with cells from the breast, cancer cells must shape the tumour microenvironment to become conducive to survival and growth (Sica et al., 2006, Friedl and Wolf, 2003). Pro-tumour inflammatory microenvironments support colonization of malignant cells in metastatic sites by enhancing neoplastic cell homing to target metastatic organs, extravasation out of vasculature, mobilization of bone marrow-derived progenitor cells, activation and sustained angiogenesis at secondary site, and enhanced neoplastic cell proliferation and survival in the ectopic site (Figure 1-3).

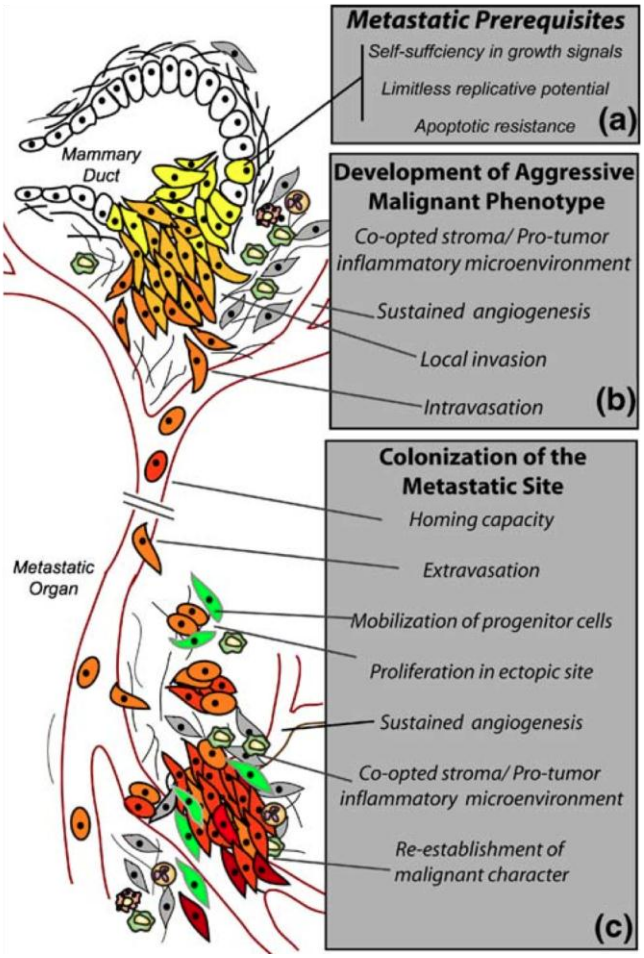


Figure 1-3: Pro-tumor inflammation potentiates metastatic progression

Pro-tumour inflammatory responses potentiate malignancy and hematogenous metastatic spread process by promoting critical steps in the metastatic process. Productive metastases require, (a) oncogenic transformation and genomic instability in initiated cells, resistance to apoptotic and cell death programs, self-sufficiency for growth signaling and limitless replicative potential. (b) Interactions between neoplastic cells and pro-tumor pro-inflammatory microenvironments facilitate malignant conversion and favors selection for malignant cells with metastatic potential by engendering development of an aggressive malignant phenotype characterized by sustained angiogenesis, local invasion and vascular intravasation by neoplastic cells. (c) Pro-tumor inflammatory microenvironments support colonization of malignant cells in metastatic sites by enhancing neoplastic cell homing to target metastatic organs, extravasation out of vasculature, mobilization of bone marrow-derived progenitor cells, activation and sustained angiogenesis at secondary site, and enhanced neoplastic cell proliferation and survival in the ectopic site.

Modified from DeNardo et al. Cancer Metastasis Rev (2008) 27:11–18

1.12 Chemokines

Chemokines are a superfamily of small secretory proteins that have many diverse functions. They apply their effects locally in a paracrine or autocrine fashion. Chemokines play a fundamental role in immune and inflammatory reactions including the trafficking of immune cells (Rollins, 1997). Chemokines have been classified in terms of their physiological features into constitutive (homeostatic e.g. CXCL12) or inducible (inflammatory e.g. CCL2, 3, 4) expression (Table 1-3).

Chemokines may be synthesised and secreted by practically all cell types. Inflammatory chemokines are usually secreted in response to stimuli such as from endogenous pro-inflammatory cytokines (e.g. TNF-α and IFN-γ (Interferon-γ) or exogenous stimuli such as mitogens and bacterial lipopolysaccharide (Palmberg et al., 1998). Chemokine cDNAs encode proteins of 92 to 130 amino acids (Garton et al., 2001) and are synthesised in precursor form with a small signal peptide sequence (Gortz et al., 2002), which is cleaved prior to secretion (Baggiolini, 2001).

1.12.1 Chemokine structure and classification

Chemokines have three-dimensional structural similarity dictated by the conserved cysteine (C) residues. Four chemokine classes are recognised based on the location of the first two cysteines in the protein sequence (CXC, CC, C and CX3C) (See Table 1-3). The majority of chemokines belong to the CXC or CC families. CXC chemokines have been further divided into two groups based on the presence of a sequence of three residues in the amino-terminal region, Glu-Leu-Arg (the so-called ELR motif). The ELR motif is an important factor in determining angiogenesis (ELR positive), or angiostasis (ELR negative) (Keane and Strieter, 1999, Strieter et al., 1995), although not an absolute determinant of angiogenic status (e.g. CXCL12 is ELR negative but is angiogenic). All ELR positive chemokines bind CXCR2 but several also bind to CXCR1, while ELR negative chemokines bind to various receptors.

New	Human ligand	Chromosome	Mouse ligand	Chromosome	Receptor(s)
CXC Chemokines					
CXCL1	GROα/ MGSAα	4q13.3	GRO//KC	5qE2	CXCR2, CXCR1
CXCL2	GROβ/MGSAβ	4q13.3	MIP-2	5qE2	CXCR2
CXCL3	GROγ	4q13.3	Dcip	5qE2	CXCR2
CXCL4	PF4	4q13.3	PF4	5qE2	CXCR3B
CXCL4V1		4q13.3			
CXCL5	ENA-78	4q13.3	LIX	5qE2	CXCR2
CXCL6	GCP-2	4q13.3			CXCR1, CXCR2
CXCL7	NAP-2	4q13.3	Ppbp	5qE2	CXCR2
CXCL8	IL-8	4q13.3			CXCR1, CXCR2
CXCL9	MIG	4q21.1	MIG	5qE3	CXCR3, CXCR3B
CXCL10	IP-10	4q21.1	IP-10	5qE3	CXCR3, CXCR3B
CXCL11	I-TAC	4q21.1	I-TAC	5qE3	CXCR3, CXCR3B, CXCR7
CXCL12	SDF-1α/β	10q11.21	SDF-1α/β	6qF1	CXCR4, CXCR7
CXCL13	BLC, BCA-1	4q21.1	BLC, BCA-1	5qE3	CXCR5
CXCL14	BRAK, Bolckine	5q31.1	BRAK, Bolckine	13qB2	Unknown
CXCL15	Unknown		Lungkine	5qE2	Unknown
CXCL16		17p13.2	CXCL16	11qB4	CXCR6
CXCL17	DMC	19q13.2	DMC	7qA3	unknown
CC Chemokines					
CCL1	I-309	17q11.2	TCA-3	11qB5	CCR8
CCL2	MCP-1/MCAF/TDCF	17q11.2	JE	11qB5	CCR2
CCL3	MIP-1α/ LD78α	17q11.2	MPI-1α	11qB5	CCR1, CCR5
CCL3L1	LD78β	17q12			
CCL3L3	LD78β	17q12			
CCL4	MIP-1β	17q12	MIP-1β	11qB5	CCR5
CCL4L1	AT744.2	17q12			
CCL4L2		17q12			
CCL5	RANTES	17q12	RANTES	11qB5	CCR1, CCR3, CCR5
CCL7	MCP-3	17q11.2	MARC	11qB5	CCR1, CCR2, CCR3
CCL8	MCP-2	17q11.2	MCP-2, MCP-5	11qB5	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin	17q11.2	Eotaxin	11qB5	CCR3
CCL13	MCP-4	17q11.2	unknown		CCR1, CCR2, CCR3
CCL14	HCC-1	17q12	unknown		CCR1
CCL15	HCC-2/ LKN1/ MIP-1γ	17q12	CCL9, MMRP2, MIP-1γ	11qB5	CCR1, CCR3
CCL16	HCC-4/ LEC/ LCC-1	17q12	pseudogene	11qB5	CCR1, CCR2, CCR5, HRH4
CCL17	TARC	16q13	TARC	8qC5	CCR4
CCL18	DC-CK 1/ PARC/ AMAC-1	17q12	pseudogene		Unknown
CCL19	MIP-3β/ ELC/exodus-3	9p13.3	MIP-13β	4qB1	CCR7
CCL20	MIP-3α/ LARC/ exodus-1	2q36.3	MIP-α / LARC	1qC5	CCR6
CCL21	SLC/6Ckine/ SLC/ exodus-2	9p13.3	CCL21a, b, C/ SLC	4qB1	CCR7
CCL22	MDC/ STCP-1	16q13	ABCD-1	8qC5	CCR4
CCL23	MPIF/ CKβ8/ CKβ8-1	17q12	CCL6/ C10	11qB5	CCR1
CCL24	Eotaxin-2/ MPIF-2	7q11.23	Eotaxin-2	5qG1	CCR3
CCL25	TECK	19p13.2	TECK	8qA1.2	CCR9
CCL26	Eotaxin-3	7q11.23	CCL26l/Eotaxin-3-like	5qG1	CCR3
CCL27	CTACK/ ILC	9p13.3	CCL27a,b/CTACK/ ILC	4qB1	CCR10
CCL28	MEC	5p12	MEC	13	CCR3, CCR10
C Chemokines					
XCL1	Lymphotactin/ATAC/SCM-1α	1q24.2	Lymphotactin	1qH2	XCR1
XCL2	SCM-1β	1q24.2			XCR1
CX_3C Chemokine					
CX_3CL1	Fractalkine	16q13	Fractalkine	8qC5	CX3CR1

Table 1-3: Chemokine and receptor nomenclature.

Adopted from Ali et al Cancer and Metastasis review 26, 3-4 (2007) 401-420

1.13 Chemokine receptors

Chemokine receptors enable the transduction of inflammatory or constitutive chemokine expression into a biological response (Hansell et al., 2006). Chemokine receptors are present on the surface of leukocytes in the peripheral blood and can bind to chemokines on the surface of endothelial cells. This enables reactivation of integrin and induces a firm adhesion of the leukocyte to the endothelial surface facilitating extravasation of the leukocyte from the blood into the surrounding tissue. When the biological response no longer exists, the rapid removal of these chemokines is vital in order to resolve inflammation and restore tissue homeostasis. The chemokine receptor is recycled in the perinuclear region to be trafficked back to the plasma membrane, or it will enter the lysosome compartment for degradation (Neel et al., 2005). Internalisation of chemokine receptors may radically reduce the membrane surface expression of the receptor and is a likely mechanism for the downregulation of chemokine receptors (Neel et al., 2005).

Chemokine receptors consist of a seven-transmembrane spanning domain composed of single polypeptide chains with three extracellular and three intracellular loops (Figure 1-4). Ten CC, seven CXC, one CX3C and one XCR receptors have been identified (Mantovani, 2004, Homey et al., 2002). An acidic N-terminal domain is localised extracellularly and is involved in ligand binding. It has a role in ligand specificity as a conserved cysteine motif within the chemokine limits it to a specific receptor subgroup. A serine/threonine-rich C-terminal domain, which is placed intracellularly and G-protein coupled, is a component of a functioning chemokine receptor. It is involved in the transduction of signalling pathways when the receptor binds to the certain chemokine. The majority of receptors can respond to more than one chemokine and most of the chemokines can activate more than one receptor (Neel et al., 2005) (Table 1-3).

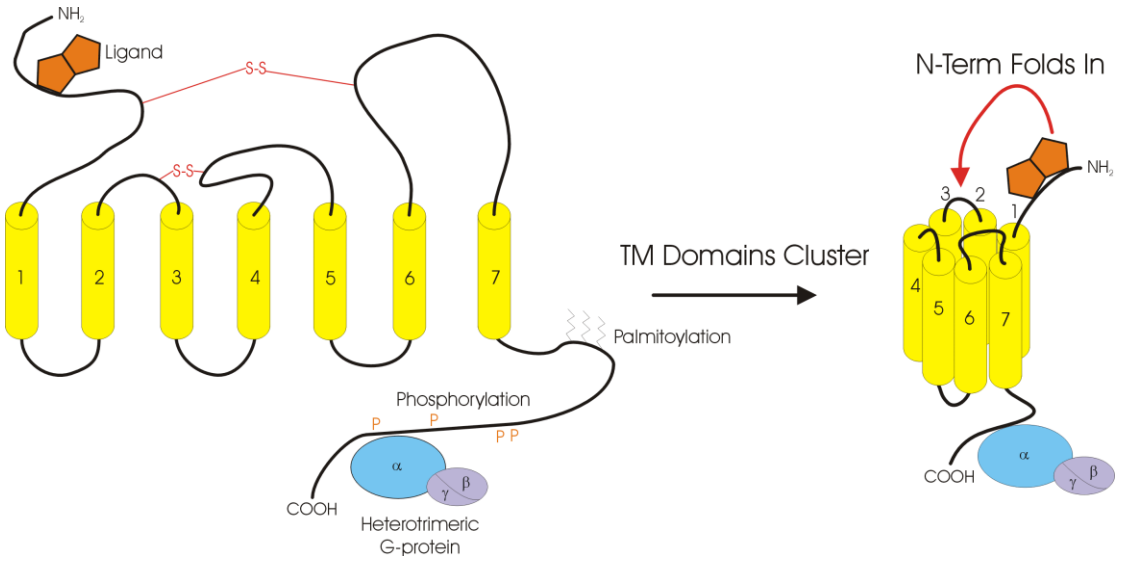


Figure 1-4: Chemokine receptor structure

The seven transmembrane domains are demonstrated along with an extracellular N-terminal region containing a generic chemokine binding pocket. The intracellular C-terminus and the approximate G-protein binding location are also shown.

1.14 CXCR4 Signalling

Upon binding of CXCL12 the CXCR4 receptor undergoes receptor dimerisation, resulting in the activation of a variety of intracellular signalling pathways and effector molecules that regulate cell survival, proliferation, chemotaxis, migration, and adhesion (Vila-Coro et al., 1999). These pathways are summarised in the Figure 1-5. The initial stages of CXCR4 receptor signalling are mediated through G-proteins. The specific downstream signalling cascades stimulated from the G-proteins are dependent on the specific chemokine.

Stimulation of CXCR4 activates the PI3K pathway that leads to activation of the protein kinase Akt (Vlahakis et al., 2002, Kayali et al., 2003). Following PI3K activation, Akt is recruited to the plasma membrane and activated through phosphorylation by phosophoinositide-dependent protein kinase (PDK)-1 and PDK2 (Sarbassov et al., 2005). The active form of Akt stimulates the transcription of genes fundamental for cell migration, proliferation and resistance apoptosis (Chinni et al., 2006, Vlahakis et al., 2002, Luo et al., 2003). NF-kB is an essential protein in the transcription of these genes and Akt activates it. NF-kB activation leads to the transcription of vascular endothelial growth faxtor (VEGF) and metaloprotein (MMP9) and increasing the expression of CXCR4 on breast cancer cells. In the breast cancer cell-line MDA-MB 231, CXCR4 expression was increased in response to activation of NF-κB, and blocking of this transcription factor genetically or pharmacologically reduces expression of CXCR4 (Helbig et al., 2003). NF-kB knockout mouse models of breast cancer demonstrated an increase in the apoptosis of breast cancer cells, linking NF-kB and hence Akt directly to the suppression of apoptosis in breast cancer (Sovak et al., 1997).

Mitogen-activated protein kinase (MAPK) pathway is another pathway activated by CXCR4. Following CXCL12 binding CXCR4 activate the kinase MEK1/2, which then phosphorylates and activates p44/42 MAPK (ERK1/2). p44/42 MAPK increases the expression of Elk-1 (Chang and Karin, 2001, Prasad et al., 2004).

The third pathway involved in CXCR4 signalling is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Following activation of CXCR4, JAK kinases activate the STAT family of transcription factors. These transcription factors then traffic to the nucleus to regulate gene expression (Vila-Coro et al., 1999).

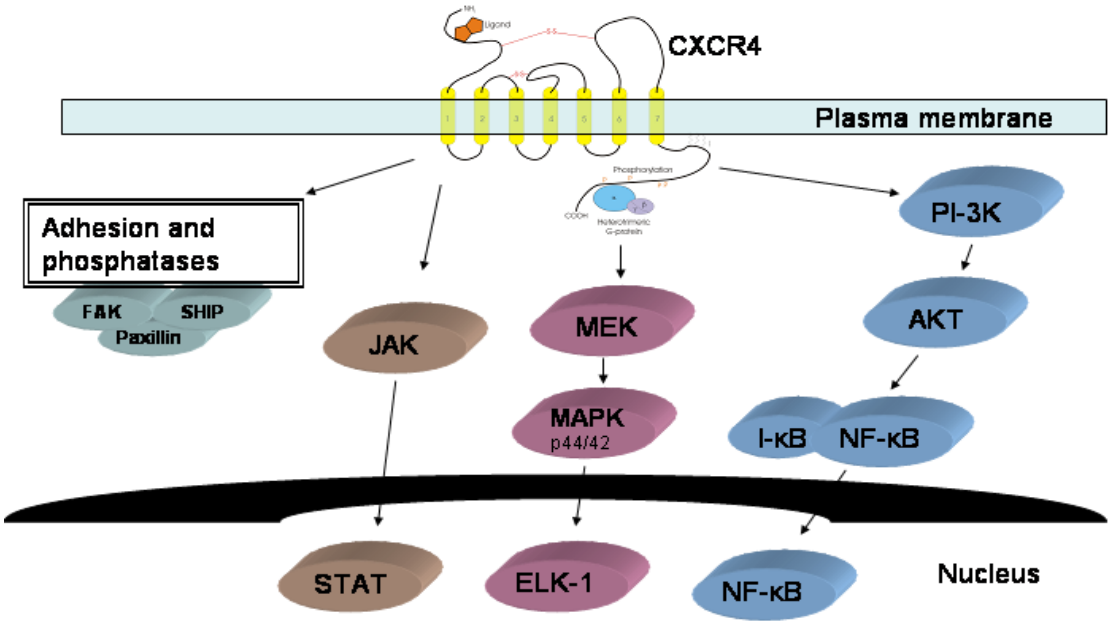


Figure 1-5: Major signal transduction pathways activated by CXCL12 stimulation of CXCR4.

SDF binding to CXCR4 leads to the activation of multiple signaling pathways, resulting in diverse biological outcomes such as migration, adhesion, and transcriptional activation. Pathways activated and outcomes elicited may differ between CXCR4+ cell types.

1.15 CXCR4 and CXCL12 interactions

Particular malignancies have a tendency to metastasise to specific distant organs, suggesting a crucial role of the organ microenvironment for the localisation and proliferation of the metastatic tumour cells (Hart and Fidler, 1980). It appears that breast cancer cells are able to metastasise to specific organs in a manner that is dependant upon the ability of the organ to mediate tumour cell adhesion, extravasation and to support subsequent viability and proliferation. In 19th century Paget described that breast cancer has a distinct metastatic pattern, with metastases commonly localising to lung, liver, lymph nodes and bone (Paget, 1889).

The pathway of tumour metastasis shares many similarities with that of leukocyte localisation and chemokines play a vital role in both of these processes (Figure 1-5). The most widely studied chemokine in breast cancer is CXCL12, and its chemokine receptor CXCR4 is expressed on primary breast tumours but not on normal breast cells (Muller A, 2001). Moreover CXCL12 is expressed at common sites of breast cancer metastasis. CXCR4 plays important roles in cancer cell adhesion and migration (Muller A, 2001). Glycosaminoglycans (GAG) bind chemokines for presentation to responsive cells (Sadir et al., 2001, Amara et al., 1999, Valenzuela-Fernandez et al., 2001, Sweeney et al., 2002, Pablos et al., 2003). CXCL12 is bound and presented by heparan sulphate on the apical surface of endothelial cells, potentially inducing immobilisation of haematogenous breast cancer cells followed by extravasation during metastasis (Harvey et al., 2007).

Work within our group explores the importance of CXCR4 in breast cancer metastasis, and demonstrate inhibition of CXCR4/CXCL12 by soluble GAG molecules. Heparinoids disrupt normal presentation of CXCL12, reducing the metastasis of CXCR4-expressing breast cancer cells (Harvey et al., 2007, Harvy, 2005, Mellor et al., 2007).

CXCL12 is expressed in bone marrow (Semerad et al., 2005), lung (Phillips et al., 2003, Muller et al., 2001), liver (Sun et al., 2005, Wald et al., 2004), adrenals (Sun et al., 2005) and the brain (Bonavia et al., 2003). CXCL12 is also produced by many different cell types within the primary tumour site, including breast cancer cells, immune cells, stromal fibroblasts and myofibroblasts (Kang et al., 2005, Lee et al., 2004, Orimo et al., 2005, Allinen et al., 2004). CXCL12 production by myofibroblasts associated with invasive breast cancer is significantly higher than that of myofibroblasts in normal breast tissue (Allinen et al., 2004). Localisation of CXCL12 to tumour myofibroblasts was confirmed by immunohistochemistry (Allinen et al., 2004). The overall significance of CXCL12 production at the site of the primary lesion is not understood. CXCL12 may stimulate tumour growth, promote survival and suppress an immune response, conversely, it may act to retain tumour cells within the tumour or stimulate an immune response (Balkwill, 2004, Dunussi-Joannopoulos et al., 2002, Luker and Luker, 2005).

CXCR4 is expressed on various cell types, including endothelial cells (Volin et al., 1998, Feil and Augustin, 1998), bronchial epithelial cells (Eddleston et al., 2002), lymphocytes (Bleul et al., 1996b, Bleul et al., 1996a), intestinal (including colonic) epithelial cells (Dwinell et al., 1999, Jordan et al., 1999), primitive haematopoietic progenitor cells (Aiuti et al., 1997), microglia, neurons and astrocytes (Bonavia et al., 2003), vascular smooth muscle cells (Schecter et al., 2003) and fibrocytes (Phillips et al., 2004). CXCR4 is also expressed in pluripotent stem cells, including mammary stem cells (Dontu et al., 2003).

Recently, several studies have demonstrated the existence of a small subset of cancer cells that share many characteristics with stem cells and named cancer stem cells (CSC). They constitute a reservoir of self-sustaining cells with the ability to maintain the tumor growth. In particular, most of them express CXCR4 receptor and respond to a chemotactic gradient of its specific ligand SDF-1, suggesting that CSC probably represent a subpopulation capable of initiating metastasis (Liu et al.,

2005). This suggests that CXCR4 expression may define the progenitors of breast cancer (Liu et al., 2005).

1.16 CXCR4 and CXCL12 in breast cancer

The role of the CXCR4-CXCL12 axis is well established in the pathogenesis of breast cancer progression (Ali et al., 2003, Ali S, 2007). CXCR4 expression in breast cancer cells is confined to a small population of cells (Kang et al., 2003b). Amongst breast cancer cell lines, MDA-MB-231 is the most commonly used CXCR4 expressing metastatic cell line (Muller et al., 2001, Lee et al., 2004, Tamamura et al., 2003, Kang et al., 2005, Matteucci et al., 2005b, Fernandis et al., 2004). Approximately 10-15% of early passage MDA-MB-231 cells demonstrate CXCR4 expression (Lee et al., 2004, Helbig et al., 2003). Immunohistochemical staining of primary breast tumours has revealed that normal breast epithelial cells do not express CXCR4, whereas, between 5 and 73% of primary breast cancers express this receptor (Muller et al., 2001, Kato et al., 2003, Cabioglu et al., 2005c) as do more than 90% of specimens with atypical ductal hyperplasia (Schmid et al., 2004). CXCR4 expression in the primary tumour has been positively correlated with the degree of lymph node metastasis, homogenous metastasis to the lungs, bone metastasis, poor patient overall survival and tumour grade (Altundag et al., 2005). High levels of CXCR4 have also been correlated with poor patient overall survival (Li et al., 2004). CXCR4 expression has been correlated with the ability of breast cancer cells to metastasise to lungs (Helbig et al., 2003). In a mouse model of breast cancer, low CXCR4 expressing parental MDA-MB-231 breast cancer cells were injected into the mammary fat pad. Primary breast tumours that grew in the mammary pad and cells that had metastasised to the lungs demonstrated very high levels of cell surface CXCR4 when compared to parental cells (Helbig et al., 2003).

The angiogenic effects of CXCR4 are also important in breast cancer. CXCR4 is expressed by endothelial cells (Heidemann et al., 2004, Molino et al., 2000) as well as breast cancer cells. CXCR4 signalling induces tube formation by endothelial cells (Chen et al., 2003, Molino et al., 2000) and is a participant in angiogenesis (Salcedo

et al., 1999, Guleng et al., 2005). Importantly, CXCR4 inhibition decreases endothelial cell migration (Salcedo et al., 1999), endothelial tube formation (Chen et al., 2003, Heidemann et al., 2004) and intratumour blood flow (Guleng et al., 2005). *In vivo*, high expression of CXCR4 by prostate cancer cells promoted tumour vascularisation, invasion and metastasis, similar effects to those occurring in CXCR4 expressing breast cancer cells (Darash-Yahana et al., 2004). The angiogenic effect of CXCR4 expressing breast cancer cells may be mediated at least in part by VEGF which has autocrine effects upon breast cancer cells (Bachelder et al., 2002). In summary, these results suggest an important role for CXCR4 in tumour angiogenesis, and growth.

CXCL12 is known to function as an important chemoattractant for the homing of multiple cell types including lymphocytes and steam cells. This suggests that CXCL12 may have a similar role upon breast cancer cells. Indeed, the chemotaxis of CXCR4 expressing breast cancer cells in response to the chemokine *in vitro* has been demonstrated by number of authors (Tamamura et al., 2003, Lee et al., 2004, Fernandis et al., 2004, Muller et al., 2001). A chemotactic response to CXCL12 can also be inhibited by anti-CXCR4 protein and RNA targeted treatments; such as anti-CXCR4 antibodies, anti-CXCR4 peptides (T140) and siRNA (small interfering RNA) (Muller et al., 2001, Tamamura et al., 2003, Lapteva et al., 2005).

CXCR4 expressing breast cancer cells grow significantly faster than CXCR4 negative cells *in vitro,* (Lapteva et al., 2005) and *in vivo*, subcutaneous implants of cells overexpressing CXCR4 demonstrated significantly faster tumour growth than cells expressing basal levels of the receptor.

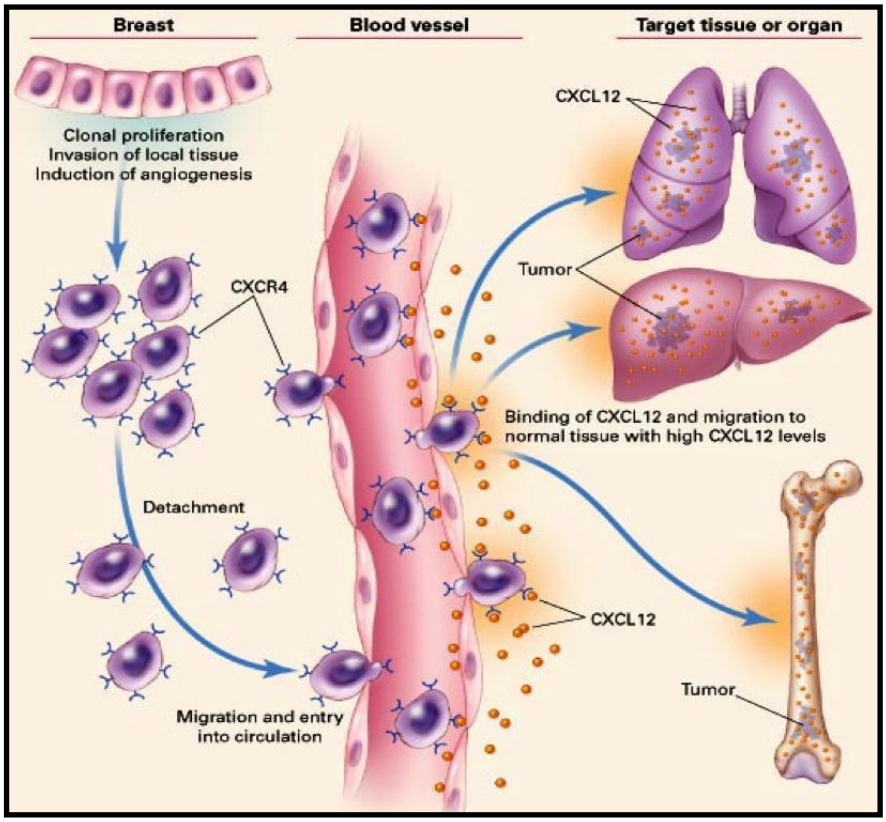


Figure 1-6: The role of CXCR4/CXCL12 interaction in breast cancer metastasis formation.

Chemokine CXCL12 has an effect upon the site-specific metastasis of breast cancer cells. Two receptors for CXCL12, CXCR4 and CXCR7, are found to be upregulated in primary breast cancers compared with normal breast tissue. CXCL12 ligand for this receptor exhibited peak levels of expression in the organs that represent the common metastatic sites for breast cancer (lung, liver, bone, brain).

Adapted from Murphy, P. M. N Engl J Med 2001; 345:833-835

1.17 CXCR4 and CXCL12 targeted therapies in breast cancer

CXCL12/CXCR4 interactions in breast cancer are targeted by anti-CXCR4 therapies. Due to the fact that CXCR4 is a major co-receptor for HIV infection, compounds that target CXCR4 to prevent infection have been developed (Hatse et al., 2005).

1.17.1 AMD3100

AMD3100 is a CXCR4 receptor antagonist which underwent Phase I and II trials in HIV, but development was terminated due to its side-effect profile and its inability to reach efficacy (Onuffer and Horuk, 2002).

1.17.2 siRNA

Small interfering RNA (siRNA) RNA is more effective than AMD3100 in preventing haematogenous breast cancer metastasis in mice (Smith et al., 2004). It is used in murine models of breast cancer as monoclonal antibody therapies (Muller et al., 2001). Silencing of CXCR4 expression using siRNA has been a successful method of inhibiting both breast cancer growth and metastasis in multiple studies (Smith et al., 2004, Lapteva et al., 2005, Liang et al., 2004, Li et al., 2004). In mice inoculated with CXCR4 expressing breast cancer cells into the mammary fat pad, RNA interference against CXCR4 prevented all growth of the primary tumours and consequently no metastases were found (Lapteva et al., 2005).

1.17.3 T140

T140 is a 14-residue peptide with high CXCR4 antagonistic activity, developed as an anti-HIV therapy. The T140 analogue 4F-benzoyl-TN14003 was shown to significantly inhibit the area of lung metastasis in a haematogenous murine model of breast cancer metastasis.

1.17.4 Heparin

Tinzaparin treatment in SCID mice, at clinically relevant dosages, was found to prevent both the number and area of metastases following intravenous injection of MDA-MB-231 cells. It was demonstrated that heparinoids inhibit the interaction

between CXCL12 and CXCR4 and may be useful as agents to prevent breast cancer metastasis (Mellor et al., 2007, Harvey et al., 2007, Abd Hamid et al., 2008).

1.18 Regulation of CXCR4 expression

In order to understand the role of CXCR4 in cancerogenesis, a fundamental understanding of the factors regulating its expression is critical. CXCR4 expression on breast cancer cells is regulated by both autocrine and paracrine factors including NF-kappa B (Helbig et al., 2003), HER2/ERBB2 (Li et al., 2004), hypoxia (Schioppa et al., 2003) and nitric oxide (Yasuoka et al., 2008), vascular endothelial growth factor (Bachelder et al., 2002), Neuropilin-2 (Nrp2) receptor (Yasuoka et al., 2009) induce functional CXCR4 expression. Thus, inhibitors of above factors should reduce breast cancer metastasis by reducing the expression of a number of prometastatic genes including CXCR4.

High expression of CXCR4 was also linked with the downregulation of six MHC class II genes, proposing a mechanism by which CXCR4 aids in the evasion of detection of cancer cells by the immune system (Sheridan et al., 2006).

1.18.1 HER2

The activation of the human epithelial growth factor receptor HER2 (erbB2, neu) has been implicated in tumour progression. HER2 is a potent oncoprotein that is overexpressed in approximately 30% of primary breast cancers and is associated with a poor prognosis and with widespread metastasis (Yu and Hung, 2000). There is evidence of a link between HER2 and CXCR4 in HER2-mediated metastasis (Li et al., 2004). Overexpression of HER2 in breast cancer cells led to an increase in expression of CXCR4, whilst inhibition of HER2 led to a corresponding reduction in CXCR4 expression. Ubiquitination of CXCR4 is a modification regulating the expression of CXCR4 post-translationally. It has been found that breast cancer cells that are HER2/neu positive have increased expression of CXCR4 as a result of inhibition of receptor ubiquitination (Yu and Hung, 2000). HER2 may therefore maintain CXCR4 expression on breast cancer cells even in the presence of the

CXCL12 ligand, thus potentially increasing their metastatic and growth potential. Immunohistochemical analysis revealed that approximately two thirds of HER2 expressing primary breast cancers also express CXCR4 (Li et al., 2004). The link between HER2 and CXCR4, both of which play critical roles in cancer metastasis furthers our understanding of the mechanisms by which CXCR4 expressing cells home to their metastatic organs.

CXCR4 and HER2 are not co-localised on the breast cancer cell surface (Cabioglu et al., 2005b). Stimulation of CXCR4 and HER2 expressing cells with CXCL12, induced HER2 phosphorylation and phosphorylation of downstream Src kinases. This effect was inhibited with the CXCR4 antagonist AMD3100 (Cabioglu et al., 2005b).

1.18.2 NF-kappa B

Extracellular signal-activated transcription factor NF-kappaB up-regulates the expression of matrix metalloproteinases, urokinase-type plasminogen activator, and cytokines in highly metastatic breast cancer cell lines (Helbig et al., 2003). NF-kappaB regulates the motility of breast cancer cells by directly up-regulating the expression of CXCR4. Overexpression of the inhibitor of kappaB (IkappaB) in breast cancer cells resulted in reduced expression of CXCR4 and a corresponding loss of SDF-1alpha-mediated migration *in vitro* (Helbig et al., 2003).

1.18.3 Hypoxia

CXCR4 expression is increased on MCF-7 and MDA-MB 231 breast cancer cells in hypoxic conditions, which are characteristic of the tumour microenvironment (Matteucci et al., 2005a). Hypoxia induces the activation of hypoxia inducible factor 1 (HIF-1) which in turn promotes expression of a number of target genes including CXCR4 (Staller et al., 2003). In addition, CXCR4 expression was increased in MCF-7 breast cancer cells following stimulation with hepatocyte growth factor (HGF) (Matteucci et al., 2005a). In both hypoxia and stimulation with HGF there were significant increases in NF-κB activation. Furthermore, studies on tumour suppressor von Hipple Lindau (VHL) reviled that inactivating mutations of VHL, which normally targets HIF-1 for degradation, account for the increased CXCR4

expression in renal cell carcinomas (Zagzag et al., 2005, Schioppa et al., 2003, Staller et al., 2003)

1.18.4 Nitric oxide

Nitric oxide can stimulate cytoplasmic CXCR4 expression *in vitro*. Cytoplasmic CXCR4 expression has been described as a significant prognostic factor for long-term survival in breast cancer (Yasuoka et al., 2008).

1.18.5 Vascular endothelial growth factor

VEGF regulates expression of the chemokine receptor CXCR4, what is essential for invasion but not for cell survival. CXCR4 mediates migration of breast carcinoma cells toward stromal-derived factor-1, and this migration is dependent on autocrine VEGF. VEGF autocrine pathway induces chemokine receptor expression in breast carcinoma cells, thus promoting their directed migration toward specific chemokines (Bachelder et al., 2002).

1.18.6 Neuropilin-2 receptor

Neuropilin-2 (Nrp2) is a receptor for VEGF-C, a lymphangiogenic factor, important in lymph node metastasis of various human cancers, including breast cancer. The evidence suggests that Nrp2 can regulate expression of cytoplasmic CXCR4 *in vitro.* Nrp2 was shown to play a role in cancer by promoting tumor cell metastasis and its expression was correlated with lymph node metastasis, VEGF-C expression, and cytoplasmic CXCR4 expression. Nrp2 expression was suggested to serve as a significant prognostic factor for long-term survival in breast cancer (Yasuoka et al., 2009).

1.18.7 FOXP3 transcription factor

FOXP3 in T cells is known to induce or repress a number of genes, including CXCR4 (Marson A, 2007, Zheng Y, 2007). It binds to the gene region upstream of the transcriptional start site of CXCR4 and CCR7 two chemokine receptors reported to play a vital role in cancer invasion and metastasis (Marson A, 2007). The expression of CXCR4 in breast cancer is linked to the expression of HER-2 (Merlo

A, 2009) and it has been proven that FOXP3 in an important regulator of this oncogene (Zuo et al., 2007c). Hence, it is also possible that FOXP3 can regulate CXCR4 expression indirectly through its action on HER-2. Foxc transcription factors are important regulators of the chemotactic motility of endothelial cells through the induction of CXCR4 expression (Hayashia H, 2008). Some members of forkhead family of transcription factors (Foxc1 and Foxc2) are able to directly induce CXCR4 expression by activating its promoter in endothelial cells. Furthermore, Foxc1-deficient endothelial cells show a significant reduction in CXCR4 expression as well as CXCL12-stimulated migration (Hayashia H, 2008).

1.19 FOXP3

The X-linked gene FOXP3 is a member of the forkhead-box/winged-helix transcription factor family. The forkhead gene product was initially identified in the fruit fly *Drosophila melanogaster* as factor required for the terminal pattern formation in the terminal regions of the embryo (Weigel and Jackle, 1989). The gene was identified during position cloning of *Scurfin*, a gene responsible for X-linked autoimmune diseases in mice and humans (Brunkow et al., 2001, Bennett et al., 2001a, Bennett et al., 2001b, Chatila et al., 2000, Wildin et al., 2001).

Since description of the first forkhead transcription factor in *Drosophila* over 10 years ago, more than 100 different family members have been identified in organisms ranging from yeast to humans. These proteins have in common a 100 amino acid "winged-helix" DNA binding domain, the forkhead (FKH) domain, with a folded motif made up of three α- helices and two loops, or "wings." Outside this conserved domain, forkhead proteins are diverse in sequence, structure, and function. The convention for naming human FKH proteins has been established as capital letters (eg. FOXP3), mouse proteins with only the first letter capitalized (Foxp3), and with the subclass denoted with a capital letter for all other chordates (FoxP3). To refer to the gene rather than the protein, the convention for the above list is FOXP3, foxp3, FoxP3 (Kim, 2007).

FKH proteins are transcriptional activators or repressors, with a broad range of cellular functions including embryonic development, speech and language development, and regulation of the immune system (Coffer and Burgering, 2004).

1.20 FOXP3 gene

The FOXP3 gene is well conserved in mammals (Ziegler and Buckner, 2006, Ziegler, 2006). The human FOXP3 genes contain 11 coding exons. The FOXP3 gene maps to the p arm of the X chromosome (specifically, Xp11.23) (Bennett et al., 2001a, Brunkow et al., 2001) (Figure 1-7).

1.20.1 FOXP3 gene mutation

In mice, a Foxp3 frameshift mutation in the forkhead domain results in lethality in hemizygous males 16 to 25 days after birth (Brunkow et al., 2001). The mutation in some human IPEX patients (Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-linked) is analogous as they cause frameshift and early termination of translation (Brunkow et al., 2001, Chatila et al., 2000, Bennett et al., 2001b, Bennett et al., 2001a, Wildin et al., 2001). In breast cancer patients, a total of 27 somatic mutations in all 11 coding exons and intron-exon boundary regions have been identified in 36% of 65 patients by PCR (Zuo et al., 2007d, Zuo et al., 2007b) (Figure 1-7 B). In these mutations, there are 18 nonsynonymous mutations, 3 synonymous mutations and 6 mutations in the intron-exon junction 12 (Zuo et al., 2007b). Interestingly, the mutations are not randomly distributed in FOXP3 gene and the overwhelming majority of them are either in the functional domains or within intron 11 which can affect the forkhead domain sequence (Zuo et al., 2007b). In prostate, the FOXP3 gene plays a critical role in suppressing cancerous transformation of this gland (Wang L, 2009). Four somatic FOXP3 mutations were identified in prostate cancer and three of them prevent nuclear localization of FOXP3 which is a major mechanism to inactivate FOXP3 tumour suppressor function (Wang L, 2009, Wang L, 2009) (Figure 1-7 B). The exact role of the somatic FOXP3 mutations in breast cancer remains unknown. The question whether, like in prostate cancer, it is responsible for failure of nuclear localization of FOXP3 protein, is yet to be answered.

1.21 FOXP3 protein

FOXP3 gene encodes FOXP3 protein, which is 431-amino acid long, and its molecular weight is 47 kDa. It contains four potential functional domains including the repressor, zinc finger, the leucine zipper and fork-head (FKH) domains (Figure 1-7 B). The FKH domain is critical for both DNA binding and nuclear localization (Ziegler, 2006). The FKH domain is the most frequent target in

immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients.

The FKH domain of FOXP3 is capable of physically interacting with DNA (Ziegler and Buckner, 2006). FOXP3 interacts with DNA at consensus sequences found within the promoters of several genes (Schubert et al., 2001). Reporter and chromatin immunoprecipitation (ChIP) assays indicated that FOXP3 binds such sequences in the promoters of IL-2, CD25, CTLA-4 and CD127 genes (Wu et al., 2006). The FKH domain also contains a nuclear localization sequence. A mutation in the forkhead domain, which affects the nuclear localization of FOXP3, leads to severe autoimmunity (Zuo et al., 2007d).

Leucine zipper structures are involved in protein-protein interactions, often leading to homo- and hetero-oligomerization. Many FKH proteins have been found to homo- or hetero-oligomerize, including FOXP3. It has been reported that FOXP3 self-associates and can complex with FOXP1, and that the ability to form these interactions is elemental for normal protein function (Li et al., 2007b). IPEX patients with deletions in this region (Δ250, Δ251) suffer from severe disease due to the inability of FOXP3 to self-associate, and/or associate with other forkhead family members such as FOXP1 (Li et al., 2007b).

1.21.1 Isoforms of FOXP3 protein

In contrast with mouse Treg, in which Foxp3 is only expressed as a full-length protein, human Treg express at least two alternatively spliced isoforms of FOXP3 in approximately equal amounts: a full-length isoform (FOXP3FL, apparent molecular weight: 58 kDa) an alternative-splicing product lacking exon 3 (FOXP3D3, apparent molecular weight: 54kDa) (Allan et al., 2005). It should be emphasized that several reports refer to exon 3 as exon 2, because exon 1 is often not considered due to its position within the 5' untranslated region (Ebert et al., 2008). FOXP3 exon 3 (aa 72–106) encodes a domain that inhibits retinoic acid receptor-related orphan receptors (ROR)a, which leads to the expression of genes that characterize Th17 cells. Consequently, the full-length isoform but not

FOXP3D3 negatively regulates Th17 differentiation in T cells (Du et al., 2008). Lately, a third splice variant has been described that in addition to exon 2 also misses exon 7 (FOXP3D2D7). Exon 7 encodes for a leucine zipper motif commonly used as structural dimerization element. Mutations in exon 7 have been linked to IPEX, a severe autoimmune disease suggested to be caused by impaired dimerization of the FOXP3 protein. FOXP3D2D7 could play a role in regulating the function of the other FOXP3 isoforms and may be involved in cancer pathogenesis (Mailer et al., 2009, Kaur et al.) (Figure 1-7 C).

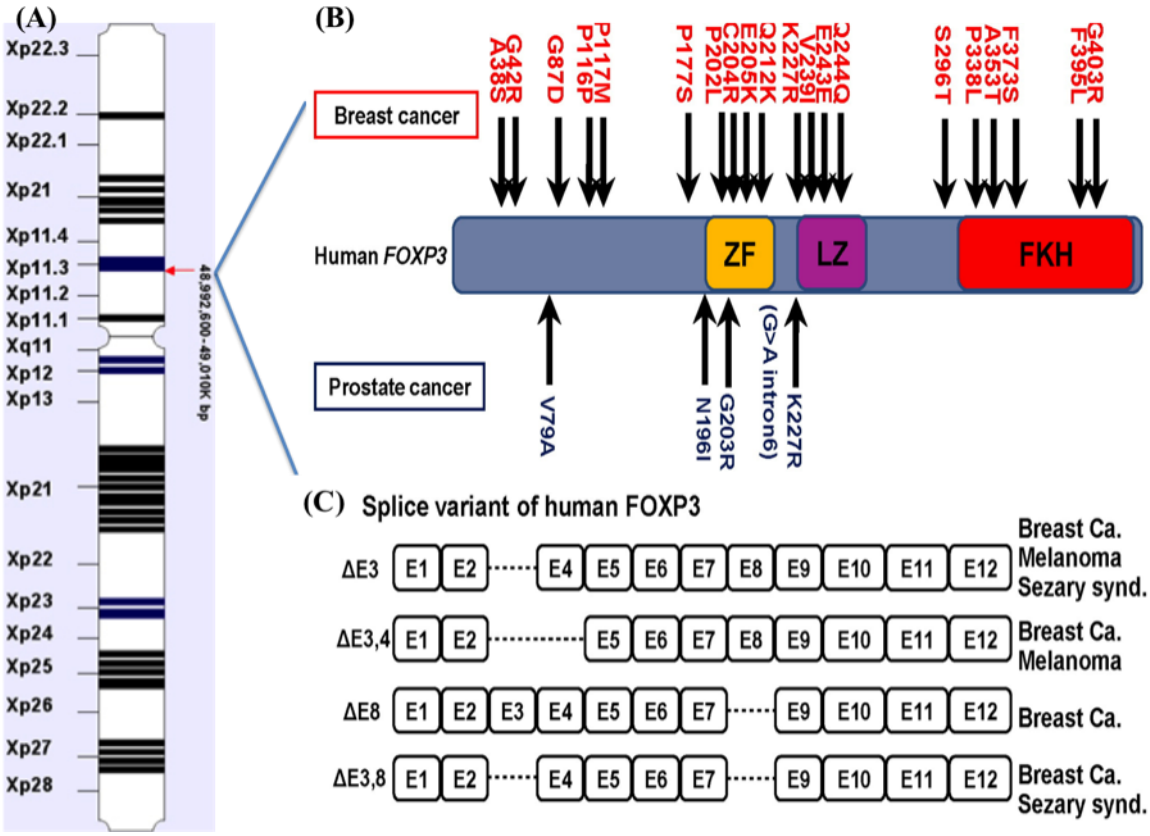


Figure 1-7: FOXP3 gene and protein

A) The location and orientation of FOXP3 gene on X chromosome; location: Xp11.23

B) Schematic diagram of the FOXP3 gene and protein. ZF: zinc finger domain, LZ: leucine zipper domain, and FKH fork-head domain, showing FOXP3 and its somatic mutations found among human breast and prostate cancers.

C) Splice variants of the FOXP3 that are predominantly expressed in human cancers. "…" represents any exons that is/are deleted in the variant forms of FOXP3. Breast Ca: Breast cancer; Sezary synd.: Sezary syndrome.

(Modified from: http://atlasgeneticsoncology.org)

1.22 Intracellular trafficking of FOXP3

Intracellular trafficking of FOXP3 to the nucleus is required in order for this protein to function and maintain cellular homeostasis. FOXP3 is synthesised outside of the cellular nuclei but functions by interacting with DNA in the nuclei and when upregulated is rapidly translocated to the nuclei to bind chromatin. There are three distinct FOXP3 domains that contribute to its nuclear transport (Hancock W, 2009). The first domain (Domain 1) comprises the C-terminal 12 amino acids. The second domain (Domain 2) is located immediately N-terminal to the forkhead domain (FHD), recently reported to be a binding site for the runt-related transcription factor 1/acute myeloid leukaemia 1 (Runx1/AML1). The third domain (Domain 3) is located within the N-terminal first 51 amino acids. FOXP3 mutant protein missing domains 1, 2, and 3 is found almost exclusively in the cytoplasm with only trace levels in the nuclei (Hancock W, 2009). Identification of factors which interact with these domains will not only facilitate our understanding of how FOXP3 achieves its nuclear transport to regulate the expression of target gens, but may lead to therapeutic modulation of these systems and provide a novel approach to cancer treatment. Indeed, it has been demonstrated that the disruption of nuclear localization is sufficient to abrogate growth inhibition by FOXP3 in prostate cancer (Wang L, 2009).

1.23 FOXP3 expression

FOXP3 is expressed at high levels in nuclei of Tregs and lymphoid cells (thymus, spleen and lymph nodes) (Brunkow et al., 2001, Hori and Sakaguchi, 2004, Hori S, 2003). Some epithelial cells express this protein at lower levels in the nuclei and cytoplasm (Chen G, 2008). In human cells, FOXP3 expression was detected in breast epithelial cells, lung respiratory epithelial cells, prostate epithelial cells and liver (Li et al.), although not in the heart, and intestine (Chen G, 2008), whereas in mice Foxp3 is expressed on epithelial cells of multiple organs (Chen G, 2008).

There are opposing reports in the literature regarding the expression of FOXP3 in breast cancer stating that it is increased (Merlo A, 2009, Gupta et al., 2007, Ohara et al., 2009), decreased (Zuo et al., 2007d, Zuo et al., 2007b) or not changed compared to the benign epithelium (Zuo et al., 2007d, Zuo et al., 2007b). It has been suggested that a significant loss of FOXP3 expression by breast cancer epithelium is associated with cancer development (Zuo et al., 2007b, Zuo et al., 2007d) and FOXP3 was named the first X-linked breast cancer suppressor gene. Paradoxically, another group has demonstrated increased FOXP3 expression level in breast cancer (Andrea Merlo and Elda Tagliabue, 2009). Using immunohistochemical staining, FOXP3 expression was assessed in 397 primary breast carcinoma specimens from two clinical trials (Milan 1 and 3). FOXP3 stained positive in the majority of the breast cancer tissues and both cytoplasmic and nuclear staining was observed (Andrea Merlo and Elda Tagliabue, 2009). Surprisingly, most recently the results of the large screening study of FOXP3 expression in breast tissue, examining 2200 cores, has reported that, in healthy and cancerous human breast tissue, FOXP3 protein expression is negligible (Wolf D, 2010). Expression of FOXP3 on cancer cells is correlated with the expression levels of IL-10 and TGF- β1 (Karanikas et al., 2008).

The nuclear expression of FOXP3 in human benign breast, respiratory and prostate tissue is well documented (Chen G, 2008). However, immunocytochemical staining of tumour cell lines revealed predominant cytoplasmic expression of breast, colon, pancreas, lung cancer cell lines (Karanikas et al., 2008). There is disagreement amongst the authors with regards to the immunohistochemistry of malignant breast epithelium: some describe nuclear staining only (Zuo et al., 2007b, Zuo et al., 2007d), others state that expression of FOXP3 in breast cancer is predominantly cytoplasmic (Andrea Merlo and Elda Tagliabue, 2009). Immunohistochemistry of other human malignancies detected nuclear FOXP3 staining in prostate cancers (Wang L, 209), mixed cytoplasmic and nuclear FOXP3 staining in the pancreatic cancers (Hinz et al., 2007) and hapatocellular cancer

(Wang et al.) and solely cytoplasmic staining in ovarian cancers (Zhang and Sun, 2010). Heterogeneous subcellular FOXP3 localization in cancer cells may reflect the presence of abnormal proportion of splice variants, somatic mutations or different post-translationally modified forms.

1.24 The function of FOXP3

FOXP3 is a gene involved in immune system responses. It functions as the master regulator in the development and function of regulatory T (Treg) cells (Fontenot et al., 2003, Hori and Sakaguchi, 2004, Khattri et al., 2001). Tregs were initially defined as immunosuppressive CD4 positive T cells expressing constitutively the subunit of the interleukin (IL)-2 receptor (CD25) on their surface (Sakaguchi, 2005, Setoguchi et al., 2005). The nuclear expression of FOXP3 is now considered as the most specific marker for these cells, which is essential for development of immune tolerance (Sakaguchi, 2005, Setoguchi et al., 2005). However, FOXP3 can be expressed transiently on human non-regulatory CD4 positive T cells upon T-cell antigen receptor (TCR) activation. Additionally, Foxp3 is also expressed in non-lymphocytic normal or cancer cells, suggesting that FOXP3 may have a broader role in cancer than initially thought.

The up-regulation of Foxp3 expression on T cells has, in animal studies, led to marked reductions in autoimmune disease severity in models of diabetes, multiple sclerosis, asthma, inflammatory bowel disease, thyroiditis and renal disease (Xia et al., 2007). Alterations in numbers of Tregs expressing FOXP3 are found in various pathological conditions. For example, patients with tumours have a local relative excess of FOXP3 positive Tregs, which inhibits the body's self defence(Mizukami et al., 2008, Paul Salama, 2008). On the other hand, patients with an autoimmune disease such as systemic lupus erythematosus (SLE) have a relative dysfunction of FOXP3 positive cells (Li et al., 2009).

1.25 FOXP3 signalling in epithelial cells

Various cascades and molecules are involved in FOXP3 signalling in epithelial cells as illustrated in Figure 1-8.

1.25.1 HER2/neu (ERBB2)

HER2 is a member of the transmembrane receptor tyrosine kinases and is involved in the regulations of various cellular functions like cell growth and survival (Blackwell et al., 2009, Hardee et al., 2009). FOXP3 can repress the transcription of HER2 in human breast cancers by binding directly to ERBB2 gene promoter (Zuo et al., 2007c). This repression of HER2 may be decisive for the tumour suppressor function of FOXP3 in the breast epithelial cells.

1.25.2 C-MYC

Overexpression of c-MYC contributes to more aggressive and poorly differentiated cancer phenotypes (Pelengaris et al., 2002a, Pelengaris et al., 2002b). c-MYC is a transcription factor which is vital for cell cycle and apoptosis. c-MYC directly activates CDK4 and CCND2 expression, while indirectly represses CDK inhibitors such as CDKN1A (p21) and CDKN2B (p15) expression (Pelengaris et al., 2002a, Pelengaris et al., 2002b). Furthermore, c-MYC directly up-regulates eIF4E and eIF2, the rate-limiting effectors of cell cycle (Pelengaris et al., 2002a, Pelengaris et al., 2002b). In prostatic epithelial cells, FOXP3 directly represses c-MYC gene expression (Wang L, 2009).

1.25.3 p21 (CDKN1A, CIP1, WAF1)

p21 is a universal CDK inhibitor and plays an important role in regulating cell cycle progression, specifically at the G1-checkpoint (Park and Koff, 2001). It is often implicated in multiple malignancies including breast cancer (Park and Koff, 2001). Cancer cells with low levels of p21 can escape from G1 arrest, and thus cells acquire a growth advantage during tumour development. FOXP3 can activate the p21 promoter on breast epithelial cells (Liu et al., 2009). There is strong evidence that the activation of p21 contributes to FOXP3's tumour suppressor function (Liu et al., 2009).

1.25.4 SKP2

High levels of expression of SKP2 have been reported in a wide variety of cancers (Fotovati et al., 2006, Nishitani et al., 2006). SKP2 is imperative in the ubiquitin

dependent degradation of p27KIP1, a CDK inhibitor especially of Cyclin-E/CDK2 and Cyclin-A/CDK2 (Fotovati et al., 2006, Nishitani et al., 2006). SKP2 is expressed during S and G2 phases of cell cycle and regulates p27 degradation. Therefore SKP2 facilitates progression of the cell cycle. Overexpression of SKP2 is frequently observed in human cancers and is correlated with poor prognosis of breast cancers (Fotovati et al., 2006, Nishitani et al., 2006). We found that FOXP3 directly represses SKP2 expression in human and mouse mammary epithelial cells (Zuo et al., 2007b).

1.25.5 LATS2

Defective expression of LATS2, a negative regulator of YAP onco-protein, has been reported in cancer of prostate, breast, liver, brain and blood origins (Li et al.). However, no transcriptional regulators for the LATS2 gene have been identified. Spontaneous mutation of the transcription factor FOXP3 reduces expression of the LATS2 gene in mammary epithelial cells. ShRNA-mediated silencing of FOXP3 in normal or malignant mammary epithelial cells of mouse and human origin repressed LATS2 expression and increased YAP protein levels. LATS2 induction required binding of FOXP3 to a specific sequence in the LATS2 promoter, and this interaction contributed to FOXP3-mediated growth inhibition of tumour cells (Li et al.).

1.26 Regulation of gene expression by FOXP3

In Tregs, there are pathways activating Foxp3 gene expression. TGF-β, Smad, IL-2 and IFN-γ signals are known to set off Foxp3 expression in the development of Tregs (Lal et al., 2009). SMAD, NFAT and Rel family molecules can occupy Foxp3 enhancer and/or promoter loci and activate Foxp3 transcription (Ruan et al., 2009). In mammary epithelial cells, ATF-2 is crucial for FOXP3 expression (Liu et al., 2009). In cancer cells, doxorubicine, which induces activation of p53 via DNA damage response pathway, significantly up-regulates FOXP3 expression (Jung et al.).

FOXP3 forms complexes with the Rel family transcription factors, NFAT and NFkappaB, and FOXP3 blocks their ability to activate IL-2 and INF-γ signal transduction (Bettelli et al., 2005, Holmes et al., 2008). By making a repressive FOXP3: NFAT complex, FOXP3 inhibits NFAT: AP-1 complex at the IL-2 promoter (Holmes et al., 2008). AML1/RUNX1, which activates endogenous IL-2 and IFN-γ expression in CD4+ T cells, is reported to make a complex with FOXP3 (Ono et al., 2007). AML1/RUNX1 could bind to the IL-2 enhancer with FOXP3 and exert optimal repression of the IL-2 in Tregs (Ono et al., 2007).

FOXP3 is able to radically change histone modifications at its binding loci (Zheng and Rudensky, 2007) and function as a molecule which recruits other co-factors to the FOXP3-bound loci. The histone modifying enzymes such as TIP60, HDACs 7 and 9, and EOS/CtBP1 have been found to be physically associated with FOXP3 to form a repressor complex (Li and Greene, 2007, Li et al., 2007a, Li et al., 2007b).

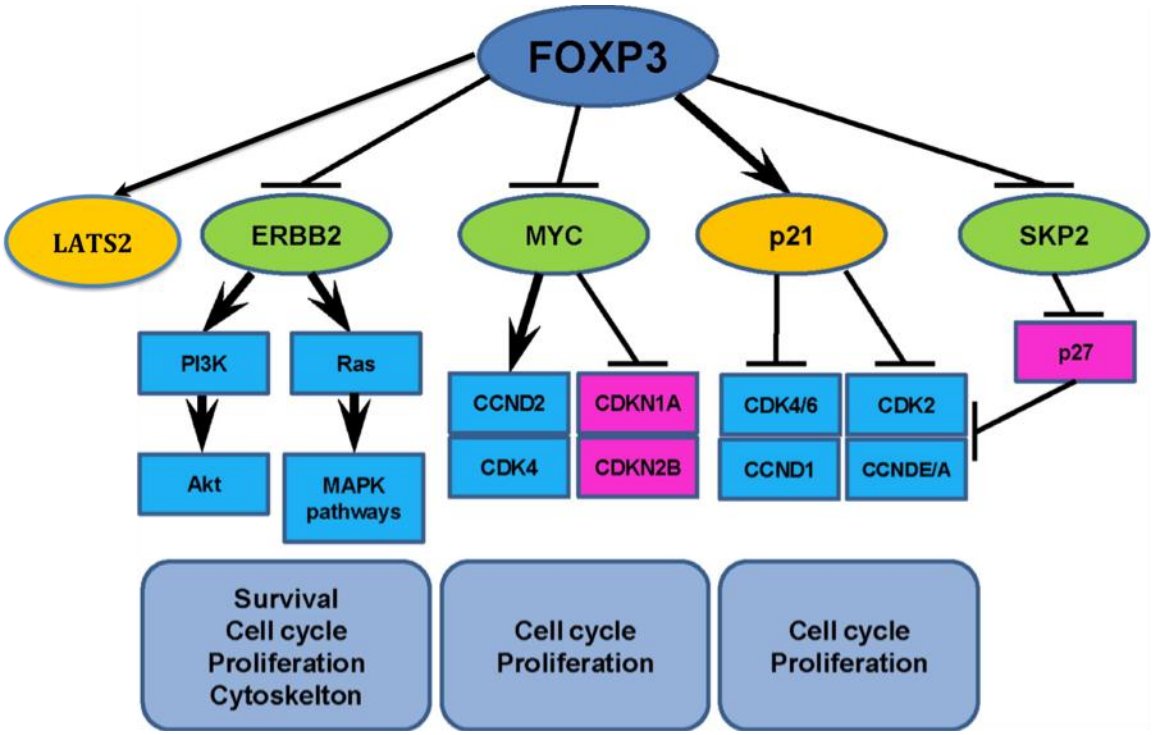


Figure 1-8: A schematic view of the signaling networks of the FOXP3 in epithelial cells

FOXP3 suppresses breast cancer and prostate cancer by inducing tumour suppressor genes while repressing oncogenes. The genes are direct targets for FOXP3 and their regulation is essential for growth inhibition by FOXP3.

1.27 Role of FOXP3 in cancerogenesis

1.27.1 Inactivation of FOXP3 gene in cancer

The identification of FOXP3 as an X-linked tumour suppressor gene has developed better understanding of the mechanism of a single genetic hit and challenge the traditional theory of "two-hit inactivation" in tumour suppressor genes. Nevertheless, the silencing mechanism of this gene in cancer cells is still not fully understood. Since females have two X chromosomes and males have only one, the inactivation mechanism of this gene in human cancers potentially ought to be different between females and males.

X-chromosome inactivation silences gene expression from one of the two X chromosomes in females. Random X-chromosome inactivation occurs during embryogenesis. The inactivated X chromosome will remain idle throughout the lifetime of the cell. Therefore, in females an X-linked tumour suppressor gene is more susceptible to additional genetic damage since one of the alleles is silenced by X-chromosome inactivation. The suggestion of heterozygosity in cancer pathogenesis has been demonstrated with the identification of two X-linked tumor suppressor genes, FOXP3 (Zuo et al., 2007c) and WTX (Han et al., 2007, Rivera et al., 2007) that are inactivated by a single genetic hit. Female mice with a FoxP3-heterozygous mutation develop spontaneous breast cancer at a higher rate than wild type mice (Zuo et al., 2007d). The majority of the mutations and all deletions of FOXP3 are heterozygous in human breast cancers (Zuo et al., 2007d).

As men have just one X chromosome, the recessive X-linked inherited diseases are more frequently found in males than in females. Literature suggests the deletion of the FOXP3 gene in 23 (14%) of 165 prostate cancer samples (Wang L, 2009). Among the 23 cases, 5 showed an increase in the number of X chromosomes and the deletion of FOXP3 was complete in these chromosomes what may be suggestive that X chromosome duplications in cancer tissues likely occurred after the deletion of FOXP3. Although FOXP3 is frequently inactivated in prostate cancer

by deletion and somatic mutation, approximately 70% of prostate cancers without nuclear FOXP3 expression are not fully explained by these identified somatic alterations (Wang L, 2009). Inactivation of FOXP3 in prostate cancer may also be caused by other additional events such as DNA methylation, histone hypoacetylation, upstream gene regulation, etc.

1.27.2 FOXP3 in the cancer inflammatory infiltrate

T cells can exert both tumour-suppressive and tumour-promoting effects, as determined by their effector functions (DeNardo et al., 2009, Langowski et al., 2007).

Cancers are able to escape CD8 positive T-cell cytotoxicity by promoting expansion of Treg cells. Treg cells normally function to protect tissue from autoimmune disease by suppressing self-reactive cells. Characterized by expression of CD4, CD25 and FOXP3, Treg cells can account for about 5–10% of all T lymphocytes in healthy tissues. In breast cancers the percentage of Treg cells, which are assessed by FOXP3 positivity, increases in parallel with the disease stage, from normal to DCIS and from DCIS to invasive carcinoma (Bates et al., 2006). In patients with invasive carcinoma the presence of high numbers of FOXP3 positive T cells predicts worse relapse-free survival and decreased overall patient survival (Bates et al., 2006), and may indicate that the presence of Treg cells promotes tumour progression by inhibiting immunosuppression.

FOXP3 expression in regulatory CD4+ T cells may result in impaired immunological function with maintenance of low-level chronic inflammation. Inflammation is essential for the effective host defence. However, chronic inflammation may predispose to cancer. Increased and sustained inflammation may provide a mechanism for involvement of FOXP3 in cancer development. Mutation of an X-linked cancer suppressor gene, such as FOXP3, in females may result in its mosaic expression. In Tregs, the mosaic FOXP3 expression produces their partially impaired function without disease and elevated expression of

inflammatory cytokines. It consequently sustains inflammation (Medema and Burgering, 2007).

Signalling pathways that mediate the protumourigenic effects of inflammation are often subject to a feed-forward loop, for example, activation of NF-kB in immune cells induces production of cytokines that activate NF-kB in cancer cells to induce chemokines that attract more inflammatory cells into the tumour (Grivennikov et al., 2010).

FOXP3-induced down-regulation of genes, e.g. PDE3B (cyclic nucleotide phosphodiesterase 3B,cGMP inhibited) has been shown to give a survival advantage and a resistance to apoptosis in T cells (Gavin et al., 2007).

It has been observed that poor prognostic factors (negative hormonal receptors, high tumour grade, and nodal involvement) were associated with a significantly higher number of CD3, CD8, and Foxp3 expressing cells in the infiltrate (Ladoire et al., 2008). Chemotherapy resulted in a decrease in Foxp3 positive cells in the infiltrate, whereas the level of CD8 and CD3 infiltrate remained unchanged (Ladoire et al., 2008). Pathologic complete response to chemotherapy was found to be associated with an absence of immunosuppressive Foxp3 cells and the presence of a high number of CD8 T cells and cytotoxic cells in cancer infiltrate. Since Tregs are potential inhibitors of anti-tumour response, the infiltration by FOXP3+ Tregs may be associated with increased relapse and shorter survival of patients with both *in situ* and invasive breast cancer (Generali et al., 2009). Oestrogen is able to induce immunotolerance by increasing the Tregs pull. The evidence suggests that aromatase inhibitors may have an indirect antitumor mechanism of action through reducing Tregs in breast tumours (Generali et al., 2009). FOXP3 in T cells has been also studied as a therapeutic target, and it has been shown that vaccination to eradicate FOXP3-expressing cells enhances tumour immunity (Nair S, 2007).

Recently, a new explanation for the association of CD4 positive T-cell and Treg-cell markers with a more aggressive behaviour in advanced breast cancers has been proposed, by demonstrating that tumour-infiltrating CD4 positive CD25 positive

FOXP3 positive Treg cells are a major source of RANKL, which stimulates the metastatic progression of RANK-expressing breast carcinoma cells (Tan et al., 2010). Furthermore, the pro-metastatic function of T cells can be replaced by exogenous RANKL (Tan et al., 2010).

1.27.3 FOXP3 as a tumor suppressor gene

FOXP3 can be expressed by not only tumour-infiltrating Tregs but also by cancer cells. This raises the possibility that tumour cells themselves modulate T-cell function through FOXP3.

Using microarray analysis, a large number of genes that are either up- or down-regulated by ectopic expression of FOXP3 have been discovered and the genes involved in cancer were the most affected group (Wang L, 2009). Therefore, FOXP3 will likely suppress development of breast cancer by targeting multiple genes, including both tumour suppressor and oncogenes.

FOXP3 is expressed in normal human breast epithelial cells but is lost in 80% of human breast cancer cells (Zuo et al., 2007c). The significance of FOXP3 as an X-linked tumour suppressor gene in humans is supported by the prevalence of somatic mutations (36%), gene deletion (13%), and lack of nuclear FOXP3 seen in the majority of breast cancer samples (Zuo et al., 2007c). The majority of these mutations are heterozygous and the deletion of the gene is heterozygous in all cases. FOXP3 inhibits breast tumour growth through directly repressing the transcription activity of two oncogenes, HER2 and SKP2, while inducing the transcription activity of tumour suppressor gene p21 (Figure 1-8) (Zuo et al., 2007b, Zuo et al., 2007c, Liu et al., 2009). A cyclin-dependent kinase inhibitor p21 is also major downstream target of other tumour suppressor genes, including p53 (el-Deiry et al., 1993) and BRCA1 (Somasundaram et al., 1997). FOXP3 is essential for p21 expression in normal epithelia and that lack of FOXP3 is associated with p21 down-regulation in breast cancer samples (Liu et al., 2009).

Ectopic expression of HER-2 completely reverses the anti-tumourigenic effect of Foxp3 *in vitro* but Foxp3 can also suppress the growth of HER-2 negative breast

cancer cells (Zuo et al., 2007d), thus Foxp3 can repress ErbB2 signalling and tumour growth by mechanisms other than ErbB2 expression. SKP2 was found to be critical for FOXP3-mediated growth inhibition in breast cancer cells that do not over-express ERBB2/HER2 (Zuo et al., 2007a).

FOXP3 is frequently inactivated in prostate cancer samples by deletion (14%) or somatic mutation (25%) (Wang L, 2009). Inactivation of FOXP3 causes strong growth inhibition of prostate cancer cell lines upon addition of FOXP3 (Wang L, 2009). Importantly, prostate-specific ablation of the FoxP3 in the mouse caused early onset of prostatic hyperplasia and prostatic intraepithelial neoplasia (PIN) (Wang L, 2009). FOXP3-mediated transcriptional repression of c-MYC is necessary to control c-MYC levels in normal prostate epithelial cells (Figure 1-8).

Recently, FOXP3 has been described as a direct transcriptional activator for Lats2 in both normal and malignant breast and prostate cells from mouse and human (Li et al.). This may be a novel mechanism of LATS2 downregulation in cancer and reveals an important tumour suppressor relay between the FOXP3 and HIPPO pathways that are widely implicated in human cancer.

In pancreatic cancer FOXP3 expression may represent a type of molecular mimicry that enables immune evasion of tumour cells. Pancreatic cancer cells share growth-suppressive effects with Tregs mimicking Tregs function (Hinz et al., 2007).

FOXP3 was shown to be weakly or not expressed in ovarian cancer cells (Hai-Yan Zhang, 2010). Transfection of ovarian cancer cells with FOXP3 inhibited cell proliferation, decreased cell migration, and reduced cell invasion. Cells with upregulated FOXP3 showed decreased expression of Ki-67 and cyclin-dependent kinases. FOXP3 can inhibit cell migration and invasion by down regulating expression of matrix metalloproteinase-2 and urokinase-type plasminogen activator in ovarian cancer cells (Zhang and Sun).

1.28 Hypothesis

The tumour suppressor FOXP3 is inactive in breast cancer cells causing an increase in expression of CXCR4 chemokine receptor and acquisition of responsiveness to chemokines such as CXCL12, thus regulating the potential for metastatic spread.

1.29 Aims

- To characterise the relationship between FOXP3 and CXCR4 expression in breast cancer
- To investigate FOXP3 translocation to the nucleus of breast cancer cells
- To assess the role of FOXP3 in CXCR4-induced migration of breast cancer cells

Chapter

2 Materials and Methods

2.1 Cell culture

2.1.1 Cryopreservation of Cells

Cells were routinely cryopreserved in liquid nitrogen. Cells were detached from flasks, pelleted, and resuspended in 0.5 ml of medium. 0.5 ml of freezing medium was added, containing 20% dimethyl sulphoxide (DMSO, Sigma) in fetal calf serum (FCS), resulting in a final concentration of 10% DMSO. The cell suspension was transferred to a cryogenic vial and then gently cooled at 1°C/min in an isopropyl alcohol containing freezing container (Nalgene, Cryo 1°C Freezing container, Nalgene, Hereford) for 4 hours, prior to transfer into liquid nitrogen for long term storage.

Recovery of the cells was achieved by rapid thawing of the cryogenic vial contents and their addition to 10 ml of complete medium in a 75 cm^3 flask. After 18-24 hours the DMSO-containing medium was replaced by appropriate complete medium.

2.1.2 Cell counting

Cells were counted prior to their use in experiments using 10 μl of resuspended cells and a Neubauer chamber haemocytometer (Reichert, Buffalo, USA). Cells in the 25 squares of the grid were counted and the total multiplied by $1x10^4$ to obtain the number of cells/ml.

2.1.3 Cell lines

Table 2-1 summarises cell lines used in protein and mRNA studies to analyse the expression of FOXP3 and CXCR4.

2.1.3.1 Human Mammary Epithelial Cells (HMEpC)

Purchased from Promocell, HMEpC are isolated from the human adult mammary gland. They were grown in the recommended Mammary Epithelial Cell Growth

Medium (Promocell). Adherent cells were cultured at 37°C in a humidified, carbon dioxide free system until 70-80% confluent and split in a ratio of 1:3.

2.1.3.2 MCF-10A

The MCF 10A cell line was a kind gift from Dr Felicity May (Northern Institute of Cancer Research, Newcastle University). It is a non-neoplastic immortal human breast epithelial cell line. The line was produced by long-term culture in serum free medium with low calcium concentration. It retains many characteristics of normal breast epithelium. Adherent cell line was cultured in a low calcium medium, at 37°C in a humidified, carbon dioxide free system until 70-80% confluent and split in a ratio of 1:3.

2.1.3.3 MCF-7

MCF-7 is a human breast adenocarcinoma cell line (European Collection of Cell Cultures – ECACC, Salisbury). These relatively non-invasive cells were derived from the pleural effusion of a 69-year-old female Caucasian. These cells demonstrate a number of features of differentiated mammary epithelium including oestradiol synthesis and express oestrogen and progesterone receptors. Adherent cell line was cultured in complete DMEM without phenol red at 37°C, 5% CO2 in a humidified atmosphere until 70-80% confluent and split in a ratio of 1:3.

2.1.3.4 MDA-MB-231

MDA-MB-231 (ECACC) is a human breast adenocarcinoma cell line derived from the pleural effusion of a 51-year-old female Caucasian. These cells have an invasive phenotype. They do not express oestrogen receptors. Adherent cells were cultured in complete Leibovitz L-15 medium, at 37°C in a humidified, CO2 free system until 70-80% confluent and split in a ratio of 1:3.

2.1.3.5 TMD-MDA-MB-231 (TMD-231)

TMD-231 cells were a gift from H. Nakshatri, Indianapolis, USA. They were isolated from the mammary fat pad of mice inoculated with MDA-MB-231. This cell line expresses high levels of CXCR4 (Helbig et al., 2003). Cells were cultured in

complete Minimum Essential Medium and complete RPMI mixed in a 50:50 ratio at 37°C, 5% in a humidified atmosphere CO2 until 70-80% confluent and were split in a ratio of 1:3.

2.1.3.6 LMD-MDA-MB-231 (LMD-231)

LMD-231 cells were also a gift from H. Nakshatri, Indianapolis, USA. This cell line was cultured from a lung metastasis of mice inoculated with MDA-MB-231. This cell line expresses high levels of CXCR4 (Helbig et al., 2003). Cells were cultured in complete Minimum Essential Medium and complete RPMI mixed in a 50:50 ratio at 37°C, 5% in a humidified atmosphere CO2 until 70-80% confluent and were split in a ratio of 1:3.

2.1.3.7 Flp-InTM T-RExTM-293

This cell line originated from HEK293 cells. These Tet repressor expressing cells contain a single, integrated Flp recombination target (FRT) site for stable specific genomic incorporation of a gene of interest. Gene expression is then controlled with tetracycline. Untransfected Flp-In™T-Rex™-293 cells were grown in complete DMEM medium in the presence of 10µg/ml Blasticidin and 100µg/ml Zeocin (Invitrogen). After transfection, Zeocin was replaced by 100 µg/ml Hygromycin B. The expression of FOXP3 was induced by Tetracycline (100 µg/ml). The cells were a kind gift from Marcin Pekalski (Institute of Cellular Medicine, Newcastle University, UK).

2.1.3.8 Jurkat

The leukaemic T cell line was (originally called JM) was established from the peripheral blood of a 14-year-old boy with T cell leukaemia. Jurkat cells were cultured in RPMI 1640 complete medium in 75 cm^2 flasks in vertical position.

Cell line	Cell type	Growth type	Media	Origin	Expression	Source
HMEpC	Primary human mammary epithelial cells	adherent	HMEpC medium	Human adult normal mammary gland	Cytokeratin	Promocell
MCF-10	Human immortalized breast epithelial cells	adherent	DMEM/ F12	Human fibrocystic mammary tissue	MMP9	ATCC
MCF-7	Human invasive breast ductal carcinoma	adherent	DMEM	Pleural effusion of 69-year old female with breast cancer	Oestrogen and progesterone receptors	ECACC
MDA-MB-231	Human adenocarcinoma	adherent	Leibovitz L-15	Pleural effusion of 51-year old female with breast cancer	EGF, TGFa, WNT7B	ECACC
TMD-MDA-MB-231	Mice metastatic adenocarcinoma	adherent	DMEM/RPMI	Mammary fat pad of immunodeficient mice inoculated with MDA-MB-231	CXCR4	H. Nakshatri, Indianapolis USA
LMD-MDA-MB-231	Mice metastatic adenocarcinoma	adherent	DMEM/ RPMI	Lung metastases of immunodeficient mice inoculated with MDA-MB-231	CXCR4	H. Nakshatri, Indianapolis USA
Flp-InTM T-RExTM-293	Human embryonic kidney	adherent	DMEM	Fetal kidney hypotriploid human cell line	FOXP3	Invitrogen
Jurkat	Human leukaemic T cell lymphoblast	suspension	RPMI	Jurkat FHCRC	CXCR4	ECACC

Table 2-1: Cell lines used in protein and mRNA studies to analyse the expression of FOXP3 and CXCR4

2.1.4 Cell media

2.1.4.1 Mammary Epithelial Cell Growth Medium

Mammary Epithelial Cell Growth Medium is a serum-free medium supplemented with bovine pituitary extract (0.004 ml/ml), Epidermal Growth Factor (EGF) 10ng/ml, Insulin (5 µg/ml), Hydrocortisone (0.5 µg/ml).

2.1.4.2 Medium for MCF-10A cells

The growth medium for MCF-10A contains several additives and is composed of DMEM/F12 supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, and antibiotics (Debnath et al., 2003).

2.1.4.3 Dulbecco's Modified Eagle Medium (DMEM)

Dulbecco's Modified Eagle Medium (with 1000 mg/l D-glucose and 110 mg/l sodium pyruvate; Invitrogen) without phenol red was supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.146 g/l L-glutamine.

2.1.4.4 Minimal Essential Medium

Minimum Essential Medium Eagle with Hank's salts, 0.292 g/l L-glutamine and 0.35 g/l sodium bicarbonate was supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2nM human insulin (Sigma).

2.1.4.5 Leibovitz L-15 Medium

Leibovitz medium (Sigma) is buffered to maintain physiological pH control in a carbon dioxide free system. Media was supplemented with 0.3 g/l L-glutamine, 10% FCS, 100 U/ml penicillin and 100µg/ml streptomycin.

2.2 Flow cytometry

Flow cytometric analysis was performed using the BD FACS scan flow cytometer. 1x106 cells were washed in 500 µl of PBS with 1% BSA. Cells were then resuspended in 100 µl of PBS with 0.1% BSA and appropriate monoclonal antibody was added at appropriate concentrations (CXCR4 10 µl/ml). The appropriate

secondary antibodies conjugated to fluorochrome were added after washing and the samples were incubated for 30 min at 4ºC. Cells were then washed and resuspended in 500 µl PBS prior to examination by flow cytometry. To control for non-specific binding, an isotype-matched primary antibody (at the same dilution as the test monoclonal antibody) was applied and subjected to the same secondary staining procedure as experimental samples.

2.3 Immunofluorescence

Cells were grown on chamber slides (90000 cells/chamber), fixed with 4% paraformaldehyde for 30 minutes, permeabilized using 0.1% Triton X100 in PBS for 15 minutes at 4°C and blocked in 5% FBS for 1 hour. Primary antibody was added to chambers and left overnight at 4°C at a concentration of 1:5 (Foxp3) and 1:300 (CXCR4) diluted in 5% FBS. Slides were covered with FITC conjugated rabbit anti-mouse secondary antibody for two hours, stained with DAPI, mounted using VectaShield and examined with a Leica fluorescent microscope.

2.3.1 Antibodies

The primary antibodies used during the course of this work are summarised in Table 2-2. Two anti-Foxp3 and one anti-CXCR4 antibodies were used:

Non-commercial monoclonal mouse anti-human Foxp3 antibody (clone 150D/E4; isotype Ig1) is a cell culture supernatant and was a kind gift from Dr. Alison H. Banham, University of Oxford. It was diluted (1:5) in blocking buffer and incubated over night at 4°C.

Monoclonal mouse anti-human Foxp3 antibody (clone 326A/E7; isotype IgG1) was purchased from Abcam. Previously, it has been successfully used for staining prostate (Wang L, 2009) and breast cancer tissues (Wang L, 209). The antibody was diluted 1:50 in blocking buffer and incubated over night at 4°C.

Monoclonal mouse anti-human CXCR4 antibody (clone: 44716, isotype IgG2b) was purchased from R&D systems. It was diluted 1:300 in blocking buffer and incubated over night at 4°C.

Name of antigen	Antigen retrieval		Source of antibody	Antibody dilution	
	Physical conditions			Primary	Secondary (Biotinylated)
	Buffer	Method			
FOXP3	Citrate	PC	Non commercial	1:5	1: 250
FOXP3	Citrate	PC	Abcam	1:50	1: 250
CXCR4	Citrate	PC	R&D systems	1:300	1:250
CD45	Citrate	MCW	DAKO	1:100	1: 250

Table 2-2: Primary antibodies used for Immunohistochemistry

MCW=microwave, PC= pressure cooker

2.4 Bacterial cell culture

Bacteria were grown on *Luria-Bertanni* (LB Sigma) media agar plates or liquid LB medium at 37°C. For positive selection appropriate antibiotics were added to media and plates. Bacterial stocks were stored at -80°C in 10% glycerol in LB (Table 2-3: Bacterial cell culture components.

LB Medium (1 Liter)		**LB Agar (1 Liter)**	
10g	bacto-tryptone	10g	bacto-tryptone
5g	bacto-yeast extract	5g	bacto-yeast extract
10g	NaCl	10g	NaCl
pH 7		30g	Agar (10g/L)

Table 2-3: Bacterial cell culture components.

2.4.1 Generation of chemically competent *Escherichia coli*

Chemical competence is competence in the ability of a cell to take up extracellular DNA from its environment. In order to make *Escherichia coli* (*XL1-Blue* cells) chemically competent, cells in 10 ml of medium were grown overnight at 37°C. Next day, 200 ml of medium was inoculated with 5 ml of overnight innoculum on shaker at 37°C. The cells were grown to optical density of OD600. Following this cells were spun for 15 minutes at 3500 rpm at 4°C and the pellet was resuspended in 50 ml of ice cold 10 mM NaCl, incubated for 10 minutes on ice and pelleted again. Following this, cells were resuspended in 50 ml of ice cold 75 mM $CaCl_2$, incubated for 35 minutes on ice, pelleted again and resuspended in 3 ml of ice cold 75 mM $CaCl_2$, aliquoted and stored in -70°C.

2.4.2 Transformation of competent *Escherichia coli*

One aliquot of competent cells was defrosted on ice and incubated with DNA on ice for 30 minutes. This allows the DNA to adsorb onto the surface of the cells. The cells were then heat shocked for 90 seconds at 42°C and returned to ice for 2 minutes. This process causes the DNA to be taken up by cells through pores in their membranes before these pores are closed by rapid cooling. Cells were then added to 1ml of LB growth medium and allowed to recover for 2 hours at room temperature with shaking, before being spread onto LB agar plates containing appropriate antibiotics (Ampicillin) as selection agents for the uptake of the desired plasmid type. Following overnight growth, individual colonies were picked off plates for further analysis. Positive (cells but no antibiotics on a plate) and negative (no cells or control cells with antibiotics on a plate) controls were used.

2.5 DNA methods

2.5.1 Recovery of plasmid DNA from bacteria culture

Plasmid DNA was isolated from *Escherichia coli (XL1-Blue* cells) cultures using the Qiagen Mini-Prep kit in accordance with the manufacturer's instructions. This technique uses silica-binding technology and spin columns. Briefly: 5 ml of overnight Escherichia coli culture was pelleted by centrifugation at 12,000 g for 1 minute. Pelleted bacterial cells were resuspended in 250 µl of the re-suspension solution containing RNase. Cells were lysed with 250 µl of alkaline-Sodium Dodecyl Sulphate lysis solution before neutralising with 350 µl of neutralising solution. The lysate was centrifuged at 13,000 g for 10 minutes to pellet cell debris, proteins, lipids and chromosomal DNA. Clear lysate was added to the prepared silica column before centrifuging for further 30-60 s. Following two wash steps with wash solution, plasmid DNA was eluted from the column using 100 µl of Elution Solution.

For the large plasmid DNA preparation Maxi-prep (Qiagen) was used in accordance with the manufacturer's instructions. This technique, similarly to Mini-Prep, uses silica-binding technology and spin columns.

2.5.2 Enzymatic digestion of plasmid DNA with restriction enzymes

Restriction enzymes are bacterial enzymes that hydrolyse DNA that normally comprise the modification defence response against foreign DNA in bacterial cells. Restriction endonucleases recognise short, symmetrical base pair sequences and therefore cut foreign DNA into small segments. Hydrolysis of DNA by endonucleases results in either 'blunt' or protruding 'sticky' 3'-OH and 5'-OH ends. Blunt-ended digestions can be ligated to any other blunt-ended digestion, but if different enzymes are used the original restriction site will be disrupted. Sticky ends will bind to any other sequences with the same overhanging sequence, usually resulting in specific annealing of complementary ends and restoration of the original restriction site.

Typically, one unit is defined as the amount of enzyme required to digest 1 μg of DNA in 1hour at 37°C. A standard 60 μl reaction to digest 5 μg of DNA would contain: DNA, 6 μl 10x Buffer (optimal for the enzyme(s) being used), 6 μl restriction enzyme, made up to 60 μl with water. Digestions were left for 3-12 hours at 37°C. Restriction enzymes used were *Xho1* and *Not1*.

2.5.3 DNA and RNA ethanol precipitation

Ethanol precipitation is a commonly used technique for concentrating and de-salting nucleic acid (DNA or RNA) preparations in aqueous solution. Briefly: the volume of the DNA sample was measured, the salt concentration adjusted by adding 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M). The sample was mixed and 2.5 volumes of cold 100% ethanol was added. The sample was placed on ice for 20 minutes, spun at a maximum speed in a microcentrifuge for 10-15 min, supernatant was removed and 1 ml of 70% ethanol added. Again, the sample was spun, air-dried and resuspended in water.

2.5.4 DNA sequencing

Automated DNA sequencing was used to determine the sequence of both DNA fragments and junctions with vectors containing inserts. Sequencing was carried out by GeneService (www.geneservice.co.uk) and chromatograms were produced

using the Geospiza's FinchTV v.1.4.0 freeware software (http://www.geospiza.com).

2.5.5 Agarose gel electrophoresis

1% agarose gel was produced by melting 0.5 g agarose (Sigma) with 50 mL 1x TAE (50x stock consists of 2 M Tris base, 1 M acetic acid and 0.5 M EDTA pH 8). To visualise DNA sample, Ethidium Bromide (Promega) at final concentration of 5 μg/ml was added to the gel and left to set in a plastic casting stand. Subsequently, the gel was transferred to an electrophoresis tank filled with TAE buffer. To establish an approximate band size 5 μl Hyperladder IV (Bioline) was added to one of the lanes. Once electrophoresis was completed, an Alpha Imager Instrument (AlphaImager®, Alpha Innotech Corp., San Leandro, Ca, USA) was used to visualise the DNA bands.

2.5.6 Gel purification of DNA

Purification of DNA from a gel was performed by the QIAquick Gel Extraction Kit (Qiagen) according to the manufacture protocol.

2.6 PCR

2.6.1 RNA extraction

Total RNA was isolated from cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Trizol Reagent promotes the formation of complexes of RNA with guanidine and water molecules, while abolishing the hydrophilic interactions of DNA and proteins. Consequently, RNA remains in aqueous phase, separated from DNA and proteins. All RNA work was carried out with ribonuclease-free and contaminant-free apparatus. All water used in RNA work and analysis was Diethyl pyrocarbonate (DEPC) treated. 0.2 ml of Trizol Reagent was added to lyse 1x106 cells, which were solubilised by passaging lysate through a pipette. Following addition of chloroform at 10% of the volume of Trizol Reagent, the homogenate was shaken vigorously for 15 seconds, incubated on ice for 2-3 minutes and centrifuged at 12,000 g for 15 minutes. The aqueous phase was removed and RNA precipitated with the addition of an equal volume of isopropanol. This was left to incubate for further 10 minutes on ice. The RNA precipitate was centrifuged at 12,000 g for 10 minutes to pellet, supernatant was removed and the pellet washed by centrifuging with 75% ethanol, prior to resuspending RNA in water. The concentration and quality of the isolated RNA was assessed using nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, USA). The integrity of the extracted RNA was assessed using agarose gel electrophoresis to identify 28S and 18S ribosomal RNA (rRNA).

2.6.2 RNA/DNA quantification

RNA and DNA preparations were quantified by nanodrop (Biophotometer, Eppendorf, Hamburg, Germany) at 260 nm and 280 nm. Small samples of RNA or DNA were diluted in water prior to spectrophotometry. The 260/280 ratio provides an estimate of nucleic acid purity. Pure preparations of DNA and RNA have OD 260/280 values of 1.8 and 2.0 respectively.

2.6.3 Reverse Transcription

First strand cDNA synthesis was performed using Superscript III RNase H Reverse Transcriptase. 5 μg of total RNA was incubated at 65°C for 5 minutes with 1 μg of Oligo (dT) and 1 μl of 10 mM dNTP (deoxy-nucleotide tri-phosphate) and distilled water was added to the volume of 13 μl. This mixture was then incubated on ice for at least 1 minute. Following this 4 μl of First Strand Buffer, 1 μl of 0.1M DTT and 1 μl of Superscript III (200 U/μl) were added and mixed by pipetting. The mixture was reverse transcribed at 50-55°C for 50 minutes, before inactivating at 70°C for 15 minutes, prior to cooling. This was performed in a thermal cycler (Hybaid, Basingstoke). The cDNA can be used as a template for amplification in PCR.

2.6.4 Primer Design

All FOXP3 primers were designed manually. Nucleotide search tool from www.ncbi.nlm.nih.gov was used to search for target sequence of nucleotides for FOXP3 locus (AF277993). "Perfect Primers Design" tool (Invitrogen), www.tools.invitrogen.com, was used to determine final primer sequence. Forward and reverse FOXP3 primers were designed to have approximately the same number of G and C components and to be 15-35 nucleotides in length. Primers were designed with a G-C content producing a melting temperature (Tm) of 60°C. Table 2-4 summarises 3 pairs of FOXP3 primers designed to sequence three nuclear localisation domains (NLD) of FOXP3.

FOXP3 primer	Where synthesised	Sequence	NLD
Forward primer	VH Bio (Gateshead)	CAAATGGTGTCTGCAAGTGG	2
Reverse primer		ATTGAGTGTCCGCTGCTTCT	2
Forward primer	Invitrogen	AAGCCAGGCTGATCCTTTTCT	2, 3
Reverse primer		TCTGCCTCCCACCAGTTTG	2, 3
Forward primer	IDT	AGATCTACCACTGGTTCACACGCA	1
Reverse primer		AGGCAAGACAGTGGAAACCTCACT	1

Table 2-4: Primer sequence used in conventional PCR

2.7 Conventional PCR

The Polymerase Chain Reaction (PCR) is a method for amplifying specific sections of a DNA molecule. PCR is based on the fact that DNA polymerases are primer-dependant. This means that a pair of primers is designed and synthesised complementary to sequences upstream to the sequence of interest on both the coding and complementary DNA strands. The polymerase will then extend these primers past the sequence of interest by incorporating new complimentary bases, thus copying it. Primers are generally 15 to 30 base pairs in length and have a large degree of sequence homology with the target. The two primers also have approximately the same ratio of G/C to A/T bases. The G/C to A/T ratio determines the annealing temperature for the reaction. The reaction can only be held at one annealing temperature. The PCR cycle comprises three phases: heating to denature DNA double helices, cooling to a low temperature allowing the specific annealing of helices carrying complementary base-pair sequences as well as primers and elongation when the polymerase extends the primers, copying the sequence of interest. A typical PCR cycle comprises 1 minute at 95°C to denature, 30 seconds at around 55°C to anneal, followed by 1 minute at 72°C for polymerase function. The temperatures used are relatively high and therefore thermostable polymerases must be used which are able to withstand the heat without denaturing. *Taq* and *Pfu* polymerases were used in the course of this work. *Taq* was originally isolated from *Hyperthermophilic archaea (Thermus aquaticus)* and is stable at high temperatures and has an error rate of about 1.3×10^{-5} and mutation frequency of 5.1 %, Thermostable *Pfu (Pyrococcus furiosus)* DNA Polymerase with 3′→5′ exonuclease proofreading activity exhibits a higher fidelity than *Taq* DNA Polymerase. It has an error rate of about 1.8×10^{-5} – 8×10^{-7} and 5.1–0.3% mutation frequency and was used to reduce error rate in the amplified products.

All polymerases used in PCR are dependant on magnesium, which stabilises DNA and RNA interactions. PCR reactions were evaluated with various concentrations

of magnesium ($MgCl_2$=1.5 mM, 2 mM, 2.5 mM) and with various annealing temperatures to increase their specificity.

A standard 40 µl reaction for a PCR would comprise: 4 µl deoxynucleoside triphosphate solution, 4 µl of 10x PCR buffer concentrate, 0.8 µl of Taq polymerase enzyme, 4 µl (10 mM), forward primer solution, 4 µl (10 mM), reverse primer solution, 1.6 µl (50 mM) $MgCl_2$, 2 µl (1 ng) template DNA, dH_2O to make up to 40 µl. Negative control included experiments with no cDNA added to the reaction. Positive controls were performed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. GAPDH is constitutively expressed and would thus be amplified if conditions of the PCR were optimal.

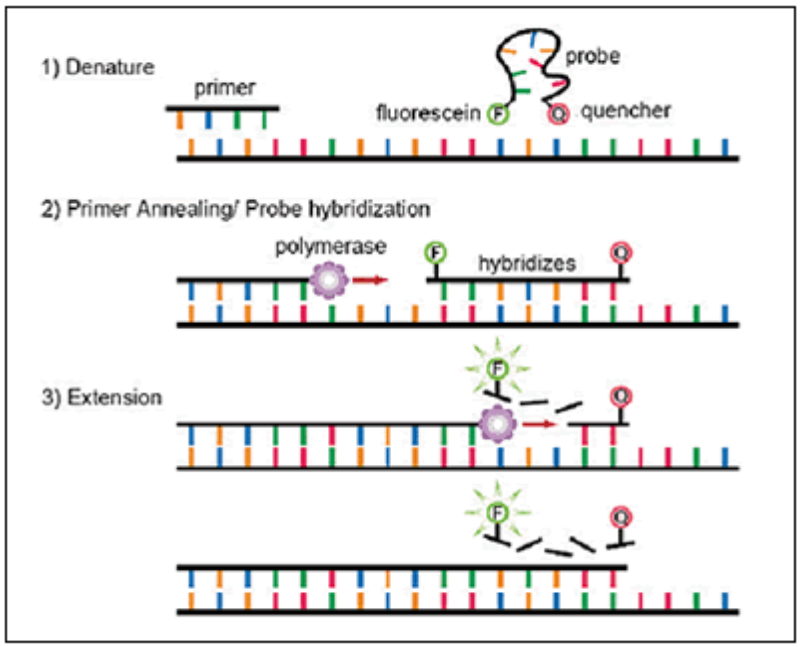


Figure 2-1: Chain extension and emission of fluorescence in real-time PCR.

The probes anneal to specific sequences in DNA between the forward and reverse primers. When excited the reporter (F) transfers energy to the quencher (Q) preventing emission of fluorescence. As *Taq* polymerase copies the DNA the 5' exonuclease activity of the enzyme cleaves the probe. This separates the reporter and quencher allowing the reporter dye to fluoresce.

Adapted from App Biosystems www.appliedbiosystems.com: Real-time PCR 2009

2.8 Real-Time PCR

In comparison to normal PCR that detects the amount of final amplified product at the end-point, real-time PCR measures the initial amount of the template precisely and reproducibly. During a real-time PCR reaction each amplification cycle emits fluorescence in real time. Real-time PCR allows measurement at the relative gene expression compared to the expression of endogenous control. The endogenous control has to be abundant and remain constant in proportion to total RNA among the samples. Similarly to normal PCR, real-time PCR uses primers and follows the same three-step reaction of denaturation, annealing and chain elongation. Real time PCR employs three main types of fluorescing systems to monitor DNA amplification, which include hydrolysis probes, hybridizing probes and DNA-binding agents. In this study hydrolysis probes were used, namely TaqMan probes. The TaqMan probe has a high-energy fluorescing dye, a reporter, at the 5' end and a low energy quencher molecule at the 3' end. When the probe is excited by a light source the fluorescent dye transfers energy to the quenching dye molecule rather than fluorescing. The probes anneal to specific sequences in DNA between the forward and reverse primers. As Taq polymerase copies a template of DNA to which a probe is bound the 5' exonuclease activity of the enzyme cleaves the probe. This separates the reporter and quencher preventing the transfer of energy allowing the reporter dye to fluoresce (Figure 2-1). This cleavage increases with every cycle and is proportional to the accumulation of PCR products. In the early cycles there is no considerable change in fluorescent signal. This is referred to as the baseline and a fixed threshold can be set above this line. A noteworthy parameter for the quantification of initial template is the threshold cycle (CT). This is defined as the cycle number at which fluorescence passes the fixed threshold. Therefore the higher the initial amount of template, the lower the CT.

In the present work 18S RNA and GAPDH were used as endogenous controls. Real-time PCR was used to quantify the amount of FOXP3 and CXCR4 cDNA in breast cell lines and cells following TGF-β1 treatment and FOXP3 amount following

transfection of MDA-MB-231 cells with FOXP3 vector. RNA was extracted and cDNA synthesised as outlined above. Random primers instead of oligo-dT have to be used during the RT-PCR reaction when working with 18S, as 18S RNA does not have a poly-A tail. Assays-on-Demand™ Gene Expression products consist of a 20x mix of PCR primers and TaqMan® probe (FAM™ dye-labelled). All Assays-on Demand™ Gene Expression products are optimized to work with TaqMan® Universal PCR Master Mix and with complementary DNA (cDNA). In the present work gene expression assays (Assays-on-Demand™) designed by Applied Biosystems were used (Table 2-5).

Each well of real-time PCR plate contained 10 µl master mix (Stratagene), 7 µl distilled water, 2 µl template cDNA and 1 µl primer probes (Applied Biosystems). Each experiment was carried out in triplicates. For each set a reaction without cDNA template was used as a negative control. Experiments were performed using an Applied Biosystems ABI Prism 7000 plate reader. Results were normalised to the expression of endogenous 18S RNA and GAPDH (ΔCT), the values of the negative controls were subtracted from that of the test samples (ΔΔCT), and those values were expressed as a fold change in relation to the negative controls. In addition, the relative expression software tool 2008 (REST2008) was used to estimate changes in gene expression.

Assay ID	Reporter Dye	Context Sequence	Gene Name
Hs99999901_s1	FAM	n/a	18S RNA human 18S rRNA
Hs99999905_m1	FAM	TTGGGCGCCTGG-TCACCAGGGCTGC	GAPDH-glyceraldehyde-3-phosphate dehydrogenase
Hs00203958_m1	FAM	ATCCGCTGGGCCA-TCCTGGAGGCTC	FOXP3 forkhead box P3

Table 2-5: Assays-on-Demand™ Gene Expression Products (TaqMan® probes, FAM™ dye-labelled).

2.8.1.1 Relative quantification of gene expression

When the comparative CT method (ΔΔCT) of real-time PCR data analysis is used, there is no need for a standard curve. Relative quantification is applied when determining the expression levels relative to the housekeeping gene.

Efficiency of both primer pairs used in the experiment has to be assessed using serial dilutions and estimated to be between 90 and 100%, which means that the slope between linear phases of amplifications graphs should be between -3.6 and -3.1. A standard method to check the efficiency of primer pairs is to examine how ΔCT varies with template dilution. The efficiency of the PCR reaction depends on some variables, including quality of the primers and length of the amplicon. Lower than 90% efficiency does not exclude valid results but it is recommended to use an optimized system. When the efficiencies of reaction of the two different dilutions are equal, the plot of logs input quantity versus ΔCT gives horizontal lines.

To calculate the relative expression of two different samples, one of them should be chosen as the examined and the other as the reference.

$$\Delta CT = CT\ (examined) - CT\ (reference=housekeeping\ gene)$$

The comparative ΔΔCT step involves finding the difference between ΔCT of the unknown sample and the sample to which expression is compared. The final calculation for the comparative expression level is performed as per following equation:

$$Comparative\ expression\ level = 2 - \Delta\Delta CT$$

2.9 Statistical analysis

The data was analyzed using the Microsoft Office Excel 2003 and Prism3 Graphpad statistical software packages. Quantitative values were expressed and plotted as mean ± standard deviation. Comparisons were evaluated by the unpaired Student's t-test and ANOVA and Bonferroni tests. Kappa statistics was used to compare inter-observer agreement in FOXP3 and CXCR4 immunohistochemical scoring for manual and digital microscopy interpretation. A linear regression analysis was used to determine the relationship between FOXP3 nuclear quick score on tissue sections and the patients' age.

A value of $P < 0.05$ was considered statistically significant.

3 Characterization of FOXP3 and CXCR4 expression in breast cancer

3.1 Introduction

3.1.1 CXCR4 expression

CXCR4 is the physiological receptor for CXCL12, which belongs to a chemokine family that has potent chemotactic activity for lymphocytes. It is well known that peripheral lymphocytes preferentially localize to peripheral lymphoid tissues, such as lymph nodes, which is called the homing phenomenon (Blackwell et al., 2009). Metastatic breast cancer cells overexpress CXCR4 and this receptor plays a critical role in homing of cancer cells at specific metastatic sites (Muller et al., 2001).

CXCR4 is expressed on various cell types, including: endothelial cells (Volin et al., 1998, Feil and Augustin, 1998), bronchial epithelial cells (Eddleston et al., 2002), lymphocytes (Bleul et al., 1996b, Bleul et al., 1996a), intestinal (including colonic) epithelial cells (Dwinell et al., 1999, Jordan et al., 1999), primitive haematopoietic progenitor cells (Aiuti et al., 1997), microglia, neurons and astrocytes (Bonavia et al., 2003), vascular smooth muscle cells (Schecter et al., 2003), fibrocytes (Phillips et al., 2004) and pluripotent stem cells, including mammary stem cells (Dontu et al., 2003). CXCR4 expression by stem cells suggests that CXCR4 may mask the progenitors of breast cancer (Liu et al., 2005).

CC and CXC chemokine receptor expression is described on a wide variety of cancer cell types (Tanaka et al., 2005, Dowsland et al., 2003, Balkwill, 2004). CXCR4 is the most common chemokine receptor expressed on both murine and human cancer cells, having been noted in at least 23 different haematopoietic, epithelial and mesenchymal tumours (Balkwill, 2004). On the other hand, within primary tumours and cancer cell lines e.g. ovarian and non small-cell lung cancer, only a subpopulation of cells express the CXCR4 receptor (Scotton et al., 2001, Phillips et al., 2003). Similarly, expression of CXCR4 in breast cancer cells is confined to a small sub-population of cells (Kang et al., 2003b).

Immunohistochemical staining of primary breast tumours has revealed that normal breast epithelial cells do not express CXCR4, whereas, between 51% and 73% of primary breast cancers express the protein (Muller et al., 2001, Kato et al., 2003, Cabioglu et al., 2005c) as do more than 90% of samples with atypical ductal hyperplasia (Schmid et al., 2004). CXCR4 expression in the primary cancer has also been positively correlated with the degree of lymph node metastasis (Cabioglu et al., 2005c, Kato et al., 2003), tumour grade (Helms et al., 2005) and poor overall survival (Li et al., 2004). CXCR4 expression as demonstrated by flow cytometry has been linked to the ability of breast cancer cells to metastasise to lungs (Helbig et al., 2003).

3.1.2 FOXP3 expression

FOXP3 controls the function of thymically derived naturally occurring regulatory T cells (Hori and Sakaguchi, 2004, Fontenot et al., 2003), where it induces or represses a number of genes, including CXCR4.

This protein is known to be expressed at high levels in the nuclei of Treg cells and lymphoid tissue (thymus, spleen and lymph nodes) (Brunkow et al., 2001, Hori and Sakaguchi, 2004, Hori S, 2003). However, epithelial cells express this protein at lower levels in the nuclei and cytoplasm (Chen G, 2008). In the human, FOXP3 expression was detected in breast epithelial cells, lung respiratory epithelial cells, prostate epithelial cells, but not heart, and intestine (Chen G, 2008), In mice Foxp3 is expressed on epithelial cells of multiple organs (Chen G, 2008). Recent publications described the expression of FOXP3 in human tumour cells (Andrea Merlo and Elda Tagliabue, 2009, Zuo et al., 2007c, Wang L, 2009). It has been found that FOXP3 expression was related to the regulation of several cytokines, such as IL-10 and TGF-β2, and FOXP3 might mediate the inhibiting efficacy of tumour cells to escape immune destruction. Those reports implicated that FOXP3 performs its functions in the regulation of tumour progression by expressing not only in Tregs, but also in tumour cells.

Widespread confusion exists in literature regarding FOXP3 expression in human cancers. Some state that FOXP3 expression is increased in cancer (Merlo A, 2009, Gupta et al., 2007, Ohara et al., 2009), some suggest a decrease (Zuo et al., 2007d, Zuo et al., 2007b) in expression of this protein or change compared to benign epithelium (Zuo et al., 2007d, Zuo et al., 2007b).

Since FOXP3 is a transcription factor, majority of authors considered the presence of nuclear FOXP3 as positive (Liu et al., 2009, Wang L, 2009, Zuo et al., 2007d). Using this criterion, FOXP3 positive tumours usually account for less than 30% of all tumour samples. On the other hand, if both nuclear FOXP3 and cytoplasmic FOXP3 are counted, then the number of FOXP3-positive samples can be considerably higher. When total expression of FOXP3 in 25 tumour cell lines was analysed, including lung cancer, colon cancer, breast cancer, melanoma, erythroid leukemia, and acute T-cell leukemia, in most cell lines, the authors observed FOXP3 expression of both mRNA and its protein (Karanikas et al., 2008). Immunohistochemical staining of the cytospins of these cell lines showed cytoplasmic expression of FOXP3 predominantly in melanoma (GERL), colon (HCA 2.6), and breast cancer (MCF7) cell lines and both cytoplasmic and nuclear expression in cell lines of lung cancer (GILI) and T lymphoblastic leukemia (JURKAT) (Karanikas et al., 2008). More recently, it has been revealed that FOXP3 is only identified in the cytoplasm in all HER2 positive breast cancer samples (Ladoire et al., 2008, Martin et al., Ladoire et al.). The analysis of somatic FOXP3 mutants indicated that the majority of the missense mutations disrupted their nuclear localization (Wang L, 2009). Crucially, the cytoplasmic localization is best correlated with the loss of growth inhibition (Wang L, 2009). Furthermore, there is evidence that FOXP3 alternative splicing forms also can disrupt localization of FOXP3 (Ebert et al., 2008, Katoh et al., Krejsgaard et al., 2008, Sun et al., Zuo et al., 2007d).

3.2 Specific Aims and Objectives

- To examine expression of FOXP3 and CXCR4 in benign and malignant human breast tissue

- To explore the relationship between FOXP3 and CXCR4 expression with clinico-pathological factors of breast cancer

- To study CXCR4 protein expression in breast cancer cells

- To compare FOXP3 and CXCR4 expression at the mRNA level in breast cancer cells

3.3 Materials and Methods

3.3.1 Immunohistochemistry

3.3.1.1 Ethical Approval

An amendment to the Ethical Approval for collection of paraffin embedded breast tumour human tissue sections was submitted to the Northumberland Local Research Ethics Committee and to Newcastle-upon-Tyne Hospitals NHS Trust. Full approval was granted for the study "06/Q0906/12- Role of Chemokine/GAG Interactions in Breast Cancer Metastasis" (Appendix: ethical approval 231).

3.3.1.2 Principles

Immunohistochemistry utilises the specificity and high affinity of antibodies to detect antigens in tissue sections. Immunohistochemistry visualises antibody-antigen complexes within the tissue allowing the location of the antigen to be viewed in relation to tissue and cellular structure.

The principle technique used in this project was avidin-biotin complex (ABC) method. This method relies on the strong affinity of avidin or streptavidin for the vitamin biotin. In avidin-biotin complex (ABC) method secondary antibodies are conjugated to both and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex. The secondary antibody conjugated to biotin, which is detected with a streptavidin-biotin-HRP (horse radish peroxidase) complex (SABPx). The SABPx system improves the sensitivity of detection by amplifying the number of HRP molecules at the site of reaction by four-fold. HRP catalyses the conversion of H_2O_2 to water and oxygen. This oxidises soluble diaminobenzadine (DAB) or nickel-enhanced diaminobenzadine (NiDAB) producing an insoluble brown and black precipitate.

Endogenous avidin and biotin activity is commonly found in human tissues and therefore endogenous biotin binding sites must be blocked prior to incubation with antibodies (Sternberger and Sternberger 1986). Endogenous peroxidase activity was quenched using methanol containing 0.45% H_2O_2.

3.3.1.3 Antigen Retrieval

Antigen retrieval was performed to unmask antigens to improve antigen antibody interactions using one of the following two methods:

In Citrate Buffer retrieval method, the slides were immersed in preheated citrate buffer (0.1 M Tri sodium citrate and 0.1 M citric acid; pH 6.0) in a pressure cooker and exposed to full pressure for 1 min. The pressure cooker was then cooled under running tap water. In EDTA retrieval method EDTA buffer (EDTA 0.37; pH 9.0) was used instead of Citrate Buffer.

3.3.1.4 Technique

Paraffin embedded sections were dewaxed in xylene for 10 minutes then rehydrated by passing slides through decreasing ethanol concentrations of 100%, 97% and 95% for 1 minute in each. Endogenous peroxidase activity was blocked for 10 min with 0.45% H_2O_2. in methanol. After a wash under tap water, the antigen retrieval was performed. Slides were then treated using Avidin/Biotin blocking kit for 15 minutes. After washing with TBS, pH 7.6, sections were covered in blocking serum for 20 minutes and optimisation of the antibody was performed using a range of concentrations of primary antibody. For CXCR4: 1:50, 1:100, 1:200, 1:300, 1:400 dilutions were evaluated. Whereas for Foxp3: 1:5, 1:10, 1:50. Incubation with the primary antibody was over night at 4^oC. Subsequently, sections were washed in two changes of TBS for 5 minutes per wash, then covered in biotinylated secondary antibody (DAKO Rabbit anti-mouse; dilution: 1:250) for 60 minutes and washed as before. Sections were covered in tertiary reagent (Avidin/Biotin peroxidase complex) for 30 minutes and washed. Colour was developed using DAB or NiDAB solution for 2-3 minutes and sections were washed afterwards for 5 minutes in running tap water. Counterstaining with Haematoxylin was performed to reveal the tissue architecture. Slides were dehydrated through a series of alcohol concentrations (50, 70, 90, 95% and absolute alcohol), cleared in xylene and mounted using DPX (Dibutylphthalate xylene).

3.3.1.5 Tissue sections

All tissue samples were collected during breast cancer surgery at the Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Trust, UK. Staining was performed on 4 μm thick serial sections of (10% neutral buffered) formalin fixed, paraffin embedded breast tissue specimens.

To estimate the statistically significant number of sections to be used in the study, a pilot study of 10 sections was performed. A power calculation based on the pilot study of 10 whole tissue sections led to selection of 100 breast tissue sections with at least 5 sections in each analysed group to determine differences in FOXP3 and CXCR4 staining patterns. In total, 100 fixed sections were examined in total with each antibody. They were: 21 normal breast, 5 carcinoma *in situ*, 37 infiltrating carcinoma, 30 corresponding normal lymph nodes and 7 corresponding metastatic lymph nodes.

3.3.1.6 Preparation of paraffin blocks from pelleted cells

Cells were grown until 70-80% confluent, detached and resuspended in 10% phosphate-buffered formalin at room temperature, fixed cells were then transferred to tissue cassettes and processed into paraffin blocks using standard tissue processing. Paraffin blocks were prepared by the Department of Histopathology (RVI, Newcastle upon Tyne).

3.3.1.7 Controls

For immunohistochemical staining positive and negative controls were used. Positive controls for FOXP3 were normal human lymph nodes and HEK293 FOXP3 transfectants; whereas for CXCR4 normal human lymph nodes and tonsils were used. Negative controls included: no primary antibody, no primary and no secondary antibody, IgG1 control for Foxp3 and IgG2b for CXCR4.

3.3.1.8 Antibodies

Two anti-FOXP3 and one anti-CXCR4 antibodies were used:

Non-commercial monoclonal mouse anti-human Foxp3 antibody (clone 150D/E4; isotype Ig1) is a cell culture supernatant and was a kind gift from Dr. Alison H. Banham, University of Oxford. It was diluted 1:5 in blocking buffer and incubated over night at 4°C.

Monoclonal mouse anti-human Foxp3 antibody (clone 326A/E7; isotype IgG1) was purchased from Abcam. Previously, it has been successfully used for staining prostate (Wang L, 209) and breast cancer tissues (Wang L, 209). The antibody was diluted 1:50 in blocking buffer and incubated over night at 4°C.

Monoclonal mouse anti-human CXCR4 antibody (clone: 44716, isotype IgG2b) was purchased from R&D systems. It was diluted 1:300 in blocking buffer and incubated over night at 4°C.

3.3.2 Immunocytochemistry

Cells were grown on chamber slides (90000 cells/chamber). These slides were fixed with 4% paraformaldehyde for 30 minutes, then permeabilized using 0.1% Triton X100 in PBS for 15 minutes at 4°C and blocked in 5% fetal bovine serum (FBS) for 1 hour. Primary antibody was added to chambers and left overnight at 4°C at a concentration of 1:5 (Foxp3) and 1:300 (CXCR4) diluted in 5% FBS. Slides were then covered with rabbit anti-mouse biotinylated secondary antibody (DAKO) at concentration 1:100. The procedure for visualisation was performed as described in immunohistochemistry.

3.3.3 Manual microscopy and IHC interpretation

The samples were analysed by two independent observers, one of whom was a clinical consultant breast pathologist, Dr Uta Kerlikowski (Department of Histopathology, RVI, Newcastle upon Tyne Hospitals NHS Trust).

3.3.3.1 Quick Score algorithm

The Quick Score pathological algorithm is a sum of the percentage of positively stained cells and the average staining intensity. It was adapted from the "quick score steroid receptors quantification in breast cancer" which is routinely used in

the Department of Histopathology in Newcastle Hospitals for assessment of hormonal receptors in breast cancer (RCOP Publication, 2005). Each section was manually scored for both the percentage of positively stained cells (from 0 to 5) and the average staining intensity of the stained cells within the sample (from 0 to 3). The Quick Score was calculated by adding the scores for both the percentage of positive cells and the staining intensity. On each section, multiple relevant regions were examined and the overall Quick Score was expressed as Mean Quick Score (MQS). Nuclear and cytoplasmic staining were scored separately for the Foxp3 antibody. Only membrane staining was considered for the CXCR4 antibody. Kappa statistics demonstrated fair strength (kappa=0.29) of agreement between the two independent assessors.

3.3.4 Digital microscopy and automated IHC image analysis

Clinical Histology and Pathology practice is undergoing a "digital revolution" (Staniszewski, 2009). In order to improve reproducibility and standardization of interpretation, IHC quantification methods have been developed. Instead of classical light microscopes, virtual microscope and onscreen diagnosis is now practiced worldwide (Khramtsov et al., 2009, Rojo et al., 2009, Bruch et al., 2009, Soenksen, 2009b, Soenksen, 2009a, Dore et al., 2009, Staniszewski, 2009, Slodkowska et al., 2009, Rexhepaj et al., 2008). Currently, there are more than 30 different systems for the Virtual Microscopy available on the market. However, none of them is as clinically established as the Aperio ScanScope system (Staniszewski, 2009). This system improves accuracy, objectivity and reproducibility for the quantification of biomarkers on tissue specimens (Rexhepaj et al., 2008, Staniszewski, 2009). The Aperio ScanScope system was used in this study for automated analysis of breast tissue immunohistochemistry. The machine and ScanScope software were kindly provided by Dr Steven Darby from Northern Institute of Cancer Research, Newcastle University.

Prior to Aperio analysis a qualified pathologist, Dr Uta Kerlikowski, outlined a set of tumour-cell only regions that are representative of the tumour. Digital images of

breast tissue were captured with the Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, CA, USA) using brightfield imaging at x20 magnification. Specimen areas were selected and individual images were saved in a 24-bit RGB TIFF file format with a resolution of 1 μm/pixel using ImageScope software (Aperio Technologies). For each of the digital slides, the areas previously selected by the pathologist, were digitally marked as regions of interest (ROI). On average, 8-10 ROIs per digital slide were analysed. The automated analysis of the TIFF image files was performed using Aperio image analysis algorithms, which were modified for the purpose of this study (Figure 3-1).

3.3.4.1 IHC Nuclear algorithm

The IHC nuclear algorithm was used to analyse FOXP3 nuclear staining. The software counted DAB negative and DAB positive nuclei and categorized them into four levels of intensity (0, 1+, 2+ or 3+). The algorithm was adjusted to segregate nuclei of specific size and shape for different cell types (Figure 3-2).

3.3.4.2 IHC Positive Pixel Count algorithm

The IHC Positive Pixel Count algorithm was used to measure the area and intensity of FOXP3 staining (positive DAB staining) in the cytoplasm. The software categorized positive pixels into four levels of intensity (0, 1+, 2+ or 3+) and counted the area occupied by each. The "negative marking pen" was used to subtract the nuclei from the image (Figure 3-3).

3.3.4.3 IHC Membrane algorithm

Originally, the IHC membrane algorithm was developed to test HER2 (Human Epidermal growth factor Receptor 2) IHC in invasive breast cancer (Bloom and Harrington, 2004). For the purpose of this project it was adapted to analyse IHC CXCR4 membrane staining. The algorithm quantified the intensity of staining into four levels of intensity (0,1+, 2+ or 3+) and percentage of stained cells (Figure 3-4).

3.3.4.4 Digital Quick Score

The Quick Score pathological algorithm is a sum of the percentage of positively stained cells and the average staining intensity. The Digital Quick Score algorithm was calculated in the same way as for the manual analysis. The sum of the digitally quantified percentage of positively stained cells (from 0 to 5) and the digitally determined average staining intensity (0-blue, 1-yellow, 2-orange, 3-red) gave a digital Quick Score. The nuclear IHC algorithm was used for calculation of the digital Quick Score of FOXP3 nuclear staining, the IHC Positive Pixel Count algorithm for the digital Quick Score of FOXP3 cytoplasmic staining and the IHC Membrane algorithm for the digital Quick Score of CXCR4 membrane staining (Table 3-1).

For each of the digital slides 8-10 regions of interests were analysed and the overall digital Quick Score was expressed as a Mean Quick Score (MQS).

The inter-rater kappa was used to compare inter-observer agreement in FOXP3 and CXCR4 immunohistochemical scoring for manual and digital microscopy interpretation. Significant improvements in inter-observer agreement (kappa = 0.29 versus 0.86 and 0.71 respectively; both: $P < 0.001$) were achieved when FOXP3 and CXCR4 immunohistochemical analysis were scored with the assistance of the digital microscope.

3.3.5 The relationship of IHC results with pathological prognostic indicators

The relationship between IHC analysis with pathological prognostic indicators of breast carcinoma (tumour size, grade, type, lymph node status, distant metastasis, oestrogen and progesterone receptors status) and the presence of early signs of metastasis (vascular and perineural invasion) was determined by an independent observer. The Mermaid Computer Patients Database was used for this purpose and it was kindly made available by RVI, Newcastle upon Tyne Hospital. Table 3-2 illustrates the clinico-pathological characteristic of the patients analysed in this study. Distribution of the pathology in this study corresponds to the distribution of breast cancer types in the general population.

Score	A. Proportion of stained cells	B. Staining intensity			
		Manual	Digital		
			Nuclear Algorithm	Positive Pixel Count Algorithm	Membrane Algorithm
0	No staining	**Negative** No staining at high magnification			
1	<1%	**Weak** Only visible at high magnification			
2	1–10%	**Moderate** Readily visible at low magnification			
3	11–33%	**Strong** Strikingly positive at low magnification			
4	34–66%	N/A	N/A	N/A	N/A
5	67–100%	N/A	N/A	N/A	N/A

Table 3-1: Calculation of Quick Score algorithm

A) Each section was manually or digitally scored for the percentage of positively stained cells (0: no staining, 1: <1%, 2: 1-10%, 3: 11-33%, 4: 34-66%, 5: 67-100%).

B) The average staining intensity of the stained cells within the sample was determined by manual microscopy (0: negative, 1: weak, 2: moderate, 3: strong) or digital image analysis (0-blue, 1-yellow, 2-orange, 3-red). N/A – not applicable.

Quick Score is a sum of the percentage of positively stained cells (0-5) and the average staining intensity (0-3). It was adopted from "quick score steroid receptors quantification in breast cancer" routinely used in NHS for assessment of hormonal receptors in breast cancer (RCOP Publication, 2005).

	Number of Patients
Age	
Age ≤48	10
Age >48	53
Tumour Stage (pTNM)	
Stage I	5
Stage II	22
Stage III	7
Stage IV	3
Histological Type	
Ductal carcinoma in situ (DCIS)	4
Lobular carcinoma in situ (LCIS)	1
Infiltrating ductal carcinoma	24
Infiltrating lobular carcinoma	13
Tumour Grade	
1	8
2	20
3	9
Lymph Node Metastasis	
Negative (LN-)	30
Positive (LN+)	7
Steroid Receptors	
Estrogen receptor negative (ER-)	6
Estrogen receptor positive (ER+)	11
Progesterone receptor negative (PR-)	6
Progesterone receptor positive (PR+)	11
HER-2 Status	
HER-2 negative	14
HER-2 positive	3
Unknown	20
Benign breast tissue	21

Table 3-2: Clinico-pathological characteristic of the patients. Age 48 was chosen as a cut off in NHS Breast Screening Programme.

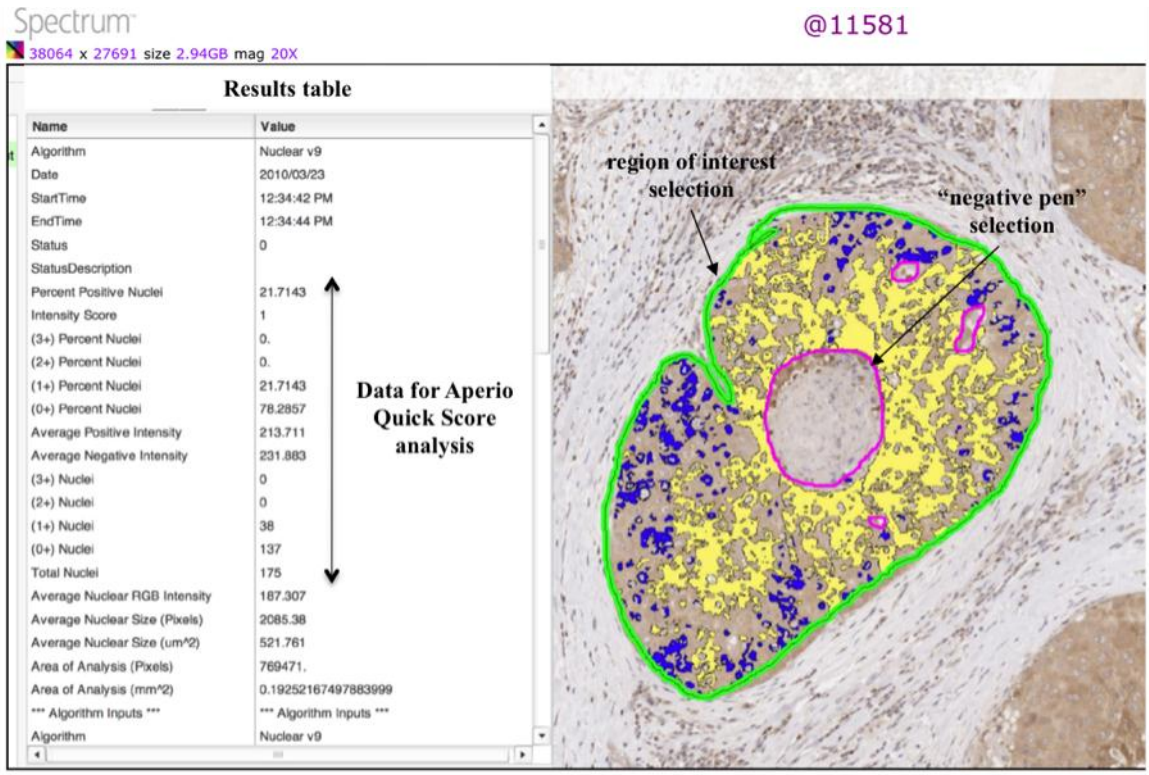


Figure 3-1: The representative example of an image processed using the IHC Nuclear algorithm

A qualified pathologist outlined a set of tumour-cell only regions and "the negative pen" was used to exclude irrelevant areas; the appropriate algorithm (here IHC Nuclear) analysed the selection. The software provided a table with results including: percentage of positive nuclei, average staining intensity of positive nuclei and percentages of 0, 1+, 2+, 3+.

In the image above, the Quick Score was calculated by adding digitally quantified percentage of positively stained nuclei (from 0 to 5) and digitally determined average staining intensity of positively stained nuclei (from 0 to 3): 38 out of 175 nuclei stained =21.7% nuclei positive for FOXP3 (score 2), the average staining intensity was weak (score 1). Digital Quick Score =2+1=3. For each of the digital slides 8-10 regions of interests were analysed and the overall digital Quick Score was expressed as Mean Quick Score (MQS).

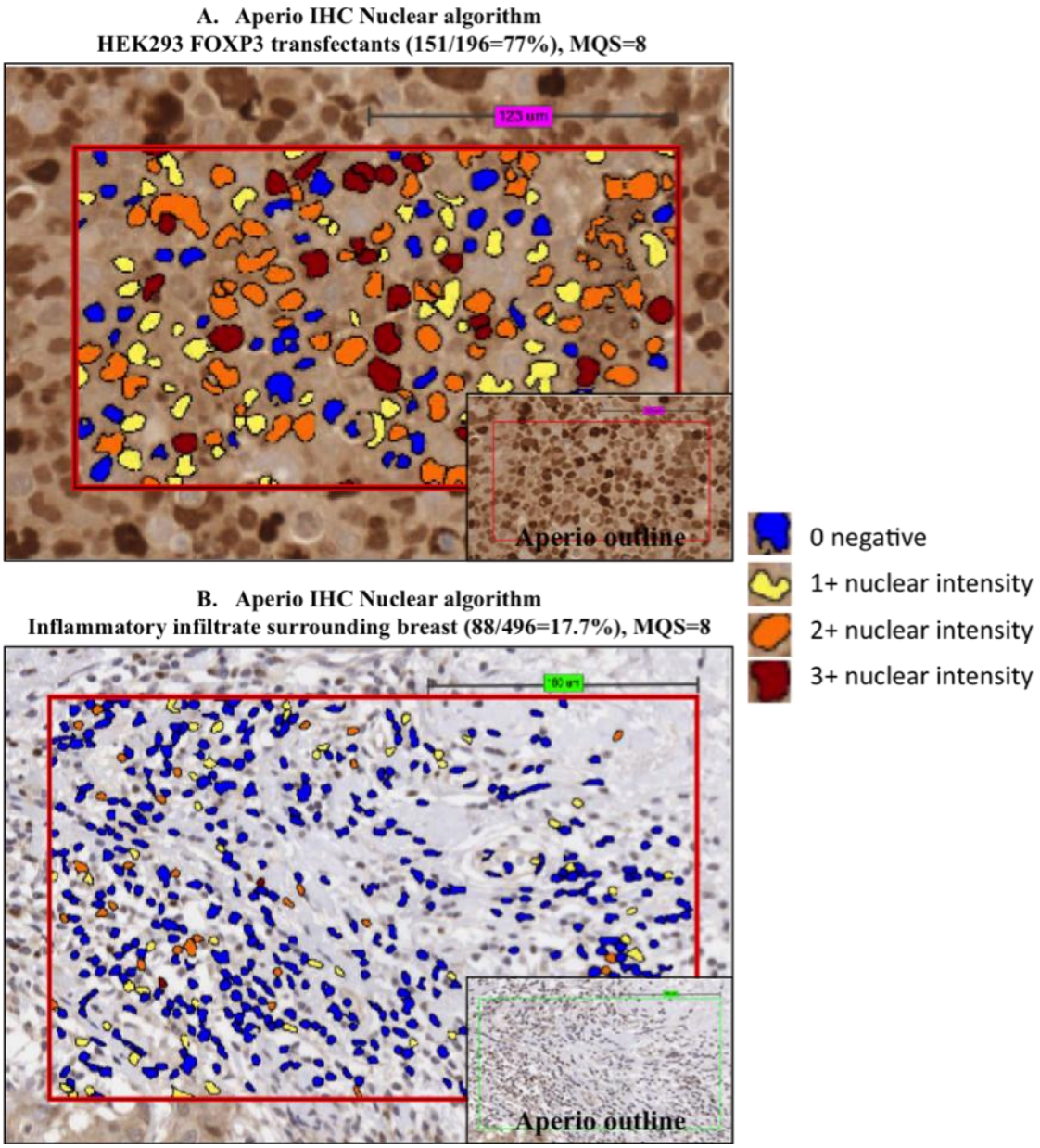


Figure 3-2: IHC Nuclear algorithm

A. HEK293 FOXP3 transfectants, B. Inflammatory infiltrate surrounding breast. The IHC Nuclear algorithm was used to analyse FOXP3 nuclear staining. It counted DAB negative and DAB positive nuclei, categorized them into three levels of intensity (0,1+, 2+ or 3+). It also allowed counting lymphocyte numbers per unit area of tissue across the section to quantify a degree of infiltration. The Nuclear IHC algorithm was used for calculation of digital Quick Score of FOXP3 nuclear staining.

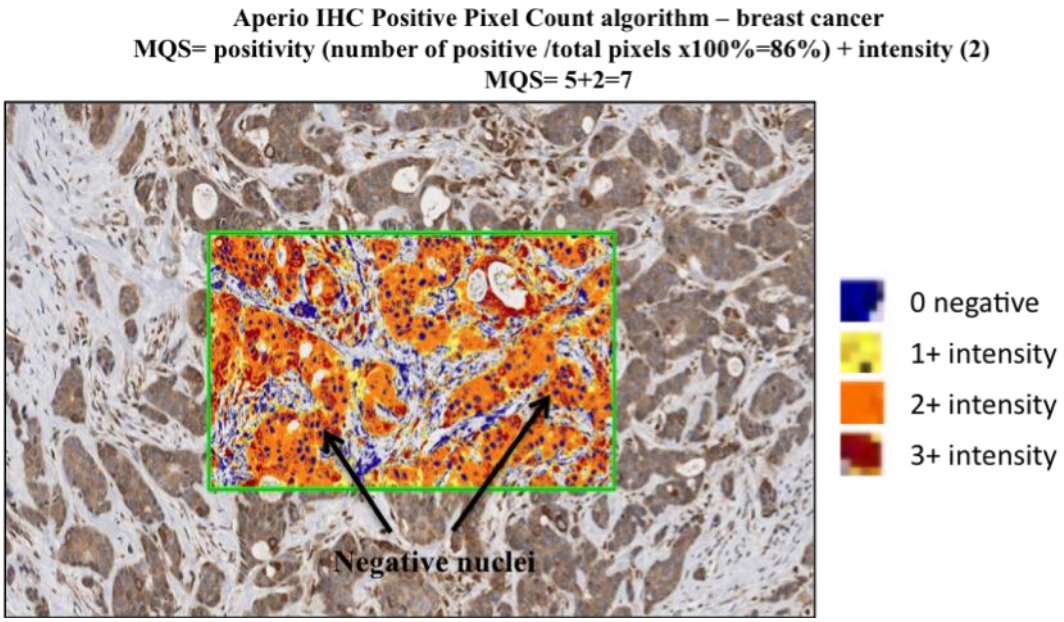


Figure 3-3: IHC Positive Pixel Count algorithm

A breast cancer section was analysed using the IHC positive pixel count algorithm to quantify FOXP3 cytoplasmic stain. The area and intensity of FOXP3 staining (positive DAB staining) was measured in the cytoplasm, positive pixels were categorised into four levels of intensity (0, 1+, 2+ or 3+) and the area occupied by each was assessed. The digital Quick Scores of FOXP3 cytoplasmic staining was then calculated. The IHC positive pixel count algorithm was useful in evaluating breast tissue IHC in particular if there is background or nonspecific DAB staining as Aperio software subtracted the background. Nuclei were also subtracted from the image (arrows).

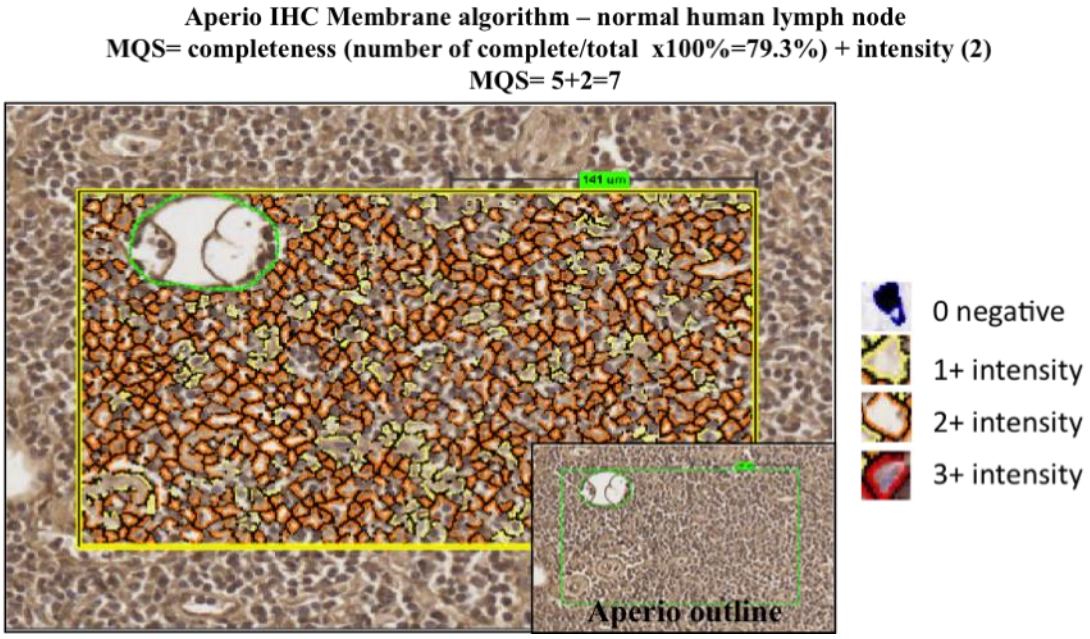


Figure 3-4: IHC Membrane algorithm

Normal human lymph node was analysed using the IHC Membrane algorithm to quantify CXCR4 membrane stain. The algorithm quantified the intensity of staining into four levels of intensity (0,1+, 2+ or 3+) and percentage of stained cells. The digital Quick Scores of CXCR4 membrane staining was then calculated.

3.4 Results

3.4.1 Manual microscopy of FOXP3 and CXCR4 IHC in human breast tissue

In order to assess expression of FOXP3 and CXCR4 by breast cancer at a tissue level, 100 paraffin-embedded sections of human breast tissue were examined (21 normal breast, 5 carcinoma in situ, 37 infiltrating carcinoma, 30 normal lymph nodes and 7 metastatic lymph nodes).

Two anti-Foxp3 antibodies were tested on the same section of ductal breast carcinoma in order to identify the one, which gives stronger IHC staining in paraffin-embedded breast tissue (Figure 3-5) The result of IHC staining using both antibodies was comparable. However, the monoclonal mouse anti-human Foxp3 antibody (clone 150D/E4; isotype Ig1, a cell culture supernatant) (A) had higher staining intensity and was chosen for further experiments. IHC was performed using a selected anti-Foxp3 antibody (Figure 3-5). FOXP3 expression has been previously established by Flp-In™ T-Rex™ -293 cells (Pekalski, 2009) which were used for positive control, demonstrating strong nuclear staining (Figure 3-6 A). Benign mammary epithelium (normal cells) had moderate to strong nuclear and cytoplasmic FOXP3 expression (Figure 3-6 D) while breast cancer cells had no or minimal nuclear staining and expression of FOXP3 transcription factor was observed mainly in the cytoplasm (Figure 3-6 F). FOXP3 positive T cells in the breast cancer infiltrate were provided as an internal positive control (Figure 3-6 C). Ductal carcinoma *in situ* (DCIS) expressed more FOXP3 in the cell nuclei than invasive cancers, but less than benign breast (Figure 3-6 E).

CXCR4 is predominantly a cell surface receptor, and IHC is likely to show cell surface expression with minimal cytoplasmic and perinuclear staining. Optimisation of the monoclonal anti-CXCR4 antibody (clone: 44716, isotype IgG2b, R&D) was carried out on human tissues (lymph node, tonsil, mammary epithelium) using various antibody dilutions (1:50, 1:100, 1:200, 1:300, 1:400) and antigen retrieval methods (no antigen retrieval, EDTA retrieval or citrate buffer retrieval). Dilution of the anti-CXCR4 antibody (1:250) without antigen retrieval

gave the best results. Positive control staining of normal human lymph node and tonsil demonstrated neat circumferential rings of CXCR4 around the cells (Figure 3-7 A and B). Benign breast epithelial cells had less CXCR4 expression than cancer (Figure 3-7). However, IHC staining of CXCR4 on breast cancer tissues had significant cytoplasmic staining and background or nonspecific DAB staining (Figure 3-7 E). Therefore, Aperio digital image analysis was used to subtract the background.

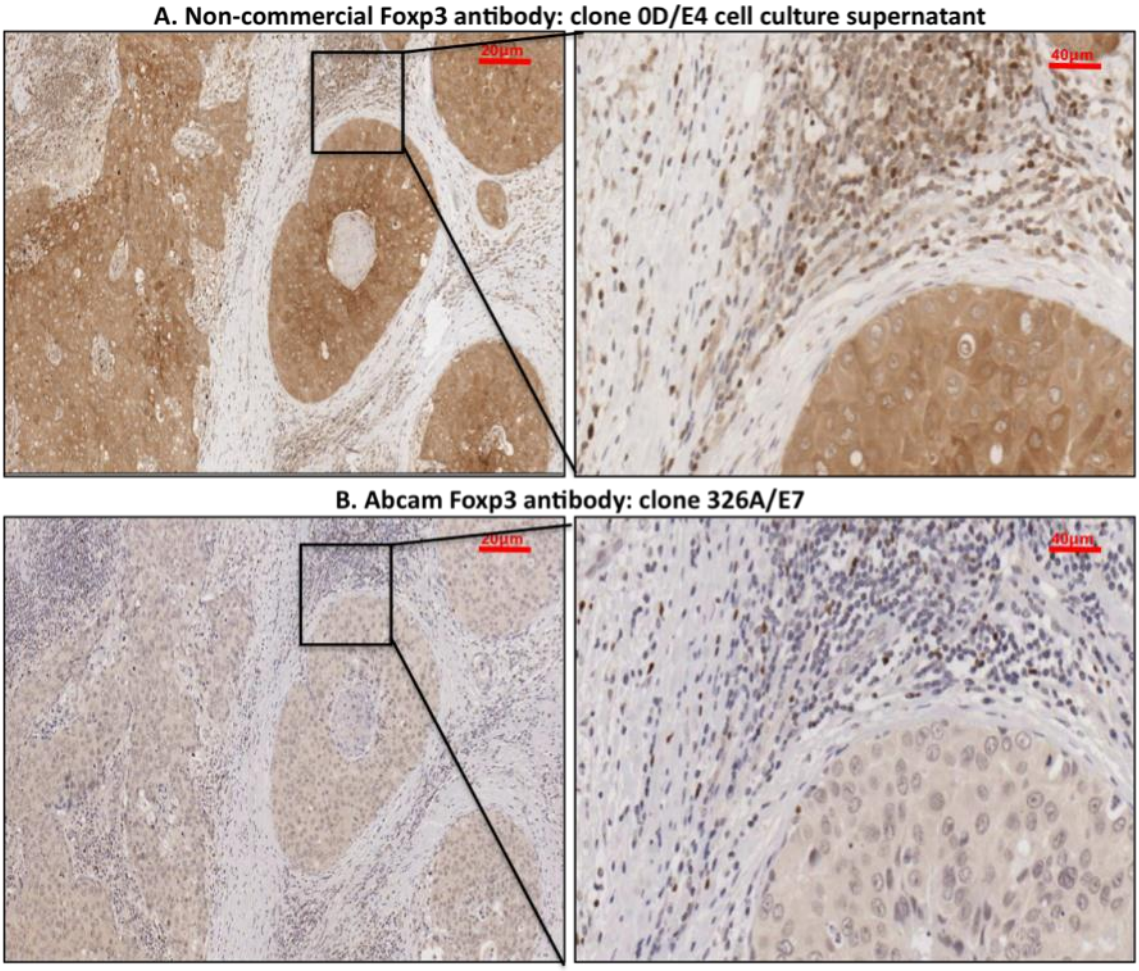


Figure 3-5: Selection of anti-Foxp3 antibody

To identify an anti-Foxp3 antibody giving the strongest staining in paraffin-embedded breast tissue, two anti-Foxp3 antibodies were tested on the same section of ductal breast carcinoma: anti-Foxp3 antibody (clone OD/E4 cell culture supernatant) at the optimum concentration of 1:5 in 20% of normal rabbit serum (A) and anti-Foxp3 antibody clone 326A/E7 from Abcam (B). The result of IHC staining using both antibodies were comparable. However, the monoclonal mouse anti-human Foxp3 antibody (clone 150D/E4; isotype Ig1, a cell culture supernatant) (A) had higher staining intensity and was chosen for further experiments. A section of invasive ductal carcinoma was used as negative control where no primary anti-Foxp3 antibody was used (Figure 3-6 B).

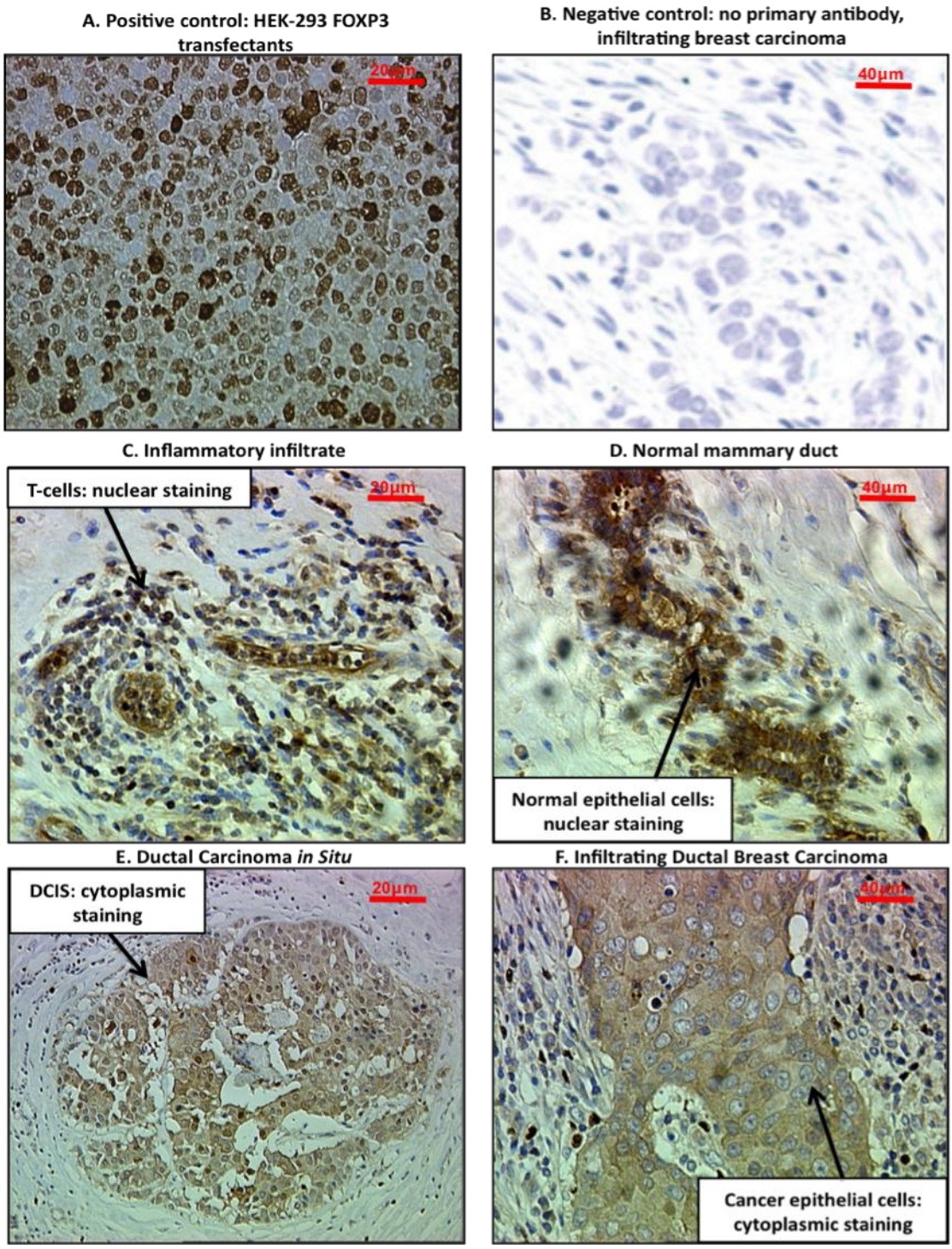


Figure 3-6: IHC manual microscopy of FOXP3 in breast tissue

A. Positive control: HEK-293 FOXP3 transfectants, B. negative control: no primary antibody, C. Internal positive control: FOXP3 positive cells in breast cancer inflammatory infiltrate, D. normal mammary duct, E. ductal carcinoma *in situ*, F. infiltrating ductal carcinoma. Tissue was stained by immunohistochemistry using monoclonal anti-Foxp3 antibody at the optimum concentration of 1:5 in 20% of normal rabbit serum.

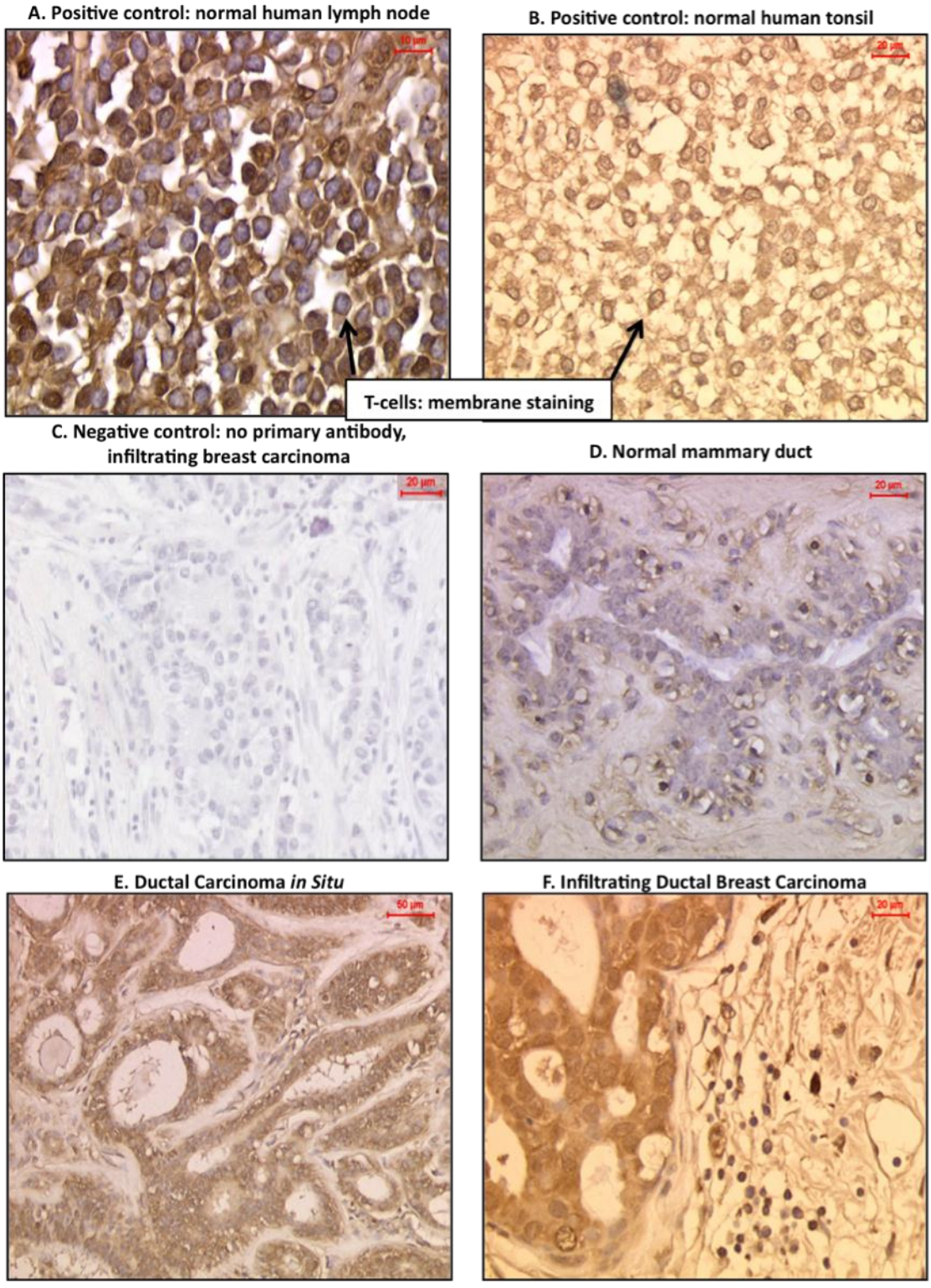


Figure 3-7: IHC manual microscopy of CXCR4 in breast tissue

A. Positive control: normal human lymph node, B. positive control: normal human tonsil, C. negative control: no primary antibody, D. normal mammary duct, E. ductal carcinoma *in situ*, F. infiltrating ductal carcinoma. Tissue was stained by immunohistochemistry using monoclonal anti-CXCR4 antibody (R&D) at the optimum concentration of 1.250 in 20% of normal rabbit serum. Benign (normal).

3.4.2 Digital microscopy of FOXP3 and CXCR4 IHC in human breast tissue

Digital microscopy and image analysis enhance accuracy and reliability of HER-2/neu IHC "quick scoring" (Bloom and Harrington, 2004). In order to validate the manual microscopy IHC interpretation and quantify FOXP3 and CXCR4 expression, Aperio digital image analysis was used.

To exclude any DAB non-specific binding or false positives in paraffin-embedded human breast tissues, no primary and no secondary antibody controls were used. Sections were then examined by digital image analysis platform demonstrating no nuclear or cytoplasmic FOXP3 and no CXCR4 membrane staining (Figure 3-8).

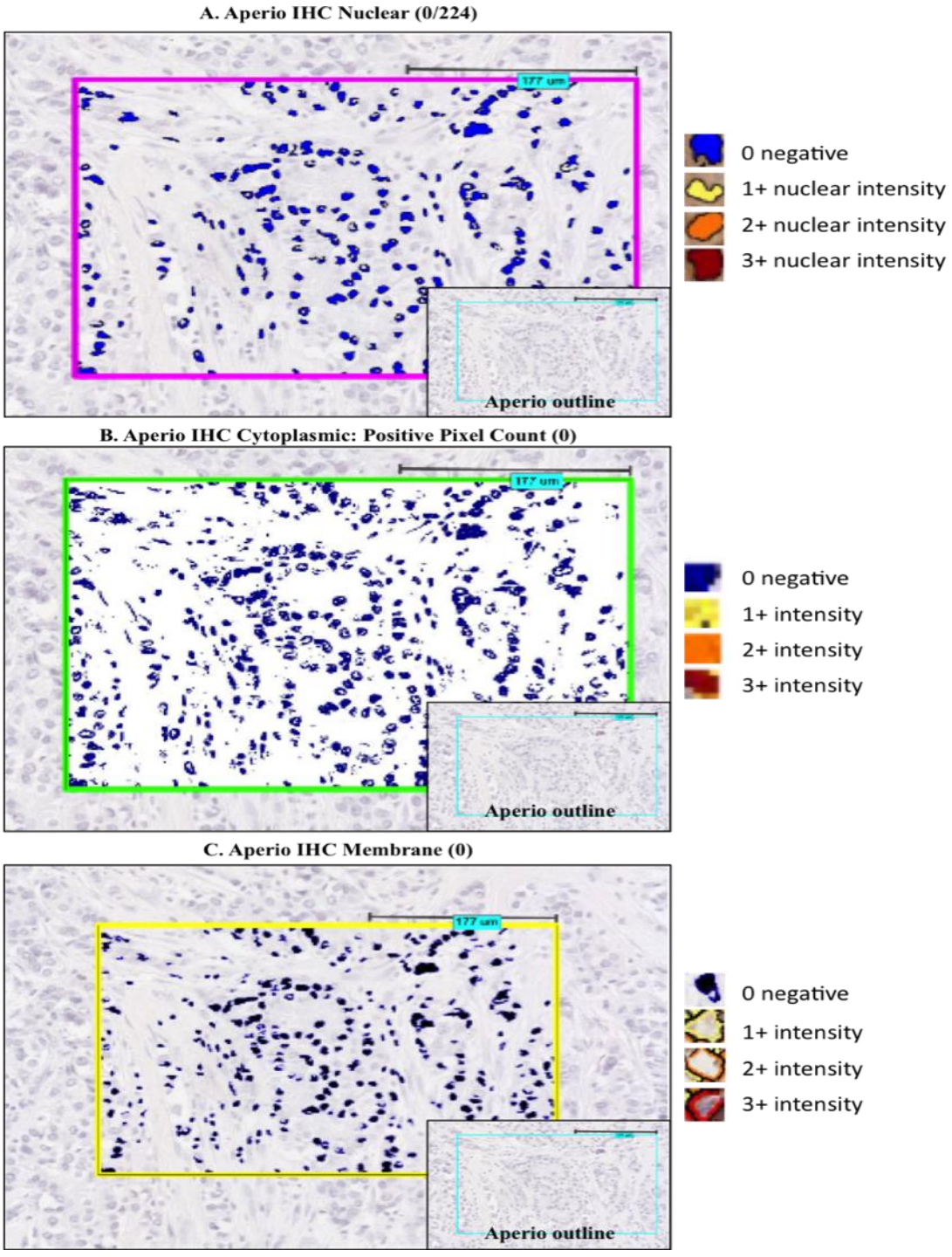


Figure 3-8: Digital image analysis: IHC FOXP3 and CXCR4 without primary and secondary antibody negative controls.

Sections of invasive ductal carcinoma were stained without primary and secondary antibodies. The ABC complex was incubated on the samples in the same way as usual. Aperio system was used to examine the section for nuclear and cytoplasmic FOXP3 and CXCR4 membrane expression. This experiment excluded any DAB non-specific binding or false positives.

FOXP3 expression was assessed in nuclei, cytoplasm and inflammatory infiltrate. No staining was shown when omitting the primary Foxp3 antibody (Figure 3-9 A top panel). Staining with IgG1 for Foxp3 antibody excluded the presence of non-specific antibody binding (Figure 3-9 A bottom panel). The nuclei of HEK293 FOXP3 transfectants and inflammatory infiltrate around breast cancer sections were positive for FOXP3 and were used as positive controls (Figure 3-9 B panel). Breast cancer epithelium had no, or minimal, nuclear staining (Figure 3-10 C) and expression of the FOXP3 transcription factor was observed mainly in the cytoplasm (Figure 3-11 C). However, where nuclear FOXP3 expression was observed, it was mainly confined to certain areas of the malignant epithelium where nuclear translocation was not lost (arrow Figure 3-10 C). Staining, in benign breast tissue cells, was heterogeneous: only cytoplasmic, nuclear and cytoplasmic or only nuclear (Figure 3-10 D and Figure 3-11 D). Cytoplasmic FOXP3 expression was similar for benign and malignant tissues (Figure 3-11).

Malignant epithelial cells, often forming ducts in a lymph node, provide evidence of metastatic disease (Figure 3-12 A). Seven metastatic lymph nodes were stained for FOXP3 expression and all analysed specimens expressed FOXP3. On average, nuclear staining (Figure 3-12 C, G) was negative or weak (score 0 or 1) and 0-10% (score 0-1) nuclei were stained. Above 80% (score 5) of cells expressed FOXP3 in the cytoplasm (Figure 3-12 D, H) and staining was weak to moderate (score 1 to 2).

FOXP3 positive cells of inflammatory infiltrate were used for internal positive control. Staining was nuclear. On average, 22% of cells within inflammatory infiltrate were stained (Figure 3-13 D). There was no difference between total numbers or percentages of FOXP3 positively stained cells in benign (Figure 3-13 B) and malignant (Figure 3-13 C) sections.

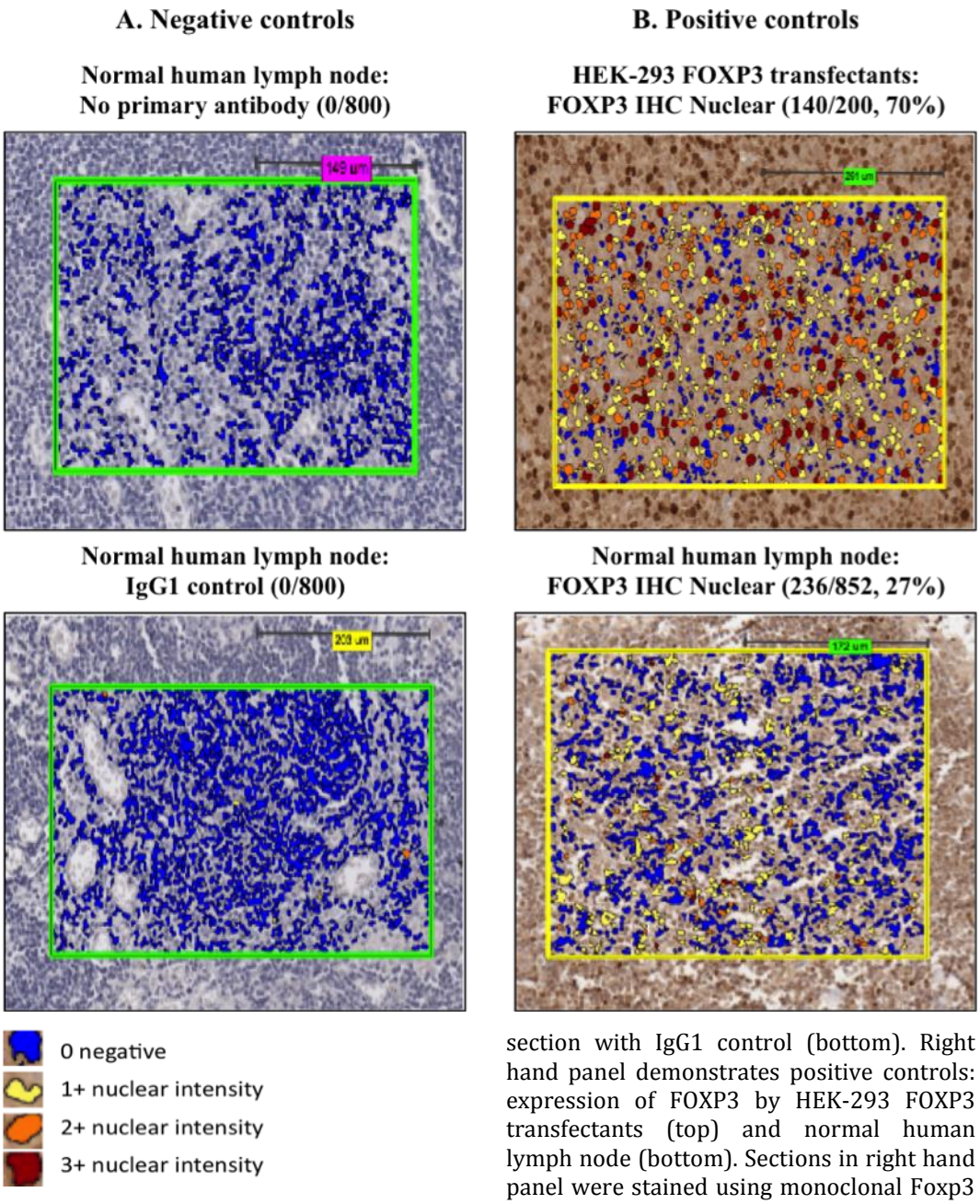


Figure 3-9: Digital image IHC nuclear analysis of controls

Panel A: FOXP3 negative control. Panel B: FOXP3 positive control. Left hand panel shows negative controls: normal human lymph node without the addition of primary anti-Foxp3 antibody (top) and the same section with IgG1 control (bottom). Right hand panel demonstrates positive controls: expression of FOXP3 by HEK-293 FOXP3 transfectants (top) and normal human lymph node (bottom). Sections in right hand panel were stained using monoclonal Foxp3 antibody (clone 0D/E4 cell culture supernatant) at the optimum concentration of 1:5 in 20% of normal rabbit serum. All sections were scanned and analysed using Aperio system.

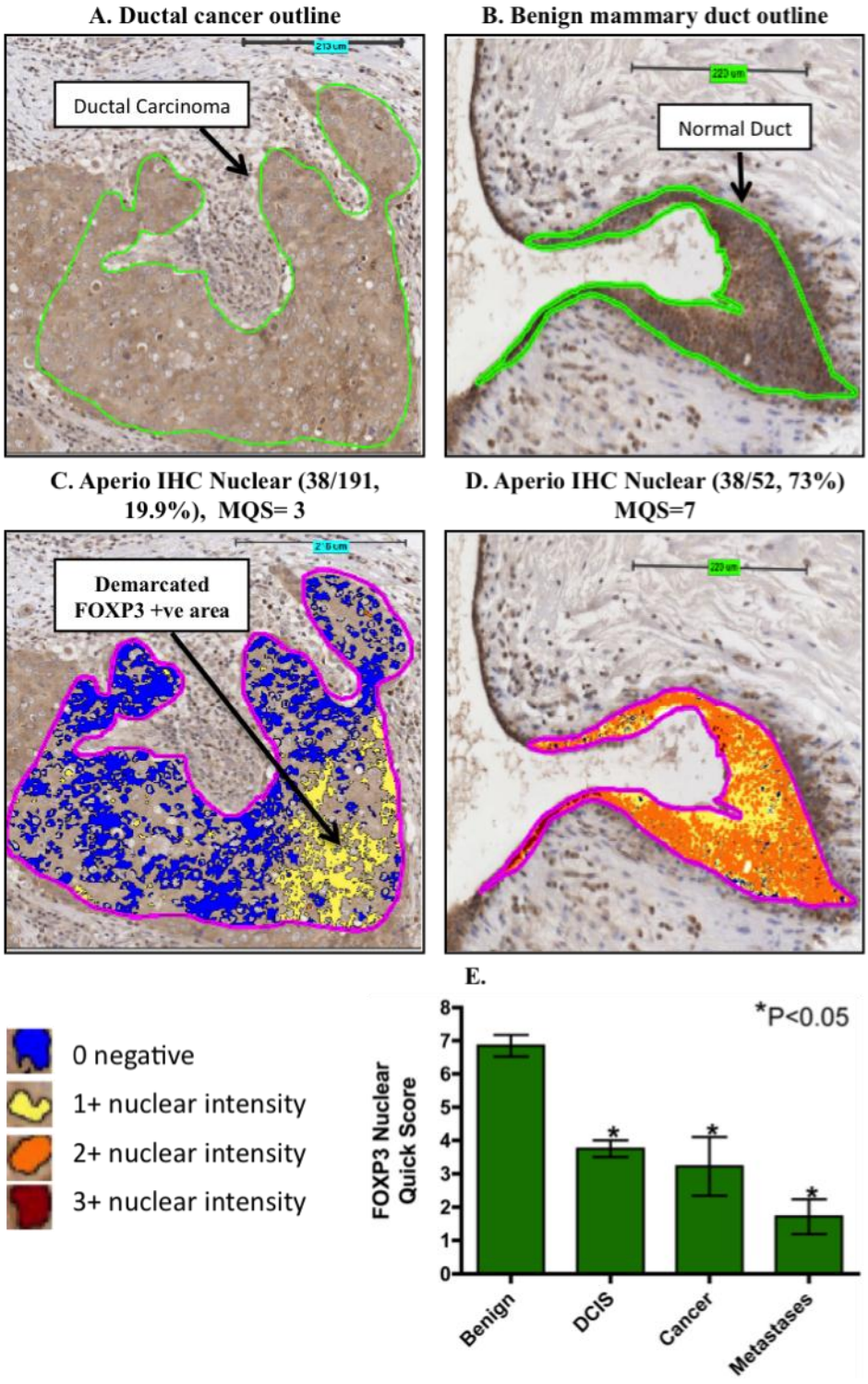


Figure 3-10: Digital image IHC nuclear analysis of human breast tissue.

A, C: FOXP3 in breast cancer. B, D: FOXP3 in benign breast tissue. A, B: Tissue sections were stained with monoclonal anti-Foxp3 antibody at the optimum concentration of 1:5 in 20% of normal rabbit serum and scanned using Aperio scanner. The digital images were analysed with the IHC nuclear algorithm and staining was automatically quantified. The quick scores were calculated and plotted (E). Error bars represent SD; the P value indicates the significance of the change from benign (normal) cells. The arrow (C) points to the nuclear FOXP3 staining in breast cancer epithelium whch were limited to certain areas (arrow).

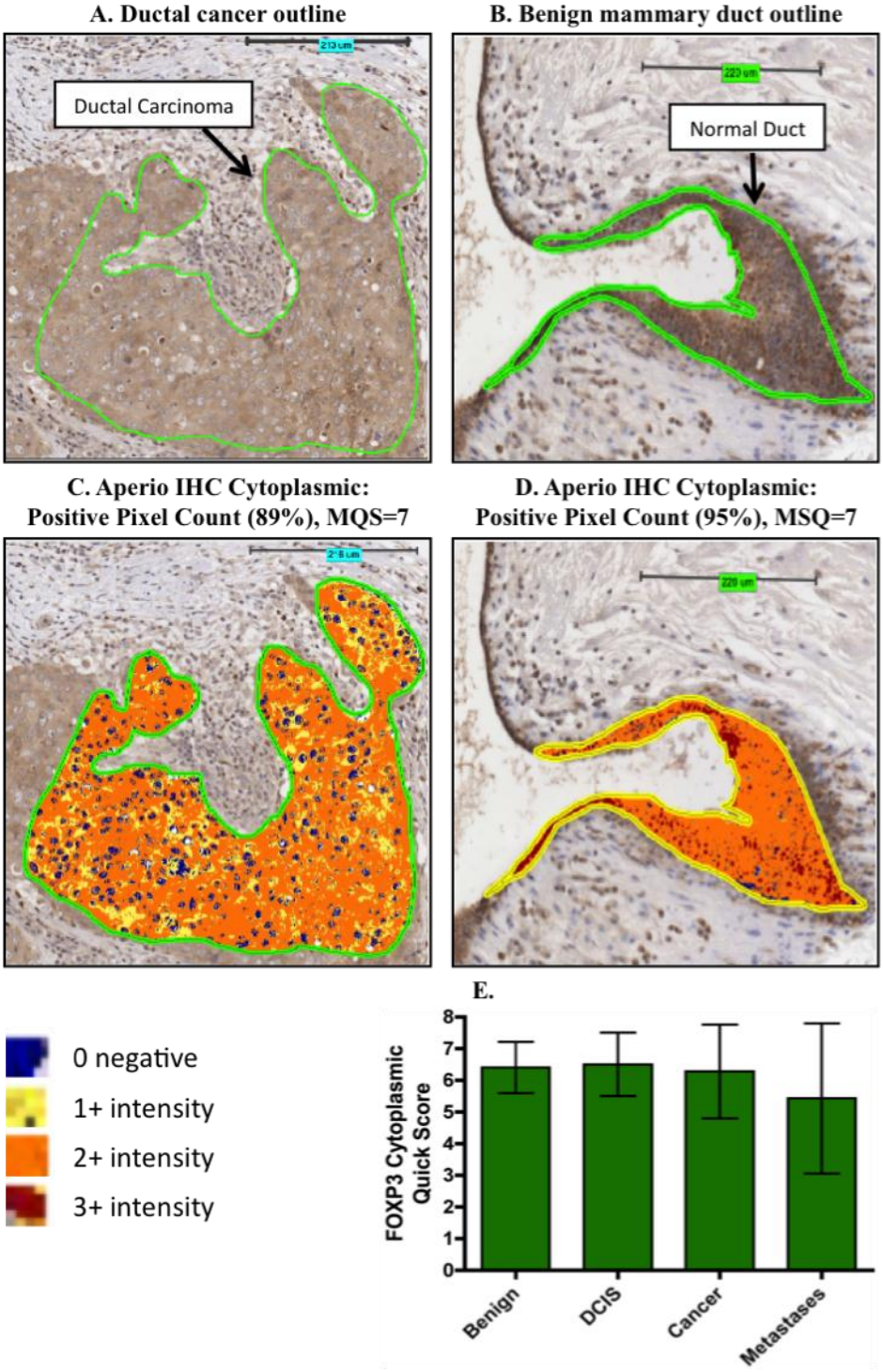
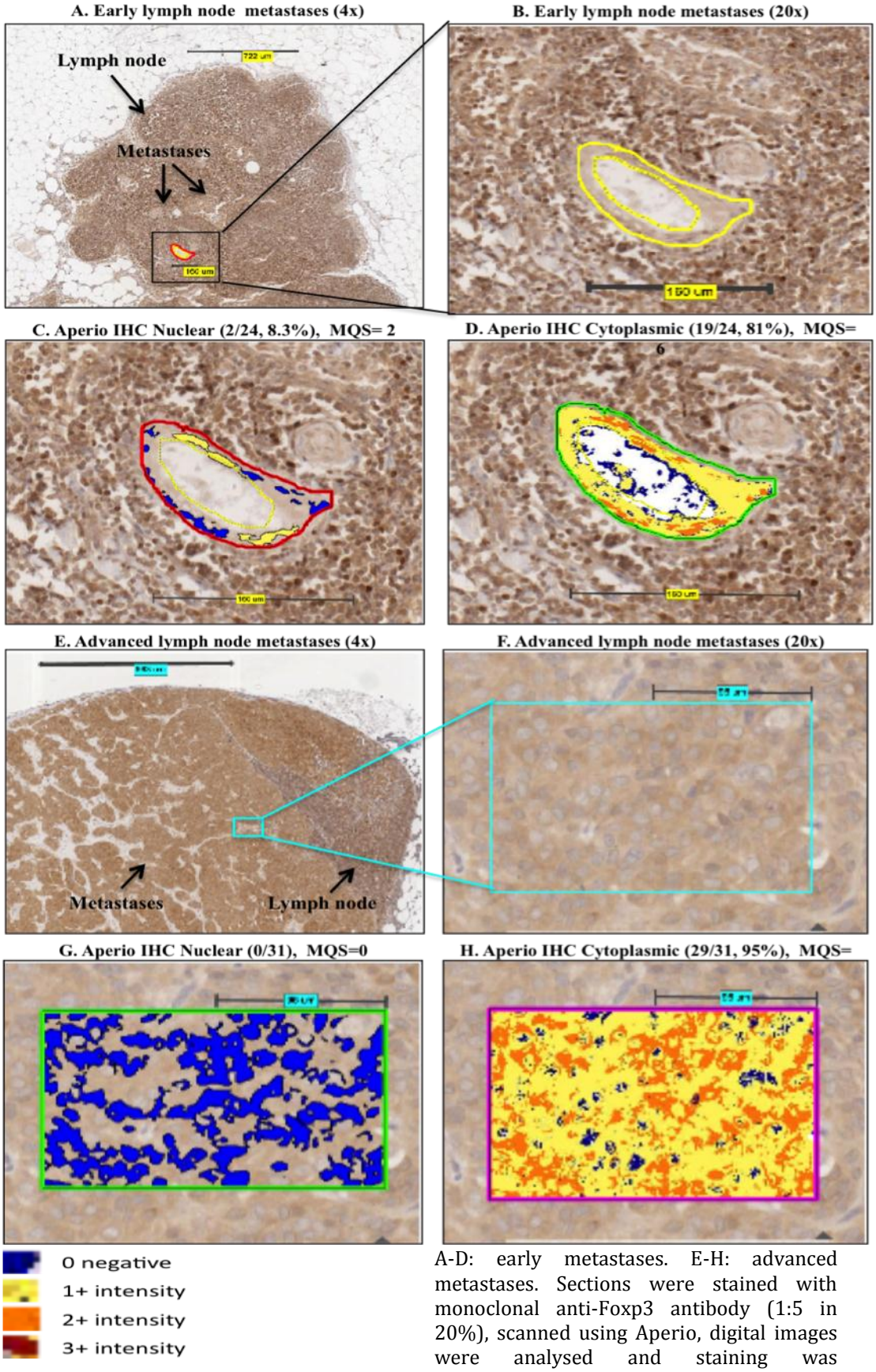


Figure 3-11: Digital image IHC cytoplasmic analysis of human breast tissue.

A, C: FOXP3 in malignant breast tissue. B, D: FOXP3 in benign breast tissue. Tissue sections were stained with monoclonal anti-Foxp3 antibody (clone 0D/E4 cell culture supernatant) at the optimum concentration of 1:5 in 20% of normal rabbit serum, scanned using Aperio digital scanner; the digital image was analysed with IHC positive pixel count algorithm and staining was automatically quantified. The quick scores were calculated and plotted (E). Error bars represent SD. Cytoplasmic FOXP3 expression was similar for benign and malignant tissues.



A-D: early metastases. E-H: advanced metastases. Sections were stained with monoclonal anti-Foxp3 antibody (1:5 in 20%), scanned using Aperio, digital images were analysed and staining was automatically quantified.

Figure 3-12: Digital image IHC analysis of lymph node metastases.

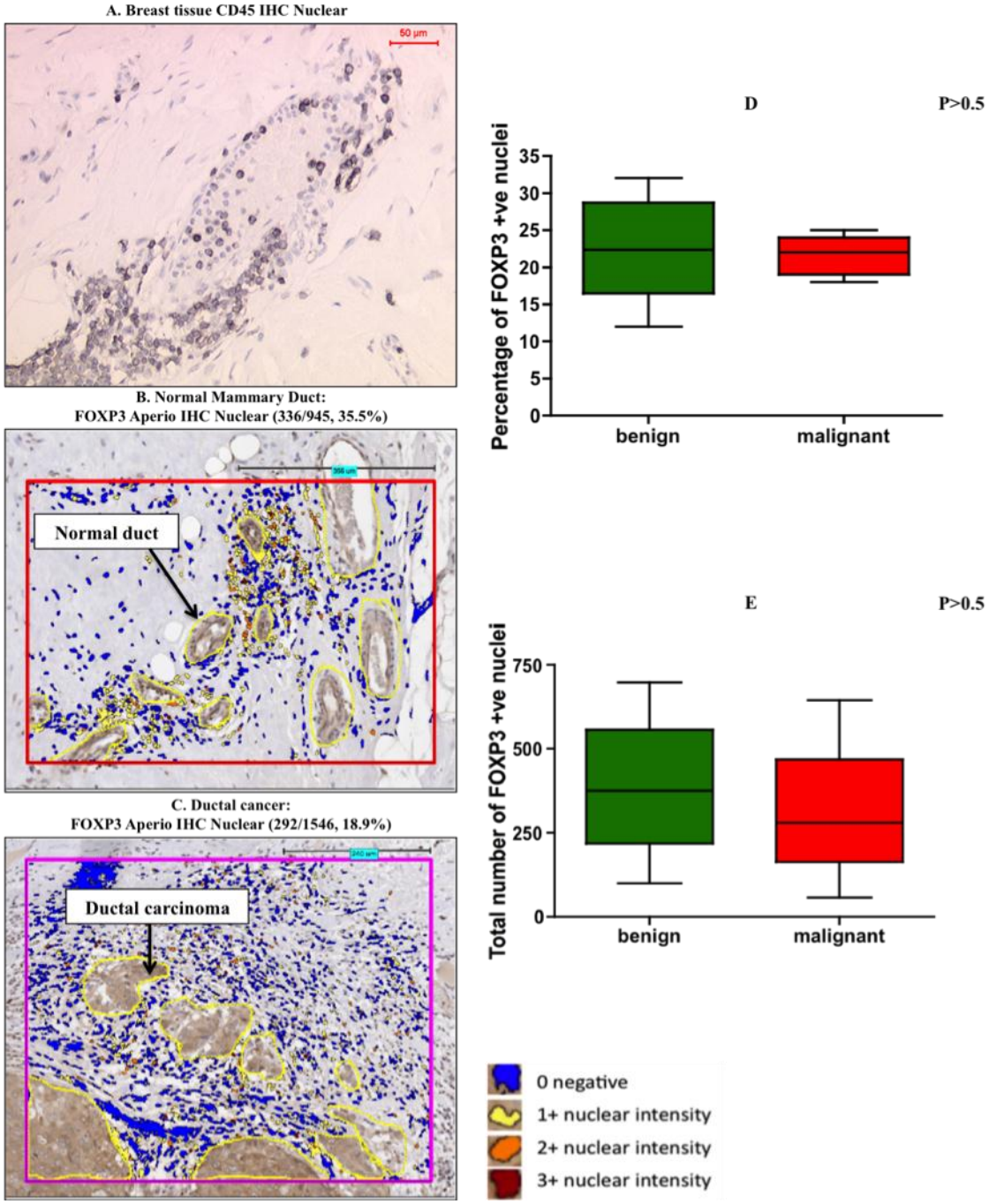


Figure 3-13: Digital image IHC nuclear analysis of FOXP3 in inflammatory cells in human breast tissue

A. Detection of leucocytes in the breast tissue, B. FOXP3 positive infiltrating cells in benign breast tissue, C. FOXP3 positive infiltrate in breast cancer, D. percentage of FOXP3 positive infiltrating cells, E. total number of FOXP3 positive infiltrating cells. Tissue sections were stained with monoclonal anti-Foxp3 antibody at the concentration of 1:5 in 20% of normal rabbit serum, scanned using Aperio digital scanner; the digital image was analysed with the IHC nuclear algorithm and staining was automatically quantified for percentage and total amount of FOXP3 positive nuclei. The results were plotted using box plots (D, E). The whiskers on the box plots represent SD; *P>0.05.

Tissue Type	Number	Percentage of stained sections	Nuclear MQS (% stained nuclei +average intensity)	Cytoplasmic MQS (% stained cytoplasm +average intensity)
Normal LN	30	62.5	4.3	-
Benign epithelium	21	90	6.8	6.4
DCIS	4	100	3.7	6.5
LCIS	1	100	3.1	6.3
Ductal Ca	24	55.55	3.2	6.2
Lobular Ca	13	100	2	3.3
Metastases	7	100	1.7	5.4

Table 3-3: Summary of the IHC FOXP3 results. The columns represent the total number of sections in each tissue type group, the overall percentage of stained sections in each group, nuclear and cytoplasmic MQS in each tissue type group.

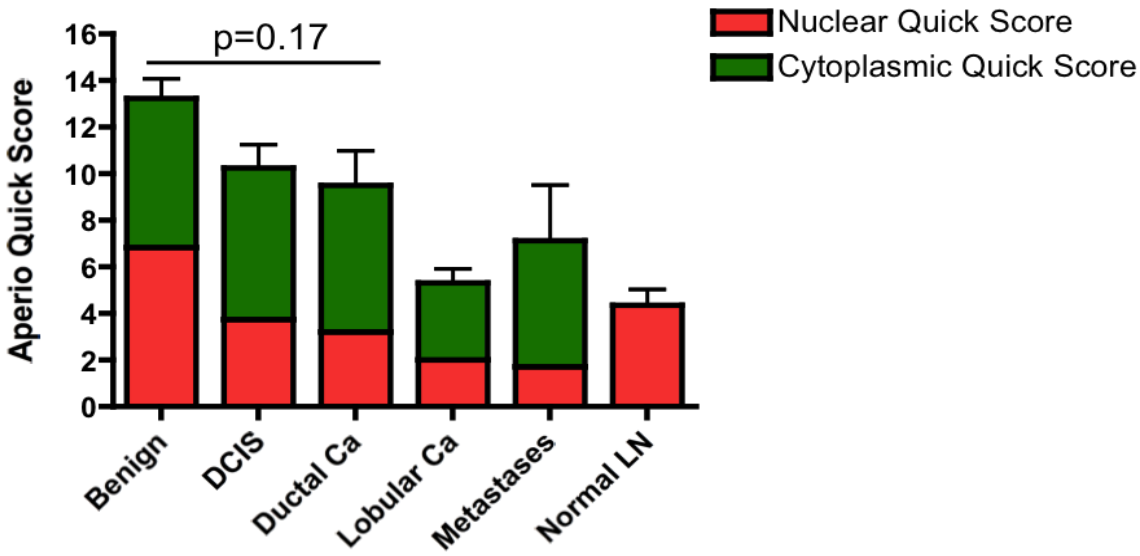


Figure 3-14: Graphic representation of FOXP3 expression in human breast tissue.

The MQS were calculated by digital microscopy: red bars represent the nuclear quick scores (0-8) stacked with green bars representing the cytoplasmic quick scores (1-8). Error bars correspond to SD.

Overall, 90% of benign and 77.77% of malignant breast samples expressed FOXP3. Out of these, about 70% of cancers and 100% of benign specimens expressed FOXP3 in the nuclei. Additionally, all samples expressed FOXP3 in the cytoplasm.

Total FOXP3 expression (nuclear and cytoplasmic) was no different ($p>0.05$) between cancer and benign breast tissue. However, when only nuclear staining was compared, there was a statistically significant difference between these two tissue types ($p<0.05$). Normal breast epithelial cells had significantly greater nuclear FOXP3 expression than invasive ductal carcinoma: digital median quick score (MQS) 6.8 versus (vs.) 3.2 ($p<0.05$) and lymph node metastases: MQS 6.8 vs. 1.7 ($p<0.05$) (Figure 3-14). Primary ductal cancers expressed more FOXP3 in their nuclei than lymph nodes secondaries: MQS 3.2 vs. 1.7 ($p<0.05$). The nuclei of ductal carcinoma *in situ* (DCIS) cells expressed FOXP3 similarly to invasive cancer cells: MQS 3.7 vs. 3.2 (Figure 3-14). On manual pathological assessment cancers showing early signs of metastasis (vascular and perineural invasion) expressed significantly less FOXP3 than tumours where vascular and perineural invasion was not observed (MQS 4 vs. 2). There was a trend towards decreasing nuclear FOXP3 expression with increase in tumour size although this was not statistically significant. There was no difference in nuclear FOXP3 expression between various cancer types ($p=0.41$). However, lobular carcinoma had generally weaker nuclear staining for FOXP3 than ductal carcinoma. Oestrogen and progesterone positivity was not correlated with nuclear FOXP3 expression ($p=0.51$, $p=0.36$ respectively). The median age of patients was 67 and linear regression analysis showed no relationship between nuclear quick score and age ($p=0.61$).

Cytoplasmic expression of FOXP3 was not significantly different between benign specimens (MQS=6.4), DCISs (MQS=6.5), ductal cancers (MQS=6.2) and lymph node metastases (MQS=5.2). However, in lobular carcinoma the cytoplasmic expression of FOXP3 was significantly ($p=0.03$) decreased (MSQ=3.3) compared to the benign breast epithelium.

There was no significant difference between total numbers or percentages of FOXP3 positively stained cells within inflammatory infiltrate surrounding normal ducts (Figure 3-13 B) and ductal cancers (Figure 3-12 C).

Aperio digital image analysis was performed in order to compare its results to the manual microscopy CXCR4 IHC and quantify CXCR4 expression. There was no staining when omitting primary anti-CXCR4 (Figure 3-15 A) antibody. Use of IgG2b isotype control for the anti-CXCR4 antibody excluded the presence of non-specific antibody binding (Figure 3-15B). Normal human lymph node was used as a positive control (Figure 3-15 C).

Normal breast epithelial cells expressed less CXCR4 than invasive cancer cells (Figure 3-15 F: cytoplasmic median quick score - MQS (3 versus (vs.) 6 ($p<0.05$) and lymph node metastatic cells: MQS 3 vs. 6.5 ($p<0.05$). Lymph node metastases expressed more CXCR4 than primary invasive cancers: MQS 8 vs. 5 ($p<0.05$). DCIS expressed more CXCR4 than normal breast epithelial cells but it was not significant ($p=0.6$). Prognostic indicators of breast carcinoma like lymph node status and distant metastasis were associated with increased CXCR4 expression ($p<0.05$). Membrane CXCR4 expression was significantly correlated with early signs of metastases (vascular and neural invasion) and established lymph nodes metastasis when analysed manually. Linear regression analysis did not show statistically significant correlation between membrane CXCR4 expression and the type or grade of tumours, receptor status or patients' age.

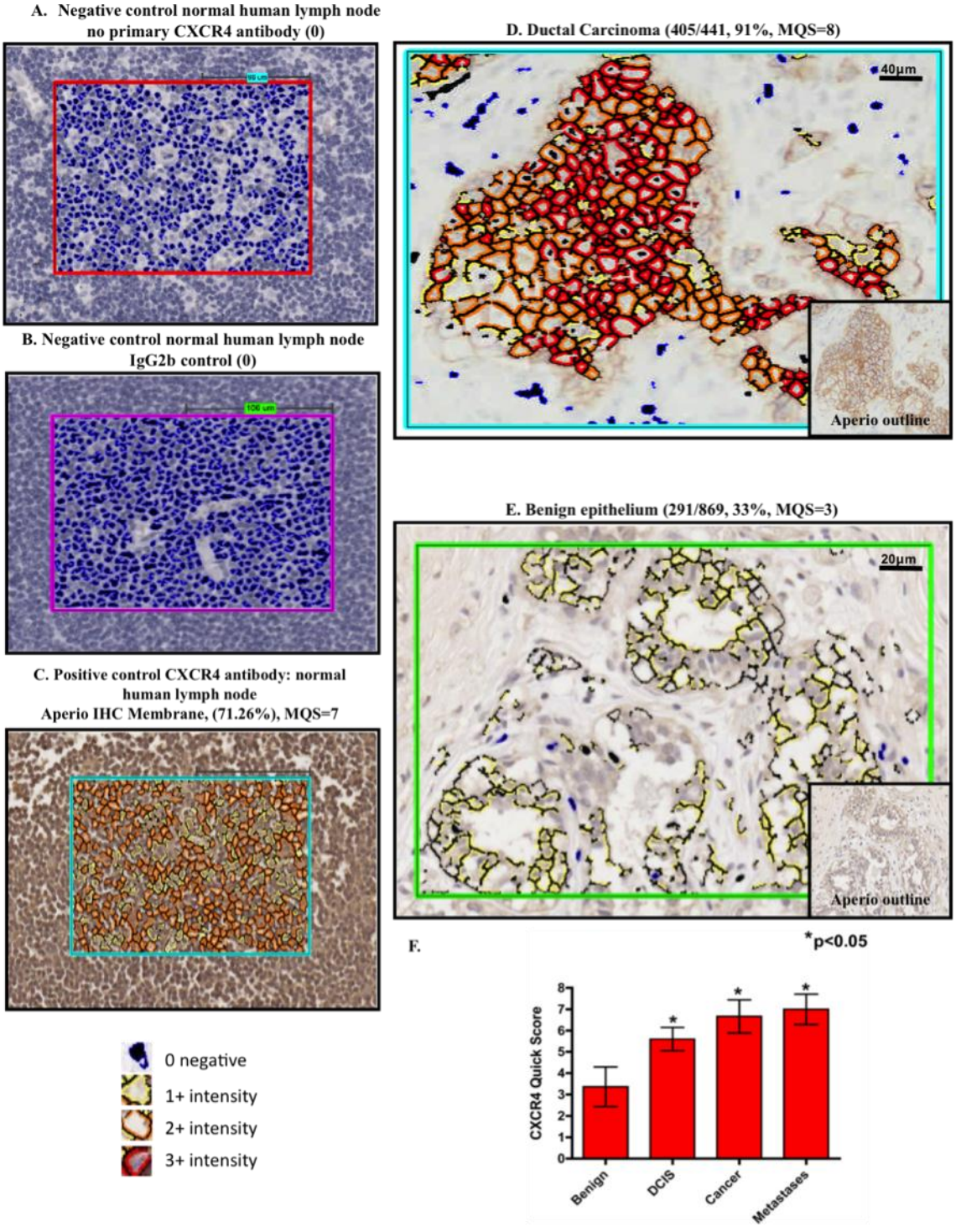
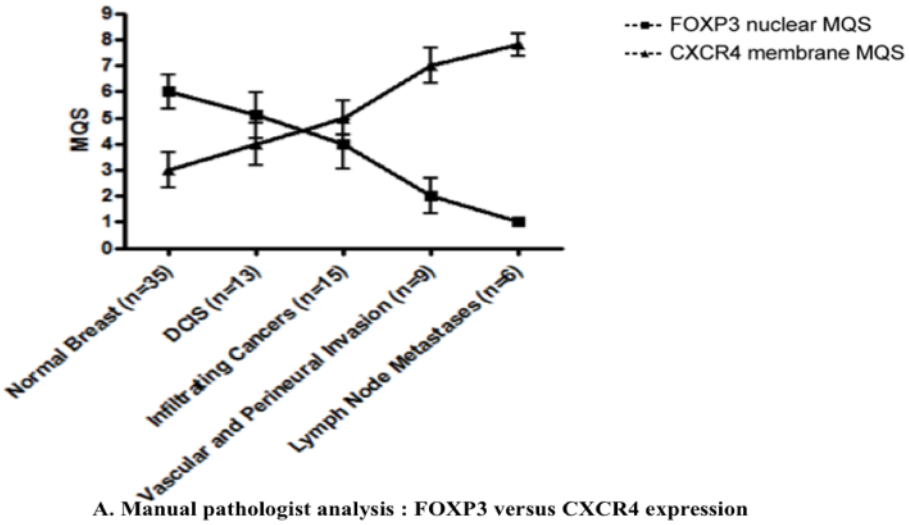


Figure 3-15: Digital image IHC membrane analysis of CXCR4

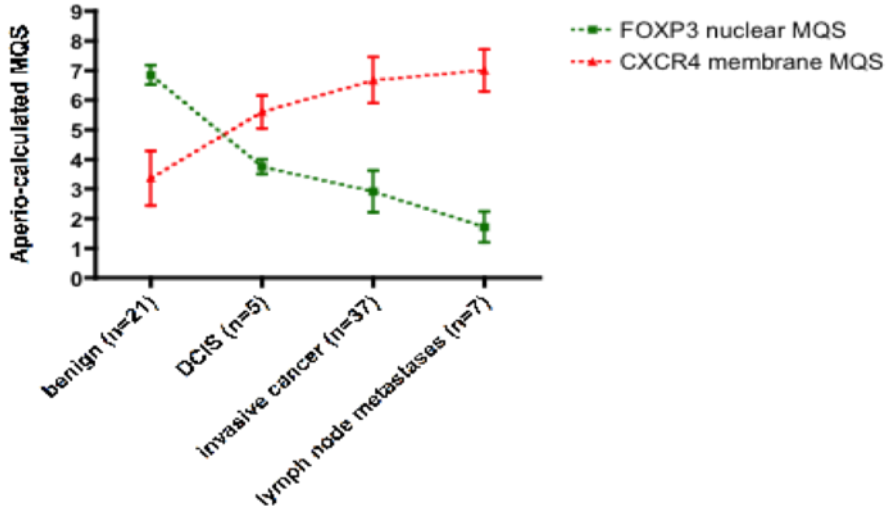
A, B: negative controls, C: positive control, D: breast cancer, E: benign breast tissue. Tissue sections were stained with monoclonal anti-CXCR4 antibody (R&D) at the optimum concentration of 1:250 in 20% of normal rabbit serum. Slides were scanned using Aperio digital scanner; digital images were analysed with IHC membrane algorithm and staining was automatically quantified. The quick scores were calculated and plotted (F). Error bars represent SD; the P value indicates the significance of the change from benign (normal) cells.

3.4.3 Comparative expression of FOXP3 and CXCR4 in breast tissue

Analysis of the correlation between FOXP3 and CXCR4 manual and digital MQS was performed for nuclear FOXP3 and membrane as well as cytoplasmic CXCR4 staining (Figure 3-16). In order to assess only the nuclear expression of FOXP3 in breast tissue epithelial cells, inflammatory cells, cytoplasmic FOXP3 staining and normal lymph nodes were excluded from calculations. More malignant sections were analysed using Aperio system than by a consultant pathologist because a number of additional sections were stained after the manual analysis. However, some sections could not be scanned using the Aperio system due to their uneven surface or excessive thickness of the sections. Early signs of metastasis (vascular and perineural invasion) were only assessed manually. The results demonstrated inverse correlation between MQS for nuclear FOXP3 and membrane CXCR4 staining.



A. Manual pathologist analysis : FOXP3 versus CXCR4 expression



B. Aperio analysis : FOXP3 versus CXCR4 expression

Figure 3-16: Manual and digital IHC analysis: FOXP3 versus CXCR4 expression in breast tissue.

Correlation of FOXP3 and CXCR4 manual (A) and digital (B) median quick scores (MQS) for nuclear FOXP3 and membrane CXCR4 staining was performed.

3.4.4 CXCR4 protein expression

Increased CXCR4 expression in breast cancer has been already confirmed at the tissue level (section 3.4.2). In order to examine CXCR4 expression at the cell level immunofluorescence and flow cytometry were performed.

For immunofluorescence analysis of breast cancer cells, CXCR4 was stained green using primary anti-CXCR4 antibody (1:250 in 20% of NRS, R&D) and FITC conjugated secondary antibody. The nuclei were counter-stained blue with DAPI. Both immortalised benign mammary epithelial cells (MCF10A) and breast cancer cells (MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) demonstrated CXCR4 membrane expression. The fluorescence intensity of CXCR4 was measured with Leica software (LSCM) and expressed graphically (Figure 3-17).

The fluorescence intensity of CXCR4 staining on TMD-MB-231, LMD-MB-231 was so high that the setting on Leica TCSSP2 UV confocal microscope had to be reset to increase the brightness threshold in order to take images and quantify the fluorescence intensity of these cells. There was a statistically significant ($p<0.05$) increase in CXCR4 expression on MDA-MB-231, TMD-MB-231, LMD-MB-231 breast cancer cells compared to MCF-10A normal breast epithelial cells (Figure 3-17).

In order to examine surface CXCR4 expression on a range of breast cancer cells, flow cytometry was used.

Lymphocytes are known to be CXCR4 expressing cells and to undergo CXCL12 induced migration and adhesion. The Jurkat T-lymphocyte cell line was screened for CXCR4 expression by flow cytometry with anti-CXCR4 antibody (Mab 173) and showed high levels of the receptor. Jurkat cells were therefore used as a positive control in FACS experiments (Figure 3-18). Flow cytometric analysis revealed that all examined cells expressed CXCR4 on their surface (Figure 3-18).

Microbeads with a known number of antibody binding sites per bead were used to compare the anti-CXCR4 antibody binding sites (proportional to CXCR4 expression) of normal breast cells MCF-10A with breast cancer cells: MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231. Each cell line was also analysed with isotype control antibodies. By analysing the Quantum Simply Cellular microbeads with a known number of anti-CXCR4 antibody binding complexes per bead at the same time, it was possible to calculate the number of anti-CXCR4 antibody binding sites on each cell line. The number of anti-CXCR4 antibody binding sites is directly proportional to the CXCR4 expression on the cell surface. Flow cytometric analysis demonstrated that MCF-10A, MCF-7 and MDA-MB-231 cells had little surface CXCR4 expression and it was not significantly different to the isotype control. TMD-231 and LMD-231 breast cancer cells expressed significantly more CXCR4 compared with MCF-10A normal breast cells and than the isotype control ($p<0.05$) (Figure 3-20).

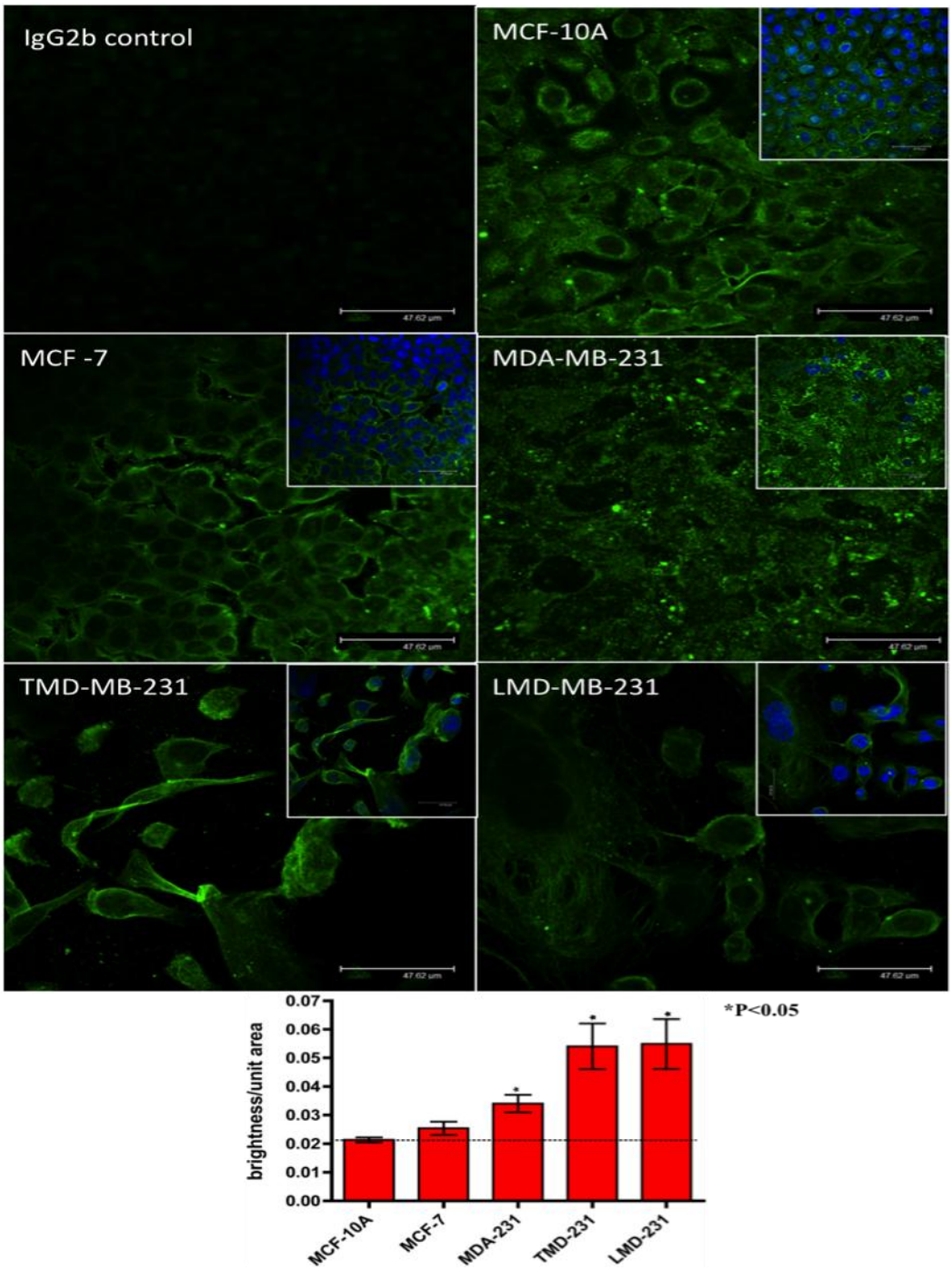


Figure 3-17: CXCR4 immunofluorescence staining of breast cell lines.

Cells were stained with primary monoclonal anti-CXCR4 antibody (1:250 in 20% of NRS, Abcam) and FITC conjugated secondary antibody. Images in the right upper corner represent addition of DAPI (blue) counter-stain to visualize nuclei. Leica TCSSP2 UV confocal microscope x20 magnification was used. CXCR4 expression was quantified by measuring fluorescence intensity (0-255) and dividing it by area. Quantified CXCR4 expression was plotted (graph). Error bars represent SD. P value indicates the significance of the change from benign MCF-10A cells (*P<0.05).

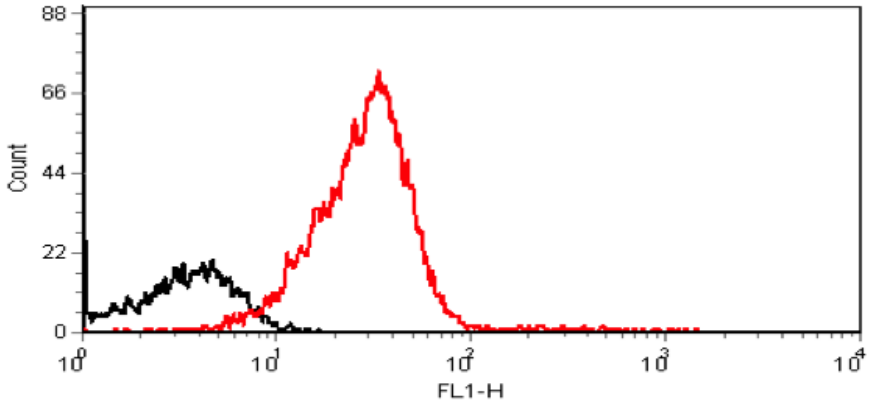


Figure 3-18: Surface CXCR4 expression of Jurkat T-cells by Flow Cytometry.

CXCR4 expression (FL1-H) is confirmed to be high on the population of Jurkat cells. Black plot – Isotype control antibody, red plot – Anti-CXCR4 antibody (Mab173), n=3.

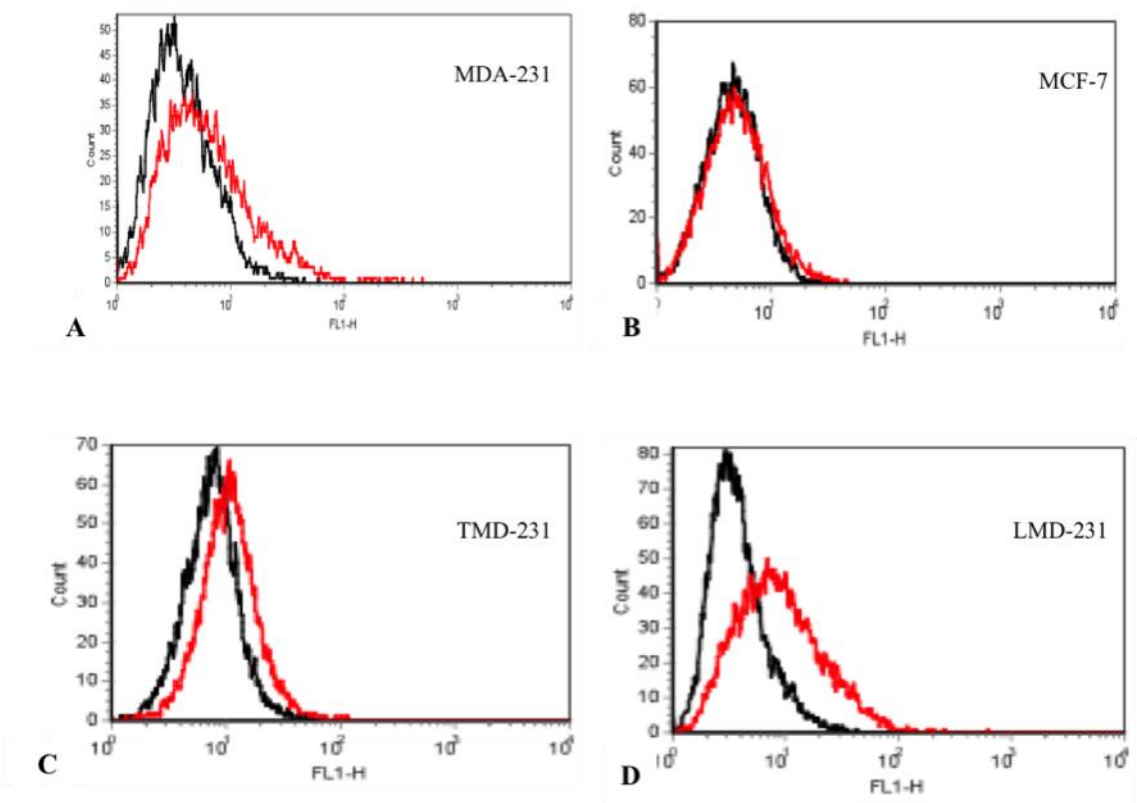


Figure 3-19: Representative histograms of surface expression of CXCR4 on breast cancer cells by flow cytometry.

MDA-231, MCF-7, TMD-231 and LMD-231 demonstrated CXCR4 expression (FL1-H) as analysed by flow cytometry using an anti-CXCR4 antibody (Mab173). For all histograms, Black plot – Isotype control, Red plot – Anti-CXCR4 antibody, n=3.

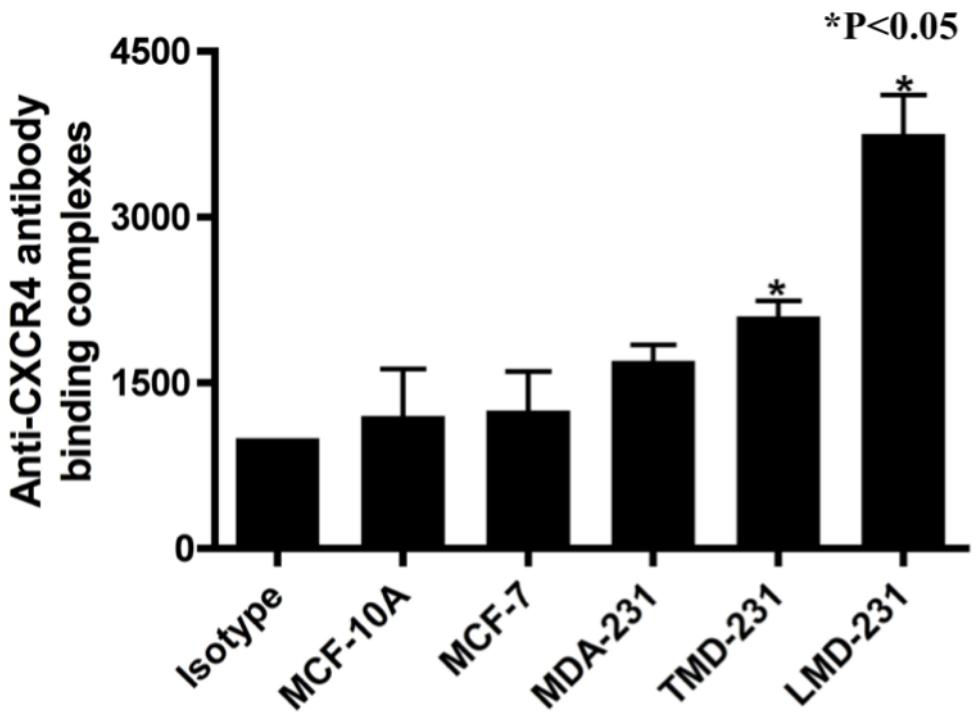


Figure 3-20: The relative number of anti-CXCR4 binding complexes on breast cancer cell lines.

MCF-10A and MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231 were analysed by flow cytometry using an anti-CXCR4 antibody (Mab173) and FITC-conjugated secondary antibody. Each cell line is also analysed with isotype control antibodies. By analysing the Quantum Simply Cellular microbeads with a known number of anti-CXCR4 antibody binding complexes per bead at the same time, it was possible to calculate the number of anti-CXCR4 antibody binding sites on each cell line. The number of anti-CXCR4 antibody binding sites is proportional to the CXCR4 expression on the cell surface. The histogram is representative data from 2 similar experiments; bars show SD of triplicate data points. MCF-10A, MCF-7 and MDA-MB-231 cells had little surface CXCR4 expression and it was not significantly different to the isotype control. TMD-231 and LMD-231 breast cancer cells expressed significantly more CXCR4 compared with MCF-10A normal breast cells and than isotype control ($p<0.05$).

3.4.5 FOXP3 and CXCR4 mRNA expression

In order to examine FOXP3 expression in breast cancer cells at the mRNA level, further experiments were carried out using conventional and qPCR.

RNA was isolated from each cell line and its integrity was verified by agarose gel electrophoresis (Figure 3-21). Following cDNA synthesis, a panel of breast cancer cell lines (MCF-10A, MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) were subjected to standard PCR and FOXP3 expression was compared to positive control, which was FOXP3 cDNA and as expected gave significant FOXP3 expression (Figure 3-22). FOXP3 expression was detected in all breast cancer cell lines examined.

To verify the specificity of manually designed FOXP3 primers, 100 µl conventional PCR reaction was performed using MCF-7 and MDA-MB-231 cDNA amplified using FOXP3 primers. The PCR products were excised from the agarose gel and sequenced by GeneService confirming 89% homology of the amplified product with the FOXP3 sequence (Figure 3-23).

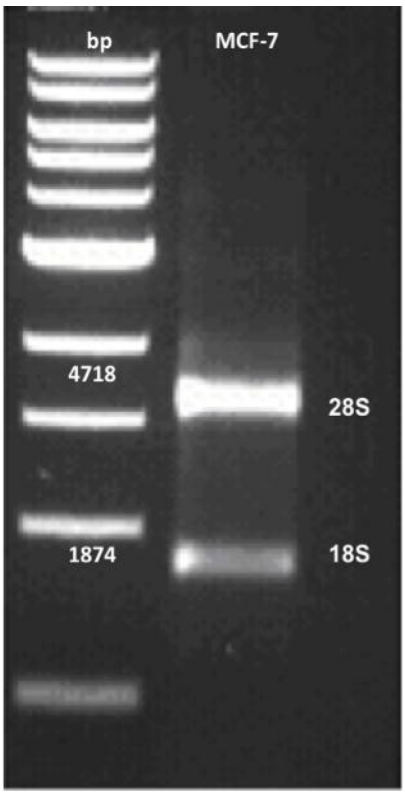


Figure 3-21: Confirmation of RNA integrity

After extraction from breast cancer cells using the Trizol Reagent (Invitrogen), RNA was quantified with nanodrop software and its quality was confirmed by electrophoresis on a 1.5% agarose gel, with a DNA ladder to determine the size of bands. Sharp 28S and 18S bands with minimal smearing indicate good integrity of the RNA.

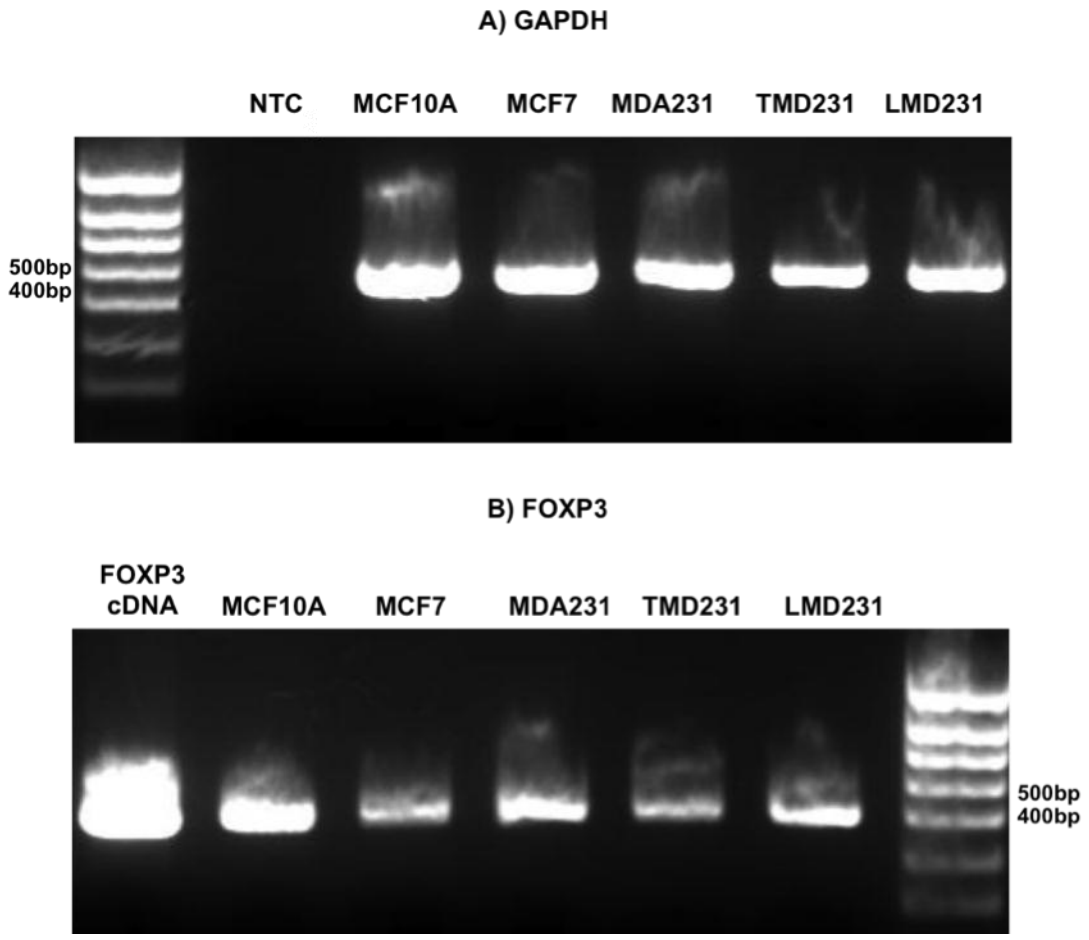


Figure 3-22: Detection of FOXP3 mRNA expression in breast cancer cell lines

MCF-10A, MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231 cell lines were tested for FOXP3 mRNA expression. First, mRNA was extracted and reverse transcribed to cDNA. Then, PCR performed using GAPDH specific primers as positive controls confirmed GAPDH size of about 550bp (A). The reaction performed using specific FOXP3 forward and reverse primers revealed (B) FOXP3 mRNA expression in all cancer cell lines studied. Primers used (VH Bio Ltd Gateshead): forward primer: 5'-CAAATGGTGTCTGCAAGTGG-3', reverse primer: 5'- ATTGAGTGTCCGCTGCTTCT 3' (product length 500bp, product region 772-1271). A reaction without cDNA template was performed as a negative control (NTC). The reaction with FOXP3 cDNA (positive control) confirmed the product size of 500bp. The result is representative of five independent experiments.

```
GENE ID: 50943 FOXP3 | forkhead box P3 [Homo sapiens]
Score =302 bits (163), Expect = 2e-79, Identities = 197/221 (89%), Gaps = 3/221 (1%)
Query  29    CGTGGCNTANGTGANAGGGGGTCGCATGTTGTGGAACTTGAAGTAGTCCATGTTGTGGAG  88
             |||||| || |||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1115  CGTGGCGTAGGTGAAAGGGGGTCGCATGTTGTGGAACTTGAAGTAGTCCATGTTGTGGAG  1056
Query  89    GAACTCTGGGAATGTGTTGTTTCCATGGCTACCCCACAGGTGCCTCCNGACAACAAACAG  148
             ||||||||||||||| |||||||||||||||||||||||||||||| |||| |||||||
Sbjct  1055  GAACTCTGGGAATGTGCTGTTTCCATGGCTACCCCACAGGTGCCTCCGGACAGCAAACAG  996
Query  149   GNTGTNAGGGGCCTCCCNGGGGCCAGACCAGGNTNGGACGACAGGGCCGNGTNNTGCCAG  208
             | ||| |||||||||| |||||||||||||| | ||||||||||||  |   ||||||
Sbjct  995   GCTGTCAGGGGCCTCCCGGGGGCCAGACCAGGCTGGGACGACAGGGCCTTGGC-TGCCAG  937
Query  209   CANCTACNATGCACCANGANCNCTT-TACGGATGATGCCAC  248
             || |||| ||||| || || | ||| | ||||||||||||
Sbjct  936   CAGCTACGATGCAGCAGGAGCCCTTGT-CGGATGATGCCAC  897
```

Figure 3-23: Determination of FOXP3 primers specificity

Normal PCR reaction was performed in 100 µl volume, using MCF-7 and MDA-MB-231 cDNA amplified using FOXP3 primers with tested FOXP3 primers. PCR products were excised from the agarose gel and sequenced by GeneService confirming 89% homology of amplified product with FOXP3 sequence.

3.4.5.1 Validation of the real-time PCR reaction for FOXP3 and CXCR4 assays

Having detected FOXP3 mRNA in breast cancer cell lines using normal PCR, further experiments to quantify the amount of FOXP3 and CXCR4 mRNA were performed using real-time PCR. FOXP3 and CXCR4 TaqMan assays were purchased from Applied Biosystems. GAPDH assay was used as internal control. It was first necessary to determine whether the pair of assays could be used together in the experiments. In order to establish that, a validation experiment was performed based on serial dilutions of the DNA template (Figure 3-24 A, B). The efficiency of qPCR reaction was determined from the equation: $Eff=10^{(-1/slope)}-1$

The qPCR efficiency should be between 90 and 100%, which means that the slope between linear phases of amplification graphs should be between -3.6 and -3.1. Slopes of -3.56, -3.27 and -3.41 indicated that efficiency of real-time PCR reaction was higher then 90%. The qPCR provided evidence that the chosen pair of assays (FOXP3 + GAPDH or CXCR4 + GAPDH), performed with almost the same efficiency during qPCR could be used for screening of breast cell lines for FOXP3 and CXCR4 mRNA expression.

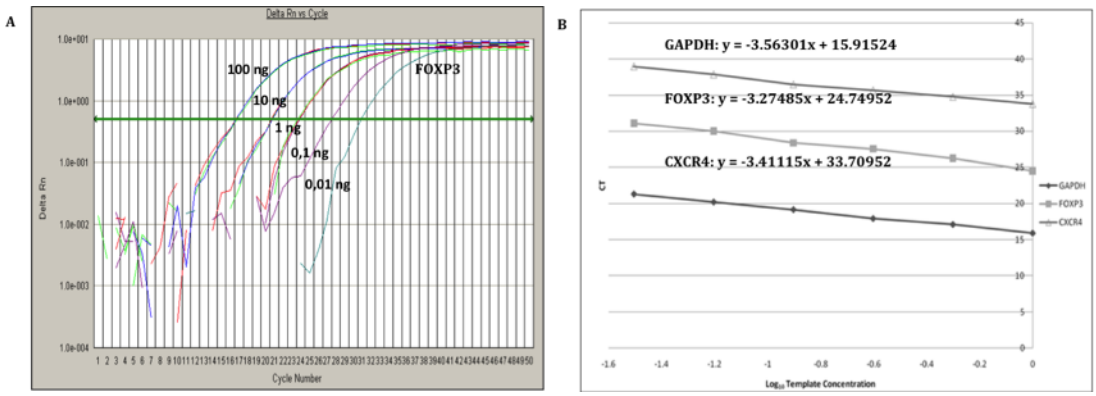


Figure 3-24: Representative amplification plots and a standard curve for real-time PCR detection of FOXP3 and CXCR4 in diluted samples.

Graph demonstrates how template cDNA concentration proportionally affects cycle threshold (CT). The gradients obtained from the optimisation experiments were acceptable for calculating ΔΔCT values. The standard curve was graphically represented as a semi-log regression line plot of CT value versus log of input nucleic acid. Slopes of -3.56, -3.27 and -3.41 indicated a qPCR reaction with >90% efficiency.

3.4.5.2 FOXP3 and CXCR4 mRNA expression in breast cells

Following the validation experiment, real-time PCR was performed to determine expression of FOXP3 and CXCR4 mRNA on a range of breast cancer cell lines. The housekeeping gene GAPDH was used as a control throughout this experiment. The pairs of verified primer/probe specific for FOXP3 and CXCR4 (TaqMan, Applied Biosystems) were used to perform absolute quantification of FOXP3 and CXCR4 mRNA expression. Expression of FOXP3 and CXCR4 mRNA was revealed in all tumour cell lines studied. FOXP3 expression in all cancer cell lines analysed was increased but not significantly different with reference to MCF10, an immortalised human mammary epithelial cell line. CXCR4 expression was significantly increased in all cancer cell lines analysed with reference to MCF10A (Figure 3-25). The results of qPCR FOXP3 mRNA expression were inconsistent with FOXP3 protein expression. FOXP3 protein expression, which was studied using immunofluorescence and immunohistochemistry, showed that cancer cell lines expressed less FOXP3 protein than normal mammary cells.

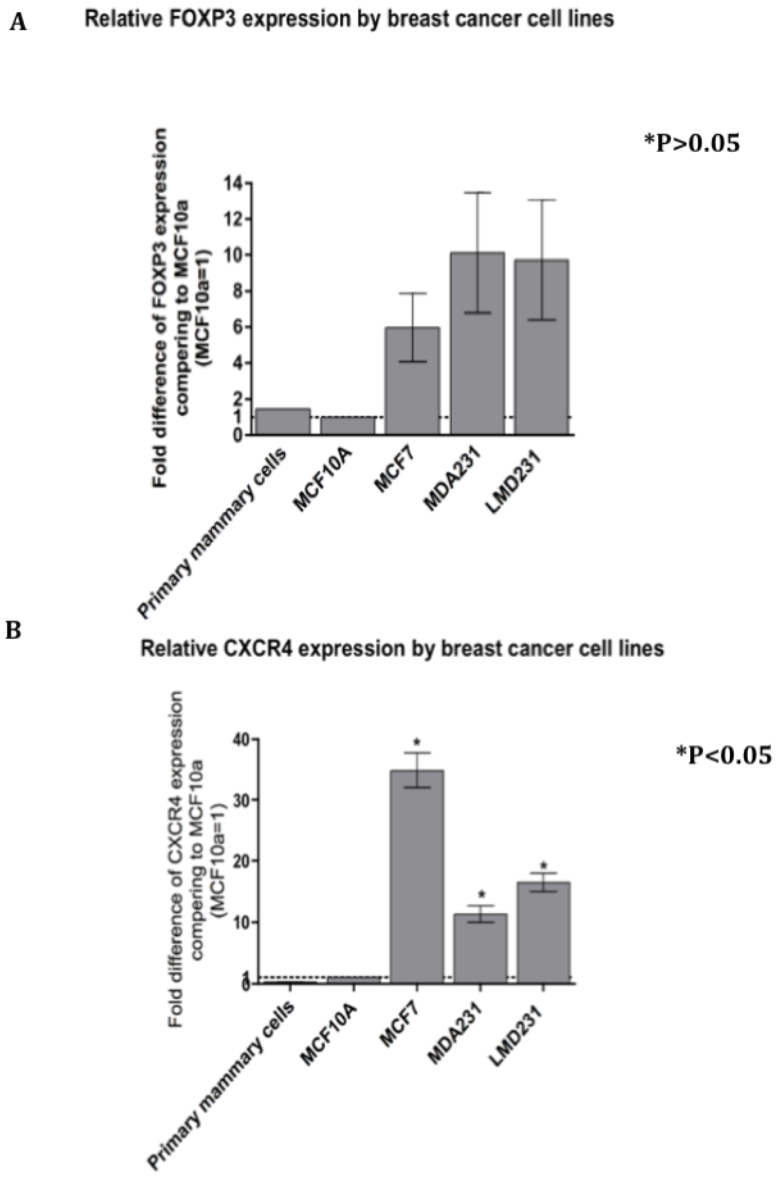


Figure 3-25: FOXP3 and CXCR4 mRNA expression in breast cells

FOXP3 (A) and CXCR4 (B) mRNA expressions in a range of breast cell lines quantified by qPCR. Results were normalised to GAPDH in relation to MCF10A as an immortalised human mammary epithelial cells. Rest 2008 and Prism 3 software was used for statistical analysis. Graph bars represent average fold expression obtained from four independent experiments. Error bars represent SD; P values indicate significant change from control cells.

3.5 Discussion

3.5.1 Expression of FOXP3 and CXCR4 in human breast tissue

The existence of FOXP3 expression in epithelial tissues remains controversial. The majority of authors describe FOXP3 expression in normal human breast tissue. However, considerable confusion persists in literature regarding FOXP3 expression in human cancers. Some describe increased FOXP3 expression in cancers (Merlo A, 2009, 2005, Gupta et al., 2007), others state the opposite (Zuo et al., 2007b, Zuo et al., 2007d). There are reports describing only negligible FOXP3 expression in breast tissue (Wolf D, 2010) and studies, based on genetic animal model, excluding expression of FOXP3 in nonhematopoietic tissues, like breast (Kim et al., 2009a).

There are at least three reasons for the contradictory evidence on FOXP3 expression in cancer tissue.

The first is whether cytoplasmic or nuclear FOXP3 is analysed. FOXP3 is a transcription factor and its functional expression is nuclear whereas role of the cytoplasmic FOXP3 remains unknown. Those who observed decreased FOXP3 expression in breast cancer (Zuo et al., 2007d, Zuo et al., 2007b) analysed only its nuclear expression; those who demonstrated equal or increased expression of FOXP3 in cancer (Wolf D, 2010, Andrea Merlo and Elda Tagliabue, 2009) examined its total expression: nuclear and cytoplasmic. To overcome this problem in this study, nuclear and cytoplasmic were analysed separately using both manual and digital microscopy. Aperio ScanScope digital microscopy system not only increased reproducibility, accuracy and objectivity, but also allowed it on precise separate analysis of FOXP3 expression in cytoplasm, nuclei and inflammatory infiltrate.

Overall, 90% of benign and 78% of malignant breast tissue samples expressed FOXP3. Out of those, about 70% of cancers and 100% of benign specimens expressed FOXP3 in the nuclei and all samples expressed FOXP3 in the cytoplasm. In malignant sections with nuclear FOXP3 expression, it was weak and in limited areas of the epithelium. It may indicate a mutation of specific cells within the cancer epithelium. Total FOXP3 expression (nuclear and cytoplasmic) was no

significantly different between cancer and benign breast tissue. However, when nuclear staining was compared, benign breast epithelial cells had significantly more nuclear FOXP3 expression than invasive ductal carcinoma.

The second reason for the confusion is the use of different anti-FOXP3 antibodies in immunohistochemical staining. A recent study showed that FOXP3 immunohistochemistry on formalin-fixed paraffin-embedded tissue has poor correlation between monoclonal antibodies 236A/E7 and Abcam 22510 used for staining sections of different organs (Woo et al., 2008). An assessment of specificity and sensitivity of several anti-FOXP3 monoclonal antibodies, including 236A/E7, hFOXY, eBio7979, and FJK-16s for immunohistochemistry staining in both human normal epithelial cells and cancer cells confirmed the specificity of 236A/E7 for FOXP3 using cells with silenced FOXP3 (Wang L, 2009).

The third reason for the controversy is the presence of FOXP3 positive T cells in the cancer inflammatory infiltrate. As a result, the increased expression of FOXP3 in breast cancer (Merlo A, 2009, Ohara et al., 2009, Bates et al., 2006) may be related to the strong FOXP3 expression of the inflammatory cells around tumours rather than FOXP3 expression on the epithelial cells. This finding makes the interpretation of the gene expression data of FOXP3 expression in tumours difficult. A FOXP3 mRNA variant lacking exons 3 and 4 has been identified in tumour cell lines but not in CD4 positive FOXP3 positive T cells (Chen C, 2006). In contrary to the previously published work (Merlo A, 2009), in this study there was not statistically significant difference between total numbers or percentages of FOXP3 positively stained cells within the inflammatory infiltrate surrounding normal duct and ductal cancer. However, it may be related to the local inflammatory response, which is a physiological reaction to the biopsy in the breast clinic.

3.5.2 CXCR4 protein expression in breast cancer cells

Cytoplasmic and nuclear CXCR4 expression in cancer cells has been observed in various cancers (Cabioglu et al., 2005a, Cabioglu et al., 2005c, Na et al., 2008,

Yoshitake et al., 2008). As expected, we observed strong membrane and cytoplasmic CXCR4 expression in cancer cells. The opposite was true for normal breast cells. Nevertheless, cytoplasmic CXCR4 expression was thought to be critical for lymph node metastasis and the patients' poor prognosis compared to nuclear expression of the receptor. CXCR4 localization at the plasma membrane and intracellular vesicle (cytoplasm) were observed in leukocyte cell lines with enforced CXCR4 expression and CXCL12 induced polarization of CXCR4 to the edge of migrating leukocyte cells (van Buul et al., 2003). Therefore, cytoplasmic CXCR4 expression may be more important for migration of cancer cells contributing to lymph node metastasis and unfavourable prognosis. While nuclear CXCR4 expression occurs in normal and cancer tissues (Salvucci et al., 2006), its function is unknown. The splice variants of chemokines without a signal peptide translocating into the nucleus have been identified (Gortz et al., 2002). Similarly, the nuclear CXCR4 accumulation may lack a signal peptide and may not be functional. There are several identified factors which may induce CXCR4 expression: NF-kappa B (Helbig et al., 2003), c-erbB-2 (Li et al., 2004), hypoxia-inducible factor 1 (Schioppa et al., 2003) nitric oxide (Yasuoka et al., 2008). In this study nuclear CXCR4 expression was also observed but it was not analysed.

3.5.3 Relationship between FOXP3 and CXCR4 expression and clinico-pathological factors of breast cancer

This study confirmed that normal breast epithelial cells had significantly greater FOXP3 nuclear expression than invasive cancers and the opposite was true for CXCR4 membrane expression. Correlation of FOXP3 and CXCR4 quick scores (MQS) were done for nuclear and membrane staining respectively and results demonstrated statistically significant inverse correlation between MQS for nuclear FOXP3 and membrane CXCR4 staining.

Recently it has been proposed that overall FOXP3 expression in breast cancer tissue is associated with worse prognosis and spread of cancer to lymph nodes (Andrea Merlo and Elda Tagliabue, 2009). However, this work suggested that

nuclear FOXP3 expression was significantly decreased in cancers showing early signs of metastasis (vascular and perineural invasion) when compared to tumours where vascular and perineural invasion were not observed. In addition, the prognostic indicators of breast carcinoma like lymph node status and distant metastasis were associated with decreased nuclear FOXP3 expression and increased cytoplasmic CXCR4 expression.

3.5.4 FOXP3 and CXCR4 expression at the mRNA level

We observed FOXP3 and CXCR4 mRNA expression in all tumour cells studied. FOXP3 expression was elevated in all cancer cell lines analysed, with reference to MCF10A as an immortalised benign human mammary epithelial cells, but it was not significant. This finding was contradictory to decreased FOXP3 expression at protein level. It may be that in cancer the production of FOXP3 is increased because of dysfunctional nature of this protein.

Recent data showed that quantification of Foxp3 mRNA in tissues represented an independent prognostic variable in terms of overall survival and progression-free survival. However, those data were mostly focused on the expression of Foxp3 in Tregs (Wolf et al., 2005). The large number of Tregs infiltrated in cancerous tissues was associated with a dismal prognosis in ovarian cancer. Quantification of Foxp3 mRNA serve as a surrogate marker for the ovarian cancer tissue infiltration grade by Tregs, and immunohistochemical staining of Foxp3 on a tissue microarray corroborated Foxp3 mRNA data in terms of the identification of tumours with Foxp3-positive Tregs (Wolf et al., 2005).

CXCR4 expression was significantly increased in all cancer cell lines analysed with reference to MCF10A.

3.6 Summary of observations

- Normal breast tissue had significantly greater FOXP3 nuclear expression than cancers and the opposite was true for CXCR4 cytoplasmic expression.

- There was an inverse correlation between nuclear FOXP3 and cytoplasmic CXCR4 staining in human breast tissue.

- Local and distal metastasis were associated with decreased nuclear FOXP3 and increased cytoplasmic CXCR4 expression.

- There was a significant increase in CXCR4 cytoplasmic expression on breast cancer cell lines.

- FOXP3 mRNA expression was not significantly elevated in cancer cells and the opposite was true for CXCR4 mRNA expression.

Chapter

4 Failure of FOXP3 translocation to the nucleus in breast cancer

4.1 Introduction

4.1.1 Nuclear translocation of FOXP3

Intracellular trafficking of FOXP3 to the nucleus is essential for expression of this protein in its active form and the maintenance of homeostasis. FOXP3 is synthesised in the cytoplasm but functions by interacting with DNA in the nucleus. Following expression it is rapidly translocated to the nucleus and binds to chromatin. There are three distinct FOXP3 domains that contribute to its nuclear transport (Hancock W, 2009). The first domain (Domain 1) comprises the C-terminal 12 amino acids. The second domain (Domain 2) is located immediately N-terminal to the forkhead domain (FHD), recently reported to be a binding site for the runt-related transcription factor 1/acute myeloid leukaemia 1 (Runx1/AML1). The third domain (Domain 3) is located within the N-terminal first 51 amino acids.

FOXP3 mutant protein missing domains 1, 2, and 3 is found almost exclusively in the cytoplasm with only trace levels in the nuclei (Hancock W, 2009). Identification of factors which interact with these domains will not only facilitate our understanding of how FOXP3 achieves its nuclear transport to regulate the expression of target gene, but may lead to therapeutic modulation of these systems and provide a novel approach to cancer treatment. Indeed, it has been demonstrated that the disruption of nuclear localization is sufficient to abrogate growth inhibition by FOXP3 in prostate cancer (Wang L, 2009).

4.1.2 Failure of nuclear FOXP3 translocation in cancer

The nuclear expression of FOXP3 in human benign breast, respiratory and prostate tissue is well documented (Chen G, 2008). However, immunocytochemical staining of tumour cell lines revealed predominant cytoplasmic expression of breast, colon, pancreas, lung cancer cell lines (Karanikas et al., 2008). There is disagreement amongst researchers with regards to the immunohistochemistry of malignant

breast epithelium: some describe nuclear staining only (Zuo et al., 2007b, Zuo et al., 2007d), others state that expression of FOXP3 in breast cancer is predominantly cytoplasmic (Andrea Merlo and Elda Tagliabue, 2009). Immunohistochemistry of other human malignancies detected nuclear FOXP3 staining in prostate cancers (Wang L, 209), mixed cytoplasmic and nuclear FOXP3 staining in the pancreatic cancers (Hinz et al., 2007) and solely cytoplasmic staining in ovarian cancers (Zhang and Sun, 2010).

4.1.3 Expression and sub-cellular distribution of FOXP3 isoforms

The identification of splice variant forms of the protein suggests an additional level of complexity surrounding the biology of FOXP3. In contrast to mice, human Treg cells always display expression of at least two FOXP3 isoforms. Both are expressed at similar levels. One represents the full-length isoform (FOXP3fl), while the other isoform lacks exon 2 (FOXP3D2). Recently, two further splice variants have been described: missing exon 7 (FOXP3D7) (Kaur et al.) and missing exons 2 and 7 (FOXP3D2D7) (Mailer et al., 2009). All isoforms are effectively translocated into the nucleus in mammalian cells (Mailer et al., 2009). Analysis by fluorescence microscopy of HEK293T cells transfected with FOXP3fl, FOXP3D2, FOXP3D7 and FOXP3D2D7 constructs showed bright staining of the nucleus, while GFP-FOXP3DFKH, a truncated version lacking the DNA binding domain was retained in the cytoplasm (Mailer et al., 2009). Splice variants FOXP3fl, FOXP3D2, FOXP3D7 and FOXP3D2D7 bind to transcription factors, including NFAT, NF-kB and RUNX1. While FOXP3D2D7 lacks its suppressive functions, it is able to inhibit FOXP3fl in a dominant negative manner (Mailer et al., 2009). Recent data obtained by crystal structure analysis and co-immunoprecipitation experiments mapped the binding site of the NFAT RelA domain to the forkhead domain of FOXP3 (Wu et al., 2006), whereas RUNX1 targets a region starting at the c-terminus of exon 7. Isoforms FOXP3D2 and FOXP3D2D7 are overexpressed by certain malignant cells, eg. in Sezary Syndrome, an aggressive variant of cutaneous T cell lymphoma, the transformed T cells express FOXP3D2 and FOXP3D2D7. Moreover, FOXP3 also

plays a role in breast cancer, as FOXP3fl acts as repressor of Her-2 (Zuo et al., 2007c). Future studies have to establish, however, whether the isoform actually plays a role in cancer pathogenesis.

4.1.4 Expression and sub-cellular distribution of FOXP3 following mutations

In mice, a Foxp3 frameshift mutation in the forkhead domain results in lethality in hemizygous males 16 to 25 days after birth (Brunkow et al., 2001). The mutation in some human IPEX patients (Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-linked) is analogous as they cause frameshift and early termination of translation (Brunkow et al., 2001, Chatila et al., 2000, Bennett et al., 2001b, Bennett et al., 2001a, Wildin et al., 2001). In breast cancer patients, a total of 27 somatic mutations in all 11 coding exons and intron-exon boundary regions have been identified in 36% of 65 patients by PCR (Zuo et al., 2007d, Zuo et al., 2007b). In these mutations, there are 18 nonsynonymous mutations, 3 synonymous mutations and 6 mutations in the intron-exon junction 12 (Zuo et al., 2007b). Interestingly, the mutations are not randomly distributed in the FOXP3 gene and the overwhelming majority of them are either in the functional domains or within intron 11, which can affect the forkhead domain sequence (Zuo et al., 2007b). In prostate FOXP3 gene plays a critical role in suppressing cancerous transformation of this gland (Wang L, 2009). Four somatic FOXP3 mutations were identified in prostate cancer and three of them prevent nuclear localization of FOXP3, which is a major mechanism to inactivate FOXP3 tumour suppressor function (Wang L, 209, Wang L, 2009). The exact role of the somatic FOXP3 mutations in breast cancer remains unknown. The question whether, like in prostate cancer, it is responsible for failure of nuclear localization of FOXP3 protein, is yet to be answered.

4.2 Specific Aims and Objectives

- To study sub-cellular location of FOXP3 in breast cancer cells

- To study sub-cellular location of FOXP3 on breast cancer cells following induction of its expression with cytokines and stable transfection

- To analyse nuclear localization sequences of FOXP3 gene in breast cancer for presence of mutations and isoforms.

4.3 Methods

4.3.1 Sequencing of FOXP3

The samples of cDNA from breast cancer lines (HMEPC, MCF10A, MCF-7, MDA-MB-231, TMD-231, LMD-231) were amplified by conventional PCR and sequenced. In the initial experiments *Taq* DNA polymerase was used. The results were subsequently verified using thermostable *Pfu (Pyrococcus furiosus)* DNA polymerase with 'proofreading' properties. *Taq* (*Thermus aquaticus*) polymerase has an error rate of about 1.3×10^{-5} and mutation frequency of 5.1 %, Therefore, it is possible that the mutations observed in the initial experiments were introduced and amplified during the course of the experiment. Thermostable *Pfu (Pyrococcus furiosus)* DNA Polymerase with 3′→5′ exonuclease proofreading activity exhibits a higher fidelity than *Taq* DNA Polymerase. It has an error rate of about 1.8×10^{-5} – 8×10^{-7} and 5.1–0.3% mutation frequency. Automated DNA sequencing was used to determine the sequence of DNA within cDNA encoding 3 domains of nuclear localization within FOXP3 gene. Sequencing was carried out by GeneService (www.geneservice.co.uk) and Bioscience (www.bioscience.co.uk) and chromatograms were produced using the Geospiza's FinchTV v.1.4.0 freeware software (http://www.geospiza.com). The sequences were compared with FOXP3 reference gene (accession number: A277993).

4.3.2 Primer Design for sequencing of FOXP3 domains

All FOXP3 primers were designed manually. Nucleotide search tool from www.ncbi.nlm.nih.gov was used to search for target sequence of nucleotides for FOXP3 locus (AF277993). "Perfect Primers Design" tool (Invitrogen: www.tools.invitrogen.com), was used to determine final primer sequence. Forward and reverse FOXP3 primers were designed to have approximately the same number of G and C components and to be 15-35 nucleotides in length. Primers were designed with a G-C content producing a melting temperature (Tm) of 60oC. Table 4-1 summarises 3 pairs of FOXP3 primers designed to sequence three nuclear localisation domains (NLD).

FOXP3 primer	Where synthesised	Sequence	NLD
Forward primer	VH Bio (Gateshead)	CAAATGGTGTCTGCAAGTGG	2
Reverse primer		ATTGAGTGTCCGCTGCTTCT	2
Forward primer	Invitrogen	AAGCCAGGCTGATCCTTTTCT	2,3
Reverse primer		TCTGCCTCCCACCAGTTTG	2,3
Forward primer	IDT	AGATCTACCACTGGTTCACACGCA	1
Reverse primer		AGGCAAGACAGTGGAAACCTCACT	1

Table 4-1: Primer sequences used in conventional PCR.

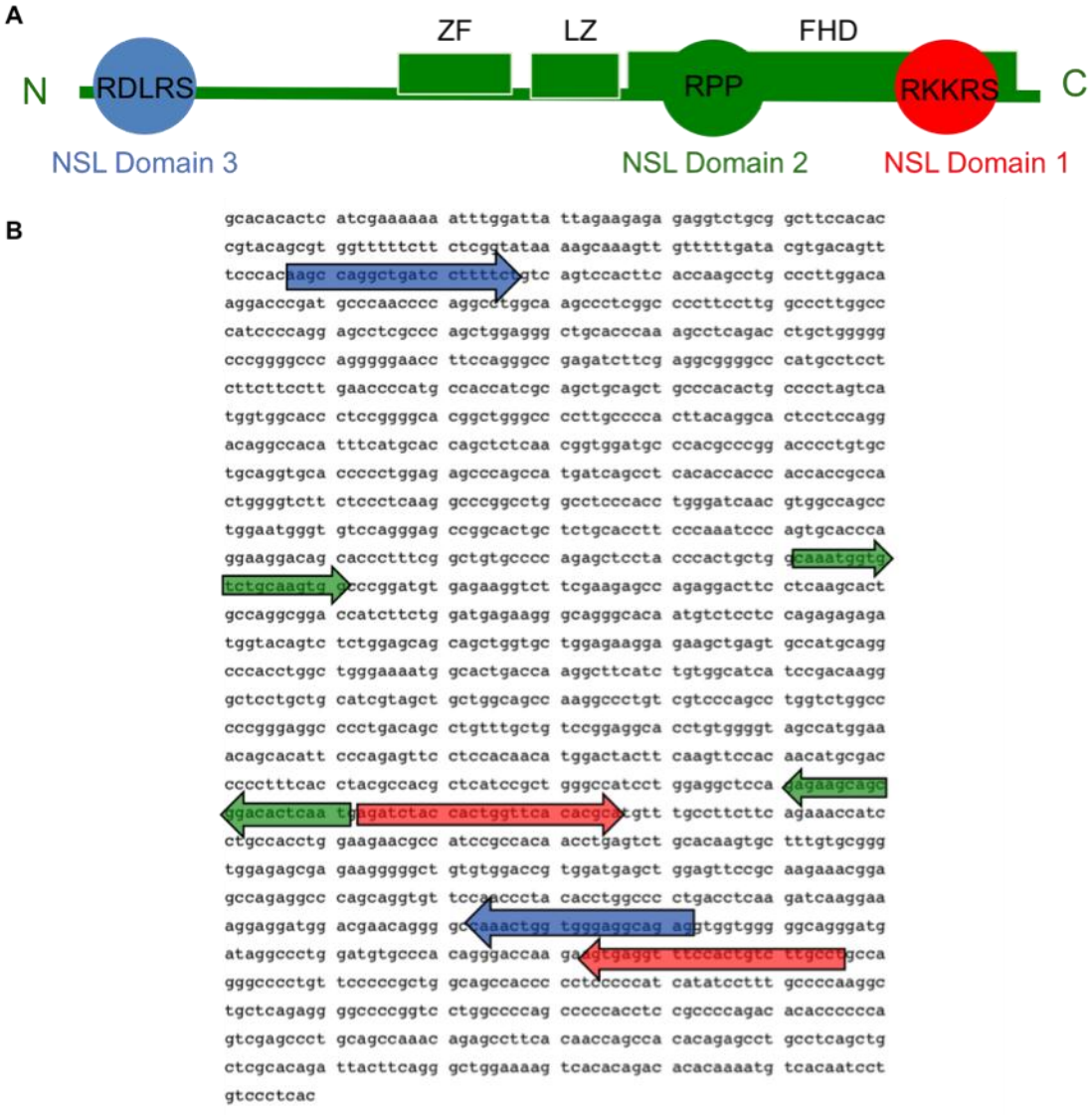


Figure 4-1: Primer design for sequencing FOXP3 Nuclear Localization Domains.

Based on the murine FOXP3 structure there are three distinct FOXP3 domains that contribute to its nuclear transport (Hancock W, 2009). The first domain (Domain 1 - red) comprises the C-terminal 12 amino acids. The second domain (Domain 2 - green) is located immediately N-terminal to the forkhead domain (FHD) and the third (Domain 3 - blue) is located within the N-terminal first 51 amino acids. FOXP3 mutant protein missing domains 1, 2, and 3 is found almost exclusively in the cytoplasm with only trace levels in the nuclei. (A) Diagram of FOXP3 protein with positions of domains of nuclear transport marked. ZF: zinc finger domain, LZ: leucine zipper domain, and FKH fork-head domain. (B) Three pairs of primers for sequencing each of three nuclear localization domains were designed manually. The primers' sequences are highlighted as coloured arrows corresponding to the colours of the domains they amplify.

4.3.3 Quantitative analysis of PCR electrophoresis products by densitometry

Amplified products were electrophoresed at 120 volts for one hour on 2 % agarose gels, then photographed by the AlphaImager 3400 gel imaging system (Alpha Innotec, Santa. Clara, CA). Densitometry of FOXP3 isoform ratios (isoform 3/ isoform 1 or 2) were performed using the imaging analyses system (Image J system, NIH). The mean pixel intensity of the analysed band of the electrophoresis gel was presented as a band plot. The size of the band was then multiplied by a mean pixel intensity of that band giving an absolute intensity. The ratio of absolute intensities of two bands in the same sample gave a relative intensity. Relative intensity quantified the mRNA expression of the FOXP3.

4.4 Results

4.4.1 Sub-cellular FOXP3 localization

Having examined the expression of FOXP3 and its distribution in breast cancer epithelium at the tissue level, further work was carried out on the cellular level using immunofluorescence, immunocytochemistry, digital microscopy and image analysis and transient transfection of MDA-MB-231 cells with human FOXP3 cDNA.

FOXP3 is a transcriptional regulator that functions by interacting with DNA in the nuclei (Zuo et al., 2007c). As the next step to test the hypothesis that FOXP3 fails to localise in the nuclei of the cancer cells, FOXP3 protein was tagged with an anti-Foxp3 antibody conjugated with FITC giving green fluorescence and cellular nuclei were counter-stained with DAPI. Slides were analysed using confocal microscopy as shown in Figure 4-2. HEK-293 FOXP3 transfectants, used as a positive control, expressed the highest levels of FOXP3 and their expression was nuclear. Immortalised benign human breast cells (MCF-10A) expressed more FOXP3 in the nuclei compared to breast cancer cells. All cancer cell lines examined (MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) revealed FOXP3 expression, which was predominantly localised outside the nuclei in the cytoplasm (arrows point to cancer cells nuclei (Figure 4.3). FOXP3 nuclear expression was quantified by measuring fluorescence intensity (0-255) and dividing it by unit area. The more aggressive the cancer cell line was, the less nuclear FOXP3 it expressed (Figure 4-2 graph). The least FOXP3 expression was found in the nuclei of MDA-MB-231, an invasive breast cancer cell line.

To further validate this finding with digital microscopy and image analysis, FOXP3 immunocytochemistry for cell lines grown on the chamber slides was carried out. All sections were scanned and analysed by the Aperio system using nuclear and positive pixel algorithms to quantify nuclear and cytoplasmic FOXP3 expression as described in chapter 3. Quick scores were then calculated in the same way as in breast tissue IHC. FOXP3 transfectants and normal primary human mammary cells

were used as positive controls (Figure B, F). All cell lines examined expressed FOXP3, with the lowest expression being observed on MDA-MB-231 cell line.

It was noticed, however, that in some sections the Aperio system did not pick up all the nuclei. For example in MCF-7 Figure 4-3 panel D black arrows) some nuclei were not detected by the computer system as negative (blue) or positive (yellow, orange, red). If all nuclei had been detected, the MCF-7 MQS would have been lower because smaller percentage of nuclei would have been scored as positive.

The results indicate that all the cancer cell lines examined (MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) fail to import FOXP3 protein into the nuclei.

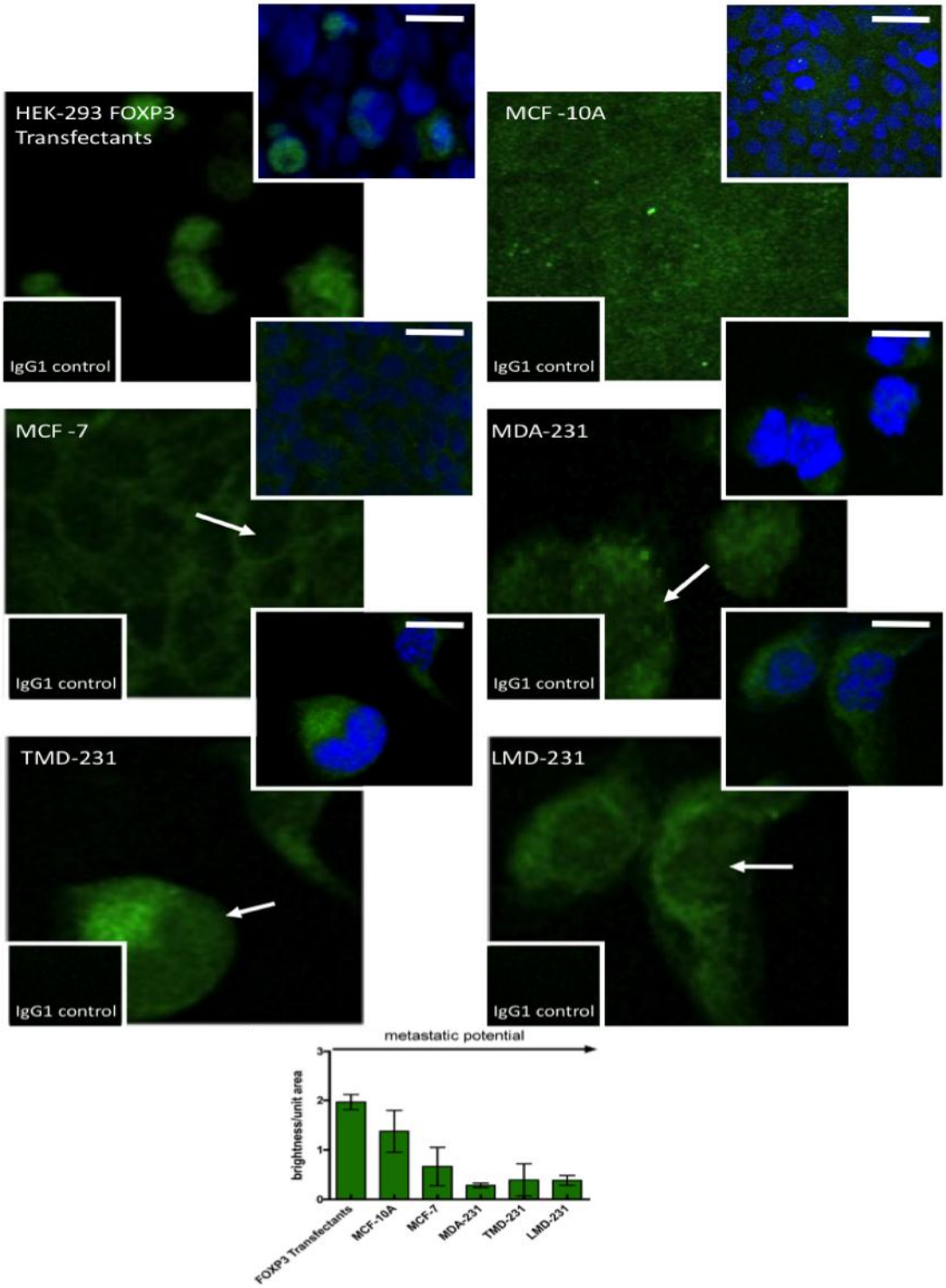


Figure 4-2: FOXP3 immunofluorescence staining of breast cell lines.

FOXP3 HEK-293 transfectants, MCF-10A, MCF-7, MDA-231, TMD-231, LMD-231 were stained with primary monoclonal anti-Foxp3 antibody (1:5 in 20% of normal rabbit serum) and FITC conjugated secondary antibody. Images in the right upper corners represent addition of DAPI (blue) staining to highlight the cellular nuclei. Leica TCSSP2 UV confocal microscope x40 magnification was used. Scale bars represent 50 µm. Arrows point to nuclei of cancer cells. FOXP3 nuclear expression was quantified by measuring fluorescence intensity (0-255) and dividing it by unit area. Results were plotted (bottom panel). Error bars represent SD; n=3.

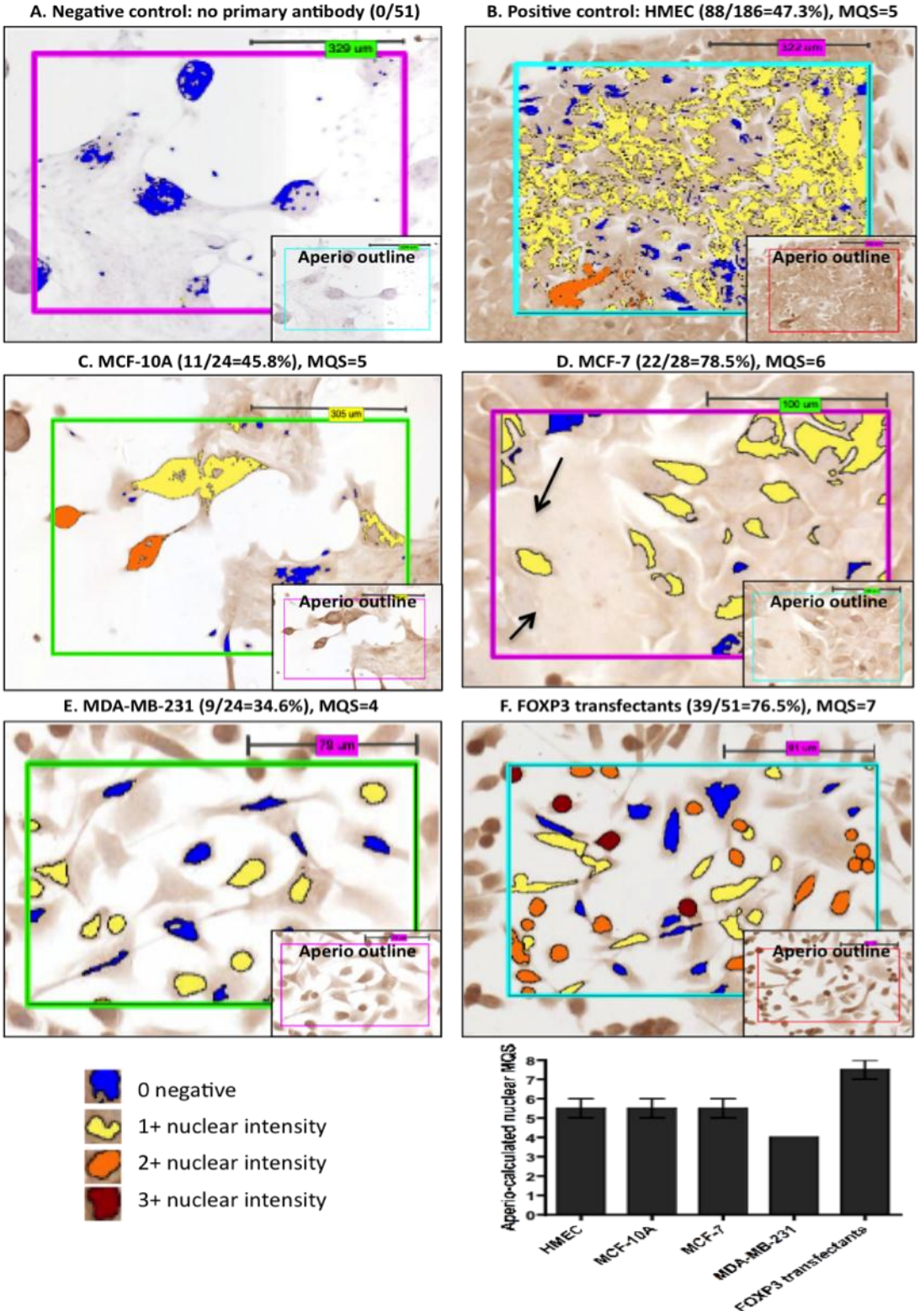


Figure 4-3: Immunocytochemistry and digital microscopy of FOXP3 nuclear expression in breast cell lines.

Cells were stained with primary monoclonal anti-Foxp3 antibody (cell culture supernatant 1:5 in 20% of normal rabbit serum) and biotinylated secondary antibody (DAKO). Slides were scanned with the Aperio scanner; digital images were analysed with IHC nuclear algorithm and staining was automatically quantified. The quick scores were calculated and plotted (bottom panel). Error bars represent SD; n=2.

4.4.2 Sub-cellular location of FOXP3 in breast cancer cells following induction of its expression.

Intracellular trafficking of FOXP3 to the nucleus of the cells is required in order to express this protein in its active form and subsequently maintain cellular homeostasis. Having observed pathological cytoplasmic localisation of FOXP3 protein in a range of breast cancer cells, the attempt to reverse this expression pattern was made by upregulating FOXP3 expression. Stable transfection of a cancer cell line with a plasmid encoding wild type FOXP3 protein was performed to determine whether it would reverse FOXP3 expression to the normal nuclear pattern. TGF-β-treated MDA-231 and stable FOXP3 transfectants of MDA-MB-231 cells (methodology is described in the chapter 5) were stained with primary monoclonal anti-Foxp3 antibody (1:5 in 20% of normal rabbit serum); FITC conjugated secondary antibody (green) and DAPI to visualize nuclei (blue). FOXP3 protein in MDA-231 breast cancer cells was localised predominantly in the cytoplasm (Figure 4-4 top column). Following treatment with TGF-β for 6h amount of FOXP3 significantly ($p<0,05$) increased but still its localization was mainly cytoplasmic. Transfection of MDA-231 cells with normal FOXP3 vector significantly increased FOXP3 expression within cellular nuclei (Figure 4-4 bottom column).

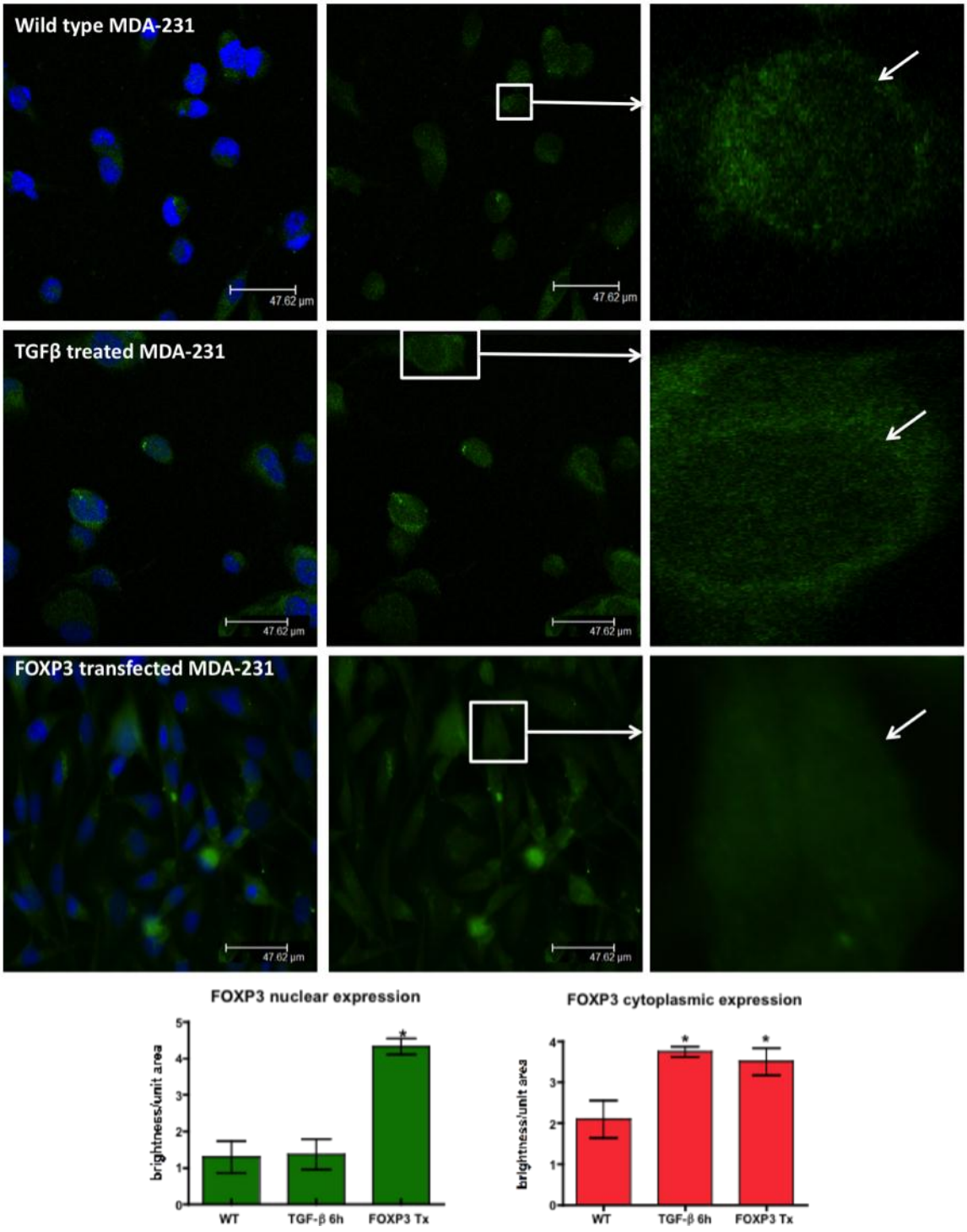


Figure 4-4: Sub-cellular localization of FOXP3 protein following upregulation of its expression.

Wild type, TGF-β treated and FOXP3 transfected MDA-MB-231 cells were stained with primary monoclonal Foxp3 antibody (1:5 in 20% of normal rabbit serum), FITC conjugated secondary antibody (green) and DAPI to visualize nuclei (blue).

Images in the middle column show green FITC immunofluorescence of FOXP3 within MDA-231 cells, whereas the left column represents merged DAPI and FITC images. Right column demonstrates selected cells in higher magnification. Leica TCSSP2 UV confocal microscope x40

magnification was used for left and middle columns, x60 for right column. Scale bars represent 47.6 µm. Arrows point to cellular nuclei.

FOXP3 nuclear and cytoplasmic expressions were quantified by measuring fluorescence intensity (0-255) and dividing it per unit area. Results were plotted and illustrated on the histograms (bottom panel). Error bars represent SD. This experiment has been repeated at least 3 times.

4.4.3 Analysis of nuclear localization sequences of FOXP3 gene

Having observed a failure of FOXP3 nuclear localisation in breast cancer cells, an attempt was made to identify possible mutations or splice variants in FOXP3 gene that may cause disruption of its nuclear localization (Figure 4-5).

Samples of cDNA from MDA-MB-231 and MCF-7 cell lines were amplified by conventional PCR using *Taq* and *Pfu* DNA Polymerases and sequenced. Finch TV software was used to assemble the sequences and compare them with FOXP3 reference gene. Figure 4-5 represent PCR electrophoresis gels of nuclear localization domains. Domain 1 gave at least two bands on electrophoresis gel: 480bp and 345bp. All the bands were cut out from the gel for sequencing. All the analysed cells analysed showed a shorter band representing FOXP3 isoform 1 or 2 with an expected product size of 344bp. The larger band with the insertion of 120bp might represent isoform 3 of FOXP3. The protein sequence of isoform 3 has been already described and published by Swissprot (Figure 4-6), however, there has been no experimental confirmation of this isoform so far. Interestingly, cancer cells (MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) had proportionally higher expression of isoform 3 compared to isoform 1 or 2 than normal cells as shown by electrophoresis densitometry (Figure 4-7).

Domain 2 gave a single band of 650bp as expected. They were sequenced confirming 100% alignment to the FOXP3 gene (AF277993).

Domain 3 PCR electrophoresis gel resulted in multiple bands shorter then expected product. Six bands for each cell line were sequenced confirming non-specific binding to myelin transcription factor and zinc finger protein. The primers for domain 3 spanned a large gene area of 1.4k and had a low melting temperature of 53.5ºC, therefore likely to give the multiple bands due to non-specific binding.

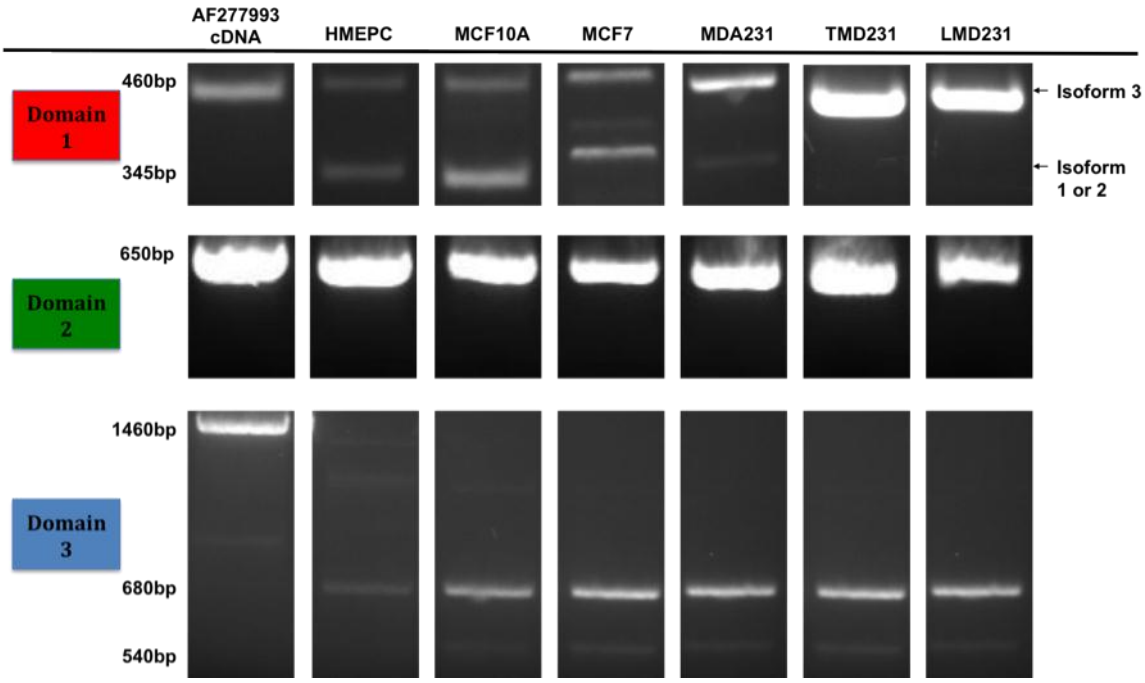
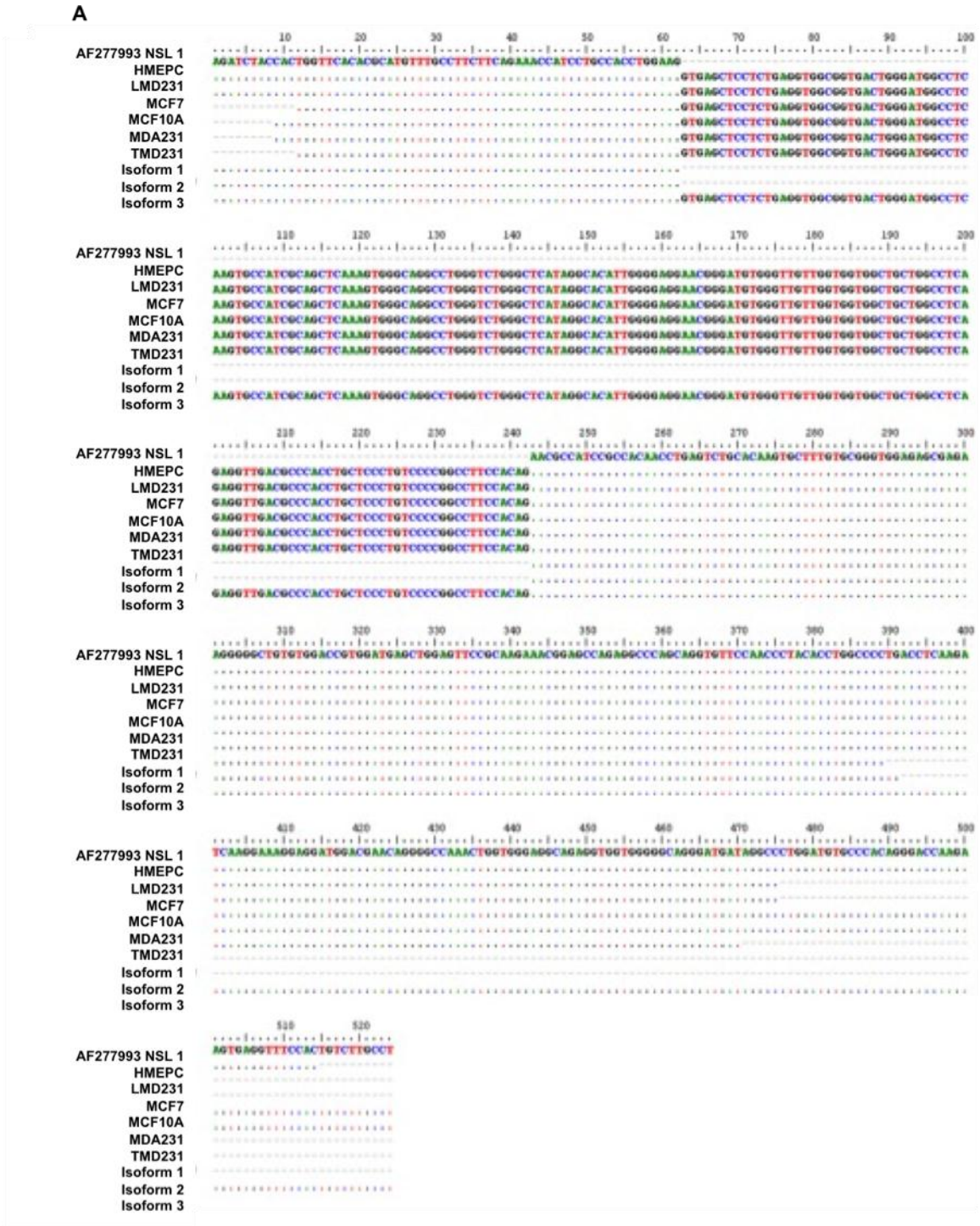


Figure 4-5: Multiple FOXP3 cDNA fragments on RT-PCR electrophoresis gel

RNA was extracted from various cell lines (primary epithelial breast cells HMEPC, benign breast cells MCF10A and breast cancer cells MCF7, MDA231, TMD231, LMD231). Samples of cDNA were amplified by conventional PCR using *Pfu* DNA Polymerase and sequenced. Finch TV software was used to assemble the sequences and compare them with FOXP3 reference gene. Amplification of domain 1 gave at least two bands on electrophoresis gel: 480bp and 345bp. The expected product's size was 344bp and the insertion of 120bp might represent isoform 3 of FOXP3. Domain 2 gave a single bands 650bp. Domain 3 resulted in multiple bands shorter then expected product. The experiment was performed 6-9 times in duplicates or triplicates.


A
AF277993 NSL 1
HMEPC
LMD231
MCF7
MCF10A
MDA231
TMD231
Isoform 1
Isoform 2
Isoform 3

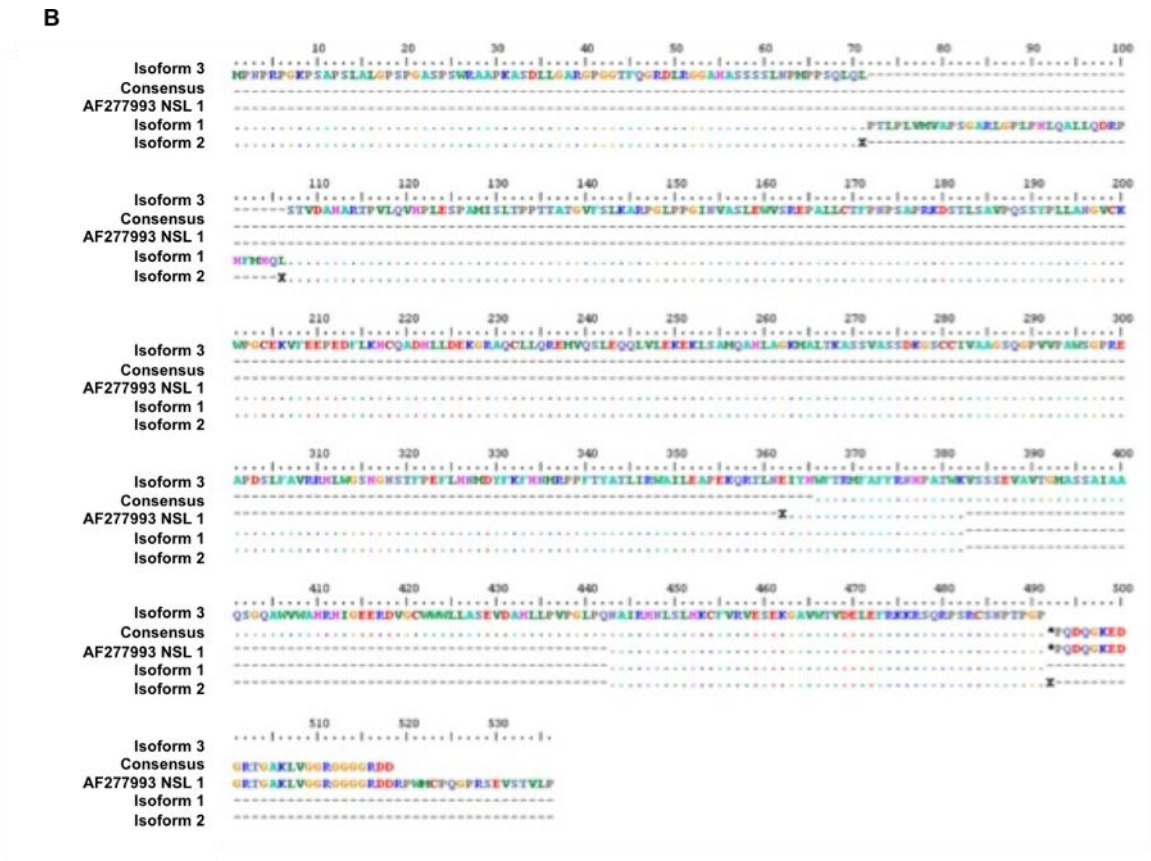


Figure 4-6: Identification of splice variant forms of the human FOXP3 gene

There are 3 isoforms: Isoform 1 (Q9BZS1), Isoform 2 (Q9BZS1-2), and Isoform 3 (Q9BZS1-3). All analysed sequences corresponded to Isoform 3. (A) The published human FOXP3 sequence (AF277993 Isoforms 1, 2 and decoded from protein "draft DNA" sequence of isoform 3) was aligned with nuclear sequence localization number 1 (NSL) on forkhead domain isolated from primary (HMEPC), benign (MCF10A) and breast cancer cells (MCF7, MDA231, TMD231, LMD231). (B) NSL 1 sequence was translated into protein and aligned with FOXP3 isoforms 1,2 and 3 published protein sequences. The experiment was repeated 7 times in duplicates or triplicates.

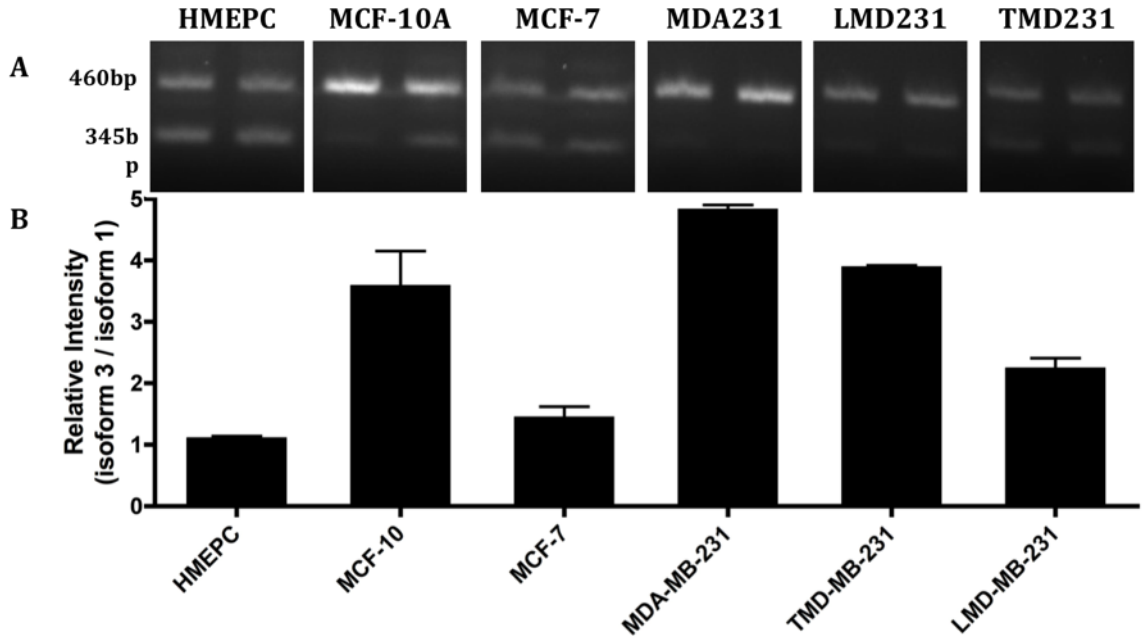


Figure 4-7: RT-PCR expression analyses and gel electrophoresis of alternatively spliced FOXP3 in breast cancer cells

A) The levels of FOXP3 expression mRNA were analyzed by RT-PCR. The product underwent agarose electrophoresis and then photographed by the AlphaImager 3400 gel imaging system (Alpha Innotec, Santa. Clara, CA). Two well-defined bands were visualized using agarose gel electrophoresis: isoform 3 (460bp) and isoform 1 or 2 (345bp). N=7

B) FOXP3 mRNA levels were determined by densitometry. Data ware expressed as ratios of longer (isoform 3) to shorter band (isoform 1 or 2) and as means ± SE of 3 independent experiments. The result suggests that at the mRNA cancer cells (MCF-7, MDA-MB-231, TMD-MB-231, LMD-MB-231) had more isoform 3 comparing to isoform 1 or 2 then HMPC.

4.5 Discussion

Intracellular trafficking of FOXP3 to the nucleus is required in order for this protein to function. FOXP3 physically interacts with the nuclear factor of activated T cells (NFAT) and forms nuclear complexes that are important to regulate the transcription of several target genes (Marson et al., 2007) thereby conferring Tregs their suppressive function (Wu et al., 2006, Rudensky et al., 2006). There are three regions on FOXP3 that contribute to its nuclear transport (Hancock W, 2009) and identification of factors which interact with these domains is crucial for studying FOXP3 regulation on cancer cells. It has been demonstrated that the failure of FOXP3 nuclear translocation is sufficient to inhibit its tumour suppressor function in prostate cancer (Wang L, 2009, Wang L, 2009). Somatic mutations detected in human prostate cancer abrogated MYC repression by preventing FOXP3's nuclear localization (Wang L, 2009, Wang L, 2009). The presence of alternative splice variants as dominant forms of human FOXP3 isoform can also lead to disruption of nuclear localization by inhibition of FOXP3fl in a dominant negative manner (Hancock W, 2009). During initial experiments, a novel isoform 3 was found to be highly expressed in cancer cells (MCF7, MDA-MB-231, TMD-231, LMD-231) and benign breast cell line MCF-10A in comparison with primary mammary cells (HMPC). In contrary to previous work (Zuo et al., 2007b), this study did not reveal any mutations in FOXP3 gene. However, the authors who described FOXP3 mutations, used genomic DNA from cancer tissue rather then cell lines.

Since FOXP3 is a transcription factor, majority of authors considered the presence of nuclear FOXP3 as an indication of its functional expression (Liu et al., 2009, Wang L, 2009, Zuo et al., 2007d). Immunohistochemical staining of the cytospins of cancer cell lines showed cytoplasmic expression of FOXP3 predominantly in melanoma (GERL), colon (HCA 2.6), and breast cancer (MCF7) cell lines and both cytoplasmic and nuclear expression in cell lines of lung cancer (GILI) and T lymphoblastic leukemia (JURKAT) (Karanikas et al., 2008). In agreement with their

findings this study demonstrated that cancer cell lines (MCF7, MDA-MB-231, TMD-231, LMD-231) failed to import FOXP3 protein into their nuclei where it is required to maintain its tumour suppressor function. Following 6h TGF-β treatment, the amount of FOXP3 protein significantly increased but still its localization was mainly cytoplasmic. FOXP3 expression was increased in MDA-MB-231 breast cancer cells transfected with FOXP3 plasmid compared to the wild type MDA-MB-231 cells. Importantly, there was an increase in intra-nuclear FOXP3 expression in the FOXP3 transfectants. This may indicate that cancer cells fail to import FOXP3 protein into their nuclei due to the loss of FOXP3 biological function. However, when transfected with the normal FOXP3 plasmid, cancer cells reverse to their physiological non-cancerous phenotype and express FOXP3 in their nuclei. FOXP3 gene may be inactivated in breast cancer by excessive proportion of FOXP3 splice variants or presence of somatic mutation, which results in disruption of nuclear localisation of FOXP3 protein and leads to development of malignant FOXP3 phenotype. A change in the subcellular localization of FOXP3 from a more cytoplasmic and perinuclear to a nuclear expression pattern, possibly due to posttranslational modifications, has recently been reported for Tregs activated with anti-CD3/anti-CD28 antibodies (Ebert LM, 2008). The question remains whether, in human breast tissue, heterogeneous subcellular FOXP3 localization reflects the presence of its different post-translationally modified forms and whether such modifications are functionally relevant.

4.6 Summary of observations:

- Breast cancer cell lines unlike non-cancerous cells failed to import FOXP3 protein into nuclei.

- TGF-β significantly increased expression of cytoplasmic FOXP3 whereas transfection of normal FOXP3 vector increased its nuclear expression.

- Nuclear localization sequences of FOXP3 contained multiple bands of shorter then expected products what might represent various splice variants.

- The increase of isoform 3 expression was observed in all cancer cell lines. Isoform 3 nucleotide sequence might provide an experimental confirmation of corresponding protein described by Swissprot.

Chapter

5 Role of FOXP3 in CXCR4-induced migration of breast cancer cells

5.1 Introduction

5.1.1 CXCR4-induced migration of breast cancer cells

Metastasis is the major cause of death associated with breast cancer (Gupta and Massague, 2006). Metastasis of cancer cells is a complex process including invasion, hemangiogenesis, lymphangiogenesis, trafficking of cancer cells through blood or lymph vessels, extravasations, organ-specific homing, and growth. Evidence suggests that CXC chemokine receptor 4 (CXCR4) plays a critical role in the homing of cancer cells to specific metastatic sites including lung, liver and bone (Muller et al., 2001).

CXCR4 is normally expressed on various cell types, including: endothelial cells (Volin et al., 1998, Feil and Augustin, 1998), bronchial epithelial cells (Eddleston et al., 2002), lymphocytes (Bleul et al., 1996b, Bleul et al., 1996a), intestinal (including colonic) epithelial cells (Dwinell et al., 1999, Jordan et al., 1999), primitive haematopoietic progenitor cells (Aiuti et al., 1997), microglia, neurons and astrocytes (Bonavia et al., 2003), vascular smooth muscle cells (Schecter et al., 2003) and fibrocytes (Phillips et al., 2004). CXCR4 is also expressed in pluripotent stem cells, including mammary stem cells (Dontu et al., 2003). This suggests that CXCR4 may define the progenitors of breast cancer (Liu et al., 2005).

CXCR4 expression in the primary tumour has been positively correlated with the degree of lymph node metastasis, homogenous metastasis to the lungs, bone metastasis, poor patient overall survival and tumour grade (Altundag et al., 2005). The CXCR4 ligand, CXCL12, is expressed in bone marrow (Semerad et al., 2005), lung (Phillips et al., 2003, Muller et al., 2001), liver (Sun et al., 2005, Wald et al., 2004), adrenals (Sun et al., 2005), brain (Bonavia et al., 2003) promoting migration of cancer cells (Fernandis et al., 2004). Reports demonstrated CXCR4/CXCL12

inhibition in preventing breast cancer cell migration to metastatic sites (Harvey et al., 2007, Harvey, 2005).

5.1.2 Regulation of CXCR4 expression by FOXP3

There is evidence that the FOXP3 transcription factor may participate in the regulation of the expression and function of the CXCR4 chemokine receptor (Merlo et al., 2009). TGF- β, SMAD, NFAT can occupy Foxp3 enhancer and/or promoter loci and activate Foxp3 transcription on Tcells (Ruan et al., 2009).

FOXP3 in T cells is known to induce or repress a number of genes, including CXCR4 (Marson A, 2007, Zheng Y, 2007). It binds to the region upstream of the transcriptional start site of CXCR4 and CCR7 two chemokine receptors reported to play a vital role in cancer invasion and metastasis (Marson A, 2007).

FOXP3 is a master regulator of T reg cells which are known to be present in the inflammatory infiltrate of breast cancer (Zheng and Rudensky, 2007). Inflammatory mechanisms influence tumorigenesis and metastatic progression even in cancers whose aetiology does not involve pre-existing inflammation or infection, such as breast and prostate cancers (Grivennikov et al.). For instance, prostate cancer metastases are associated with the infiltration of lymphocytes into advanced tumours and the upregulation of two tumour-necrosis-factor family members: receptor activator of nuclear factor-kB (RANK) ligand (RANKL) and lymphotoxin (Luo et al., 2007). Most recently, study of a mouse model and humans revealed that RANKL-producing T cells expressed FOXP3 (Tan et al.). The dependence of pulmonary metastasis on T cells was replaceable by exogenous RANKL, which also stimulated pulmonary metastasis of RANK positive human breast cancer cells (Tan et al.).

Up-regulation of FOXP3 inhibits the activity of NF-kappa B. FOXP3 might inhibit cell migration and invasion partly through the inhibition of NF-kappa B and its target genes MMPs (Grant et al., 2006, Hai-Yan Zhang, 2010). NF-kappa B is known to induce CXCR4 expression and therefore induction of CXCR4 fails with upregulation of FOXP3 (Helbig et al., 2003).

CXCR4 and CCR7 are two chemokine receptors which play a vital role in cancer invasion and metastasis (Marson A, 2007). The expression of CXCR4 in breast cancer is linked to the expression of HER-2 (Merlo A, 2009) and it has been proven that FOXP3 is an important regulator of this oncogene (Zuo et al., 2007c). Hence, it is also possible that FOXP3 can regulate CXCR4 expression indirectly through its action on HER-2. Foxc transcription factors are important regulators of the chemotactic motility of endothelial cells through the induction of CXCR4 expression (Hayashia H, 2008). Some members of the forkhead family of transcription factors (Foxc1 and Foxc2) are able to directly induce CXCR4 expression by activating its promoter on endothelial cells. Furthermore, Foxc1-deficient endothelial cells show a significant reduction in CXCR4 expression as well as CXCL12-stimulated migration (Hayashia H, 2008).

5.2 Specific aims and objectives

- To examine FOXP3 expression in breast cancer cells following stimulation with TGF- β1

- Construction of FOXP3 overexpressing breast cancer stable transfectants

- To study the chemokine responsiveness of breast cancer cells following induction of FOXP3 expression

- To analyse the functional significance of FOXP3 nuclear localisation on CXCR4-induced migration of breast cancer cells

5.3 Methods

5.3.1 Induction of FOXP3 expression with TGF-β1

Cells were incubated in their normal growing conditions with or without TGF-β1 (R&D Systems) at final concentration of 10 ng/ml for 6h, 12h, 24h, 48h and 72h. At the protein level FOXP3 expression was measured using immunofluorescence staining and Leica TCSSP2 UV confocal microscope by quantifying the fluorescence intensity (0-255). At mRNA level, FOXP3 expression was measured by real-time PCR.

5.3.2 Transient transfection of MDA-MB-231 cells with a reporter gene construct

In order to establish the optimum conditions for stable transfection, a plasmid encoding a green fluorescence protein (GFP) was used to transiently transfect MDA-MB-231. Cells were transiently transfected with EGFP reporter plasmid (encodes enhanced green fluorescent protein) for 24, 48 or 72 hours using Effectene Transfection Kit (Qiagen). The day before transfection, 0.9–4.0 x 10^5 cells were seeded into each 6-well plate and 1600 µl of standard growth medium containing serum and antibiotics was added. The cells were incubated under their normal growth conditions (37°C and 5% CO2) until 70-80% confluent on the day of transfection. Various amounts of EGFP plasmid were dissolved in TE buffer, pH 7 (DNA concentration: 325 ng/µl) with the DNA-condensation buffer, Buffer EC, to a total volume of 100 µl; enhancer and effectene reagent were added keeping the ratio of DNA to enhancer constant (1:25 and 1:50). Cells in 6-well plates were washed once with 4 ml of PBS, 1600 µl of standard growth medium containing serum and antibiotics was added and after 15 minutes of incubation, transfection-complexes were added. To avoid cytotoxicity, after 10 hours the medium containing effectene–DNA complexes was replaced by 5 ml of standard growth medium.

5.3.3 Construction of FOXP3 overexpressing breast cancer stable transfectants

Human FOXP3 vector was cloned within a pcDNA3 backbone (Invitrogen). It was a kind gift from Prof S Sakaguckhi from Kyoto University, Japan. The sequence was confirmed using T7 forward and bGHR reverse primers by GeneService (www.geneservice.co.uk). Once the sequence was verified, plasmid DNA was isolated following initial transformation into *Escherichia coli*. Cells were selectively isolated based upon their Ampicillin resistance. The target plasmid was eluted using Qiagen Mini-Prep kit, and this was sequenced again to ensure the plasmid expressed the target FOXP3 sequence. 1µg of the plasmid was linearised using the restriction enzymes *Xho1* and *Not1* and run on a 1% agarose gel to confirm the plasmid size of 6.9 kbp containing FOXP3 sequence 1.4 kbp + plasmid sequence 5.5 Kbp. Large quantities of FOXP3 plasmid DNA were isolated using plasmid DNA isolation kit Maxi-Prep (Sigma).

Two different methods of transfection depending on cell type were used to obtain stably transfected cell lines.

5.3.3.1 Effectene Reagent

Based on the results of the transient transfection with the EGFP reporter plasmid, the optimum DNA/effectene/enhancer combination was determined. The day before transfection, 2.0–8.0 x 10^5 cells were seeded into 60 mm dish and 4000 µl of standard growth medium containing serum and antibiotics was added. The cells were incubated under their normal growth conditions (37°C and 5% CO2) until 70-80% confluent on the day of transfection. The transfection was performed as per Qiagen Effectene reagent protocol. The DNA/effectene/enhancer combinations used are shown in Table 5-1. To avoid cytotoxicity effectene–DNA complexes were removed after 10 hours, cells were washed once with PBS and 5 ml of fresh growth medium was added to each well. After 48h the cells were trypsinised and transferred to 25 mm^3 flasks. Previously optimised G418 concentration was added to the flasks (1200 µg/ml) for generation of stable transfectants.

60 mm dish	FOXP3 DNA (µg)	Enhancer (µl)	Final volume of DNA in Buffer EC (µl)	Volume of Effectene Reagent (µl)	Volume of medium added to cells (µl)	Volume of medium added to complexes (µl)
Dish 1	2.0	16.0	100	50	4000	1000
Dish 2	2.0	16.0	100	100	4000	1000

Table 5-1: Transfection of MDA-MB-231 with FOXP3 plasmid

5.3.3.2 Amaxa system

Jurkat cells were transfected with the pcDNA 3.1(+)-FOXP3 vector using the Amaxa nucleofection system (Amaxa Biosystems, Germany). Prior to poration, cells were were grown in the 75cm^2 flasks. For each sample, 5 µg DNA (in 1-5 µl H_2O) was prepared. Six-well plates were prepared by filling the appropriate number of wells with 1 ml of complete medium. Cells were harvested with trypsin-EDTA, counted and centrifuged (1x10^6 cells per nucleofection sample), at 200×g for 10 min. The supernatant was completely removed and the cell pellet was resuspended. Nucleofector Solution V (Amaxa) was added to make a suspension of 1x10^6 cells/100 µl. 5 µg DNA was added to prepared 100 µl of cell suspension and then transferred into an Amaxa cuvette. The Cuvette was inserted into the cuvette holder of the Amaxa machine and the program Q-01 was used. The sample was removed from the cuvette immediately after the program was finished. Subsequently, 500 µl of the pre-warmed culture medium was added and the sample was transferred into 6-well culture plates. Cells were then incubated as required.

5.3.3.3 Generation of antibiotic resistant clones

It is necessary to determine the lowest concentration of antibiotic required to kill untransfected cells in order to generate a stable line containing integrated plasmid DNA. MDA-MB-231 cells were plated in 6-well plates. At 60-70% confluence Neomycin (G418 Calbiochem) selection media were added to the plates at following concentrations: 500 µg/ml, 800 µg/ml, 1000 µg/ml, 1200 µg/ml, 1500 µg/ml, 2000 µg/ml, 3000 µg/ml. Media replaced every 3-4 days and cells were examined. 800 µg/ml G418 was chosen as optimal concentration for selection of MDA-MB-231 transfected cells (Figure 5-7).

To select stable cell lines of MDA-MB-231 transfectants, 48 hours after transfection the cells were split into 24-well plates at 1/10 dilution in fresh medium containing an appropriate concentration of antibiotic required for this cell line. Antibiotic-

resistant clones were generally visible after 14 days culture in the presence of antibiotic. Clones were then isolated and cultured in selective medium.

5.3.4 Functional studies

5.3.4.1 Proliferation assay

The proliferation assay allows measurement of the number of cells growing in the absence or presence of proliferation-affecting agents. 10000 cells were seeded in the wells of a 12 well plate. At the same time TGF-β1 was added to half of the wells. The cells were incubated in their normal growing conditions for 24, 48 and 72h, after which time cells were detached from the flask, resuspended in PBS and counted.

5.3.4.2 Chemotaxis Assays

The chemotactic potential of cells was assessed using a transwell filter system developed using 8μm pore polyethylene terepthalate chemotaxis filters (Falcon, Fahrenheit Laboratory Supplies, Rotherham) and 24-well companion plates (Falcon). Human breast cancer cells require a Fibronectin matrix on the filter insert upon which to migrate. The underside of filter was coated with 150μl of 2.5μg/ml of Fibronectin (Sigma) at room temperature for 60 minutes prior to the assay. After removal of excess fibronectin, filters were allowed to dry for a further 30 minutes at room temperature before use. 1ml of 0.1% BSA-containing media was added to the lower well along with CXCL12 (0-200 nM), which was assessed for its chemotactic potential. Mammalian cells were resuspended in media containing 0.1% BSA at a concentration of $1x10^6$ cells/ml. 300μl of the cell suspension was then added to the filter before carefully placing the filter into the well. Chemotaxis assays were then incubated at 37°C, 5% CO_2 for a period of time optimised for the individual cell line used.

Filters were removed and fixed in methanol overnight at -20°C, dehydrated through increasing ethanol concentrations and then stained with haematoxylin (Sigma). Filters were mounted on glass microscope slides prior to counting. Migrant cells on the undersurface of the filter were assessed by counting 9 high

power fields per filter. Triplicates of each assay were counted with the identity of each filter being blinded to the assessor.

5.4 Results

5.4.1 Analysis of FOXP3 expression following TGF-β1 stimulation

TGF-β1 is able to stimulate FOXP3 expression on activated T-cells (Pekalski, 2009). A series of experiments was performed to examine the possibility of induction of the expression of FOPX3 transcription factor in a range of breast cancer cell lines. Real-time PCR and immunofluorescence were used to examine regulation of the expression of FOPX3 transcription factor by TGF-β1.

5.4.1.1 Real-time PCR examination

To examine regulation of FOXP3 expression by TGF-β1 at RNA level a real-time PCR was performed. A range of breast cancer cell lines was screened for up-regulation of FOXP3 mRNA expression following treatment with TGF-β1. T-cells activated with CD3/CD28 Dynabeads were used as positive control (Figure 5-1 A) and 18s RNA as a housekeeping gene. TGF-β1 significantly induced FOXP3 expression after 6 hours in all cancer cell lines analysed (MCF-7, MDA-MB-231, LMD-MB-231). Longer periods (24 hours, 48 hours- data not shown) of incubation with TGF-β1 did not lead to a further increase of FOXP3 expression (Figure 5-1 B). However, the large standard deviation indicated that the data were spread over a large range of values. Therefore, the MDA-MB-231 cell line, which gave the highest FOXP3 up-regulation, was nominated to verify the initial results. In order to improve the results further experiments were performed with two housekeeping genes: GAPDH (Figure 5-1C) and 18S RNA (Figure 5-1 D) and repeated four times in triplicates. TGF-β1 significantly increased FOXP3 expression after 6 hours in MDA-MB-231 and did not change FOXP3 expression after 24 hours.

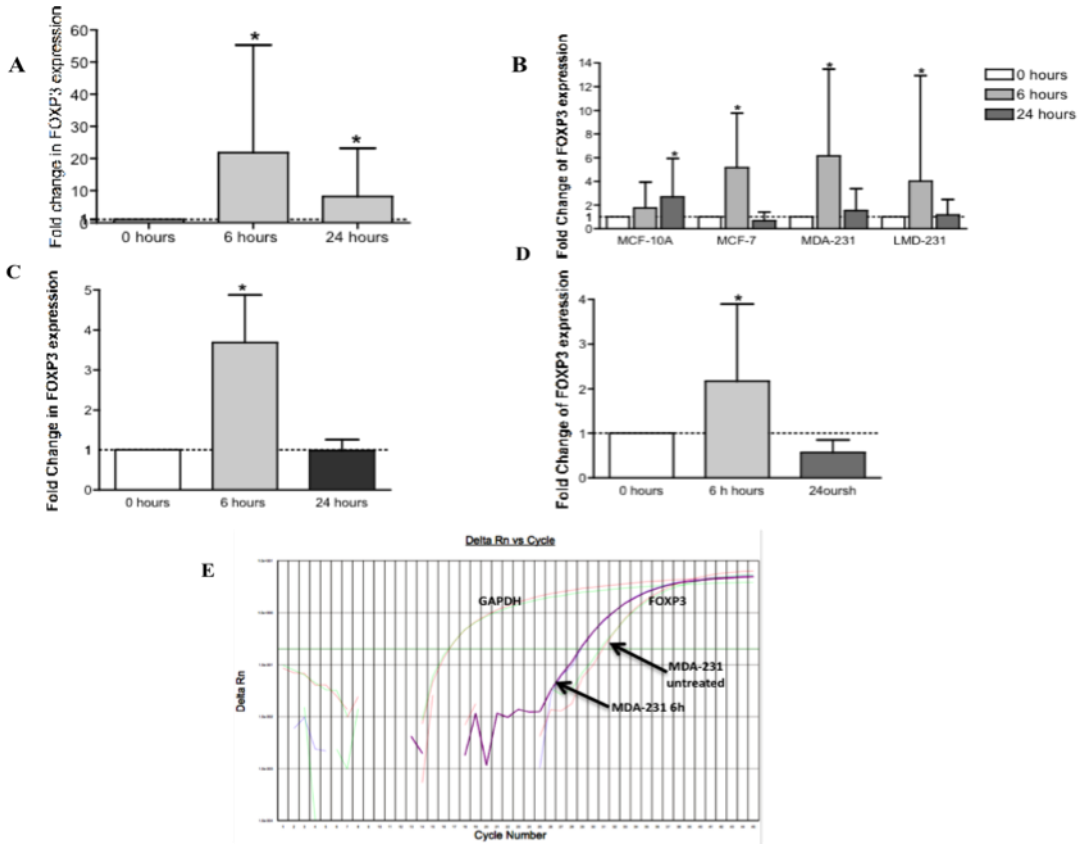


Figure 5-1: Real-time PCR analysis of FOXP3 expression in breast cancer cells following treatment with TGF-β

Positive control: A.) FOXP3 expression on T cells activated using CD3/CD28 Dynabeads after TGF-β1 treatment (housekeeping gene: 18s RNA, n=2). B) FOXP3 expression on breast cancer cell lines after TGF-β1 treatment (housekeeping gene: GAPDH, n=2), C) FOXP3 expression on MDA-MB-231 after TGF-β1 treatment (housekeeping gene: GAPDH, n=4), D) FOXP3 expression on MDA-MB-231 after TGF-β1 treatment (housekeeping gene: 18s RNA, n=4); *P<0.05, error bars indicate SD, Rest 2008 and Prism 3 software was used for statistical analysis. E) Representative real-time PCR graph showing amplifications of FOXP3 mRNA.

5.4.1.2 FOXP3 Immunofluorescence

Expression of FOXP3 by activated T cells and breast cancer cells stimulated with TGF-β1 was also examined at the protein level using primary monoclonal anti-Foxp3 antibody (1:5 in 20% of normal rabbit serum) and FITC conjugated secondary antibody. The 6-hour incubation with TGF-β1 was used in this experiment as it gave the highest FOXP3 upregulation at RNA level. FOXP3 expression was induced after 6-hour incubation with TGF-β1 in the cellular nuclei of T-cells (Figure 5-2 B) and cytoplasm of MCF-10A, MCF-7 and MDA-MB-231 cells (Figure 5-2 C).

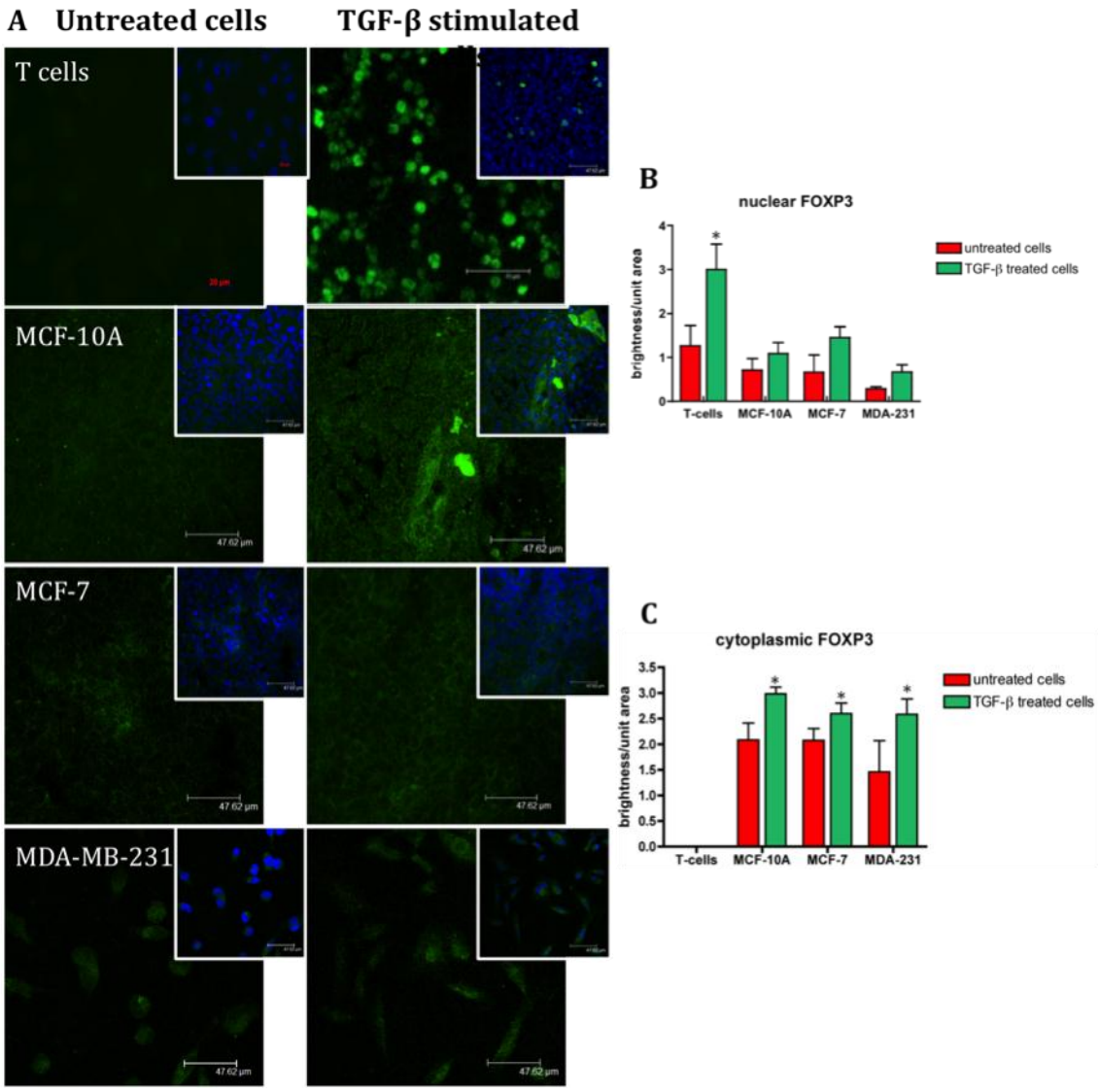


Figure 5-2: Immunofluorescence analysis of FOXP3 expression in breast cells following treatment with TGF- β.

A) Cells were stained with primary monoclonal Foxp3 antibody (1:5 in 20% of normal rabbit serum) and FITC conjugated secondary antibody. Images in the right upper corners represent addition of DAPI (blue) staining to highlight the cellular nuclei. Leica TCSSP2 UV confocal microscope x40. FOXP3 nuclear expression was quantified by measuring fluorescence intensity (0-255) and dividing it by unit area. B) Expression of FOXP3 in nuclei was quantified and plotted, C) Expression of FOXP3 in cytoplasm was quantified and plotted. *p<5 values indicate significant change from control cells.

5.4.2 Generation of FOXP3 breast cancer stable transfectants

Following observation of reduced FOXP3 nuclear expression in breast cancer cells, stable transfection of a cancer cell line with plasmid encoding wild type FOXP3 protein was performed to determine whether this would increase nuclear FOXP3.

Along with FOXP3 stable transfectants, MDA-MB-231 were transfected with the empty vector pcDNA3.1+/Zeo to generate mock stable transfectants for subsequent experiments and controls including mock transfectants.

A human FOXP3 sequence was cloned within a pcDNA3.1+/Zeo (Invitrogen) (Figure 5-3). *Escherichia coli* colonies were used to expand the DNA plasmid. Plasmid was isolated using a Mini-Prep kit (Qiagen). The mammalian expression vector was a kind gift from Prof S. Sakaguckhi from Kyoto University, Japan. The plasmid DNA was sequenced, confirming 100% homology with target FOXP3 sequence (AF299773 Genebank FOXP3 cDNA). To generate stable transfectants with DNA integrated into the chromosomes, use of linearised plasmid is recommended. Hence, the plasmid was linearised using *Xho1* restriction enzyme and its size was determined by agarose gel electrophoresis to confirm correct product size of 6.9kbp (FOXP3 sequence 1.4 kbp + plasmid sequence 5.5 Kbp) (Figure 5-4).

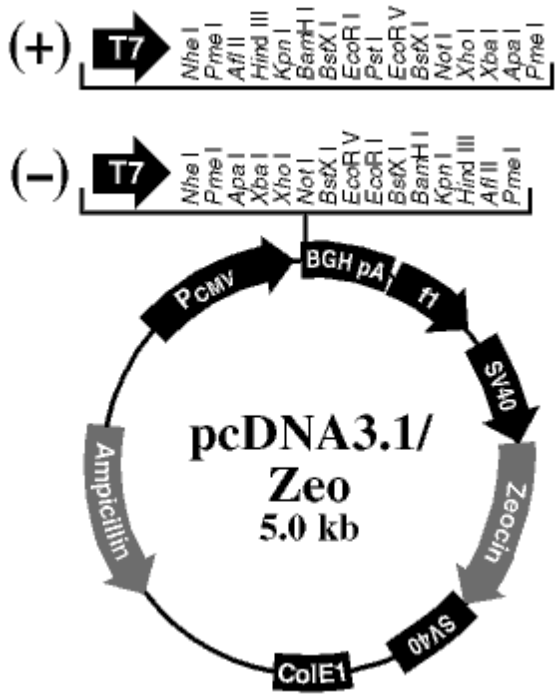


Figure 5-3: pcDNA3.1+/Zeo eukaryotic plasmid map.

This vector is designed for high-level stable and transient expression in mammalian hosts using the human cytomegalovirus (CMV) promoter. The unique restriction endonuclease recognition sites on either side of the cloning site are listed. The vector also contains resistance genes for both Ampicillin and Zeocin.

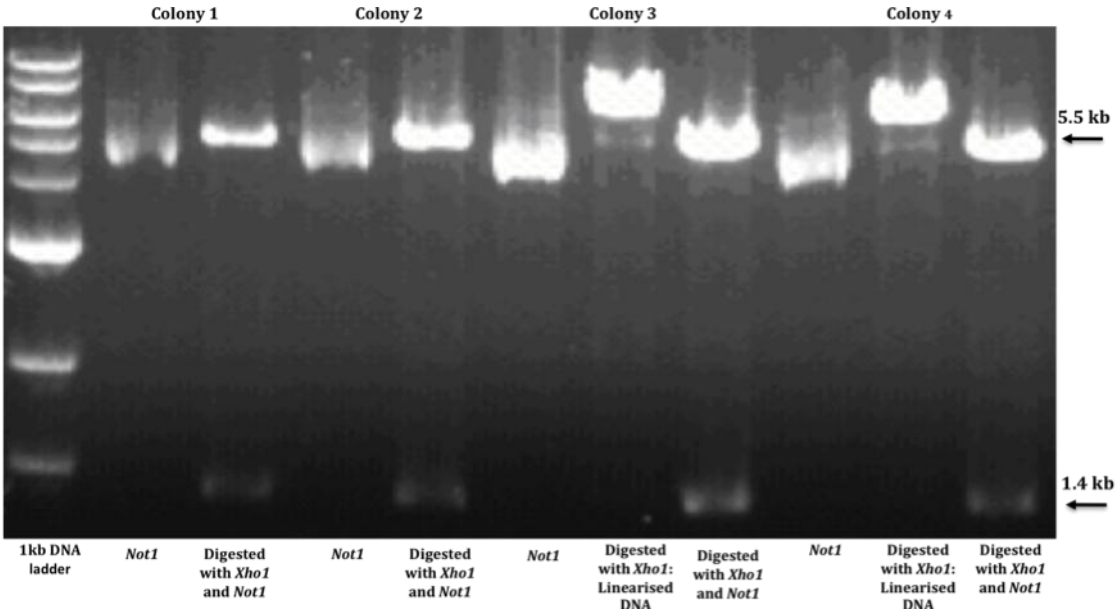


Figure 5-4: Agarose gel demonstrating restriction enzyme digestion of plasmid containing FOXP3 cDNA

Four *Escherichia coli* colonies were used to expand the DNA plasmid. Plasmid was isolated using a Mini-Prep kit (Qiagen). To produce transfectants that express FOXP3 DNA, the plasmid containing FOXP3 was sequenced and results confirmed 100% homology with AF299773 Genebank FOXP3 cDNA. DNA underwent enzyme digestion with *Xho1* and *Not1.* Plasmid was linearised with *Xho1* and its size was determined by agarose gel electrophoresis to confirm correct product size of 6.9kbp (FOXP3 sequence 1.4 kbp + plasmid sequence 5.5 Kbp).

5.4.2.1 Transient transfection of MDA-MB-231 cells

Prior to stable transfection, transient transfection was carried out. To determine optimum transient transfection efficiency a control study was performed with the reporter plasmid EGFP (Figure 5-5). The greatest transfection efficiency of around 65% of MDA-MB-231 cells was demonstrated after 48 hours. Based on these results, 48-hour transfection was done to produce MDA-MB-231 transient transfectants that expressed FOXP3.

Slides with transfectants were scanned and analysed with digital microscopy (Figure 5-6). The Quick Scores were calculated for nuclear FOXP3 expression. FOXP3 nuclear expression increased in FOXP3 transfected MDA-MB-231 cells (Median Quick Score = MQS=7) compared to the wild type MDA-MB-231 cells (MQS=4). Importantly, there was an increase in intra-nuclear FOXP3 expression in FOXP3 transfectants (Figure 5-6 D, arrows).

Following successful transient transfection, stable MDA-MB-231 FOXP3 transfectants were generated. MDA-MB-231 cell line was used for the stable transfection because it revealed low nuclear FOXP3 expression on immunofluorescence and immunocytochemistry (Figure 4-2, Figure 4-3) as well as high CXCR4 expression, which was mentained with passaging the cells (Harvey et al., 2007). A dose response curve for antibiotic selection was performed. 850 μg/ml of G418 was chosen as optimal concentration for selection of colonies (Figure 5-7). Empty vector pcDNA3.1+/Zeo was used as a negative control for stable transfection. HEK293 were transfected along with MDA-MB-231 as a positive control.

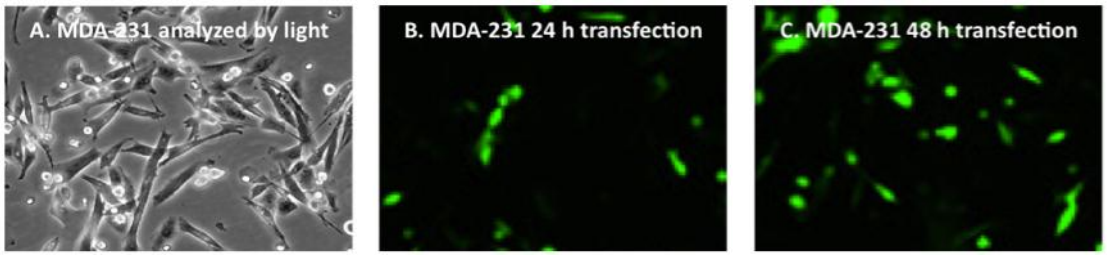


Figure 5-5: Optimization of transient transfection for MDA-MB-231 cells using the reporter plasmid encoding EGFP

MDA-MB-231 cells were transfected with EGFP plasmid over and expression analysed over a range of time points. A) Untransfected cells analysed by light microscopy; B) 24-hour transfection; C, 48-hour transfection. Optimum transfection efficiency was observed at 48 hours.

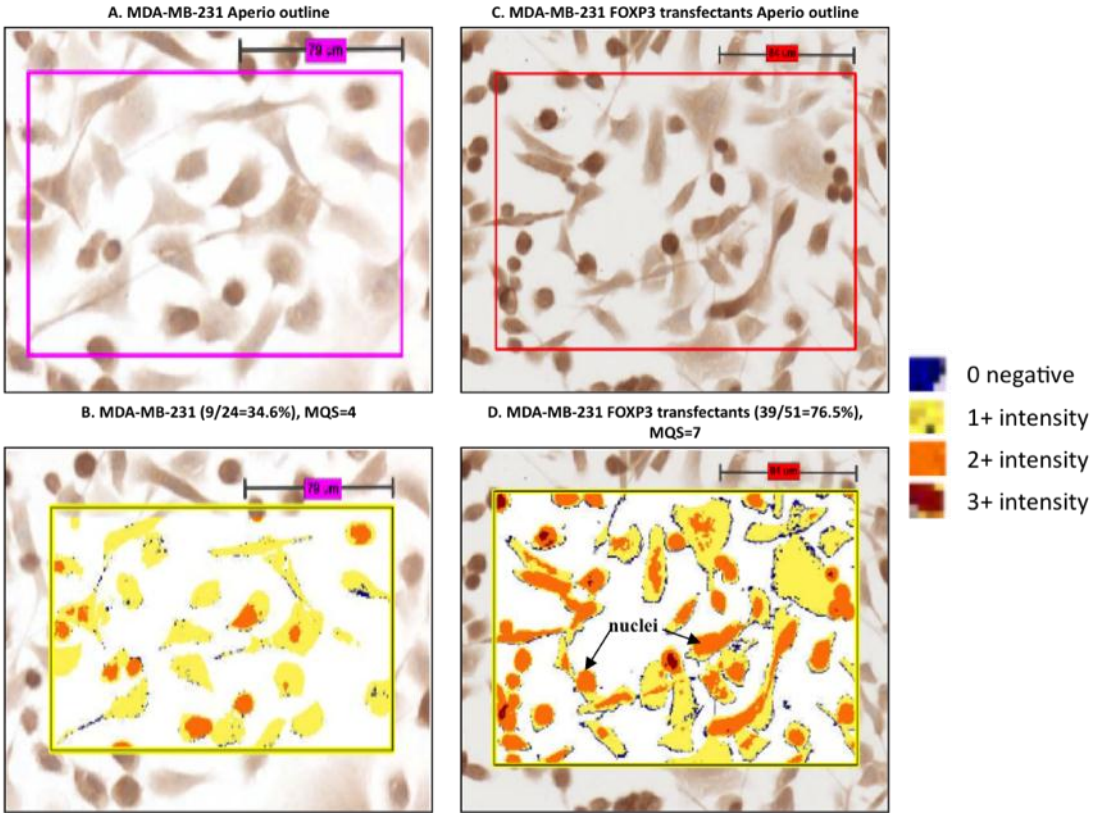


Figure 5-6: Transient transfection of MDA-MB-231 with FOXP3

A: WT MDA-MB-231 cells growing on the chabber slides were fixed, permeabilized, and stained with primary monoclonal Foxp3 antibody (cell culture supernatant) at the optimum concentration of 1:5 in 20% of normal rabbit serum and biotinylated secondary antibody (DAKO). The images were acquired and generated by Aperio digital scanner.

C: Transient transfection of cells growing on chamber slides was performed. Effectene (Qiagen) was used to transiently transfect MDA-MB-231 with FOXP3 plasmid. Again, cells were fixed, permeabilized, and stained with primary monoclonal Foxp3 antibody (cell culture supernatant) at the optimum concentration of 1:5 in 20% of normal rabbit serum and biotinylated secondary antibody (DAKO). The images were acquired and generated by Aperio digital scanner.

The digital images of WT (B) and stable transfectants (D) were analysed with IHC nuclear and positive pixel count algorithms and staining was automatically quantified. Each section was manually or digitally scored for the percentage of positively stained cells (0: no staining, 1: <1%, 2: 1-10%, 3: 11-33%, 4: 34-66%, 5: 67-100%). Results are representative of two independent experiments.

5.4.2.2 Real-time PCR analysis of stable FOXP3 transfectants

Individual stable clones were expanded: out of 9 isolated colonies, 3 were further cultured and examined for FOXP3 expression. Using real-time PCR, FOXP3 expression was significantly increased in all 3 colonies analysed ($P<0.05$). The maximal FOXP3 upregulation was observed in colony 3 (Figure 5-8).

5.4.2.3 Immunofluorescence analysis of FOXP3 transfectants

HEK-293 FOXP3 transfectants, wild type (WT) MDA-MB-231, MDA-231 FOXP3 transfectants and MDA-MB-231 transfected with empty vector pcDNA3.1+/Zeo grown on the chamber slides were stained with primary monoclonal Foxp3 antibody (1:5 in 20% of normal rabbit serum), FITC conjugated secondary antibody (green), and DAPI to visualize nuclei (blue). FOXP3 protein in FOXP3 transfected HEK-293 and MDA-MB-231 cells was localised predominantly in the nuclei. HEK-293s, as a type of T-cells, don't have the cytoplasm so the FOXP3 localizes only in the nuclei. Transfection of MDA-MB-231 cells with the FOXP3 vector significantly increased FOXP3 expression within cellular nuclei compared to negative control (transfectants with the empty vector) and WT cells (Figure 5-9 E). However, there was no significant difference in cytoplasmic FOXP3 expression between WT MDA-MB-231, MDA-231 FOXP3 transfectants and MDA-MB-231 transfected with empty vector pcDNA3.1+/Zeo (Figure 5-9 F).

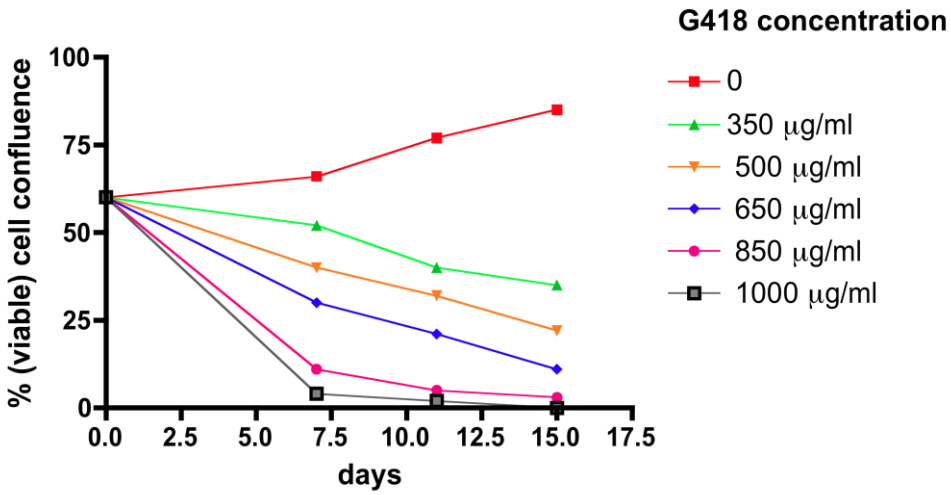


Figure 5-7: G418 killing curve for MDA-MB-231 cells.

MDA-MB-231 cells were seeded onto 6-well plates and cultured with different concentrations of the antibiotic. Daily assessment of confluence was made by visual inspection of the growth area and the antibiotic reapplied every 4 days. 850 μg/ml of G418 was determined as the optimum concentration for selection of colonies

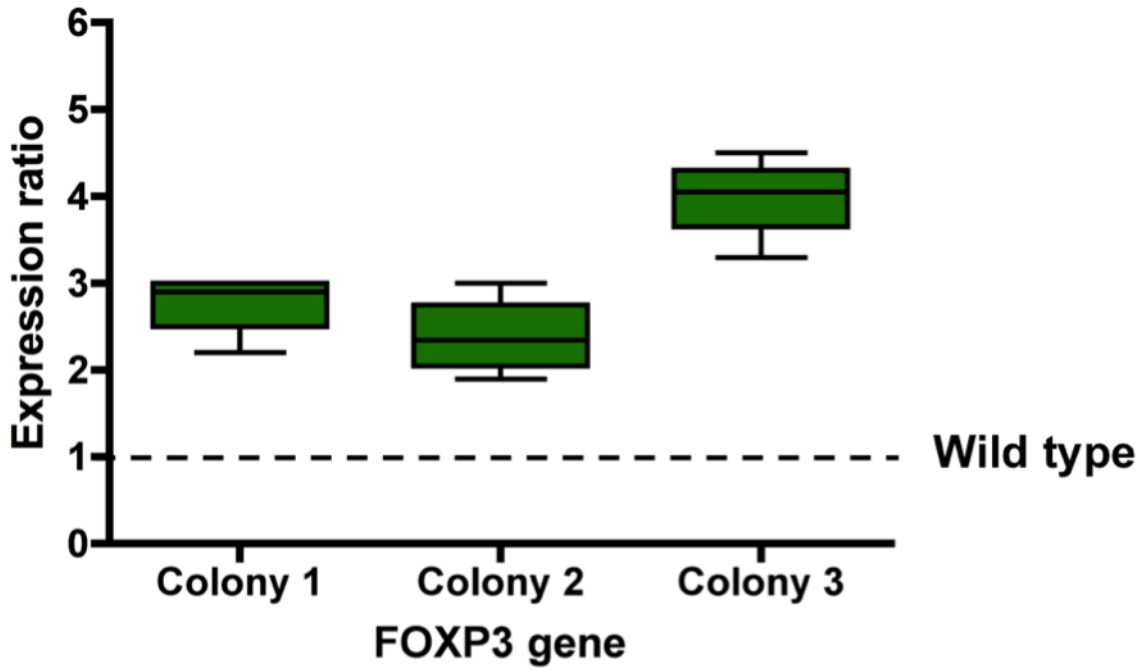


Figure 5-8: RT-PCR analysis of FOXP3 expression in MDA-MB-231 FOXP3 stable transfectants

Representative real-time PCR graph showing amplification of FOXP3 mRNA in relation to wild type MDA-MD-231. FOXP3 expression was significantly induced in all 3 colonies analysed ($P<0.05$). The maximal FOXP3 upregulation was observed in colony 3.

Boxes represent the interquartile range, or the middle 50% of observations. The line in the boxes represents the median gene expression. Whiskers represent the minimum and maximum observations. Housekeeping gene was GAPDH. The experiment was repeated 3 times in triplicates.

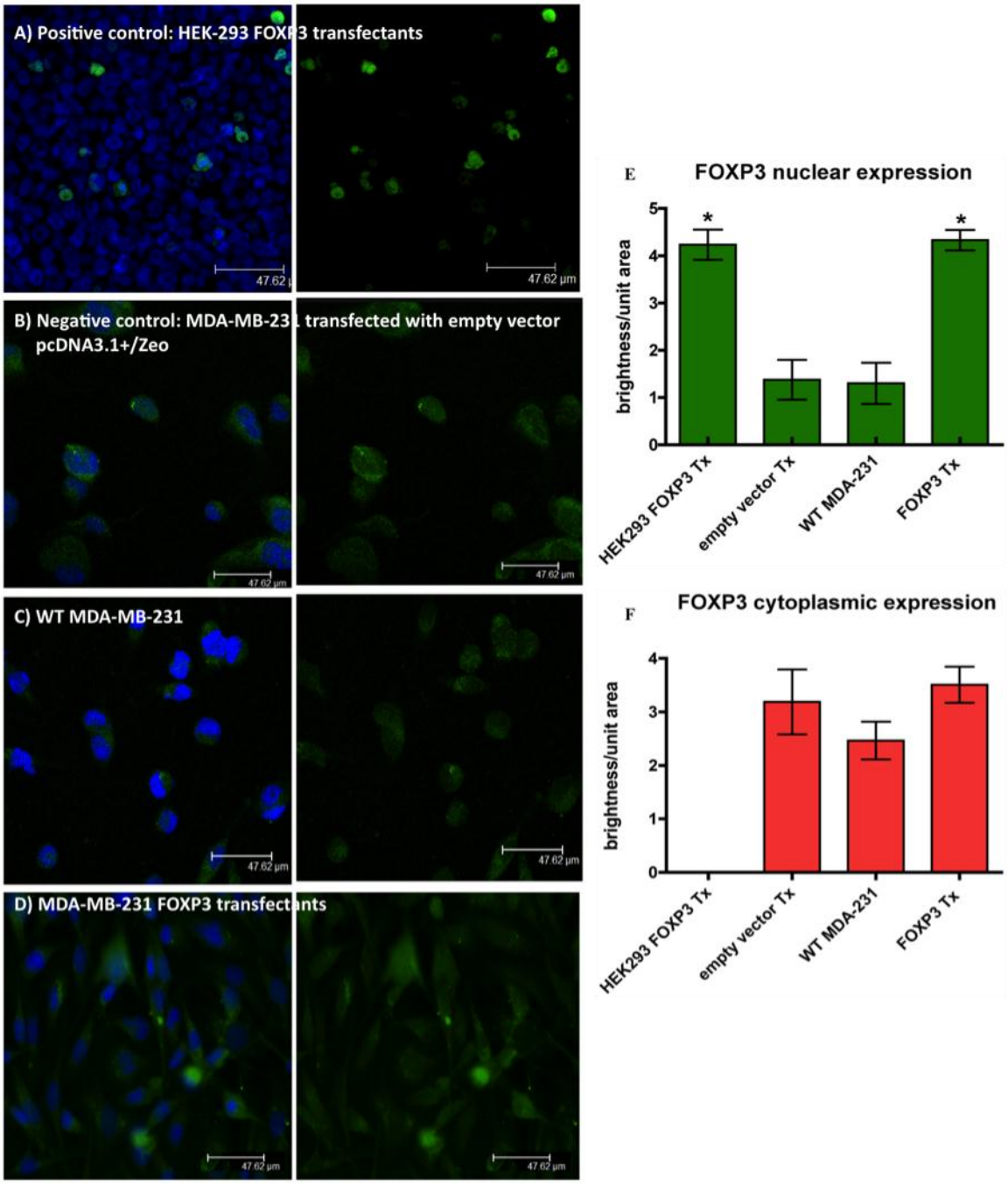


Figure 5-9: Immunofluorescence analysis of FOXP3 expression in MDA-MB-231 FOXP3 transfectants

HEK-293 FOXP3 transfectants (A), MDA-MB-231 transfected with empty vector pcDNA3.1+/Zeo (B), wild type MDA-MB-231 (C), MDA-231 FOXP3 transfectants (D) were stained with primary monoclonal Foxp3 antibody (1:5 in 20% of normal rabbit serum), FITC conjugated secondary antibody (green) and DAPI to visualize nuclei (blue). Expression of FOXP3 in nuclei (E) and cytoplasm (F) was quantified (fluorescence intensity/ unit area) and plotted. Images in the right column show green FITC IF of FOXP3, whereas the left column represents merged DAPI and FITC images. Leica TCSSP2 UV confocal microscope at x40 magnification was used. Scale bars represent 47,6 µm. Error bars represent SD. This experiment has been repeated at least 3 times.

5.4.3 CXCR4 expression of breast cancer cells following induction of FOXP3 expression.

Following validation of stable transfection of cells with FOXP3 vector, changes of CXCR4 expression were investigated.

Six weeks after transfection of MDA-MB-231 levels of the FOXP3 and CXCR4 transcripts were quantitated by real-time PCR. For comparison, results for MDA-MB-231 stimulated with TGF-β (10 ng/mL) for 6h were added to the graph. There was significant downregulation of CXCR4 in FOXP3 transfected breast cancer cells. Stimulation of FOXP3 expression with TGF-β did not have a significant effect on CXCR4 expression levels (Figure 5-10 A, arrows).

Immunofluorescence of cells transfected with FOXP3 vector decreased CXCR4 expression within cellular membrane (Figure 5-10 B arrows) comparing to negative control transfectants with the empty vector. Stimulation of MDA-MB-231 with TGF-β did not influence the CXCR4 expression.

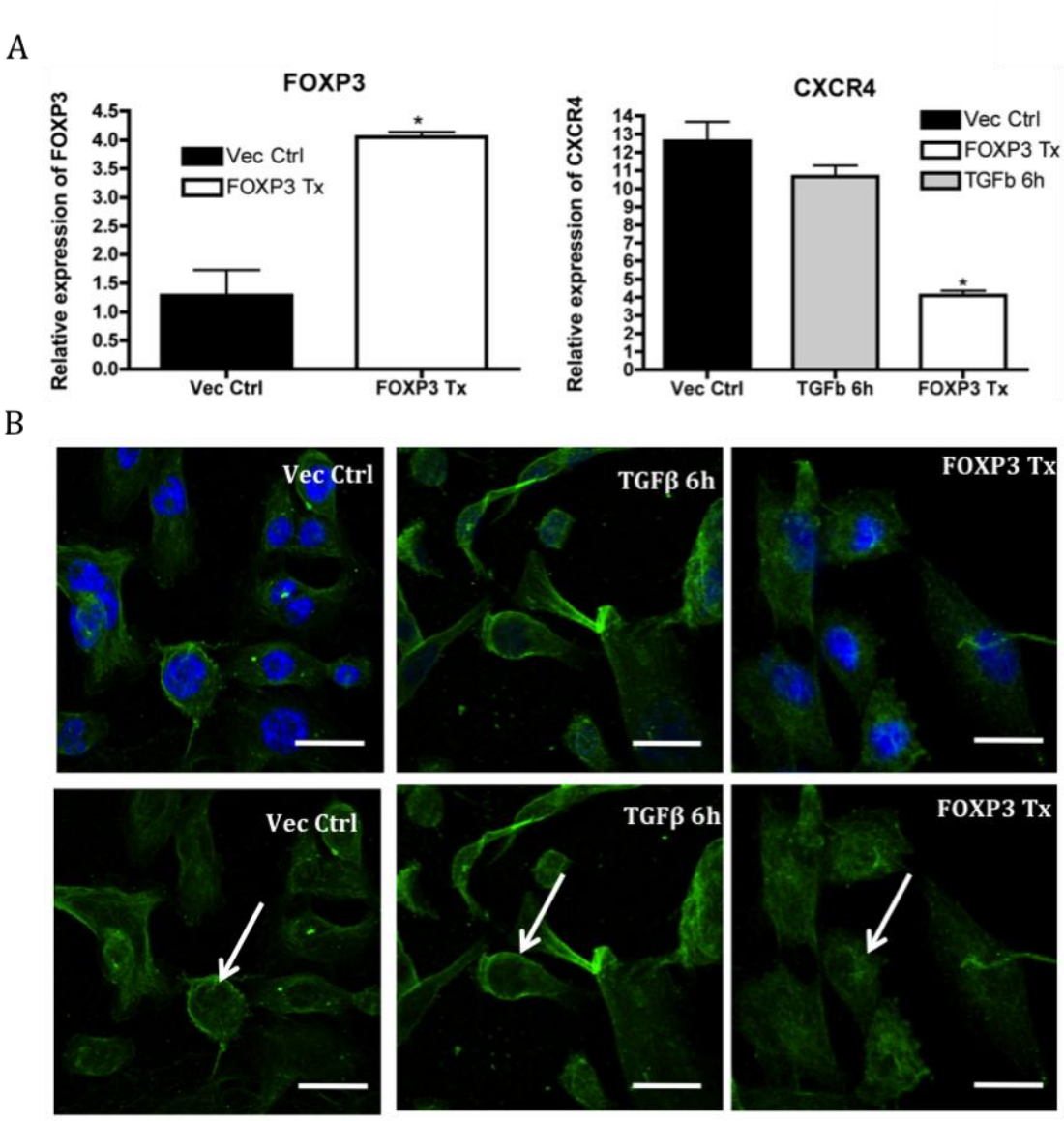


Figure 5-10: Analysis of CXCR4 expression in breast cancer cells following FOXP3 transfection

A) Overexpression of FOXP3 resulted in downregulation of CXCR4 in MDA-MB-231 breast cancer cells. MDA-MB-231 were transfected with either normal FOXP3 vector or control vector. Six weeks after transfection levels of the FOXP3 and CXCR4 transcripts were quantitated by real-time PCR (GAPDH was used as a house-keeping gene; error bars represent SD, *$p<0.05$); representative results of 3 similar experiments. For comparison, MDA-MB-231 stimulated with TGF-β (10 ng/mL) for 6h were added to the graph.

B) CXCR4 immunofluorescence staining: FOXP3 Tx - MDA-MB-231 FOXP3 transfectants, Vec Ctrl - MDA-MB-231 transfected with empty vector pcDNA3.1+/Zeo (control vector transfectants), TGF-β 6h – cells treated with TGF-β (10 ng/mL) for 6h. Cells were stained using primary monoclonal CXCR4 antibody (1:250 in 20% of NRS) and FITC conjugated secondary antibody. The top panel Images represent addition of DAPI (blue) counter-stain to visualise nuclei. Leica TCSSP2 UV confocal microscope x20 magnification was used. Arrows poin to cellular membrane.

5.4.4 Chemokine responsiveness of breast cancer cells following induction of FOXP3 expression

5.4.4.1 Validation of chemotaxis assay

Following confirmation that MDA-MB-231, TMD-MB-231 and LMD-MB-231 demonstrated surface CXCR4 expression (chapter 3), it was necessary to investigate whether this resulted in functional difference between the cell lines. In order to optomise chemotaxis assay MDA-MB-231, and cell lines dervived from MDA-MB-231 (TMD-MB-231, LMD-MB-231) were used alomng T-cells for positive controls.

The chemotaxis assay was optimised for incubation time and both the presence and absence of fibronectin prior to definitive assays. Cells were incubated with varying concentrations of chemokine at 37^{o}C for 24 hours in 0.1% BSA medium. Migration of cells through the 8μm filter was assessed after 12 and 24 hours. After 24h MDA-MB-231, TMD-MB-231 and LMD-MB-231 cells demonstrated significant chemotaxis towards 6-50nM CXCL12 compared to the "No CXCL12" control. TMD-MB-231 and LMD-MB-231 cells demonstrated significant chemotaxis towards 12.5-50nM CXCL12 compared to the "No CXCL12" control after 24h, whereas MDA-MB-231 cells failed to demonstrate consistent and significant chemotaxis towards CXCL12 after 12h (Figure 5-11). A concentration of 12.5 nM CXCL12 was considered optimal and was used in further experiments.

Despite significant chemotaxis towards CXCL12, TMD-MB-231 and LMD-MB-231 were not used earlier for the transfection experiments because previous studioes in our group demonstrated reduction of CXCR4 expression following culture (Harvey et al., 2007).

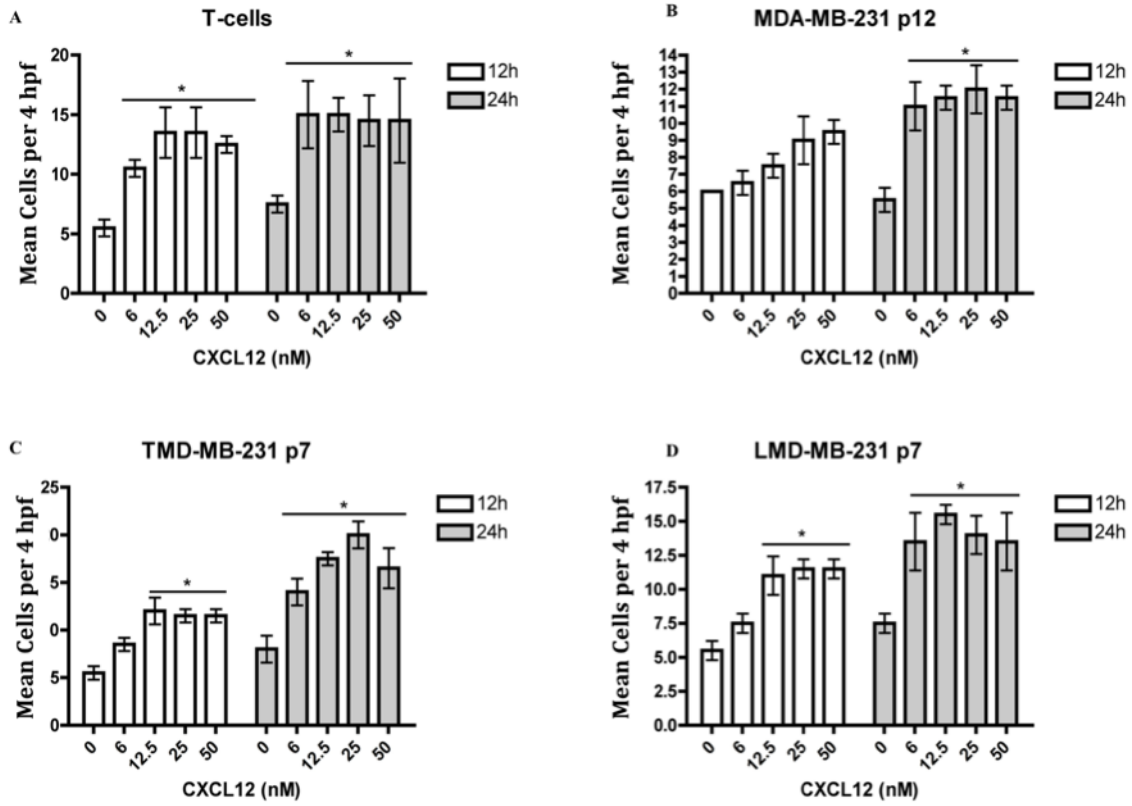


Figure 5-11: Chemotaxis of breast cancer cells following FOXP3 transfection

T-cells, MDA-MB-231, TMD-MB-231 and LMD-MB-231 cells underwent chemotaxis in an 8µm transwell filter assay coated with fibronectin, in presence of increasing concentrations of CXCL12. Representative data from 2 similar experiments; bars show Mean ± SE of triplicate data points. * $p<0.05$

5.4.4.2 Chemotaxis of breast cancer cells following FOXP3 transfection

MDA-MB-231 cells were able to migrate in response to CXCL12. Following successful transfection of MDA-MB-231 cells with vector encoding FOXP3 cDNA and demonstration of nuclear expression of FOXP3 proteins; the stable transfectants were used to investigate the potential of FOXP3 to reduce chemotaxis of MDA-MB-231 breast cancer cells in vitro in relation to stable mock transfectants. Chemotaxis was reduced in transfected colony 1, 2 and 3 after 24h of incubation with 12.5 nM CXCL12. Migration of colony 1 was reduced by 30% ($p<0.05$), colony 2 by 38% ($p<0.05$) and colony 3 by 76% ($p<0.01$) (Figure 5-12).

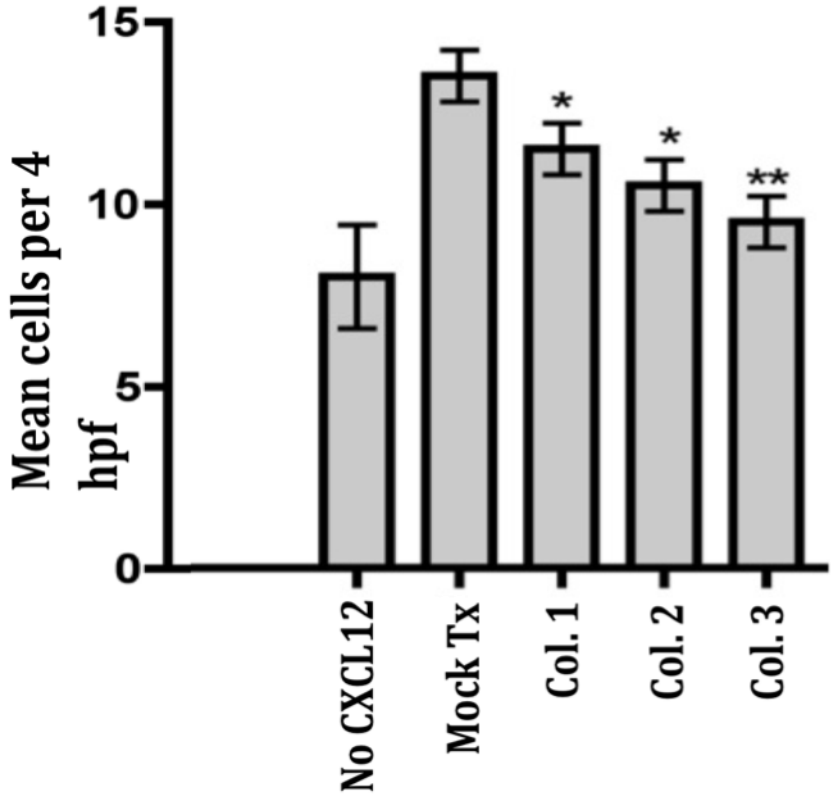


Figure 5-12: Effect of FOXP3 transfection upon chemotaxis of breast cancer cells towards CXCL12

Over 24h of incubation WT MDA-MB-231 cells and three colonies of MDA-MB-231 stable transfectants underwent chemotaxis in an 8μm transwell filter assay coated with fibronectin, in presence of 12.5 nM CXCL12. "No CXCL12" represents the negative control. The histograms are representative of data from 2 similar experiments; bars show Mean ± SE of duplicate data points. * $p<0.05$, ** $p<0.01$.

5.4.5 Functional significance of FOXP3 subcellular localization on migration and proliferation

Nuclear expression of FOXP3 is required in order to express this protein in its active form and subsequently maintain homeostasis. The results of this study have already demonstrated that TGF-β treatment significantly increased expression of cytoplasmic FOXP3 whereas transfection of normal FOXP3 encoding vector increases its nuclear expression in MDA-MB-231 cells. In order to investigate whether subcellular localization of FOXP3 protein mediates a functional difference chemotaxis assay was performed using FOXP3 MDA-MB-231 colony 3 transfectants and TGF-β1 (6 h, 10 ng/mL) treated MDA-MB-231 cells.

5.4.5.1 Effect of FOXP3 subcellular localization on migration

As shown in Figure 5-12 and Figure 5-13, MDA-MB-231 showed significant chemotactic response to CXCL12. Furthermore, the chemotactic responses of MDA-MB-231 cells were significantly blocked in cells overexpressing FOXP3 in the nuclei (MDA-MB-231 FOXP3 transfectants colony 3) ($p<0.05$). Treatment of MDA-MB-231 with TGF-β1 had no effect on migration of cells towards CXCL12.

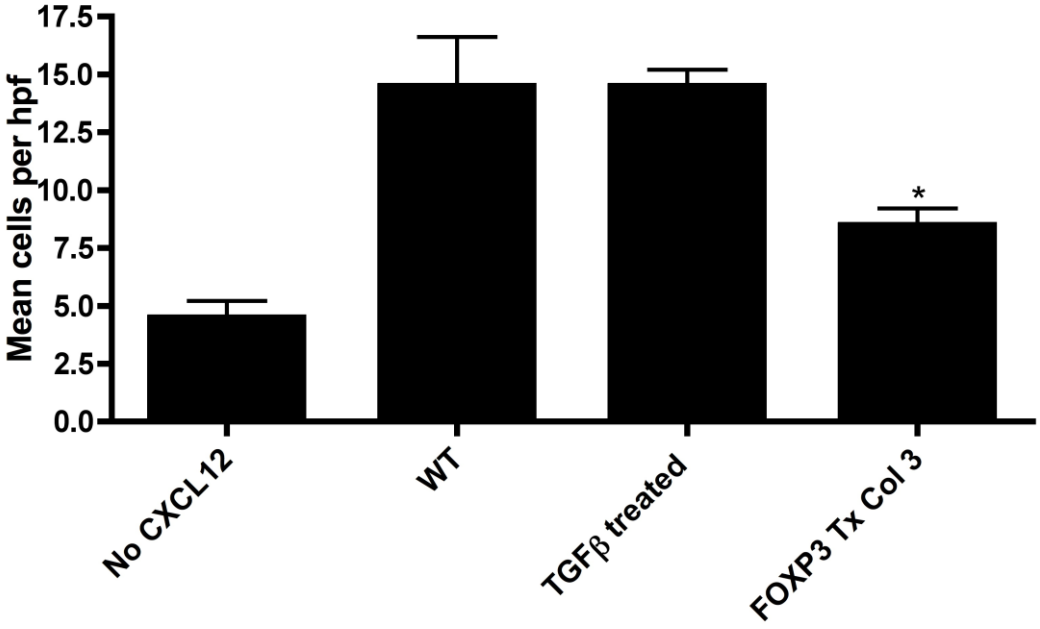


Figure 5-13: Effect of FOXP3 cellular localization upon chemotaxis of breast cancer cells towards CXCL12.

WT MDA-MB-231, TGF-β1 (10 ng/mL) treated (6h) MDA-MB-231 and FOXP3 transfected MDA-MB-231 cells underwent chemotaxis over 24h in an 8μm transwell filter assay coated with fibronectin, towards 12.5 nM CXCL12.

The histogram is representative of data from 2 similar experiments; bars show Mean ± SE of triplicate data points. * $p<0.05$

5.5 Discussion

Metastasis is facilitated by up-regulation of particular chemokine receptors (such as CXCR4) on the tumour cells, which enables them to migrate to secondary tissues where the ligands are expressed (such as CXCL12) (Balkwill and Mantovani, 2001).

There are several ways in which FOXP3 may be involved in regulation of CXCR4. First of all, FOXP3 on T cells induces or represses a number of genes, including CXCR4 (Marson A, 2007, Zheng Y, 2007). FOXP3 binds to the region upstream of the transcriptional start site of CXCR4 and CCR7 two chemokine receptors reported to play a vital role in cancer invasion and metastasis (Marson A, 2007). FOXP transcription factors can directly induce CXCR4 expression by activating its promoter on endothelial cells. (Hayashia H, 2008).

At the post-transcriptional level, changes in receptor translation and desensitization by internalization and degradation provide mechanisms for regulating chemokine- receptor expression. Enhanced CXCR4 translation in breast cancer cells is associated with the oncogene HER2, which may help to protect CXCR4 from ligand-induced ubiquitination and degradation (Li et al., 2004).

In this study, the effects of FOXP3 up-regulation and the implications of its cellular location on the migration and proliferation of MDA-MB-231 cells were examined. Cytokine TGF-β1 was used to induce FOXP3 expression in cancer cells. It has been previously demonstrated in Tregs. FOXP3 might prolong nuclear retention on Tregs rather than influx of Smad2 and 3. Thus, FOXP3 can directly influence TGF-β1 signalling, even in non-T cells. In this study, significant induction of FOXP3 expression on the epithelial breast cancer cells following short period (6h) of incubation with TGF-β1 was demonstrated. However, longer TGF-β1 stimulation (24h, 48h) decreased FOXP3 expression. Stimulation for up to 72h with TGF-β1 had no significant effect on proliferation of MDA-MB-231 cells. Results of TGF-β1 stimulation beyond 12 hours are not presented.

Up-regulation of nuclear FOXP3 reduced CXCR4 expression on the cancer cells. Furthermore, the clone with the highest FOXP3 nuclear expression (clone 3) demonstrated the most significant inhibition of chemotaxis of cancer cells.

The above chemotaxis results are provisional and should be validated by futher experiments. The limitations of chemotaxis experiments are only 3 colonies of MDA-MB-231 stable transfectants, which only increased FOXP3 expression 3-4 fold. Multiple attempts of producing more stable transfectants resulted very slow growing colonies (at least 6 weeks). It has been previously observed that FOXP3 inhibits proliferation in ovarian cancer and subsequently generated slowly growing transfected colonies (Zhang and Sun). In futher studiues the transfectants of MDA-MB-231 with inducible FOXP3 vector could be used. It would be easier to generate these transfectants, as the proliferation of their colonies would not be inhibited.

It is known that MMPs were critically involved in the processes of tumour cell invasion and metastasis (Sillanpaa et al., 2007), and MMP-9 and MMP-2 were directly associated with metastatic processes in ovarian and colorectal cancers (Curran et al., 2004, Belotti et al., 2003, Manenti et al., 2003). In addition, upregulation of FOXP3 inhibits the activity of NF-kappa B, which confirms that FOXP3 might inhibit cell migration and invasion partly through the inhibition of NF-kappa B and its target genes MMPs (Ogawa et al., 2004, Fujioka et al., 2003).

Up-regulation of FOXP3 on the epithelial cells has been previously described on ovarian cancer, and demonstrated that it inhibited cell proliferation, decreased cell migration, and reduced cell invasion (Hai-Yan Zhang, 2010). These and our findings suggest that up-regulation of the nuclear FOXP3 could be a novel approach for inhibiting cancer progression.

5.6 Summary of observations

- TGF-β stimulated FOXP3 cytoplasmic expression in breast cancer cells
- FOXP3 overexpressing breast cancer stable transfectants had normal (predominantly nuclear) FOXP3 expression
- Nuclear FOXP3 expression significantly reduced migration of breast cancer cells *in vitro*
- Cytoplasmic FOXP3 expression had no effect on migration of breast cancer cells *in vitro*

6 Discussion

This general discussion is designed to address the extent to which the generated data has met the initial aims, which were set out at the beginning of this thesis. The results of this project have explored and partially answered the research question. Therefore, I have set out to discuss the salient points, and the future directions that this work may require to further our understanding. Finally, after addressing each aim, I have set out a simplified model to summarise our up to date understanding and outline the limitation of this project.

Evidence suggests that the CXCR4 chemokine receptor is critical for the formation of metastases in breast cancer (Balkwill and Mantovani, 2001, Muller et al., 2001). Its multifunctional implications in breast tumor biology make it an optimal candidate as a diagnostic marker and as a therapeutic target (Muller et al., 2001). Mechanism of CXCR4 regulation in normal and cancerous epithelial cells may hold the keys to the molecular mechanism of mutagenesis.

FOXP3 can have conflicting function in breast cancer depending if it is expressed on Treg cells or on epithelial breast cells. In breast cancers the percentage of Treg cells, which are assessed by FOXP3 positivity, increases in parallel with the disease stage, from normal to DCIS and from DCIS to invasive carcinoma (Bates et al., 2006). The opposite is true for the epithelial cells, where the expression of a functional FOXP3 is decreased proportionally to the breast cancer stage. On the epithelial cells, FOXP3 transcription factor plays important role in molecular pathogenesis of breast cancer development, including both upregulation of oncogenes, such as ERBB2/HER2 (Zuo et al., 2007c), MYC (Wang L, 2009), SPK2 (Zuo et al., 2007b), and inactivation of tumor suppressor genes, such as p21 (Liu et al., 2009). It is likely that Foxp3 may affect other pathways involved in breast cancer (Zuo et al., 2007b, Zuo et al., 2007c).

This study was an attempt to provide experimental evidence of CXCR4 as a downstream target of FOXP3 transcription factor. The data presented in this study

indicate that defect of FOXP3 nuclear expression is a potential mechanism for CXCR4 upregulation in breast cancer and mutagenesis.

6.1 To characterize relationship between FOXP3 and CXCR4 expression in breast cancer

CXCR4 is the physiological receptor for CXCL12 with potent chemotactic activity for lymphocytes. It has been established that peripheral lymphocytes preferentially localize to peripheral lymphoid tissues, such as lymph nodes, which is known as the homing phenomenon (Cabioglu et al., 2005c). The evidence suggests that metastatic breast cancer cells overexpress CXCR4 chemokine receptor protein and that this receptor plays a critical role in homing of cancer cells at specific metastatic sites (Muller et al., 2001). Despite vast evidence of the importance of CXCR4 in breast cancer, we aimed to confirm the CXCR4 expression in human primary breast cancers and its relation to clinico-pathological factors. The CXCR4 expression in primary breast cancers is based on CXCR4 mRNA expression levels (Muller et al., 2001) and immunohistochemical studies of tumours (Cabioglu et al., 2005c, Muller et al., 2001). As verified by our CXCR4 immunostaining of CXCR4 transfected cells, CXCR4 is predominantly localised on a cell-surface. A small degree of cytoplasmic staining is likely, due to the recycling of the receptor within the cell. In contrary to this view, there are reports describing immunohistochemistry of CXCR4 on paraffin embedded tissues demonstrating little cell surface expression, with predominantly cytoplasmic and nuclear staining (Cabioglu et al., 2005c, Muller et al., 2001). In this study, a similar pattern of membrane and cytoplasmic CXCR4 staining of paraffin embedded primary tumours to those already published has been demonstrated. However, non-specific staining was also demonstrated. Our work demonstrated that cytoplasmic CXCR4 expression, lymph node metastasis, and distant organ metastasis were significantly inversely correlated with FOXP3 transcription factor expression in human breast cancer.

The next aim was to examine the existence of FOXP3 expression in breast epithelial tissues and cancer, which have been hotly debated in the recent literature. Majority of studies describe FOXP3 expression in human breast tissue. However, their reports are opposing stating that it is increased (Merlo A, 2009, 2005, Gupta et al., 2007) or decreased (Zuo et al., 2007b, Zuo et al., 2007d). Controversially, there is also evidence from a genetic animal model excluding expression of FOXP3 in non-haematopoietic tissues, like breast tissue (Kim et al., 2009a). Additionally, negligible FOXP3 expression in benign and malignant human breast tissue has been described (Wolf D, 2010). Those who observed decreased FOXP3 expression in breast cancer (Zuo et al., 2007d, Zuo et al., 2007b) analysed only its nuclear expression; whereas those who demonstrated equal or increased expression of FOXP3 in cancer (Wolf D, 2010, Andrea Merlo and Elda Tagliabue, 2009) examined its total expression: nuclear and cytoplasmic. In this study instead of examining overall FOXP3 expression, we separately analysed nuclear, cytoplasmic and Tcells FOXP3 expression in human breast tissues. Significant majority of malignant and benign breast tissue samples expressed FOXP3. Although, all benign and significant majorities of malignant specimens expressed FOXP3 in the cellular nuclei, all samples expressed FOXP3 in the cytoplasm. In malignant sections where nuclear FOXP3 expression was observed, it was weak, and it was confined to certain areas of the malignant epithelium. It may indicate a mutation of specific areas within cancer epithelium. Total nuclear and cytoplasmic FOXP3 expression was not significantly different between cancer and benign breast tissue. However, when nuclear staining was compared, benign breast epithelial cells had significantly greater nuclear FOXP3 expression than invasive ductal carcinoma. Heterogeneous subcellular localization of FOXP3 transcription factor may be an explanation for confusing reports in the literature regarding expression of this protein in human breast tissue.

Increased expression of FOXP3 in breast cancer is thought to be related not only to the tumour cells but also the strong FOXP3 expression on the Tregs cells in inflammatory infiltrate around tumours (Merlo A, 2009, Matsuura et al., 2009, Wu

et al., 2006). FOXP3 expressed in tumours might be a different transcript than FOXP3 expressed in Tregs. FOXP3 mRNA variant lacking exons 3 and 4 has been identified in tumour cell lines but not in CD4+FOXP3+ T cells (Chen C, 2006). The fact that both tumour cells and tumour-infiltrating Tregs can express FOXP3, makes the interpretation of the gene expression data of FOXP3 expression in tumours complicated as the increased levels of FOXP3 mRNA expression in cancer may be a result of increased expression of FOXP3 in tumour cells and an influx of Tregs which is amplified in cancer tissue. In contrast to the previously published work (Merlo A, 2009), in this study there was not statistically significant difference between total numbers or percentages of FOXP3 positively stained cells within the inflammatory infiltrate surrounding normal duct and ductal cancer. We suspect that it may be due to increased local inflammatory response and influx of Tregs to the tissue around the biopsy site of both benign and malignant specimens.

Additionally, we observed that the nuclei of ductal carcinoma *in situ* (DCIS) cells expressed FOXP3 similar to invasive cancer cells even though DSIC is a non-invasive breast cancer. However, it has a potential for aggressive progression to invasive breast cancer. It may indicate that FOXP3 downregulation is widespread in breast cancer and that such downregulation may have occurred at an early stage of breast cancer (DCIS). Similarly, the development of prostate intraepithelial neoplasia, a probable precancerious condition, demonstrates clearly that inactivation of FOXP3 in prostate cancer patients contributes to prostate cancer development (Wang L, 2009). Interestingly, statistically significant downregulation of FOXP3 occurred in the prostate epithelial neoplasia (PIN) of human samples (Wang L, 2009). Moreover, somatic mutations of FOXP3 were found in both PIN and cancerous lesions which suggests that FOXP3 inactivation is an early event in prostate carcinogenesis.

Recently, it has been proposed that overall FOXP3 expression in breast cancer tissue (infiltrate and epithelium) is associated with worse prognosis and spread of cancer to lymph nodes (Andrea Merlo and Elda Tagliabue, 2009). However, our work suggested that nuclear FOXP3 expression was significantly decreased in

cancers showing early signs of metastasis, namely vascular and perineural invasion, when compared to tumours where vascular and perineural invasion were not observed. Furthermore, the prognostic indicators of breast carcinoma like lymph node status and distant metastasis were associated with decreased nuclear FOXP3 expression and increased cytoplasmic CXCR4 expression.

Expression of FOXP3 and CXCR4 was also compared on mRNA level. FOXP3 expression was detected in all cancer cell lines analysed but it was not significantly different with reference to MCF10A as a benign human mammary epithelial cell line. This result was contradictory to FOXP3 protein expression on immunostaining. It may be that FOXP3 mRNA is overproduced as a response to the functionally inactive FOXP3 protein in breast cancer. CXCR4 mRNA expression on all cancer cell lines analysed was significantly elevated with reference to MCF10A. In summary, in this study normal breast epithelial cells had significantly greater FOXP3 nuclear expression than invasive cancers and the opposite was true for CXCR4 membrane expression. Correlation of FOXP3 and CXCR4 demonstrated significant inverse correlation between nuclear FOXP3 and membrane CXCR4 staining.

6.2 To investigate FOXP3 translocation to the nucleus in breast cancer

The functionally active FOXP3 is expressed in the nucleus of normal epithelial cells of breast, colon, pancreas, prostate and bronchus (Chen G, 2008). The expression of this molecule in the tumour cells revealed predominant cytoplasmic expression (Karanikas et al., 2008). There is disagreement amongst the authors with regards to the immunohistochemistry of malignant mammary epithelium: some describe nuclear staining only (Zuo et al., 2007b, Zuo et al., 2007d), others state that expression of FOXP3 in breast cancer is predominantly cytoplasmic (Andrea Merlo and Elda Tagliabue, 2009). In this study all cancer cell lines studied fail to express FOXP3 protein in the cellular nuclei where it is required to maintain its tumour suppressor function. The expression of FOXP3 in the cancer cells was cytoplasmic and perinuclear, whilst normal mammary cells expressed FOXP3 in their nuclei.

The human cancer cells exhibit distinct FOXP3 staining profile, e.g. nuclear expression in prostate cancer (Wang L, 2009), mixed cytoplasmic and nuclear in the pancreatic cancer (Hinz et al., 2007) and solely cytoplasmic staining in ovarian cancer (Zhang and Sun). FOXP3 protein expression was recently described in hepatoma cell lines (SMMC-7721 and Hepa-G2) (Wang et al.). In addition, the authors found that FOXP3 was mainly expressed in the nucleus in well-differentiated hapatocellular carcinoma (HCC) tissues (less aggressive) and cytoplasm in moderately and poorly differentiated HCC tissues (more aggressive). In agreement with these findings, our study also indicated a relationship between aggressiveness of the cancer and FOXP3 staining at the tissue and cell level. Lobular breast cancer, which is known to be one of the most aggressive, invariably had no nuclear FOXP3 expression. It may implicate that the factors coming from microenvironment and differentiation of tumour might induce a change in the subcellular localization of FOXP3 between cytoplasmic and nuclear expression patterns, which would result from post-translational modifications (Chen C, 2006). As a result, the heterogeneous subcellular localization of FOXP3 in breast cancer cell lines and tissues may be a sign of the different post-translationally modified forms of FOXP3.

It is difficult to interpret the significance of FOXP3 cytoplasmic localization, which could be due to a mutation or a deletion of the nuclear localization signals surrounding its forkhead domain. For example, three of the four FOXP3 mutants obtained from human prostate carcinomas disrupted their translocation into nuclei but localized in the cytoplasm (Wang L, 2009). Our study hasn't demonstrated any FOXP3 mutations within its three domains of nuclear localisation, leucine zipper and forkhead domains which were previously described on prostate cancer (Wang L, 2009) and breast cancer (Zuo et al., 2007d).

In the course of this work, breast cancer cells, which were transfected with wild-type FOXP3 expressing vector, recovered their physiological nuclear FOXP3 expression, whereas following TGF-β treatment; FOXP3 expression was induced but still cytoplasmic. It may indicate that cancer cells fail to import FOXP3 protein

into their nuclei due to dysfunctional FOXP3 protein. Similarly, the baseline FOXP3 mRNA level was increased in four cancer cells compared to the normal cells possibly due to increased demand on functioning FOXP3 protein or due to a modulation exerted mainly at post-transcriptional level. These results were opposing to the previous observations where cancer cell lines had a different degree of reduction in FOXP3 mRNA levels in comparison to HMEC and MCF-10A (Zuo et al., 2007c). On the other hand, a similar change in the subcellular localization of FOXP3 possibly due to posttranslational modifications, has recently been reported for Tregs activated with anti-CD3/anti-CD28 antibodies (Ebert LM, 2008).

Finally, the heterogeneous subcellular FOXP3 localization has been thought to be due to the alternatively spliced transcript variants encoding different FOXP3 isoforms. At least five FOXP3 splice variants: Foxp3FL, Foxp3Δ2, Foxp3Δ7 (Kaur et al.), Foxp3Δ2Δ7 (Mailer et al., 2009), Foxp3Δ3Δ4 (Zuo et al., 2007c) have been identified on the normal mammalian T-cells (Kaur et al., Mailer et al., 2009). They are expressed simultaneously (Mailer et al., 2009) and no functional difference between them has been demonstrated. These splice variants were all effectively translocated into the nucleus (Mailer et al., 2009). A change in the proportions of alternatively spliced Foxp3 transcripts might affect the ability of Foxp3 to interact with other transcriptional regulators and hence influence cell function. In line with this, the E251 mutant Foxp3 protein lost its ability to homo-associate or hetero-associate with Foxp1, although it still assembled as part of a large protein complex (Li et al., 2007b, Lopes et al., 2006, Carson et al., 2006). During the course of this study, nuclear localization sequences of FOXP3 contained multiple bands of shorter then expected products which might represent various splice variants.

It has been previously reported that the primary mammary cells (HMEC) expressed the same two isoforms as observed in the T cells, while MCF-10A expressed the exon 3-lacking isoforms (Zuo et al., 2007c). The same isoform was also found in four tumour cell lines at much lower levels (Zuo et al., 2007c). In addition, three breast cancer cell lines expressed an isoform lacking both exons 3

and 4 .We could not confirm these findings in our experiments as our sequences predominantly included domains of nuclear localisation. However, our results were contradictory to the previously described that some tumour cell lines (MCF-7) expressed a FOXP3 isoform lacking exon 8 which encodes the leucine-zipper domain that is frequently mutated in IPEX patients (Ziegler, 2006, Ziegler and Buckner, 2006). All our sequences demonstrated exon 8 identical to full length FOXP3.

We identified a possible novel isoform 3 as a predominant form in cancer cells compared to primary mammary cells. The protein sequence of isoform 3 has been previously described and published by Swissprot (http://expasy.org); however, there has been no experimental confirmation and no scientific references of that isoform so far. Although functional differences between isoforms 1, 2 and 3 could not be identified in this study, identification and quantification of these alternative transcripts in future studies may shed light on their physiological significance. Interestingly, cancer cells in our study had proportionally more isoform 3 compared to isoform 1 or 2 what may suggest inactive FOXP3 in breast cancer due to pathological proportion of splice variants.

The question remains whether, in human breast tissue, heterogeneous subcellular FOXP3 localization reflects the presence of abnormal proportion of splice variants, somatic mutations or different post-translationally modified forms.

6.3 To access the role of FOXP3 in CXCR4-induced migration of breast cancer cells

Little is known about CXCR4 regulation by FOXP3. Metastatic breast cancer cells overexpress CXCR4 and this receptor plays a critical role in homing of cancer cells at specific metastatic sites (Muller et al., 2001). There are several factors regulating the expression of CXCR4 receptor. According to the previous reports NF-kappa B (Helbig et al., 2003), HER2/ERBB2 (Li et al., 2004), hypoxia (Schioppa et al., 2003) and nitric oxide (Yasuoka et al., 2008), vascular endothelial growth factor (Bachelder et al., 2002), Neuropilin-2 (Nrp2) receptor (Yasuoka et al., 2009)

induce functional CXCR4 expression. Thus, inhibitors of above factors should reduce breast cancer metastasis by reducing the expression of a number of prometastatic genes including CXCR4. NF-kappa B inhibitors reduce metastasis of several cancers including melanoma, ovarian, prostate, brain, and pancreatic cancers and are dependent on CXCR4 for migration, survival, and metastasis (Edwards and Murphy, 1998, Chambers and Matrisian, 1997, Koshiba et al., 2000, Sehgal et al., 1998, Murakami et al., 2002, Scotton et al., 2002). Indeed, receptor activator of nuclear factor-kB (RANK) signalling in mammary carcinoma cells that overexpress the proto-oncogene Erbb2, which is frequently amplified in metastatic human breast cancers (Tiwari et al., 1992) was important for pulmonary metastasis (Tan et al., 2011). Recent evidence demonstrates that Foxp3 can block retroviral transcription by targeting key inducible proteins such as NF-kappa B and cAMP-responsive element binding protein (CREB) (Grant et al., 2006) and therefore it may inhibit CXCR4 by inhibiting NF-kappa B. FOXP3 has been identified as a transcriptional repressor of the HER2/ERBB2 oncogene (Zuo et al., 2007d), hence indirectly represses the expression of CXCR4. Additionally, FOXP3 suppressed growth and induced the cell death of MCF-7, a breast cancer line without HER2/ERBB2 overexpression (Zuo et al., 2007d). Therefore, it is likely that Foxp3 may affect other pathways involved in breast cancer.

Evidence suggests that expression of FOXP3 is associated with survival but not with local relapse suggesting that this molecule might be related to metastatic potential of breast cancer (Merlo et al., 2009). FOXP3-induced downregulation of genes such as PDE3B (cyclic nucleotide phosphodiesterase 3B, cGMP inhibited) has been shown to confer a survival advantage and a better resistance to apoptosis in T cell (Gavin et al., 2007). Furthermore, in T-cells FOXP3 binds to the gene region upstream of the transcriptional start site of CCR7 and CXCR4 (Zheng and Rudensky, 2007), two chemokine receptors recently reported to play an important role in cancer invasion and metastasis (Kodama et al., 2007b, Kodama et al., 2007a, Pitkin et al., 2007). Thus, FOXP3 expressed in breast cancer cells might influence the development of metastasis by modulating the expression of these chemokine

receptors or of other genes encoding cell surface or secreted molecules that alter tumour cell response to the environment.

Very recently, FOXP3 positive Treg cells showed similar distribution to cytokine RANKL (receptor activator of NF-kappaB ligand) positive cells in breast tumours (Tan et al.). Notably, Treg cells in breast tumours are associated with an invasive phenotype and poor prognosis. CD41CD25 tumoral T cells, which are FOXP3 enriched (Fontenot et al., 2003), expressed fourfold more *Rankl* mRNA than CD41CD252 T cells. Furthermore, FOXP3 and RANKL were co-localized in tumours inflammatory cells in humans and mice (Tan et al., 2011). These results suggest that tumour-infiltrating CD4 positive FOXP3 positive Treg cells are the most critical cells for maintaining RANKL expression in the micro-environment of metastatic mammary tumours (Tan et al., 2011). On the other hand, expression of RANKL was found in human breast cancer cells and tissues and was found to trigger migration of human epithelial cancer cells and melanoma cells that express the receptor RANK (Jones et al., 2006). Previously, loss of expression of RANKL in 86% of breast cancers was found to be associated with oestrogen receptor negative cancers (Cross et al., 2006). Those cancers are known to have high histological grade, poor prognosis and potential for metastasis (Cross et al., 2006).

In this study, significant expression of FOXP3 expression on cancer cells was achieved by stable transfection and TGF-β1 stimulation as previously demonstrated on Tregs. It has been suggested that in Tregs FOXP3 might prolong nuclear retention rather than influx of Smad2 and 3. Thus, FOXP3 can directly influence TGF- β signalling, even in non-T cells.

Up-regulation of nuclear FOXP3 by transfection reduced CXCR4 cellular expression as well as inhibited migration rate of breast cancer cells. FOXP3 nuclear expression levels were correlated with degree of chemotaxis inhibition. Conversely, the expression of cytoplasmic FOXP3 had no effect on CXCR4 expression, growth and migration of cells.

Our findings suggest that up-regulation of the nuclear FOXP3 could be a novel approach for inhibiting cancer progression.

In summary, this study presented experimental evidence that supported the role of FOXP3 up-regulation as anti-tumour and anti-metastatic mechanisms in breast cancer. From these data, we could expect that up-regulation of FOXP3 might potentially be an effective therapeutic approach for the inhibition of cell growth and migration in breast cancer. However, further detail studies are needed to ascertain the precise molecular regulation of FOXP3 and the role of FOXP3 in cell growth, and migration in animal models of human breast cancer.

6.4 Clinical implications

6.4.1 Gene therapy

Unlike autosomal tumor suppressor genes that are usually inactivated by mutations in both alleles, X-linked FOXP3 mutations in cancer samples are usually heterozygous, with the wildtype allele selectively inactivated in cancer. This skewed X-inactivation suggests a new approach to reactivation of FOXP3 for cancer therapy. One of the most difficult challenges in cancer therapy is to restore the function of inactivated tumor suppressors. Mutation of X-linked tumor suppressor genes, such as FOXP3 may be heterozygous in female cancer cells (Zuo et al., 2007c). Since one allele of an X-linked tumor suppressor gene has not undergone selection during carcinogenesis, it may be possible to restore the wild type allele for cancer therapy. Indeed, anisomycin treatment induced FOXP3 expression in both mouse and human breast cancer cell lines (Zhang et al., 2009) resulting in increased apoptosis of cancer cells and reduced growth of established mouse mammary tumors (Zhang et al., 2009). It raises the intriguing question whether functional FOXP3 can be restored in cancer cells. Global reactivation of X-linked genes could potentially have serious side effects. About 10% of X-linked genes show variable patterns of inactivation and are expressed from some inactive X chromosomes in humans (Carrel and Willard, 2005). Therefore, less than precise X-chromosome inactivation is tolerated in humans. DNA methylation, X-inactive

specific transcript (XIST), and histone hypoacetylation maintain inactivation of X-linked genes (Pageau et al., 2007, Plath et al., 2002). In the breast cancer samples loss of DNA methylation and XIST on the chromosome X reinforces the need for selective reactivation of X-linked tumor suppressor genes (Richardson et al., 2006).

6.4.2 Transduction of mammary epithelium with FOXP3

The other potential way to restore physiological FOXP3 phenotype on the cancer cells is to transfect them with normal FOXP3 vector using e.g. intraductal microinjection of adenovirus vectors (Russell et al., 2003). Since adenovirus transduction appears to be confined to the mammary epithelium, this method provides a technique to target genes of interest to this tissue compartment.

6.4.3 FOXP3 as a molecular marker

Increasingly, molecular-based cancer diagnostics are supporting standard clinical diagnosis. Molecular markers are used for confirming diagnosis, monitoring patient prognosis, pre-symptomatic screening, guiding treatment options, monitoring treatment efficacy and disease progression, and identifying disease recurrence after treatment. In one study, FOXP3 expression was found to discriminate the prognostic risk of patients within node-negative patients: overall survival of FOXP3-positive patients was found to be significantly poorer than that of FOXP3-negative patients (Merlo et al., 2009). These findings might aid in identifying subgroups of patients who are more likely to have a poor outcome and to whom specific therapies might be directed.

6.5 Limitations of the study

6.5.1 Immunostaining and its interpretation difficulties

Because FOXP3 is expressed at lower levels in epithelial cells than in the regulatory T cells, the conditions typically used for detecting Treg cells in tumor samples do not give reproducible staining in the epithelial cells. Higher concentration of anti-FOXP3 antibody was used to evaluate epithelial staining.

Immunostaining of CXCR4 on breast cancer tissues had significant cytoplasmic staining and background or nonspecific DAB staining. Therefore, Aperio digital image analysis was used to subtract the background. However, some sections were not possible to be scanned on the Aperio scanner due to their uneven surface, dirt, and excessive thickness of the sections. Early signs of metastasis (vascular and perineural invasion) were only assessed manually. In some sections Aperio system did not pick up all the nuclei. For example in MCF-7 some nuclei were not detected by the computer system as negative (blue) or positive (yellow, orange, red). If all nuclei had been detected, the MCF-7 score would have been lower because smaller percentage of nuclei would have been scored as positive.

6.5.2 Real-time PCR results

During initial phase of experiments, the real-time PCR of FOXP3 expression following TGF-β1 stimulation gave very unpredictable and unreproducible results. The high standard deviations indicated that the data were spread out over a large range of values. Therefore, MDA-MB-231 cell line, which gave the highest FOXP3 up-regulation, was nominated to verify the initial results. Further experiments were performed with two housekeeping genes: GAPDH and 18S RNA and repeated four times in triplicates.

6.5.3 Sequencing problems

In the initial experiments Taq DNA polymerase was used for amplification of gene of interest prior to sequencing. The results were different when verified using thermostable *Pfu (Pyrococcus furiosus)* DNA polymerase with 'proofreading' properties. *Taq (Thermus aquaticus)* polymerase has an error rate of about 1.3 x 10^{-5} and mutation frequency of 5.1 %. Therefore, it is possible that the mutations observed in the initial experiments were introduced and amplified during the course of the experiment. Thermostable *Pfu (Pyrococcus furiosus)* DNA Polymerase with 3′→5′ exonuclease proofreading activity exhibits a higher fidelity than *Taq* DNA Polymerase. It has an error rate of about 1.8 x 10^{-5} – 8 x 10^{-7} and 5.1–0.3%

mutation frequency. No mutations were observed when Thermostable *Pfu (Pyrococcus furiosus)* DNA Polymerase was used.

Sequencing of FOXP3 domain 2 of nuclear sequence localisation resulted in multiple bands shorter then expected product. The primers for domain 3 spanned a large gene area of 1.4k and had a low melting temperature of 53.5°C therefore; the multiple bands probably were due to non-specific banding rather then true splice variants. It was not sufficient for sequence analysis.

This study did not demonstrated mutations within FOXP3 gene, which were previously shown by other authors (Zuo et al., 2007), who used genomic DNA in their experiments. This study was based on DNA reverse transcribed from RNA derived from cell culture. Futher experiments using genomic DNA sould be performed. The genomic DNA may be obtained from breast FNA (fine needle aspiration) or tumours dissected from paraffin embedded breast tissue.

6.5.4 Difficulties with generation of stable transfectants

The limitations of chemotaxis experiments are only 3 colonies of MDA-MB-231 stable transfectants, which only increased FOXP3 expression 3-4 fold. Multple attempts of producing more stable transfectants resulted very slow growing colonies (at least 6 weeks). It has been previously observed that FOXP3 inhibits proliferation in ovarian cancer and subsequently generats slowly growing transfected colonies (Zhang and Sun, 2010). In futher studiues the transfectants of MDA-MB-231 with inducible FOXP3 vector could be used. It would be easier to generate these transfectants, as the proliferation of their colonies would not be inhibited.

6.6 Future directions

Results presented in this work provide a better understanding of the role of FOXP3 in the development of breast cancer metastases. Here, I outline a few possible directions to futher develop my observations.

6.6.1 Identification of mutation in FOXP3 gene in breast cancer

During the course of this study loss of FOXP3 nuclear expression in breast cancer cells have been observed. It was successfully reversed to physiological nuclear expression following transfection with the normal FOXP3 plasmid. Somatic mutations of FOXP3 gene in breast cancer cells or expression levels of FOXP3 isoforms may lead to development of malignant phenotype deficient in nuclear FOXP3 expression. The sequencing analyses of FOXP3 three nuclear localization sequences did not demonstrate any mutations. Future work could involve extending this study to the full FOXP3 nucleotide sequence to investigate presence of mutations within FOXP3 gene on breast cancer. This work provided an experimental confirmation of nucleotide sequence of FOXP3 isoform 3 that may play a role in nuclear FOXP3 localization.

6.6.2 Examination of functional significance of mutations of FOXP3 gene and FOXP3 isoform 3 on FOXP3 nuclear localization

If mutations of FOXP3 gene in breast cancer cells are identified, the mutants DNA should be isolated in order to construct a plasmid with mutant FOXP3 DNA inserted. It could be used for transfection of cells and comparative analysis of FOXP3 intracellular location in cells transfected with normal and mutant FOXP3 plasmid. The same experiment could be performed with the isoform 3 DNA sequences.

6.6.3 Examination of functional significance of mutations of FOXP3 gene and FOXP3 isoform 3 on FOXP3 regulated genes expression

The study could also be extended to investigate functional significance of mutations in FOXP3 gene and presence of isoform 3 on FOXP3 regulated genes expression. In breast cells FOXP3 can regulate the expression of multiple genes, including both tumour suppressors and oncogenes. The genes involved in cancer are the most affected group. The functional significance of mutations of FOXP3 gene in breast cancer cells could be studied by examination of the expression of FOXP3 regulated genes (e.g. p21 and cMYC).

6.6.4 Examination of differences between cytoplasmic and nuclear FOXP3

In order to investigate FOXP3 separately in nucleus and cytoplasm the proteome studies could be used in the future experiments. The successful approach could be the fractionation of cellular compartments.

6.6.5 Investigation differences in FOXP3 function on Treg cells and epithelial cells

One of ways to investigate differences in FOXP3 function in cancerogenesis on Treg cells and epithelial cells is RANKL-CXCR4-FOXP3 signalling. RANK signallimg in mammary carcinoma cells that overexpress the proto-oncogene Erbb2, which is frequently amplified in metastatic human breast cancers, was important for pulmonary metastasis (Tan et al., 2011). Metastatic spread of Erbb2- transformed carcinoma cells also required CD4 positive CD25 positive T cells, whose major pro-metastatic function was RANKL production. Most RANKL-producing T cells expressed FOXP3. The dependence of pulmonary metastasis on T cells was replaceable by exogenous RANK, which also stimulated pulmonary metastasis of RANK human breast cancer cells (Tan et al., 2011). Our study could be expanded by investigating interactions of RANK with CXCR4 and FOXP3 not only on T-cells, as described above, but also on cancer cells.

6.6.6 Investigation of the correlation between CXCR4 and CXCR7 in breast cancer

Since CXCL12 is expressed preferentially in lymph nodes, this may support our data that CXCR4 expression was significantly correlated with lymph node metastasis in human breast tumour samples. As CXCR7 expression also promotes cancer metastasis in breast cancer and is a receptor for CXCL12 (Miao et al., 2007), it would be important to investigate the correlation between CXCR4 and CXCR7 in breast cancer in future studies.

6.6.7 Further functional studies to ascertain effect of the FOXP3 expression in breast cancer metastasis

Having demonstrated upregulation of FOXP3 expression as well as its functional effect upon the migration of CXCR4 expressing cancer cells, further investigations of the functional potential of FOXP3 in breast cancer metastasis in a series of *in*

vitro studies should be performed. This could be examined by comparing the invasion of extracellular matrix (Matrigel) and ability to modulate signal transduction pathways (pSMAD2/3). The other way to confirm FOXP3 –CXCR4 relationship on the breast cancer could be to silence FOXP3 gene *in vitro* in order to detect changes in CXCR4 expression.

6.6.8 Use of MDA-MB-231-luc-D3H1 Bioware® cell line as in-vivo metastasis model

This study has suggested the role of FOXP3 in CXCR4 driven chemotaxis of breast cancer cells by the series of *in vitro* experiments. *In vivo* experiments could be perform to examine the effect of FOXP3 up regulation on the tumour metastasis formation. The MDA-MB-231-luc-D3H1 Bioware® cell line can be used *in vivo* to establish an experimental metastasis model. Throughout the experiments bioluminescent imaging could be used to detect metastatic sites, monitor tumour progression, metastases growth rate and any changes following induction of FOXP3 expression.

6.6.9 Investigate stem cells niche expression of CXCR4

Breast cancer cells express the chemokine receptor CXCR4 and frequently metastasize to organs with an abundant source of the CXCR4 ligand, stromal cell-derived factor 1 (SDF-1). The chemokine receptor CXCR4 plays an active role in the metastasis of breast cancer. CXCR4 molecule is a potential target to control breast tumor growth as well as metastasis and could be further explored.

6.6.10 New approaches for cancer therapy

Considering that the majority of tumors have at least one wild type FOXP3 allele, and given the detrimental effect of loosing FOXP3 expression on tumor cell growth, it would be of great value to develop a drug that can reactivate the FOXP3 gene in cancer. Therefore, better understanding of FOXP3 expression in normal and cancer cells may provide new approaches for cancer therapy.

6.7 Conclusion

In conclusion, the current study confirms failure of FOXP3 nuclear localisation in breast cancer and an inverse correlation between this failure and CXCR4 expression. Out of the nucleus, FOXP3 transcription factor cannot maintain its physiological properties. Transfection of breast cancer cells with FOXP3 reversed its expression to the physiological nuclear pattern and subsequently inhibited migration and proliferation of breast cancer cells *in vitro.* Hence, inactivation of FOXP3 may suggest a mechanism of cancer invasion and metastatic spread. The study gives further evidence of FOXP3 splice variants in breast cancer. It also provides the first description of nucleotide sequence of FOXP3 isoform 3 that may play a role in localization of FOXP3 in cellular nuclei. These results of this work would need to be further confirmed by additional *in vitro* and *in vivo* studies.

6.8 Model of CXCR4-FOXP3 interactions in breast cancer

On the basis of the findings obtained in this study and the current evidence, the following simplified model of the interactions between FOXP3 and CXCR4 in breast cancer has been proposed (Figure 6-1).

FOXP3 suppresses breast cancer by inducing tumour suppressor gene p21 while repressing oncogenes HER2 and SKP2. The genes indicated are all direct targets for FOXP3 and their regulation is essential for growth inhibition by FOXP3 (Liu et al., 2009, Zuo et al., 2007c, Zuo et al., 2007b). Therefore, FOXP3 is an X-linked breast cancer suppressor gene in both mice and humans.

Amongst others they are several factors inducing the expression of CXCR4 receptor: NF-kappa B (Helbig et al., 2003), HER2/ERBB2 (Li et al., 2004), SKP2 (Zuo et al., 2007b), Neuropilin-2 (Nrp2) receptor (Yasuoka et al., 2009). Thus FOXP3, the inhibitor of above factors should reduce breast cancer metastasis by reducing the expression of a number of prometastatic CXCR4.

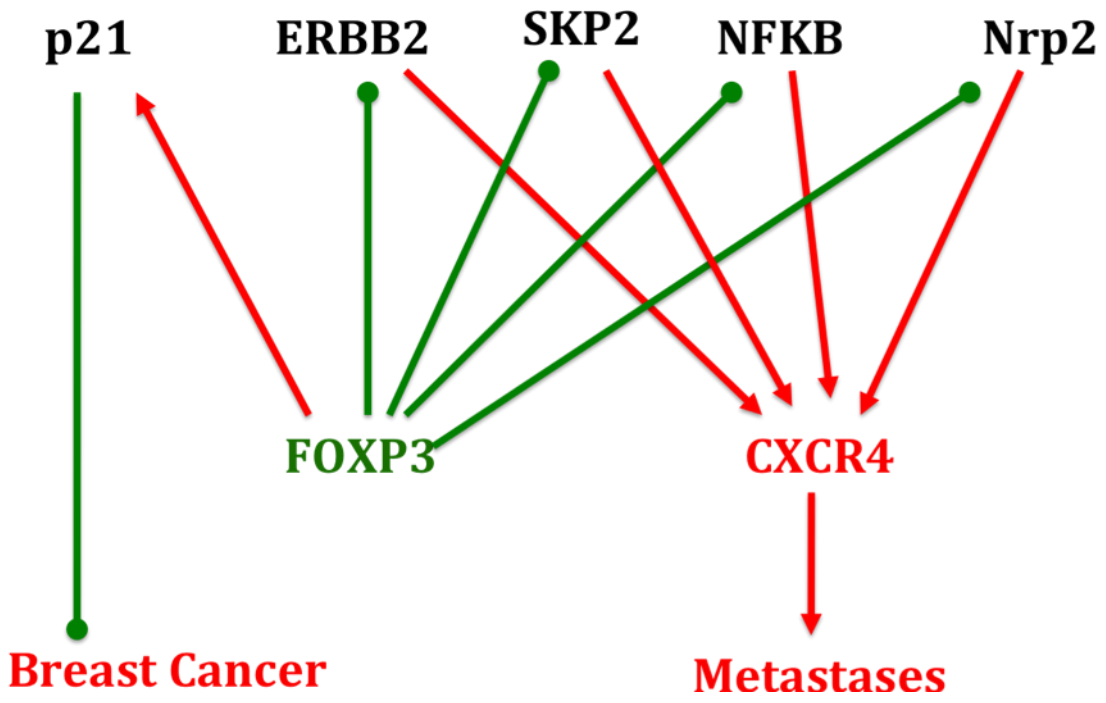


Figure 6-1: Model of CXCR4-FOXP3 interactions in breast cancer.

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Publications, presentations and prizes

- **Publications**

Journals:

Overbeck-Zubrzycka D, Douglas S., Ali S, Kirby JA, Browel D., Lennard TW. FOXP3 Regulates Spread of Breast Cancer via Control of CXCR4 Receptor. In submission to **Cancer Res.**

Abstracts:

Overbeck-Zubrzycka D, Kirby JA, Lennard TW, Ali S. FOXP3, breast cancer suppressor gene can regulate its metastatic spread. **BJS** 2011; 98 (S3) 5

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Overbeck-Zubrzycka D, Ali S, Kirby JA, Lennard TW. FOXP3 Regulates Metastatic Spread of Breast Cancer. **Cancer Res** 2010; 70 (24)

Overbeck-Zubrzycka D, Ali S, Kirby JA, Lennard TW. Expression of FOXP3 in human breast cancer is inversely correlated with disease progression. **BJS** 2009; 96(S4)

Overbeck-Zubrzycka D, Ali S, Kirby JA, Lennard TW. Expression of FOXP3 in human breast cancer is inversely correlated with disease progression and the metastatic process: an immunohistochemical study. **Ann Oncol** 2009 20 (2): ii37-II44

Overbeck-Zubrzycka D, Kirby JA, Lannard T, Ali S. "The inverse correlation of FOXP3 and CXCR4 expression in human breast cancer is associated with metastatic spread of the disease." **British Association of Cancer Research, Transcription and Cancer** 2009

- **Presentations**

International:

Overbeck-Zubrzycka D, Ali S, Kirby JA, Lennard TW. FOXP3 Regulates Metastatic Spread of Breast Cancer via Control of Expression of CXCR4 Chemokine Receptor. **33rd Annual CTRC-AACR San Antonio Breast Cancer Symposium**, San Antonio 2010

Overbeck-Zubrzycka D, Kirby JA, Lennard TW, Ali S. FOXP3 Regulates Metastatic Spread of Breast Cancer via Control of Expression of CXCR4 Chemokine Receptor. **AACR Frontiers in Cancer Prevention Research**, Philadelphia 2010

Overbeck-Zubrzycka D, Ali S, Kirby JA, Lennard TW. Expression of FOXP3 in human breast cancer is Inversely correlated with disease progression and the metastatic process: an immunohistochemical study. **IMPAKT Breast Cancer**, Brussels 2009

National:

Overbeck-Zubrzycka D, Kirby JA, Lennard TW, Ali S. FOXP3, breast cancer suppressor gene can regulate its metastatic spread. **The Association of Surgeons of Great Britain and Ireland International Surgical Congress**, Bournemouth 2011

Overbeck-Zubrzycka D, Lim A, Kirby JA, Lennard T, Ali S. "The inverse correlation of FOXP3 and CXCR4 expression in human breast cancer is associated with metastatic spread of the disease." **British Association of Cancer Research**, Cambridge 2009

Overbeck-Zubrzycka D, Ali S, Kirby JA, Lannard T; Expression of FOXP3 in human breast cancer is inversely correlated with disease progression and the metastatic process: an immunohistochemical study. **The Association of Surgeons of Great Britain and Ireland International Surgical Congress**, Glasgow 2009

Regional:

Overbeck-Zubrzycka D. Inactivation of FOXP3 transcription factor on breast cancer regulates its metastatic spread via control of expression on CXCR4 chemokine receptor. Institute of Cellular Medicine, **Newcastle University Research Day** 2010

Overbeck-Zubrzycka D. FOXP3 - a new prognostic marker of metastases in breast cancer. **North of England Surgical Society Meeting**, Newcastle upon Tyne 2010

Overbeck-Zubrzycka D. FOXP3 transcription factor and breast cancer progression. **Science Forum of Institute of Cellular Medicine**, Newcastle University 2010

Overbeck-Zubrzycka D. Is FOXP3 transcription factor the first X-linked breast cancer suppressor gene? **Transplant Immunology Forum**, Newcastle University 2009

Overbeck-Zubrzycka D. FOXP3 regulates metastatic spread of breast cancer via control of expression of CXCR4 chemokine receptor. **Science Forum at Newcastle University** 2009

Overbeck-Zubrzycka D. Inverse correlation of FOXP3 and CXCR4 in breast cancer is associated with metastatic spread of the disease. **Applied Immunology Forum**, Newcastle University 2009

- **Prizes**

Scholar-in-Training Award, American Association for Cancer Research, 09.2010
Research Grant Award, Royal College of Surgeons of Edinburgh, 02.2009
Special Trustees Award, Newcastle Healthcare Charity and NHS Charity, 01.2009

Appendix: ethical approval

NHS

Newcastle & North Tyneside Local Research Ethics Committee 2
Room G14
Dental School
Framlington Place
Newcastle upon Tyne
NE2 4BW

Telephone: 0191 222 3581
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27 April 2006

Mr M I Ahmed
MD Student
University of Newcastle upon Tyne
School of Surgical and Reproductive Sciences
William Leech Building, 3rd Floor,
The Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH

Dear Mr Ahmed

Full title of study: **Role of Chemokine/GAG Interactions in Breast Cancer Metastasis.**

REC reference number: **06/Q0906/12**

Thank you for your letter of 10 April 2006, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.0	03 February 2006
Investigator CV Muhammad Ahmed	1	
Investigator CV Professor T W J Lennard	1	

6/Q0906/12

Protocol	1	
Covering Letter		02 February 2006
Summary/Synopsis Flowchart	1	
Letter from Sponsor	JEV/PB/3659	05 January 2006
Participant Consent Form	Consent Form 1	
Participant Consent Form	Consent Form 1- Adult Generic	16 May 2005
Response to Request for Further Information		10 April 2006
Letter from T W J Lennard		31 March 2006
Offer of appointment from Northumbria Healthcare NHS Trust		11 August 2005

Research governance approval

You should arrange for the R&D department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0906/12	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

A. Taylor

Dr Philip M Preshaw
Chair

Email: anne.taylor2@ncl.ac.uk

Enclosures: *Standard approval conditions*

Copy to: Dr Craig Mackerness
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Research and Development Department
Royal Victoria Infirmary
Queen Victoria Road
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SF1 list of approved sites