The Investigation of Rab25 protein expression in early breast cancer

Thesis submitted in partial fulfilment of the requirements for the award of Doctor of Medicine

Dr Claire Kelly **BSc (Hons) MBChB (Hons) MRCP**

Faculty of Medicine

University of Glasgow

October 2010

Abstract

More than a million women worldwide are diagnosed with breast cancer every year and breast cancer is the most common cancer in women in the UK. Despite improvements in the treatment of breast cancer, it remains the second most common cause of death from cancer in women after lung. Research into the aberrant molecular pathways which characterise neoplasia is necessary to highlight potential therapeutic targets with the aim of improving the survival of women with breast cancer.

Rab25 is a member of the Rab superfamily of small GTPases, which are involved in the regulation of intracellular vesicular trafficking. A number of studies have suggested that dysregulation of Rab25 protein expression may be implicated in breast, ovarian and colorectal cancer. However, the published data are conflicting and further research is required to improve the understanding of the role of Rab25 in neoplasia.

At the commencement of this project, there was no commercially available anti-Rab25 antibody. The data presented here describe the affinity purification and validation of a rabbit polyclonal anti-Rab25 antibody suitable for use in Western blotting, immunofluorescence and immunohistochemistry. Furthermore, an immunohistochemical scoring system was devised and validated prior to the investigation of Rab25 expression in a large breast cancer cohort.

The data presented here show that loss of Rab25 expression correlates with decreased breast cancer related survival of patients who have tumours which have also lost the expression of the oestrogen receptor. Furthermore, loss of Rab25 expression has maximal negative effect on the survival of patients with tumours which have lost both oestrogen and HER2 receptor expression.

To investigate the effect of Rab25 knockdown in vitro, the MCF7 cell line was stably transfected with a short hairpin siRNA vector targeted against Rab25. Knockdown of Rab25 had no demonstrable effect on cellular proliferation or colony formation, but Rab25 knockdown cells can migrate to heal a scratch wound at a greater rate than cells transfected with a non-targeted control

vector. Knockdown of Rab25 protein expression increases total surface and intracellular ß1 integrin protein levels in MCF7 cells, but this increase is not as a result of increased transcription.

The findings presented here suggest that loss of Rab25 in patients with ER negative/HER2 negative breast cancers confers a negative effect on survival of these patients. These findings are in direct contrast to previously reported work and suggest that perturbation of Rab25 levels, either increase or decrease, may be important in breast cancer.

Author Declaration

I hereby declare that I am the sole author of this thesis. Unless expressly stated otherwise, all work described herein was performed by me.

Table of contents

List of Tables

List of Figures

Acknowledgements

This thesis would not have been possible without the guidance and support of many people. I would like to thank my supervisors, Dr Jim Norman and Professor Jeff Evans, for their guidance, time and advice, and Professor Jim Cassidy for the opportunity to undertake this period of research. My thanks go to Dr Joanne Edwards and Dr Karen Oien for providing sections of the breast cancer TMA.

I am extremely grateful to my colleagues in R20 who took a useless medic under their wing with grace and patience. Particular thanks go to Marc Jones and Kim Moran-Jones for taking the time to teach me many of the techniques used in this project and for their support when things did not go according to plan. I am also grateful to Patrick Caswell for technical advice.

Little did I know when I embarked on this journey, that I would gain not only a thesis but a great friend. My deep gratitude goes to Marta Dozynkiewicz whose friendship and support has been enduring and invaluable.

I am indebted to my parents for their many years of practical and financial support and for instilling in me the importance of perseverance. They, and my sister Nicola and a small army of friends, have provided hours and hours of fun for my children which gave me the time to write. I will always be grateful.

The practical and emotional support of my husband Grant has been unwavering and fundamental to producing this work. I could not have done it without him.

To my children Michael and Grace owe my biggest debt of gratitude, for allowing me to see what is really important in life and for providing the focus to go and get it.

This work was funded by Cancer Research UK

Definitions/Abbreviations

3D	3-dimensional
APC	adenomatous polyposis coli
BRAC1	breast cancer 1 early onset
cDNA	complementary DNA
CK	cytokeratin
cm	centimetre
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research and Treatment of Cancer
ER	oestrogen receptor
fmol	femtomole
g	gram
GAPHD	glyceraldehyde 3-phosphate dehydrogenase
GDF	GDI displacement factors
GDI	GDP dissociation inhibitor
GDP	guanosine-5'-diphosphate
GEF	GDP/GTP exchange factor
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
HA	haemagglutinin
HER2	Human epidermal growth factor receptor 2
HERA	Herceptin Adjuvant Trial
hr	hour
IHC	immunohistochemistry
KD	knockdown
kDa	kilo Dalton
mg	milligram
min	minutes
ml	millilitre
mm	millimetre
mM	millimolar
MMTV	mouse mammary tumour virus
mRNA	messenger RNA
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinases
PR	progesterone receptor
PyVMT	polyomavirus middle T
qPCR	quantitative PCR
Rab	Ras-related in brain
RCP	Rab-coupling protein
RISC	RNA-inducing silencing complex

RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
shRNA	short hairpin RNA
siRNA	small interfering RNA
TMA	tissue microarray
V	volts
µg	microgram
µl	microlitre
µm	micrometer

1 Introduction

Breast cancer remains a major health problem in the 21st century and more than a million women worldwide are diagnosed with breast cancer every year. Breast cancer is the most common cancer in women in the UK and in 2006 45,508 women were diagnosed with the disease, which equates to around 125 women every day. 80% of breast cancers are diagnosed in women aged over 50. Breast cancer can also affect men and 314 men were diagnosed in the UK in 2006. The incidence of breast cancer continues to rise and the incidence rates in the UK have increased by over 50% in the last twenty five years (1).

Despite the rising incidence of breast cancer, more women are surviving the disease than ever before. Survival rates have improved from 50% survival at 5 years in the 1970's to around 80% in the present day. Survival rates are better the earlier the cancer is diagnosed and in women from the most affluent areas. The improvements seen in survival rates are due to mainly to advances in healthcare, such as earlier and more accurate detection of breast cancer and more effective treatment. The NHS breast screening programme in England is responsible for the early detection of 10,000 breast cancers every year, saving an estimated 1,400 lives annually. A recent French study demonstrated an improvement in survival from metastatic breast cancer from 27% to 44% over a 14 year period, due in part to advances in chemotherapy and hormone therapy (2).

Whilst these figures are encouraging, in the UK each year 12,000 women die from breast cancer and around 1300 of these deaths are in women under the age of 50. Breast cancer is the second most common cause of death from cancer in women after lung (1). Traditional systemic treatments for breast cancer have included cytotoxic chemotherapy and hormonal manipulation, but more recently therapeutic agents have been developed that exploit the aberrant molecular pathways which characterise neoplasia, such as Trastuzumab which is a monoclonal antibody directed against the HER2 receptor. Investigation of molecular processes which have been shown to be disregulated in cancer may lead to the development of novel therapies of clinical benefit, further improving the outcome for women with breast cancer.

1.1 Prognostic and predictive factors in early stage breast cancer

1.1.1 Breast cancer is a heterogeneous group of diseases

Whilst the term "breast cancer" is used to describe malignant tumours arising from the mammary gland, breast cancer is not a homogenous disease. Rather, breast cancer is a disease which encompasses a wide range of morphological, biochemical and clinical features which result in marked variation in response to treatment and survival from patient to patient. The importance of classifying this group of diseases in order to tailor treatment plans and predict outcome for an individual patient has long been understood. Historically the clinical features used to predict prognosis and inform treatment decisions have included age, size of tumour at presentation, involvement of axillary lymph nodes, histological grade of the tumour, hormone receptor status and HER-2 status and these are discussed in detail below.

It is important to distinguish between prognostic factors and predictive factors. A prognostic factor can be defined as any measurement which provides information on disease outcome irrespective of treatment, and hence correlates with the natural history of the untreated disease. A predictive marker is a marker which correlates to the responsiveness of a particular treatment and selects patients likely to benefit from one treatment over another. The Nottingham Prognostic Index (3)and Adjuvant Online (4)are the most commonly used algorithms used clinically to estimate the risk of disease recurrence and response to treatment respectively.

It is becoming increasingly accepted that combining these two methods of estimating outcome may be useful in terms of defining residual risk, i.e. defining markers which predict disease outcome following adjuvant treatment. The Oncotype DX assay was developed in an attempt to quantify the residual risk of disease recurrence in patients with node negative, ER positive breast cancer, following adjuvant tamoxifen treatment. This assay uses the levels of expression of 16 outcome related genes and 5 reference genes to calculate a recurrence

score. The algorithm produced a continuous measure of risk, which has been used to identify 3 distinct risk groups; low risk (risk of distance recurrence <10%), intermediate risk (risk of distant recurrence 10-30%) and high risk (risk of recurrence >30%). (5) The algorithm has been shown to be predictive of the magnitude of benefit of chemotherapy in patient with ER positive, node negative disease. (6)

1.1.2 Tumour size and grade

The size of the tumour at presentation is an independent prognostic factor and also correlates with the number of involved axillary lymph node deposits (7). Survival decreases with increasing tumour size with overall 5 year survival rates of 86% in patients with tumours between 3 and 5 cm, 89% when the tumour is between 1 and 3 cm, and 99% in patients with tumours less than 1 cm in size.

Tumour grade is assessed by classifying the mitotic index, differentiation and pleomorphism observed within the tumour. The system most widely used is the Scarff-Bloom-Richardson classification which scores each of these categories from 1 to 3 and the total of these scores defines the grade; Grade 1 (score 3 to 5), Grade 2(score 6 to 7) and Grade 3 (score 8 to 9) (8). Tumour grade has been shown to have independent prognostic significance (9), however, it is primarily useful in making treatment decisions in patients who have small tumours and no involved lymph nodes.

1.1.3 Axillary lymph node status

The extent of involvement of the axillary nodes at the time of initial surgery is the most significant prognostic indicator in breast cancer. The extent of nodal involvement is categorised into four groups based on the data from the National Surgical Adjuvant Breast and Bowel Project. The groups are negative nodes, 1 to 3 nodes positive, 4 to 9 nodes positive or 10 or more positive nodes. The five year survival for patients with node negative disease is 82.8% compared with 28.4% in patients with the most extensive nodal involvement (5), and hence accurate staging of the axilla is of the utmost importance when considering the benefit of adjuvant treatment.

The classification of lymph node status in this way is reliant on a traditional axillary clearance. More recent surgical techniques have involved the sampling of the sentinel lymph node (10). The concept of sentinel node sampling is based upon the theory that if the first lymph node in the draining lymphatics (the sentinel node) does not contain metastatic disease (visible by hematoxylin and eosin staining) then it is unlikely that nodes further along the lymphatic chain will be involved.

The advantage of sentinel node sampling is that it reduces the number of unnecessary axillary clearances and hence reduces the associated risk of lymphodema (11). However, there remains some controversy in relation to the optimal assessment of the sentinel node and the importance of the presence of micrometastatic disease.

The AJCC definition of micrometastatic disease has recently been changed. A micrometastasis is defined as a metastasis from 0.2mm (or >200 cells) to 2mm, with a macrometastasis defined as greater than 2mm in size. Clusters of cancer cells less than 0.2 mm or 200 cells are referred to as isolated tumour cell clusters. (12)

A recent retrospective study demonstrated an increased risk of disease recurrence in patients with micrometastatic disease detected by immunohistochemistry compared with patients whose lymph nodes were truly negative ($p=0.001$) (13). However, a recently published prospective trial has shown no difference in the 8 year overall or disease free survival between patients with micrometastatic disease detected by immunohistochemistry and patients with node negative disease (14). It should be noted that both of these studies predate the new AJCC classification of micrometastatic disease.

While the involvement of lymph nodes remains the most important prognostic indicator in breast cancer, further research is required to resolve the controversy surrounding the importance of micrometastatic disease. At the present time, decisions about the need for adjuvant therapy in patient with micrometastatic disease may need to be made on the basis of the other prognostic factors available to the clinician.

1.1.4 Lymphatic and vascular invasion

Lymphatic and vascular invasion refers to the presence of tumour emboli in the surrounding lymphatic and blood vessels. In a large study of 1704 patients who did not receive adjuvant systemic therapies, lympho-vascular invasion was shown to be present in 22% of tumours and was of independent prognostic significance for local recurrence of tumour and breast cancer related survival (15). Similar to tumour grade, the presence of lymphatic or vascular invasion is used to inform treatment decisions in patients with node negative disease with tumours of borderline size.

1.1.5 Expression of the oestrogen receptor

The oestrogen receptor exists in two main forms, ERα and ERβ, and is a steroid hormone nuclear receptor containing a highly conserved DNA-binding domain (16). The classically described mechanism by which binding of oestrogenic molecules to the receptor initiates gene transcription and cell proliferation involves binding of the activated receptor to oestrogen response elements (ERE) in the promoters of target genes (17). Treatment of breast cancer cells *in vitro* with physiological concentration of estradiol results in a marked increase in cellular proliferation and the converse is seen when the same cells are treated with tamoxifen, an oestrogen receptor antagonist (18).

Expression of ER has long been known to be one of the most important prognostic factors in breast cancer (19, 20) and the clinical investigation of tamoxifen, a competitive antagonist of the oestrogen receptor in breast tissue, as an anti-cancer treatment in the 1960's has arguably had the single biggest effect on survival of women with oestrogen positive breast cancer. In this regard ER is both a prognostic and predictive factor. The early trials of tamoxifen included oestrogen receptor positive and negative breast cancers and results of the efficacy of tamoxifen in breast cancer were often mixed, with effects noted on recurrence but not overall survival (21). Once the relevance of oestrogen receptor positivity was recognised and oestrogen receptor testing became routinely available in clinical practice, the effects of tamoxifen on survival became apparent. Meta analysis of the early tamoxifen trials showed a 26%

reduction in mortality with 5 years of adjuvant tamoxifen treatment in patients with ER positive disease (22). The underlying survival benefit of ER positivity per se, coupled with the beneficial effects of tamoxifen, means that women who have cancer which expresses the ER have the best chance of surviving their breast cancer in the shorter term. However, the risk of recurrence and death vary over time in patients with ER positive and ER negative disease. A recent metaanalysis of data generated from three consecutive clinical trials of chemotherapy showed that the risk of recurrence in ER negative patients was highest in the first two to three years after treatment, which then decreased dramatically with increasing time from diagnosis.

In patients with ER positive disease, the risk of an event in the first few years after diagnosis was low, but the long term the hazards were slightly higher for patient with ER positive disease when compared with ER negative disease. (23) This demonstrates that, with the development of modern chemotherapeutic regimens, patients with ER negative cancer, who survive the first 5 years after diagnosis, have at least as good long term survival as patients with ER positive cancer.

1.1.6 Expression of HER2

The gene encoding HER2 has been shown to be overexpressed in 20-30% of breast cancers (24), and overexpression has been shown to be associated with significantly poorer clinical outcome when compared to cancers which do not overexpress HER2 (25), and this has been confirmed by the gene expression profiling studies, further discussed in Section 1.2. HER2 is a member of the type 1 transmembrane tyrosine kinase receptor family which includes the closely related epidermal growth factor receptor (EGFR) (26). HER2 has been described as an “orphan” receptor as no high affinity ligand has been demonstrated (27). It forms homo- and heterodimers with the other members of the family, namely EGFR, HER3 and HER4 (28).

A recent study has suggested that the higher levels of HER2 homodimers and a higher ratio of homodimers to HER2 total expression were significantly correlated with time to first recurrence in a cohort of breast cancer patients who had not been treated with adjuvant Trastuzumab. (29) Furthermore,

treatment of an *in vitro* model of MCF10A expressing a chimeric HER2 protein which could be induced to form either HER2 homodimers or heterodimers with EGFR or HER3 with Trastuzumab, inhibited homodimer-mediated cell growth but not heterodimer-mediated cell growth. (30) Further investigation of the clinical relevance of these observations is currently underway.

Activation of the receptor by ligand binding results in the phosphorylation of discreet tyrosine sites located in the carboxyl terminus of the receptor which in turn leads to activation of multiple intracellular second messenger systems (31). Dysregulation of these second messenger systems, such as the Ras/MAP and PI3K/AKT pathways, have been shown to be implicated in carcinogenesis (32, 33). The precise mechanism by which overexpression of HER exerts its effects are unknown, but the effect of increased activation of these pathways secondary to overexpression of HER2 upstream is thought to be the mechanism by which the HER2 exerts its effects on cancer cell growth and proliferation.

1.1.6.1 Effect of HER2 overexpression on chemosensitivity

Several studies have reported that the overexpression of HER2 may predict benefit from adjuvant chemotherapy. The widely accepted chemotherapy regimes for the adjuvant treatment of breast cancer in the last 20 years include either the combination of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) or doxorubicin and cyclophosphamide (AC) (34). The choice of regime for a particular patient is usually based on the relative toxicity of the regimen and the duration of treatment, with AC being considered less toxic and shorter to administer.

Retrospective analysis of a large adjuvant breast cancer trial has suggested that patients with tumours which express HER2 may derive preferential benefit from anthracycline-containing chemotherapy regimes, showing a relative risk reduction for death of 0.66 (p= 0.01) in favour of anthracyclines (35). A further study confirmed this trend but failed to reach statistical significance (36). In addition, there is evidence to suggest that patients with HER2 positive tumours

may be relatively resistant to CMF chemotherapy (37), but this finding has not been substantiated by further trials (38, 39).

The addition of sequential taxane treatment following anthracycline-based chemotherapy has been shown to improve survival with a hazard reduction for death of 18% in favour of the addition of paclitaxel to AC (p=0.01) (40). An unplanned subgroup analysis suggested that this effect was greater in ER negative tumours compared with ER positive tumours. A further study assessing specifically the effect of paclitaxel on HER2 positive tumours showed a hazard ratio for death of 0.59 (p=0.01) in favour of the addition of paclitaxel in HER2 positive tumours (41). On the findings from these studies, the standard treatment for HER2 positive disease is accepted to contain an anthracyline regime followed by sequential taxane.

However, as HER2 overexpression has not been shown to confer improved chemosensitivity in *in vitro* systems, (42) research has considered the hypothesis that HER2 may be a surrogate for topoisomerase II alpha (Topo2A) expression. Topo2A has been shown to be a major target of anthracycline chemotherapy, (43) and co inhabits chromosome 17 with the HER2 gene. One question has therefore been "Are HER2 positive tumours more sensitive to anthracycline chemotherapy because of associated expression of Topo2A?"

All of these studies are limited by the retrospective nature of the analysis, or unplanned subgroup analysis of small subgroup. The National Epirubicin Adjuvant Trial (NEAT) included a prospective analysis of predictive biomarkers of epirubicin sensitivity, which included HER2 and Topo2A and demonstrated no significant correlation between HER2 expression or Topo2A expression and survival benefit of epirubicin. However, in multivariate analyses, duplication of chromosome 17 centromere enumeration probe (Ch17CEP) correlated with improved relapse free and overall survival with anthracycline treatment. Given that HER2 and Topo2A are both expressed on chromosome 17, it is possible that the conflicting results in relation to sensitivity to anthracycline chemotherapy and HER2 expression are, in part, explained by this finding. Further investigation of Ch17CEP as a biomarker for anthracycline sensitivity is required.

1.1.6.2 Effect of HER2 overexpression on Tamoxifen sensitivity

Both pre-clinical and clinical studies have suggested that the HER2 and ER pathways interact. MCF7 cells that overexpress the HER2 receptor show decreased expression of the oestrogen receptor and increased resistance to tamoxifen treatment (44). A metaanalysis of clinical studies investigating the relationship between HER2 expression and tamoxifen resistance in metastatic breast cancer concluded that overexpression of HER2 does confer resistance to tamoxifen (45). However, results in early breast cancer have been conflicting with a number of studies supporting the view that overexpression of HER2 correlates with tamoxifen resistance (46-48), with other studies failing to show any effect of HER2 expression on tamoxifen sensitivity (23).

These studies have limitations that mean that the results should be interpreted with caution. All of the studies were retrospective and in some the subset analyses were small and unplanned. Furthermore, the methods used to assess HER2 status were not uniform and the reporting of HER2 status was not standardised at the time of these studies. The data presently available has not been considered strong enough to withhold tamoxifen treatment from patients with HER2 positive/ER positive breast cancer.

1.1.6.3 The role of Trastuzumab in HER2 positive early breast cancer

With the development of trastuzumab (Herceptin), a humanised monoclonal antibody against the Her2 receptor, Her2 positivity has now come to be regarded as a favourable predictive factor as opposed to a negative prognostic factor. There have been five large, multicentre randomised controlled trials investigating the adjuvant use of trastuzumab. The HERA trial, the largest of the trials, involved one year of adjuvant treatment with trastuzumab following chemotherapy in patients with HER2+ breast cancer. The results, which were published after only two years of follow up, demonstrated that trastuzumab reduced that rate of recurrence, particularly of distant metastases by 46% (hazard ratio 0.54 p=0.0001) (49). The rate of cardiotoxicity , which has been linked to use of trastuzumab therapy, particularly in combination with anthracycline treatment (50), was shown to have a slightly increased incidence in the trastuzumab treated group, but the overall rates remained low at 0.5%.

The NSABP-B31, NCCTG N9531, BICGR 006 and FinHer trials reported similar improvements in disease recurrence to that seen in the HERA trial (51-53). There was some differences between the trials with respect to chemotherapy regimes and dosing schedules of trastuzumab, however the overwhelming effect of adjuvant trastuzumab on breast cancer recurrence was described as "revolutionary" in the editorial accompanying the publication of the HERA trial (54), and the outlook for patients whose cancers overexpress HER2 is therefore improving.

1.1.7 Current prognostic and predictive factors have limitations

Whilst the histopathological features described above have all been correlated to prognosis, they provide only very limited information on the underlying biology of the disease and provide no explanation for the very wide ranging difference in survival seen in patients displaying identical clinical characteristics. Two patients with identical clinical parameters can demonstrate very different clinical courses with respect to response to treatment, development or site of local or distant recurrence and survival.

As a consequence of the limitations of these clinical predictive factors, it has become necessary to investigate further the underlying biological features of this diverse disease in an attempt to produce an improved taxonomy of breast cancer which is a more robust prognostic tool. The use of high throughput gene analysis has identified distinct subclasses of breast cancer which may be clinically useful.

1.2 Breast cancer can be classified by gene expression profile

Using gene expression profiling it has been shown that breast cancer can be subdivided into 4 main groups:- normal breast-like, luminal, HER2+ and basal-like, with each group demonstrating a distinctive molecular signature (55, 56). These studies used complementary DNA microarrays for 8102 human genes to provide a distinct molecular signature for each cancer sample. In total, 65 breast cancers were sampled, and the DNA signature of each sample was compared to

pooled RNA from 11 breast cancer cells lines as the reference sample. Hierarchical clustering was used to identify groups of tumours with similar patterns of over or under expression in relation to the reference dataset. Immunohistochemistry was performed for clinically relevant parameters such as ER and HER2 expression to corroborate the gene expression findings.

The luminal group can be further subdivided into A, B and C subgroups and is characterised by high expression of many of the genes expressed by breast luminal cells, including the oestrogen receptor and genes associated with oestrogen receptor expression, such as GATA binding protein 3, oestrogen regulated LIV-1 and X-box binding protein 1 and also express cytokeratins 8 and 18. These tumours do not overexpress HER2 and this group accounts for around 60-70% of breast cancers studied.

The Her2+ group overexpress HER2 and other genes also involved in the 17q22.24 amplicon. These tumours are generally ER negative and show low expression of the ER cluster genes described above. The basal-like group also show low expression of the ER cluster and Her2, but show high expression of cytokeratin 5 and 17 and fatty acid binding protein, which are genes that are expressed in breast basal epithelial cells.

The normal breast subtype has recently been reassessed and it has been suggested that this may not be a true subtype, rather that the analysed specimens contained a significant proportion of normal breast tissue which affected results (57). Further work is needed to substantiate this hypothesis and more recent papers have excluded the normal-like subgroup from analysis until this issue is clarified.

Gene expression classification of tumour subtypes has clinical relevance as overall survival has been shown to be significantly different between subgroups (55, 58). Furthermore, there is a correlation between subgroup and response to neo-adjuvant chemotherapy (57). Patients with luminal cancer have a highly significant increase in median survival when compared with the Her2+ and basal-like subtypes. However, within the luminal subgroups there is a significant difference between the overall survival of patients with luminal subtype A, who have best survival outcome, and those with luminal subtypes B and C, suggesting

that the differences observed are not due entirely to the presence or absence of ER expression.

The basal-like subgroup has been shown in many studies to account for a disproportionate number of breast cancer deaths. In one study overall 5 year survival for hormone receptor positive/HER2 receptor negative breast cancer patients was 94%, hormone receptor positive/HER2 positive patients was 91% and hormone receptor negative/HER2 negative was 81% (59). The gene expression profile analysis confirms that the basal –like cancers have the worst outlook and, coupled with the lack of a treatment target, this group of patients warrant further investigation.

1.3 Basal-like and triple negative breast cancer

1.3.1 Basal-like breast cancer cell resemble the cells of the normal myoepithelial layer of the breast.

The basal-like subgroup defined in the gene expression studies outlined above accounts for approximately 15% of all breast cancers and the prevalence of basal-like cancer is significantly greater in premenopausal African American women (60). The genetic signature of this subgroup is the most homogeneous and in addition to the pattern of hormone receptor and HER2 negativity, these cancers frequently express high molecular weight cytokeratins CK5/6, CK17, in addition to vimentin and the epidermal growth factor receptor, which are usually found in myoepithelial cells of the normal breast (55).

The normal breast epithelium is stratified and is comprised of two distinct layers the luminal, milk producing epithelium and the contractile myoepithelial layer. The current “stem cell” theory of breast cancer suggests that there may be early progenitor cells which can differentiate into either luminal epithelial cells or myoepithelial cells and that breast cancer heterogeneity may arise as a result of the neoplastic transformation of cells at any point along this differentiation spectrum. Experiments using mice have shown that an entire functioning mammary gland can be formed when a stem cell epithelial preparation is inoculated into a cleared mammary fat pad (61). Cellular extracts from human

breast obtained from reduction mammoplasty specimens contain three distinct progenitor cell types, luminal cell restricted, myoepithelial cell restricted and bipotent cells which can produce colonies of both cell lineages when grown *in vitro* (62). Examination of the steroid hormone status of the mouse equivalent stem cell population has shown that these cells are ER, PR and HER2 negative, in keeping with the profile demonstrated by human basal-like breast cancer, suggesting the possibility that basal-like breast cancer may arise from such progenitor cells (63).

While the current gene expression classification of breast cancer subdivides the disease into luminal, HER2+ and basal subtypes, there is evidence to suggest that breast cancer may arise from earlier progenitor cells which give rise to the differentiated components of the breast. Strategies to target the stem cell population in human breast cancer, with the intention of improving clinical outcome, are currently the focus of research.

1.3.2 Clinical identification of basal-like breast cancer

The DNA microarray technology which has provided a useful tool in breast cancer research and provided the clinically relevant classification system described above is not readily available in everyday clinical pathology practice, nor can it be used in retrospective studies using formalin-fixed, paraffin embedded tissue. The lack of a robust and clinically usable assay to define basal-like breast cancer in the clinic has resulted in the use of the triple negative phenotype as a surrogate marker.

Triple negative breast cancer is defined by a lack of expression of oestrogen, progesterone and HER2 receptors using standard immunohistochemical techniques and accounts for approximately 15% of all breast cancers (64, 65). Clinical studies confirm that the triple negative phenotype correlates with poor overall survival, shorter disease-free interval after primary treatment and increased incidence in premenopausal African – American women (60, 66). Because of the obvious similarities between triple negative breast cancer and basal-like breast cancer the two terms have become, somewhat erroneously, interchangeable.

However, while there is significant concordance between the triple negative phenotype and the basal-like gene signature, the two groups are not entirely synonymous. A number of studies have shown that a significant proportion of triple negative tumours are not basal-like and vice versa. 7.3% of non-triple negative breast cancers express basal markers (67) and 16-44% of triple negative cancers are negative for EGFR, CK5/6 and CK14 (66, 68). Conversely, oestrogen receptor expression has been demonstrated in 15-45% of basal-like breast cancer and HER2 expression in 14% (54, 55).

In an attempt to better identify basal-like breast cancers within the triple negative cohort, a "five marker" method has been proposed which includes immunohistochemistry for EGFR and CK5/6. When combining ER/PR and HER2 negativity with EGFR and CK5/6 positivity, this five marker method has been shown to have a sensitivity of 76% and a specificity of 100% for the identification of basal-like tumours in cases identified by gene expression analysis (69). Furthermore, this five marker method has significantly better prognostic value than the triple negative phenotype and identifies as subgroup of high risk patients whose survival more closely mimics that seen in the basal-like group identified by gene expression (70).

1.3.3 Improved identification of basal-like breast cancers in the triple negative subgroup is of clinical importance

The triple negative subgroup is in itself a heterogeneous group of breast cancers. Further identification of the basal-like breast cancers within this group is important, as those cancers which are truly basal-like have the poorest prognosis and, at the present time, there is a lack of targeted therapies for this group of patients. In developing new treatments, particularly molecularly targeted treatments, it will be important to identify a molecularly distinct group of tumours which are likely to be sensitive to such treatments. The concern is that the beneficial effect of a targeted therapy can easily be lost if the study population is not accurately selected. This is illustrated by the original studies of gefitinib, an EGFR tyrosine kinase inhibitor, in lung cancer in which the patient population was unselected (71, 72). These trials did not show any benefit of the addition of gefitinib to standard chemotherapy, despite encouraging early phase

clinical trials. Subsequent retrospective analysis has confirmed that a distinct subset of patients with EGFR mutations have an increased response to gefitinib (73).

The gefitinib experience confirms that in the era of specifically targeted therapies, it is essential in clinical trials that the correct subgroup of patients is identified prospectively. With regard to breast cancer, and basal-like breast cancer in particular, the identification of novel markers and molecular pathways which have clinical significance in terms of outcome is important both in identifying potential therapeutic targets and in identifying the patients most likely to benefit from such treatments.

1.4 The relationship between ER and PR expression in breast cancer.

1.4.1 The synthesis of the progesterone receptor is under oestrogen control

In the presence of oestrogen, progesterone receptors are synthesised in tissues such as the endometrium and breast which contain functional oestrogen receptors (74, 75). This has led to the hypothesis that PR positivity is a more reliable indicator of response to hormonal treatment of breast cancer than ER status alone, as the presence of PR suggests that there is some oestrogenic activity which may be abrogated by anti-oestrogen therapy, albeit that the ER receptor may not be detected by conventional laboratory techniques.

Given the relationship between oestrogenic stimulation and progesterone receptor expression, the observation of ER negative/PR positive phenotype in a small minority of breast cancers has remained an anomaly. If progesterone receptor is indeed under the control of oestrogen activity, how is it possible for this phenotype to arise?

There are a number of possibilities to explain this anomaly. The standard diagnostic laboratory technique to assess hormone receptor status is immunohistochemistry and as such there is the potential for multiple variables

which contribute to a high false negative rate. The fixation process, choice of antibody and threshold for positivity can all have an effect on the eventual immunohistochemical result (76, 77). These factors lead to a wide variation in the reporting of oestrogen receptor status. A study of inter-laboratory variation in diagnostic centres across the UK showed that 67% of the centres involved in the study failed to demonstrate oestrogen positivity in a confirmed low-expressing oestrogen receptor positive tumour (78). This significant false negative rate may provide some explanation for the persistence of the ER-negative/PR-positive phenotype.

More recently, the importance of external quality assurance provided by an independent reference agency has been recognised. In the UK this is provided by the United Kingdom National External Quality Assessment Service and provides a comprehensive external quality assessment service in many aspects of laboratory medicine, including steroid hormone and HER2 receptor testing in breast cancer. Membership of such a scheme has benefits with regard to the quality of testing with more experienced participants (defined as those with 6 or greater submissions for assessment) demonstrating the highest quality. (79)

1.4.2 Does the ER-negative/PR-positive phenotype truly exist?

The observed rate of tumours which are ER-negative/PR-positive is reported as 1-5% of the total breast cancer population (80, 81). Clinical outcome data suggests that in women with ER-negative disease, the disease-free interval and metastasis-free survival in ER-negative/PR-positive and ER-negative/PR negative is not significant at 10 years, but significantly different to those women with ER-positive disease (81, 82). Given that these studies are relatively old, and that the methods used to determine hormone receptor status have since been refined, there is significant debate as to whether the ER-negative/PR-positive phenotype is a true pathological entity (83). Current IHC methods, external quality assurance and a low threshold cut off, where any ER staining is reported as positive, have resulted in some studies reporting no evidence of ER-negative/PR-positive breast cancers within the total breast cancer cohort (83). This suggests that knowledge of the PR receptor status in cancers which are otherwise ER and HER2 negative, may add very little in terms of determining the

basal nature of the tumour. Current thinking suggests that a cancer which is reported as ER negative is likely, by virtue of the relationship between ER and PR expression, to be also PR negative. Furthermore, the hierarchical clustering patterns, on which the subgroup classification is based, does not include progesterone receptor expression, suggesting that PR expression is of less importance in differentiating between the subgroups.

1.5 The Rab protein family

The Rab (*Ras-related in brain*) proteins constitute a diverse superfamily of over 50 small GTPases which are involved in the regulation of intracellular vesicular trafficking (84). Many of these Rab proteins have been shown to be involved in distinct stages of endocytic or exocytic membrane trafficking pathways, demonstrating a complex system in which Rab proteins act as the "couriers" enabling the right "package" to reach the correct intracellular "destination" (85).

1.5.1 The Role of Rab proteins in vesicular transport

Members of the Rab family are localised to discrete membrane compartments and the highly hydrophobic double geranylgeranylation at the C-terminal end of the proteins leads to their membrane association. As Rab proteins are GTPases, the proteins cycle between inactive GDP-bound forms and active GTP-bound forms. GDP dissociation inhibitor (GDI) binds the inactive GDP-bound form of Rab proteins and delivers the inactive proteins to the appropriate membrane compartment (86). Once at the correct membrane, GDI is displaced from the Rab protein by membrane associated GDI displacement factors (GDF) which facilitate the tethering of the Rab to the membrane.

Once membrane associated, the Rab protein is activated by the exchange of GDP for GTP by a GDP/GTP exchange factor (GEF)(87, 88). Now in its active form, the Rab protein can interact with its downstream effectors. A number of Rab effectors have the ability to maintain the Rab protein in the activated GTP-bound state, leading to the hypothesis that it may not be necessary for all Rab

proteins to complete the membrane release cycle after each GTP hydrolysis (89). Figure 1-1 shows a graphical representation of the cycling of Rab proteins.

Individual Rab proteins are closely related to a specific compartment in the endocytic and exocytic vesicular trafficking pathways within the cell. For example, vesicular transport of cargo from the endoplasmic reticulum to the Golgi apparatus depends upon Rab1 and Rab2 (90, 91) and Rabs 6 and 8 are involved in transport of vesicles from the trans-Golgi network to the plasma membrane (92, 93). In the endocytic pathway Rab 5 is associated with early endosomes and Rab7 with late endosomes (94).

Rab proteins interact with a wide range of effectors such as motor proteins (95), elements of the vesicle fusion machinery (96) and tethering factors (97) to orchestrate a very tightly controlled cellular transport system in eukaryotic cells.

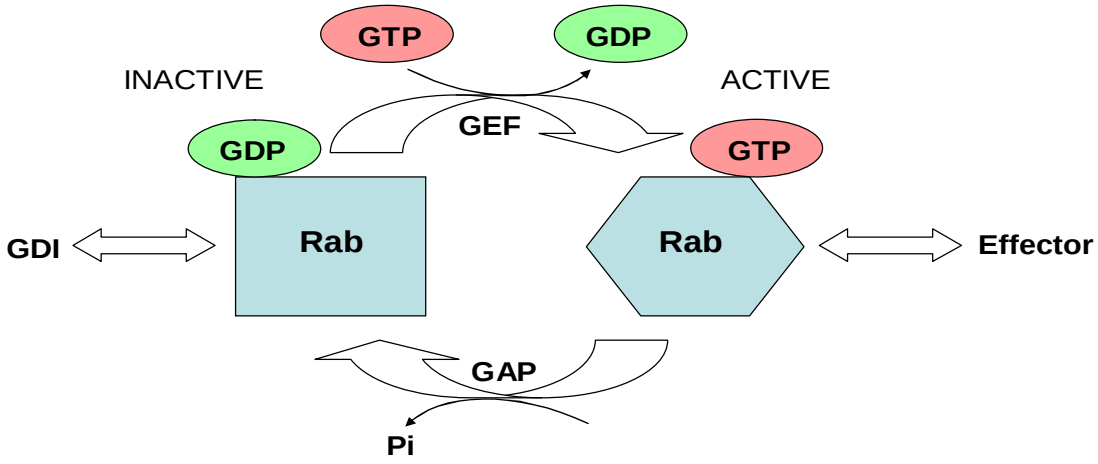


Figure 1-1 The Rab proteins cycle between two conformational states.
GDP-bound forms are coupled to GDP-GTP dissociation inhibitor (GDI) and are delivered to the correct cellular compartment. Nucleotide exchange, catalysed by GDP-GTP exchange factor (GEF) results in the GTP-bound form which can interact with effector molecules. GTPase-activating protein (GAP) converts the GTP- to the GDP-bound form with the release of inorganic phosphate (Pi).

1.5.2 Rab proteins in polarised epithelial cells.

Polarised epithelial cells, when organised into sheets of epithelial tissues, are responsible for both absorption and secretion in mammalian physiological systems. These highly specialised functions rely on the specific directional processing of signals which are being received by the cell from both the apical and basolateral surfaces (98). Epithelial cells maintain distinct apical and basolateral compartments and this intracellular conformation is reliant on regulated trafficking systems, of which the Rab proteins are part.

Whilst the majority of Rab proteins appear to be ubiquitous, which is in keeping with the function of these proteins in the vesicular transport systems common to all cells, some Rabs, such as Rab25, exhibit a defined pattern of tissue distribution. Rab25 has been shown to be differentially expressed in polarised epithelial cells such as gastrointestinal mucosa, kidney and lung tissue (99). This pattern of distribution suggests that Rab25 may be involved in the regulation of aspects of trafficking which are unique to epithelial cells.

1.5.3 The Rab11 subfamily

The Rab proteins are further subdivided into groups which demonstrate approximately 40% homology with each other. The Rab11 subfamily consists of Rab11a, Rab11b and Rab25. Rab11a and Rab11b differ from each other in only the final 30 amino acids, but are coded for by different genes (100). They are ubiquitously expressed and are associated with the apical recycling system in polarised epithelial cells (101), and the plasma membrane recycling system in non-polarised cells (102).

Rab25 was identified in the parietal cells of the gastric mucosa and shares 68% sequence homology with Rab11a, but differs in both the amino and carboxyl ends of the protein (Figure 1-2). The amino acid sequence of Rab25 shows a single amino acid substitution of leucine for glutamine in the G-3 WDTAGQE GTP-binding domain (99). The WDTAGQE sequence is the consensus sequence which is shared by the majority of small GTP-binding proteins (103). The leucine to

glutamine substitution seen in Rab25 is also seen in Ras protein, where it conveys an transforming phenotype, preventing the hydrolysis of GTP, rendering the Ras protein constitutively active (104). Despite the presence of the WDTAGLE sequence in Rab25, recombinant Rab25 protein can bind GTP on a blot, suggesting that this sequence is of physiological significance in the normal functioning of this protein (99).

In addition to the unusual WDTAGLE sequence, Rab25 contains a two amino acid difference in its effector domain. The effector domain sequence has been the focus of Rab subfamily definition and hence it was not certain that Rab25 did indeed belong to the Rab11 family. However, the exon structure of Rab11a, Rab11 b and Rab25 is very highly conserved , supporting the hypothesis that these three proteins are indeed part of the same gene family, albeit with specialised functions (105).

Figure 1-2 Amino acid sequence of Rab25, Rab11a and Rab11b.
Deduced amino acid sequence obtained from Uniprot and aligned to show areas of homology. Sequences coloured aqua are homologous to all three proteins. Further areas of homology between Rab11a and Rab11b are shown in magenta

1.6 Rab25 expression in cancer

There is increasing evidence that alterations in Rab protein expression may be implicated in human disease. Of the Rab proteins, Rab25 is the most reported.

1.6.1 Overexpression of Rab25 in ovarian cancer is associated with poor prognosis

Rab25 is located on chromosome 1q22 and comparative genomic hybridization has revealed that gains in the chromosomal region 1q21-22 are associated with a drug resistant phenotype in ovarian cancer (106). More recent work has concentrated on defining the regions of recurrent copy number increase in this amplicon. There are twenty two known genes and twelve hypothetical proteins contained within the region from position 152,377,895 to 153,495,551, based on the July 2003 human reference sequence. Eighteen candidate genes did not show a difference in expression levels between ovarian cancers and normal ovarian epithelium. The messenger RNA levels of the remaining sixteen candidates were assessed using quantitative PCR. Rab25 levels were increased in 88.7% of ovarian cancers when compared with normal ovarian epithelium. Linear regression analysis confirmed a direct relationship between copy number and expression of Rab25 mRNA. Furthermore the expression of Rab25 has been shown to be closely correlated with clinical outcome with high levels of Rab25 expression associated with decreased survival (107).

More recent work investigating the relationship between androgen stimulation and the expression of Rab25 in ovarian cancer demonstrated that exposure of ovarian cancer cell lines to androgen resulted in only a marginal upregulation in Rab25 expression, which is of interest given that the antisense strand of the Rab25 gene contains an androgen response element (108). This study also investigated the relationship between Rab25 expression in ovarian cancer samples and overall survival, using immunohistochemistry with a commercially available Rab25 antibody, in a clinical cohort of 154 patients. No correlation was seen between Rab25 expression and overall survival in this cohort, which is in direct contrast with the findings of Cheng et al. outlined above (107).

Further work attempting to differentiate the genomic differences between the morphologically similar ovarian cancer, primary peritoneal cancer and diffuse malignant peritoneal mesothelioma has shown that Rab25 is overexpressed in ovarian and primary peritoneal cancer compared with diffuse malignant peritoneal mesothelioma, suggesting that Rab25 may play a role in the pathogenesis of ovarian cancer (109). In addition, *in vitro* work investigating the effect of knockdown of Rab25 expression in the ovarian A2780 cancer cell line using a small interfering RNA expression vector has demonstrated decreased cellular proliferation rates, increased apoptosis and inhibition of tumour growth in xenograft models (110). However, it should be noted that the A2780 cells used in the work described here have been shown by Western blotting and RT-PCR not to express Rab25 and therefore the results of this study should be interpreted with caution.

These data highlight that the clinical relevance of Rab25 expression in ovarian cancer is far from clear cut and further work to investigate the relationship between Rab25 expression and survival in ovarian cancer is needed.

1.6.2 The relationship between Rab25 expression and survival in breast cancer is unclear

Approximately 50% of breast cancers contain an amplification in the 1q region, but in contrast to the specific 1q22 amplification seen in ovarian cancer, the amplification seen in breast cancer is broader and encompasses the majority of the 1q region (107). Comparative gene hybridisation analysis revealed that 67% of breast cancers showed an increase in Rab25 expression of at least 1.7 fold that seen in normal breast tissue, suggesting that increased Rab25 expression is either an important initiating event in carcinogenesis, or a consequence of such.

In this study, survival analysis of 109 patients was performed dividing the cohort into two groups – those with at least two-fold higher than normal Rab25 RNA expression and those with less than 2-fold higher than normal, as assessed by quantitative PCR. When the cohort was divided in this way, those patients with high expression of Rab25 showed a decrease in overall survival and disease free survival, compared with low expressors. In addition, Rab25 levels were an

independent prognostic factor when data were adjusted for tumour grade, tumour size and ER status. It should be noted that the breast cancer population used is this study included only patients with locally advanced disease at the time of diagnosis and included a higher than proportion of patients with a BRCA1 mutation than would be expected in an unselected breast cancer population, and therefore caution should be exercised when extrapolating these results.

Subsequent further comment on unpublished data from this study suggests that the expression of Rab25 may be related to the subtype of breast cancer, with ER positive tumours showing high levels of Rab25, basal tumours showing intermediate expression and metaplastic tumours showing low or absent expression. These findings are in keeping with data which has shown that Rab25 expression was detected in 6 nontumorigenic breast cell lines but lost in 4 out of 9 tumorigenic breast cell lines, all of which had also lost expression of the oestrogen receptor. RT-PCR for Rab25 in breast cancer samples demonstrated loss of Rab25 in 83% of ER/PR negative breast cancer, compared with loss of Rab25 in only 8% of ER or PR positive tumours (111).

Further work investigating the relationship between hormone receptor status and loss of Rab25 has shown that Rab25 expression, detected by RT-PCR, is lost in about 44% of breast cancers when compared with matched normal tissue. 69% of ER negative breast cancer samples showed loss of Rab25 compared with just 21% of ER positive cancer specimens ($p=0.005$), suggesting preferential loss of Rab25 in ER negative tumours. Furthermore, immunohistochemical investigation of frozen sections of breast cancer confirmed reduced or absent Rab25 expression in ER negative cancer samples, compared with those cancers which express the oestrogen receptor (112).

There is an element of conflict between these data which requires further investigation. The data presented by Mills et al. suggests that overexpression of Rab25 is associated with poorer survival, irrespective of the hormone receptor status of the tumour, while the data presented by Rao et al. suggests that loss of Rab25 is far more common in ER negative tumours which, as described above, have a poorer clinical outcome than ER positive tumours. It is possible that the role of Rab25 is dependent on the subtype of breast cancer, and that

perturbations of Rab25 levels from the norm may have different effects in different subtypes of breast cancer.

1.6.3 Loss of Rab25 is associated with poor survival in colon cancer

Survival analysis of patients with colorectal cancer has shown that low gene expression of Rab25, described as Rab25 gene expression below the median for the cohort, correlates with significantly poorer overall survival when compared with high expression (113). Furthermore, Rab25 expression was significantly decreased in primary colonic adenocarcinomas and lung and liver metastases compared with normal colon. The decrease in Rab25 was independent of the Dukes' stage of the cancer, suggesting that loss of Rab25 occurs at an early stage in the progression of colorectal cancer.

Further investigation into the effect of Rab25 loss in colorectal cancer has been undertaken utilizing mouse models of colorectal cancer. The adenomatous polyposis coli (Apc) *min* mouse contains a truncation of the Apc gene, which is the mouse homologue of the human adenomatous polyposis coli (APC) gene. Mice with the Apc^{min} gene develop adenomas within the gastrointestinal tract, predominately in the small intestine (114).

Crossing the $Apc^{Min/+}$ mouse with a Rab25 deficient mouse resulted in a 4 fold increase in tumour number throughout the intestine, but no increase in the invasive character of the lesions. Mice which were heterozygous for Rab25 showed an increase in intestinal tumour number which was intermediate between the wild type and deficient mice, suggesting haploinsufficiency.

To assess the effect of Rab25 loss on the invasive potential of colonic tumours, the Rab25 deficient mouse model has been crossed with *Smad3* heterozygous model. Homozygous loss of *Smad3* in mice results in proximal colonic tumours, whereas the heterozygous model shows minimal pathology. $Smad3^{+/-}$, $Rab25^{-/-}$ mice developed large invasive lesions in 80% of mice, suggesting that Rab25 acts as a tumour suppressor in the colonic epithelium (113).

1.6.4 Rab25 in other cancer types

With the advent of cDNA microarray technology, that allows the investigation of thousands of different genes simultaneously, many markers of carcinogenesis can be identified. Using this technology, Rab25 has been identified as being upregulated in testicular germ cell tumours (115) and transitional cell carcinoma of the bladder (116) when compared with their respective normal tissues. In lung cancer cell lines, Rab25 has been shown to be upregulated in squamous, large cell and small cell carcinoma cell lines when compared with a normal lung cell line (117).

Conversely, loss of Rab25 expression, as demonstrated by immunohistochemistry, has been reported with increasing dysplasia in biopsy specimens of Barrett's oesophagus (118).

The role and effect of Rab25 dysregulation in epithelial cancers remains unclear, but preliminary evidence suggests that altered Rab25 expression is seen in many cancer types and further investigation to elucidate the mechanism by which Rab25 exerts its effects may prove useful in the development of novel anti-cancer agents.

1.7 Putative mechanisms of Rab25 effect in cancer

1.7.1 Rab25 promotes invasive migration in 3D matrices

Rab25 has been shown to interact specifically and directly with the ß1 integrin cytoplasmic tail. In addition, A2780 ovarian cancer cells transiently expressing Rab25 invade fibronectin -containing 3D matrices to a significantly greater degree than control cells and this effect is dependent upon the interaction between Rab25, α5ß1 integrin and fibronectin. Furthermore, the morphology of Rab25-expressing cells is altered and is characterised by the extension of long pseudopods, at the tip of which a discreet pool of actively cycling α5ß1 integrin is maintained by Rab25 (119).

Expression of Rab25 in A2780 cells slows the speed of migration of the cells, but increases the persistence of migration. Taken in combination with the finding of

elongated, persistent pseudopodial extension, these data give rise to the hypothesis that increased Rab25 expression promotes invasive migration by maintaining α5β1 integrin at the tip of the invading pseudopod.

1.7.2 The effect of Rab25 on cell proliferation and apoptosis

Exogenous expression of Rab25, by transfection with a Rab25 expression vector, results in increased colony forming activity and increased proliferation in a number of ovarian and breast cancer cell lines, which can be abrogated by RNAi transfection of Rab25. There is no significant change in cell cycle progression as a result of increased Rab25 expression, but cell survival is increased, suggesting a role for Rab25 in the regulation of cell survival (107). Furthermore, protein levels of BAK1 and BAX, which are part of the apoptotic signalling pathway, are decreased in the presence of increased Rab25 levels. This may be as a result of increased signalling through the PI3K-AKT pathway as higher levels of AKT phosphorylation are seen with increased expression of Rab25.

Further evidence to support the hypothesis that Rab25 affects proliferation and cell survival is demonstrated by the increased rate of tumour growth *in vivo* of Rab25-transfected cells in mice xenograft models. 100% of mice injected with Rab25 expressing A2780 cells developed tumours compared with 50% of controls. In addition, the tumours formed were larger in the Rab25 expressing group and developed in a shorter time (107).

Conversely, exogenous expression of Rab25 in two ER negative breast cancer cell lines reduces cell proliferation and anchorage independent growth *in vitro*. Cells are arrested in G2/M phase and there is a significant increase in early and late apoptosis compared with control cells (111, 112). As previously described, an *in vivo* mouse model of Rab25 loss in colon cancer demonstrates larger and more aggressive tumours, consistent with increased cellular proliferation and deceased apoptosis (113).

The contradictory nature of these results suggests that the effect of Rab25 on cell proliferation and apoptosis may be dependent on the cellular context and further investigation is needed to elucidate the mechanisms by which Rab25 gain or loss exerts its effects.

1.7.3 Rab25 and growth factor receptor signalling

Overexpression of the epidermal growth factor receptor is a commonly observed event in human cancer. Lung, oesophageal, gastric, colon, pancreatic and bladder cancer have all been shown to overexpress the EGF receptor, and overexpression correlates with poor survival (120-125).

In A2780 cells overexpressing Rab25 the pattern of cell surface expression of EGFR following treatment with EGF differs from that seen in control cells, with higher surface expression of EGFR at 60 minutes in Rab25–expressing cells. In addition, AKT phosphorylation following exposure to EGF is increased in Rab25-expressing cells compared with controls (126).

The hypothesis that Rab25 may have a role in EGFR recycling is further supported by the finding that the presence of the αvß3 integrin blocking agents osteopontin and cilengitide promote the association of Rab-coupling protein (RCP), α5ß1 integrin and EGFR1 in a coprecipitatable complex and that this physical association is necessary for the effective recycling of EGFR1 under these conditions. Furthermore, this enhanced recycling of EGFR1 to the plasma membrane is associated with enhanced EGFR signalling as demonstrated by enhanced autophosphorylation of EGFR1 and activation of the PKB/AKT pathway (127).

RCP, also known as Rab11 family interacting protein 1, is one of a family of four effector proteins which can associate with the Rab11 family members, including Rab25 (128). Whilst the physiological role of RCP is not entirely understood, it has been shown to be upregulated and positively associated with metastatic recurrence in breast cancer (129). Furthermore, it has been shown that RCP and Rab25 mRNA levels are highly correlated in breast cancer samples, suggesting that there may be cooperation between these two genes in tumorigenesis (130).

Colocalisation of Rab25 and RCP has not been investigated in the studies outlined above, but given the known interaction between the two and the strong evidence to support the individual interactions between Rab25 and RCP with the α5ß1 integrin, it can be hypothesised that Rab25 is involved in the RCP/ α5ß1 integrin/EGFR complex which confers an invasive migratory phenotype to cancer

cells which may contribute to the poorer clinical outcome seen in patients with tumours which overexpress Rab25. Figure 1-3 illustrates the hypothesised role of Rab25 in growth factor receptor signalling.

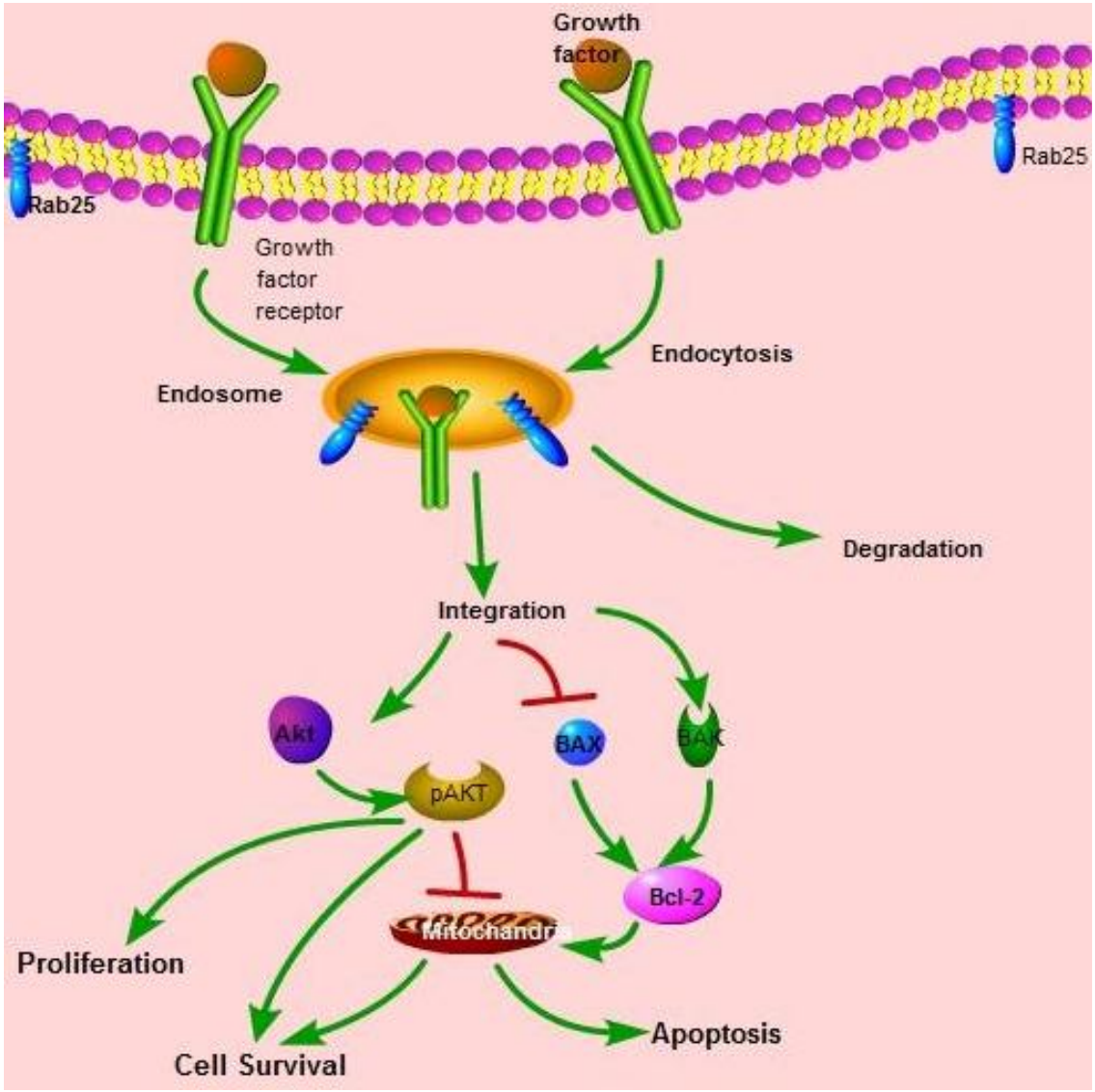


Figure 1-3 Putative role of Rab25 in growth factor receptor signalling, proliferation and cell survival.
Rab25 may have a role in the internalisation and trafficking of growth factor receptors from the cell surface to endosomes. Rab25 activates AKT and down-regulates BAK1 and BAX resulting in decreased apoptosis.
From: Subramani and Alahari *Molecular Cancer* 2010 **9**:312. (131)

1.8 The role of β1 integrin in breast cancer

1.8.1 The extracellular environment plays a role in tumorigenesis

The malignant transformation of normal cells to invasive cancer cells has been shown to involve multiple genetic events, altering growth and apoptotic pathways and providing a survival advantage to transformed cells. Increasingly, attention is being turned to the effect of the extracellular stromal environment on the behaviour of cancer cells, in an attempt to identify potential pathways that may be amenable to disruption by novel therapeutic agents.

The discovery that, when exposed to medium containing basement membrane components, normal mammary and breast cancer cells grown in *in vitro* culture systems can recapitulate the structural and functional characteristics of these cells *in vivo*, highlighted the importance of the interaction between the extracellular matrix (ECM) and cells (132).

The architecture of epithelial tissue is maintained by cell adhesion complexes which involve components of the extracellular matrix, cadherins, the actin cytoskeleton and integrins. Epithelial tumours are characterised by disordered tissue structure and loss of cell polarity which results, in part, from perturbations in these interactions.

β1 integrins are essential for the development and maintenance of polarised epithelial tissues (133, 134), and inactivation of the β1 integrin gene in mice results in embryonic lethality (135). Furthermore, in the investigation of the role of the integrin family in breast cancer, the β1 integrin subfamily has emerged as potentially important in the initiation and maintenance of breast cancer (136).

1.8.2 Increased β1 integrin is associated with decreased survival in breast cancer

Immunohistochemical examination of a cohort of 250 invasive breast cancers for β1 integrin has demonstrated that patients with high expression of β1 integrin have a significantly decreased 5 year overall survival rate (45%) compared with

patients who have intermediate or low expression (85%). In addition, ß1 integrin expression significantly correlates with fibronectin expression (137).

ß1 integrin, when associated with one of the α integrins with which it forms a heterodimer, binds to extracellular ligands which include fibronectin and laminin, expression of which have both been shown independently to correlate with poor survival in breast cancer (9, 138). However, in the study of ß1 integrin expression in breast cancer described above, fibronectin was not an independent prognostic factor, suggesting that the interaction between ß1 integrin and fibronectin may be of greater importance in the progression of breast cancer than the expression of fibronectin alone. Further evidence to support the hypothesis that the interaction between fibronectin and ß1 integrin is of significance in cancer is demonstrated by the enhanced resistance of lung cancer to chemotherapy when the two are coexpressed (139).

1.8.3 Disruption of β1 integrin function results in malignant reversion of breast cancer cells in vitro and in vivo

When "normal" mammary epithelial cells are grown in a 3D culture of reconstituted basement membrane they form organised acinar structures which deposit a basement membrane, indicating polarisation of the acini (132). When a spontaneously tumorigenic cell line derived from this parent line is grown in a similar fashion the cells form loosely associated, disorganised colonies with no basement membrane. In addition, ß1 integrins are basally distributed in the normal acini, but randomly distributed in the tumour cell colonies with a large increase in total ß1 integrin protein levels, but only a minor increase in cell surface levels (140).

Treatment of the tumorigenic progeny with a ß1 integrin blocking antibody results in the reversion of the malignant phenotype to the extent that after 12 days incubation in a 3D matrix, colonies of the two cell types are morphologically indistinguishable and the tumour cells deposit a basement membrane. Incubation of the tumorigenic cell line with the ß1 integrin blocking antibody prior to injection of the cells into nude mice results in reduced tumour size and number when compared with untreated cells, suggesting that once

"normalised" these cells can maintain a normal phenotype and their malignant potential is held in check by the normal tissue architecture. Furthermore, systemic treatment of nude mice bearing established xenografts of a number of different breast cancer cells lines with the anti- ß1 integrin blocking antibody resulted in significantly less tumour growth over the 4 weeks of treatment compared with controls, with no obvious toxicity (141). These findings suggest that inhibition of ß1 integrin function using monoclonal antibodies may be a useful therapeutic approach in the treatment of breast cancer.

1.8.4 β1 integrin is required for the induction of mammary tumours in a mouse model of breast cancer

As described above, germline deletion of the ß1 integrin gene results in embryonic lethality in mice (135). Using the Cre/LoxP system, ß1 integrin expression in mammary tissue can be disrupted by breeding a conditional LoxP1 ß1 integrin mouse with a polyomavirus (PyV) middle T (MT)/Cre recombinase mouse using mouse mammary tumour virus (MMTV) as the transcriptional promoter (142). The MMVT PyV MT strain of mice is a well developed mouse model of breast cancer which recapitulates many aspects of human breast cancer progression, including atypical hyperplasia, adenocarcinomas and development of distant metastases. Mammary tumours appear with complete penetrance and female carriers develop palpable mammary tumours by week 5. By 3 months of age 90-100% of these animals will have developed pulmonary metastatic disease (142).

Ablation of ß1 integrin in MMTV PyV MT animals does not affect the mammary ductal outgrowth which occurs during puberty, and lactation and fertility are not affected by the deletion of ß1 integrin from mammary tissue. However, at 10 weeks mammary tumours are not palpable in the ß1 – deleted, $ß1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT animals compared with $ß1^{LoxP1/wt}$ MMTV/Cre/PyV MT control animals, which develop mammary tumours at the same rate and to the same extent as described for the parental MMTV/Cre/PyV MT strain as described above, with 100% of animals demonstrating palpable tumour burden at 10 weeks.

Furthermore, at 4 months of age ß1$^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT animals develop palpable mammary tumours which retain expression of ß1 intergrin, which is consistent with lack of excision of the ß1$^{LoxP1/LoxP1}$ alleles due to the absence of MMTV/Cre expression in these cells (136). This is in keeping with the reported stochastic expression of MMTV/Cre in this tumour model system.

Taken together these data suggest that expression of ß1 integrin is not necessary for the normal mammary gland development in mice, but is required for mammary tumour development in the MMTV/Cre/PyV MT breast cancer mouse model, further highlighting the potential therapeutic potential of anti ß1 integrin agents in breast cancer.

1.8.5 Clinical trials of the β1 integrin inhibitor Volociximab

While there is growing evidence to suggest that ß1 integrin expression on tumour cells is clinically relevant, the approach adopted with regard to ß1 integrin inhibition has focused on inhibition of angiogenesis as the target, rather than direct targeting of tumour cells per se.

As previously described, the ß1 integrin forms a number of heterodimers with α integrin subunits. One of these heterodimers, the α5ß1 integrin, has been extensively investigated as a potential therapeutic target in a number of solid tumours.

The α5ß1 integrin is the main fibronectin receptor and is upregulated in tumour vasculature and in proliferating vascular endothelial cells *in vitro (143)*. Inhibition of α5ß1 integrin function in *in vitro* and *in vivo* systems results in apoptosis of endothelial cells and inhibits angiogenesis (144). Robust preclinical data to support the hypothesis that the α5ß1 integrin may be a suitable therapeutic target has led to the development of Volociximab (M200), which has entered early phase clinical trials.

Volociximab is a chimeric humanised anti-α5ß1 integrin function blocking antibody and results of Phase II clinical trials in pancreatic and renal cancer and melanoma have been presented (145-147). These show that volociximab is well tolerated in a heavily pre-treated population and may be clinically efficacious.

Despite this promising early data, subsequent robust clinical efficacy data from Phase III trials have not yet emerged. There are a number of potential reasons for this, not least that the conventional efficacy measures used in clinical trials, namely response rate and survival, may not be adequate markers of efficacy for anti-angiogenic drugs, which do not have a direct effect on tumour cells and hence tumour volume. A method to quantify and monitor angiogenesis, either directly or using surrogate markers will be required, as it is likely that such agents will be cytostatic rather than cytotoxic. In addition, combination therapies will have to be considered in a rational way in order to maximise benefit from integrin-based therapy.

While the increased expression of ß1 integrin has been shown to be associated with poorer clinical outcome in breast cancer, neither the nature of the integrin heterodimers involved, the factors which potentiate the increase nor the downstream effects of the increase are understood. If drugs which target intergrins are to be clinically successful, these mechanisms will need to be elucidated.

1.9 Aims of this thesis

Current prognostic markers for breast cancer are of value in informing treatment decisions with respect to adjuvant therapy following primary surgical removal of the tumour. However, current markers which can be routinely used in high throughput clinical laboratories have significant limitations. Much research activity is focussed on identifying other prognostic factors which may improve the ability to discriminate further those patients who might benefit from adjuvant treatments both currently available and in pre-clinical development.

As has been discussed, the nature of the relationship between Rab25 expression and survival in breast cancer in unclear. There is some clinical data to support the hypothesis that increased expression of Rab25 is associated with a poorer clinical outcome in terms of survival, but conflicting data suggests that loss of Rab25 may be associated with ER negative tumours, which have a poorer clinical outcome than ER positive disease.

The aims of this project were to investigate if Rab25 expression can be reliably detected by immunohistochemistry in a clinical large cohort of breast cancer patients, and to ascertain if there is any relationship between Rab25 expression and breast cancer related survival. In order to undertake this investigation, it was first necessary to purify and validate a mouse polyclonal antibody for use in immunohistochemistry, and establish if a robust and reproducible immunohistochemical scoring system for Rab25 staining could be obtained.

The currently available data gave rise to the hypothesis that Rab25 loss may be important in breast cancer, but the mechanism by which loss of Rab25 exerts its effect is unclear. To investigate the effect of Rab25 loss on breast cancer cell growth and motility, a Rab25 knockdown MCF7 breast cancer cell line was generated and characterised.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents, solutions and media

Solution	Ingredients	Source
AEBSF		Sigma
Affi-gel 15		Bio-Rad
Aprotinin		Sigma
Bradford reagent		Sigma
Coomassie Blue		Thermo Scientific
Dulbecco's Modified Eagle Medium (DMEM)		Gibco
Fetal Bovine Serum (FBS)		Autogen Bioclear
Glutathione Sepharose Beads		GE Healthcare
Hybond-P		GE Healthcare
IPTG		Melford Laboratories
LB broth and agar		Beatson Institute Central Services
Leupeptin		Sigma
L-Glutamine		Gibco
Lipofectamine		Invitrogen
Milk		Marvel
MOPS		Sigma
Na Bicarbonate		Gibco
NDLB Cell Lysis Buffer	50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 5 mM EDTA,10 nM Na Orthovanadate, 10 mM NaF, 0.5%, Igepal CA-630, 10 µg/ml aprotinin, 2 mM AEBSF, 10µg/m leupeptin	
NuPAGE MOPS SDS Running Buffer (20X)		Invitrogen
NuPAGE Sample Reducing Agent (10X)		Invitrogen
Opti-MEM		Gibco
PBS/EDTA (PE)	PBS + 1 mM EDTA	Beatson Institute Central Services
Penicillin and ampicillin		Sigma
Phosphate Buffered Saline (PBS)	170 mM NaCl, 3.3 mM KCl, 1.8 mM Na_2HPO_4, 10.6 mM	Beatson Institute Central Services

	H_2PO_4	
RPMI-1640 Medium		Gibco
RTL buffer		Qiagen
Streptavidin-HRP		GE Healthcare
Streptomycin		Sigma
TBS Tween (TBST)	TBS + 0.1% Tween-20	
Transfer Buffer	50mM Tris, 40mM glycine, 0.04% SDS, 20% methanol	Beatson Institute Central Services
Tris		Sigma
Tris Buffered Saline (TBS)	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	Beatson Institute Central Services
Tris-acetate-EDTA (TAE)	40 mM Tris, 0.1% glacial acetic acid, 1 mM EDTA	Beatson Institute Central Services
Triton -X100		Perkin Elmer
Trypsin	0.25% Trypsin in PE	Gibco
Vectashield-DAPI		Vector Laboratories

2.1.2 Antibodies

Antibody	**Dilution**	**Source**
Western blotting		
Anti-Rab25	1:5000	In house
Anti- ß-actin	1:10 000	Cell Signalling Technology
Anti-Rab11	1:1000	Cell Signalling Technology
Anti-ITGß1	1:500	BD Transduction Lab
Anti-GST	1:10 000	Sigma
Anti-GFP	1:10 000	Sigma
Anti-mouse HRP-linked	1:5000	Cell Signalling Technology
Anti-rabbit HRP-linked	1:5000	Cell Signalling Technology
Immunohistochemistry		
Anti-Rab25	1:1000	
Immunoprecipitation		
Anti-ITGß1		Chemicon
Immunofluorescence		
Anti-Rab25	1:500	
Anti-haemagluttinin	1:250	Sigma
Anti-mouse Cy3	1:100	Sigma
Anti-rabbit Cy5	1:100	Sigma

2.1.3 Enzymes and kits

Kit/Enzyme	Application	Source
BglII	Restriction enzyme	Invitrogen
Dako Envision	Immunohistochemistry	Dako
HindII	Restriction enzyme	Invitrogen
Improm II	RT-PCR	Promega
Maxi-Prep	Plasmid preparation	Qiagen
p-SUPER-GFP.neo	Expression vector	Oligoengine
Quantitect SYBR Green	qPCR	Qiagen
RNeasy	Isolation of RNA	Qiagen

2.1.4 Tissue culture plasticware

Falcon tissue culture dishes and multi-well plates were used throughout.

2.1.5 Tissues used for immunohistochemistry

2.1.5.1 Mouse tissue

Two virgin female Friend leukaemia virus B stain mice were culled by cervical dislocation and dissected. Portions of small intestine, colon, stomach, pancreas, spleen, liver, lung, kidney, muscle and brain were fixed in 10% neutral buffered formalin for 48hours. Tissues were then paraffin embedded, orientated and sectioned by Colin Nixon, Histology Service, Beatson Institute.

2.1.5.2 Human tissue

Commercial breast cancer tissue microarrays BR804 and BR1001 were purchased from US Biomax. BR804 consisted of single cores of paired invasive breast cancer and matched normal adjacent tissue from 40 patients. BR1001 consisted of single cores of paired invasive breast cancer and matched lymph node metastases from 50 patients.

The West of Scotland Breast Cancer TMA consisted of a single 0.6mm tissue core sampled from each formalin-fixed paraffin embedded archival breast cancer specimen using a manual arrayer and mounted into a recipient paraffin block. 6 TMA's in total were constructed. Ethics approval was obtained from the West of Scotland Research Ethics Committee, title "Molecular profiling and clinical validation of breast cancer biological subtypes" REC reference number 07/S0704/61.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Origin of cell lines

The cell lines used were kindly donated from individuals in the Beatson Institute of Cancer Research. A2780-Rab25, A2780-DNA3, SNU-601 and SNU-484 cells were a gift from Jim Norman, MCF10a cells were a gift from Adele Hannigan and MCF7 cells were a gift from the group of Walter Kolch.

2.2.1.2 Maintenance of cell lines

A2780, SNU-484, SNU-601 cells were grown in RMI-1640 supplemented with 10% FBS, 2 mM glutamine, 10U/ml penicillin and 10µg/ml streptomycin. MCF7 and MCF10a cells were grown in DMEM supplemented with 10% FBS, 2mM glutamine and 10U/ml penicillin and 10µg/ml streptomycin. Cells were maintained in a humidified 37°C /5% CO_2 incubator. To sub-culture adherent cells, the medium was removed by aspiration, the monolayer rinsed twice with PBS then 10% trypsin/PE solution was added until cell detachment. Upon detachment the cells were resuspended in the appropriate growth medium and then transferred into tissue culture plates.

2.2.2 Protein production and purification

2.2.2.1 Transformation of bacterial cells

10-20ng of plasmid DNA was added to 50μl of competent cells, mixed gently, and incubated on ice for 20 min. The cells were then heat shocked by incubation at 42° C for 45 sec in a water bath, and then for a further 2 min at 0° C. 450μl of LB medium was then added, and the cells allowed to recover at 37° C, shaking for 1 hr. 50-200μl of the transformation mix was spread evenly onto LB agar plates containing ampicillin, and the plates were incubated at 37° C overnight. Colonies from the plates were used to inoculate 3.5ml LB, and grown for 6 hours at 37° C. 0.5ml of the resulting bacterial culture was added to 100ml LB broth containing 100μg/ml ampicillin and incubated at 37° C overnight. Glycerol stocks (40 % glycerol, 60 % overnight culture) of all transformed plasmids were made, snap frozen, and stored at -80° C.

2.2.2.2 Protein expression

450ml of sterilised LB media, supplemented with 100ug/ml ampicillin, was inoculated with 50ml of the overnight culture described above and grown in an orbital shaker at 37^{o}C for 1-2hr. A 1ml sample of culture was removed to a plastic cuvette and the optical density measured at 600nm (OD_{600}) against an LB media control. Expression of the protein was induced with 0.25mM isopropyl-β-D-thiogalactopyranoside (IPTG) when an OD_{600} of 0.5-0.6 was achieved. An OD_{600} at this level ensured that the culture was in the logarithmic phase, where bacteria will grow exponentially. Expression of the target protein was undertaken for 2hr in an orbital shaker set at 37^{o}C. The cells were then harvested by centrifugation at 1915g for 10min. The cell pellet was resuspended in 10ml PBS containing 0.1% Triton and protease inhibitors. The re-suspended cells were then frozen at –80^{o}C and thawed on ice. Lysozyme was added at a final concentration of 1mg/ml and the cell suspension subjected to sonication to achieve sufficient cell lysis and the subsequent cell debris was removed by centrifugation at 20235g for 15 min.

2.2.2.3 Purification

Glutathione sepharose beads were prepared as per the manufacturer's instruction. The cell supernatant was applied to the prepared beads and incubated end-over-end for 1hr 30 min at 4°C to bind the expressed fusion protein. The beads were collected by centrifugation at 120g for 4 min at 4°C and washed three times with 1ml PBS 1% Triton followed by three washes in 1ml PBS. The protein was eluted from the glutathione beads with 250µl of 100mM NaCl, 50mM Tris-HCl, 3mg/ml glutathione; pH 8.0 by incubating end-over-end for 20min at 4°C. This was repeated up to four times. The eluted fractions were pooled and dialysed using Pierce® (Rockford, IL, U.S.A.) Slide-A-Lyser® dialysis cassettes against 5l of PBS pH 7.4. overnight 4°C. The purified protein was frozen on dry ice, and stored in aliquots, at –80°C. The expression time-course and final purification to homogeneity was analysed by SDS-PAGE and Coomassie® staining, described in Section 1.5.

2.2.3 Antibody purification

2.2.3.1 Preparation of Affi-gel column

0.5ml Affi-gel 15 immunoaffinity support was washed twice in distilled water and incubated with 0.8mg of purified GST-Rab25 protein and 1ml 0.1M MOPS buffer pH 7.5 at 4°C and tumbled overnight. Then 50µl of glycine ethyl ester pH 8 was added to block any un-reacted groups on the Affi-gel column and tumbled for 1 hr. The column was placed upright, allowed to settle and then washed twice with PBS, once with 20mM Na Citrate HCl pH 2.5 and twice more with PBS. The column was kept at 4°C until ready for use.

2.2.3.2 Purification of antibody

A 2ml aliquot of crude serum HF126B, from the terminal bleed of a rabbit inoculated with full-length Rab25 protein, was thawed on ice, added to the prepared Affi-gel column and tumbled overnight at 4°C. The column was then placed upright and allowed to settle for 30 min. The depleted serum was drained and the column washed five times with MOPS buffer. The antibody was eluted from the column in 5 fractions, using 0.5ml 20mM Na Citrate pH 2.5 per fraction.

The eluted fractions were neutralised with 35µl 1M Tris pH 9.0, combined and the protein content measured. The purified antibody was divided into 20µl aliquots and stored at -80^{o}C.

2.2.4 Protein extraction and analysis

2.2.4.1 Preparation of cell lysates

Protein extracts were made from cells when approximately 80% confluent. Cells were washed twice with ice cold PBS then lysed with NDLB buffer containing the protease inhibitors aprotinin, leupeptin and AEBSF. Lysed cells were scraped with a cell scraper and lysate transferred to a 1.5ml tube on ice for 15 min. Lysates were then centrifuged at 17949g at 4°C for 15 min to remove particulate matter, and cleared lysate was placed in a fresh 1.5ml tube. Samples were then processed immediately, or frozen and stored at -20°C.

2.2.4.2 Determination of protein concentration

Protein concentrations were estimated using the Bradford colorimetric assay. Protein-containing solutions were mixed with Bradford assay solution and the absorbance at 595nm measured. The protein concentration was then determined based on a standard curve derived from protein standards of known concentration.

2.2.4.3 Coomassie® staining

Polyacrylamide gels were removed from the pre-cast gel cassette and washed with sterile water to remove residual running buffer. Coomassie® stain consisting of 1.25g Coomassie® Brilliant Blue R_{250} (omit for de-stain), 444ml methanol, 56ml acetic acid in a final volume of 1000ml sterile water was added to the gel with gentle shaking for 2hr at room temperature. The Coomassie® stain was removed and replaced with de-stain. De-stain removed all background staining with the Coomassie® stain remaining bound to the proteins resulting in the detection of all proteins present in the sample. The gel was then washed with sterile water and kept hydrated by covering with Saran wrap.

2.2.4.4 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

The required amount of lysate to give typically 30µg protein was mixed with 5µl sample buffer and 2µl sample reducing agent, and diluted to a total volume of 30µl with NDLB lysis buffer. Samples were then heated at 95˚C for five min, briefly centrifuged to collect the whole sample, and resolved on denaturing pre-cast polyacrylamide gels by electrophoresis in gel tanks containing 1X running buffer at 120V for approximately 1 hr 30min. The separating gel contained between 8% and 12% acrylamide depending on the molecular weight of the protein to be visualised. Molecular weight markers were run adjacent to the lysate samples.

2.2.4.5 Western blotting

Following electrophoresis, the proteins were transferred onto a PVDF membrane, while being buffered by 3mm filter paper saturated in NuPAGE® Transfer Buffer containing 20% methanol, in a BioRad semi-dry blotting apparatus at 10V for 1hr 30 min. Following transfer, the membrane was blocked for 1hr at room temperature in 5% milk/TBS-T. The primary antibody in 5% milk/TBS-T was then added overnight at 4°C. The blots were then washed three times in TBS-T (5 min each) and incubated with the appropriate horseradish peroxidase conjugated secondary antibodies for 1 hr. The blots were again washed three times in TBS-T (15 min each). All washes and incubations were performed with gentle agitation. Proteins on membranes were visualised using an enhanced chemiluminescent detection reagent followed by autoradiography using Fuji Super RX medical X-ray film and a Kodak X-Omat 480 RA X-Ray processor.

2.2.5 RT-PCR

2.2.5.1 Isolation of RNA

Total cellular RNA extracts were made from cells when approximately 80% confluent. Cells were washed twice in ice-cold PBS (pH 7.4), residual PBS was allowed to drain and was aspirated. Cells were then lysed on ice, in 350µl of RLT buffer supplemented with ß-mercaptoethanol (10µl per 1ml of the buffer). Cell

lysates were homogenised using QIAshredder columns and RNA was extracted and purified using the RNeasy kit, according to the manufacturer's instructions. Residual genomic DNA was removed by incorporating an on-column DNAse I digestion step. RNA was eluted from the column in two sequential steps, each in 30µl of sterile water.

RNA concentration and purity was measured using a NanoDrop spectrophotometer. Samples with a concentration less than 0.6µg/µl were concentrated by centrifugation in SpeedVac concentrator for approximately 1.5 hr. Those samples with an $A_{260/280}$ measurement less than 1.7 were subjected to an additional clean-up step using the Qiagen RNeasy kit.

RNA was then frozen and stored at -80ºC.

2.2.5.2 cDNA generation from total RNA

cDNA was synthesised from total RNA using Improm II kit according to the manufacturers instructions. 1µg of RNA was incubated with 0.5µg Oligo dT primer and the final reaction volume made up to 10µl with RNAse/DNAse-free water. The mixture was heated to 70ºC for 5 min in the Biorad DNA Engine Peltier Thermal Cycler and then chilled on ice for two min.

0.5µl of RNAse/DNAse-free water, 4µl of 5x Reaction buffer, 3µl of 25mM $MgCl_2$, 1µl of 10mM dNTPs and 0.5µl of 40U/µl RNasin were added to the RNA mixture, mixed gently and incubated at room temperature for 2 min. 1µl of Improm Reverse Transcriptase was then added, the sample was then incubated at 25ºC for 5 min and then at 42ºC for 1hr. The reaction was terminated by incubation at 70ºC for 15 min and the sample was cooled to 4ºC and stored.

2.2.5.3 PCR amplification

Oligo Name	**Sequence (5'-3')**
Rab25-#1Forward	AGACCAATCTACTCTCCCGA
Rab25-#2Forward	CCGGACCAATGCCATCACTC
Rab25-#1Reverse	GACCCTGAGGGTGCTGTGAAC
Rab25-#2Reverse	GAGGGTCCTAGTCTGTGAGG

Rab25-#3Reverse	CGCAAAGATTTCTTTCAGGAC
Rab11a-Forward	CACCATATCCACCAGCAGGC
Rab11a-Reverse	GCCCCACACATCATGAGCAC
Rab11b-Forward	CGCAGACCGCGCTGCCCACG
Rab11b-Reverse	GGAGGACGTGCACGCACGCTG

Primers for Rab25, Rab11a and Rab11b were designed with help from Marc Jones. cDNA sequences were retrieved from NCBI , and suitable regions were chosen based on their size. Sequences were examined visually, to exclude areas containing high GC content or those potentially likely to fold into hairpins. Regions with a good balance of bases were chosen and primer sequences were chosen to have a melting point between 60 and 70°C. The proposed sequences were checked by performing a BLAST search.

2µl of cDNA template, 5µl of 5x Reaction buffer, 1µl of 10mM each dNTP's, 1µl each of 20µM forward and reverse primer and 0.2µl of Taq polymerse were made up to a final reaction volume of 50µl with RNAse/DNAse-free water. The cycling reaction was then performed in a Biorad DNA Engine Peltier Thermal Cycler, using the following conditions;

95° C	5 min	
95 ° C	30 s	x30
55 ° C	30 s	
72° C	30 s	
72° C	10 min	
4° C	until use	

2.2.5.4 DNA agarose gel electrophoresis

DNA electrophoresis was carried out in 1% (w/v) electrophoresis grade agarose dissolved in 1xTAE buffer. Prior to gel solidification ethidium bromide was added at the final concentration of 0.5µg/ml. DNA samples were diluted in 6x DNA loading dye before electrophoresis and loaded on to the gel alongside a suitable DNA ladder. Electrophoresis was conducted in 1xTAE buffer at 100 V for 1hr. DNA samples were visualized using UV transillumination.

2.2.5.5 qRT PCR

All primers used to perform real time quantitative PCR were purchased from Qiagen as QuantiTect Primer Assay kits. The primer sequences are not disclosed by the manufacturer and therefore the corresponding catalogue numbers are listed below:

Rab25 - Hs_RAB25_1_SG QuantiTect Primer Assay QT00087941

ß1-Integrin - Hs_ITGB1_1_SG QuantiTect Primer Assay QT00068124

ß-actin Hs_ACTB_2_SG QuantiTect Primer Assay QT01680476

GAPDH Hs_GAPDH_2_SG QuantiTect Primer Assay QT01192646

Quantitative PCR was performed using the QuantiTect SYBR Green PCR kit on a BioRAD DNA Engine thermal cycler fitted with a Chromo4 Engine (Bio-Rad) and coupled to Opticon Monitor 3 software.

The cDNA template was diluted 1:8 in sterile water and 8µl of the diluted cDNA was added to 10µl of 2x SYBR Green Mix and 2µl of 10x Primer Assay on a cooling block. The cycling reaction was performed using the following conditions:

95°C	15 min	
95 °C	30 s	x40
60 °C	30 s	
72°C	30 s	
Plate read		
72°C	5 min	
70-90°C	in 0.3ºC steps	

Data was extracted from the Opticon Monitor 3 software and the ΔΔC(t) method (Livak and Schmittgen 2001), or one that also incorporates amplification efficiencies (148), was used to calculate changes in gene expression with ß-actin and GAPDH serving as reference genes. Each experiment was performed in triplicate and each replica incorporated three technical repeats.

2.2.6 Immunofluorescence

Cells were plated on glass coverslips under standard culture conditions, at densities that produced a 50% confluent monolayer the following day. Culture medium was removed from cells which were fixed in 4% formaldehyde for 15 min at room temperature. Cells were then permabilised in 0.2% Triton PBS and washed twice in PBS. After blocking with 100µl of 1% BSA PBS for 1 hr, coverslips were washed in PBS and then incubated with primary antibody for 1hr at room temperature. Following three washes in PBS, slides were incubated with secondary antibody for 1 hr at room temperature, washed three times in PBS and stained with FITC-phalloidin. Coverslips were washed a further three times in PBS and mounted using DAPI Vectashield.

Cells were then examined using an Olympus BX51 confocal microscope.

2.2.7 Immunohistochemistry

2.2.7.1 Thrombin clot preparation

Cells from two confluent 10cm plates were detached using trypsin and re-suspended in 10ml culture medium. Cells were then pelleted by centrifugation at 106g for 5 min at 4°C. Following aspiration of the culture medium, the cell pellet was washed in 1ml PBS and centrifuged again. The pellet was then re-suspended in 100µl PBS and 50µl of the cell suspension was added to 200µl of citrated bovine plasma. 50µl of thrombin solution was added to the plasma and cell and the resulting clot was fixed in 10% neutral buffered formalin and embedded in wax.

2.2.7.2 Immunohistochemical staining

Commercial or gifted paraffin embedded TMA blocks were sectioned by microtome prior to transfer to the Beatson Institute for Cancer Research. All other paraffin-embedded tissue was sectioned by the Beatson Institute for Cancer Research Histology Service.

Immunohistochemistry was performed using the following protocol:

Procedure	Time
Place sections in 60°C oven then cool	2 hr
Place sections in Xylene	5 min
Place sections in 100% Ethanol	1 min
Place sections in 100% Ethanol	1 min
Place sections in 70% Ethanol	1 min
Wash sections in running tap water	
Heat induced epitope retrieval; 1 mM pH 8 EDTA at full pressure in microwaveable pressure cooker	4 min
Leave sections in buffer to cool	20 min
Wash sections in 10 mM Tris buffered saline Tween (TBST) pH 7.5	5 min
Block endogenous peroxidise (Dako EnVision)	5 min
Wash sections in TBST	5 min
Apply Rab25 Antibody (1:1000)	1 hr
Wash sections in TBST	5 min
Apply secondary antibody (Dako EnVision)	30 min
Wash sections in TBST	5 min
Apply 3.3'-Diaminobenzidine tetrahydrochloride	10 min
Terminate reaction with deionised water	
Gills haematoxylin	2 min
Wash sections in deionised water	
1% acid alcohol	3 dips
Wash sections in deionised water	
Scotts tap water substitute	1 min
Wash sections in deionised water	
Dehydrate, clear and mount	

2.2.8 Statistical analysis

All statistical tests were performed using the Statistical Package for the Social Sceinces (SPSS) version 15 software, with the exception of power calculations which were performed using GPower 3.1 software.

Test for normality of distribution of data was performed using the Kolmogorov-Smirnov test.

Pearson's coefficient and the Wilcoxon Signed Ranks test were performed to calculate correlation in parametric and non-parametric data respectively.

Survival data were calculated using Kaplan Meier life table analysis and the relationship between prognostic factors was investigated using the Cox's multiple regression analysis.

The student's t test was performed to investigate the difference between two means.

2.2.9 Design of shRNA expression plasmid

2.2.9.1 Oligonucleotide design

The oligonucleotide hairpin was designed using the OligoEngine software. The hairpin was designed containing BglII and HindIII 5' and 3' ends respectively and the proposed sequence checked by performing a BLAST search. The hairpin sequence is shown in Figure 2.1. The oligonucleotide strands were dissolved in nuclease – free water to a concentration of 3mg/ml and 1µl of each oligo was mixed with 48µl 100mM NaCl and 50mM HEPES pH 7.4.

The mixture was incubated at 90°C for 4 min, 70°C for 10 min and then cooled slowly to 10°C. The annealed inserts were then kept at -20°C until needed.

2.2.9.2 Preparation of vector

The p-SUPER.neo+GFP vector was linearised by sequential digestion with HindII and BglII enzymes. 1µl of vector was incubated with 1µl of enzyme and 5µl of the appropriate buffer and 43µl of water. The digest was performed at 37°C and heat inactivated by raising the temperature to 80°C and the linearised vector stored at 4°C.

The ligation reaction was performed using 2µl annealed oligonucleotide insert, 1µl T4 ligase, 1µl T4 ligase buffer, 1µl linearised vector and 5µl of water and incubated overnight at room temperature. 1µl BglII was added for 30min at 37°C to reduce the level of unligated background vector.

2.2.9.3 Sequencing

The resulting construct was transformed into DH5α bacterial cells as described in section 1.3.1. Six colonies were grown up in a small scale culture and submitted to Beatson Technical Services for confirmatory DNA sequencing. Glycerol stocks of culture were prepared as described in section 1.3.1.

2.2.9.4 Plasmid DNA isolation

Following confirmation of the correct DNA sequence, a large scale culture was performed using a sample of glycerol stock of transformed cells in 500ml LB growth media containing ampicillin 100mg/ml. This was transferred to a 2l flask of culture medium by means of a sterile pipette. The culture was incubated at 37°C overnight with agitation. The cells were harvested the next day by centrifugation at 6000g for 15 min using the JA-14 rotor in the Beckman refrigerated centrifuge and the DNA extracted using the Wizard Maxi-prep kit according to the manufacturer's instructions.

2.2.10 Mammalian cell transfection with plasmid DNA

The Lipofectamine method of transfection was used for transfection. Cells were plated in 6 well plates 24hr before transfection at a density which would ensure 90-95% confluence on the day of transfection.

10µl of Lipofectamine and 4µg of plasmid DNA were each diluted separately in 250ml of Opti-MEM and incubated at room temperature for 15 min, then mixed together. The culture medium was aspirated from the cells and replaced with 1.5ml Opti-MEM. 500µl of the DNA/Lipofectamine complex was added to each well and incubated at 37°C for 6 hr, following which the complex was removed and replaced with 2ml growth medium.

24hr after transfection each 6 well plate was harvested and split into two 15cm plates and grown for a further 24 hr. Selective medium containing neomycin was added the following day.

Cells were allowed to grow for 10-14 days and colonies selected on the basis of GFP expression. Further sub-culturing of clones was performed and the effect of the vector on protein expression was determined by Western blotting as described in section 1.5.

2.2.11 Cell growth assay

2.2.11.1 Growth on plastic

$5x10^3$ cells were plated per well of a 12 plate in triplicate. Four plates were prepared per cell line. 1ml of medium was added and cells were allowed to grow for eight days. Cells were counted every 2 days by trypsinising each well with 350µl trypsin and neutralising the activity of trypsin with 50µl of foetal calf serum. Cells were counted using an automated cell counter.

2.2.11.2 Growth in soft agar

1.8% LMP agarose and 2x DMEM were warmed to 40°C. 1ml of agarose and 1ml of 2xDMEM were mixed and plated per well of a six well plate and allowed to set at room temperature.

0.9% LMP agarose and 2x DMEM were mixed in equal proportions. Cells were trypsinised and counted. $2x10^4$ cells were mixed with 2ml of the 0.9% agarose/DMEM mixture and plated on top of the set 1.8% agarose layer and allowed to set.

Cells were fed with growth medium three times weekly for 3 weeks and then counted. Total colony number per $6.25mm^2$ and total large colony number (colonies greater than 62.5µm in diameter) were counted in nine positions per well.

2.2.11.3 Wound healing assay

$1x10^6$ cells were plated in wells of a 6-well tissue culture plate at and allowed to grow in 2ml DMEM for 48 hr. Once confluent, one scratch was made in the monolayer using a sterile 10µl pipette tip and the media changed to remove de-

adhered cells. Time lapse images were recorded of four fields per wound every 15 min for 20 hr on a Nikon TE2000 microscope using a 10X objective. Wound closure was quantified using Image J software.

2.2.12 Cell surface labelling and immunoprecipitation

Cells were plated and allowed to grow to 80% confluence. Plates were washed twice in PBS, incubated with 3ml 0.2mg/ml NHS-SS-Biotin in PBS for 30 min followed by two further washes with PBS. Cells were then lysed with NDLB containing protease inhibitors and the protein concentration measured. The protein concentration of the lysates was then corrected to ensure that the same amount of protein was added to each subsequent step. 50µl of each lysate was kept for Western blotting.

Magnetic beads pre-coupled with sheep anti-mouse antibody were incubated with either 1.025µg of mouse anti-integrin ß1 or 1.65µg of RG-16 per lysate. The beads were washed twice in 0.1% BSA PBS and re-suspended in 1ml PBS. The appropriate antibody was added and beads tumbled for 1hr at 4°C.

The beads were then washed twice in 0.1% BSA PBS and re-suspended in PBS. 450µl of prepared lysate was added to 50µl of prepared beads and tumbled for 2 hr at 4°C.

The beads were then washed three times in NDLB, re-suspended in 50µl of non-reducing sample buffer and boiled for 5 min. The supernatant was removed and Western blotting performed. The resulting blot was incubated with streptavadin-HRP in 1% BSA overnight and proteins visualised using a chemiluminescent reagent. Western blots of reduced whole cell lysates were also performed to assess total protein levels and loading.

3 Affinity purification and validation of an anti-Rab25 antibody

3.1 Introduction

To investigate the expression of Rab25 protein in archival clinical material, it was first necessary to obtain a reliable antibody that specifically recognised Rab25 and that was suitable for use in immunohistochemistry. As there is no effective commercial anti-Rab25 antibody currently available, it was necessary to generate an in-house antibody for this purpose. In previous work undertaken in the laboratory by Dominic White, a rabbit had been immunised with a purified preparation of full length human Rab25 protein and the resulting serum obtained from a terminal bleed was in storage at -20 °C.

In general, crude antisera are unsuitable for use in immunohistochemistry owing to the large number of antibodies that recognise proteins other than the protein of interest. The resulting background staining produced when crude antiserum is used in immunohistochemistry can entirely obscure the protein of interest to the extent that the staining patterns are meaningless. To obtain an anti-Rab25 antibody with high specificity, it was therefore necessary to purify the crude anti-Rab25 antiserum by the process of affinity chromatography.

Affinity chromatography is a well established purification technique in general laboratory use. The process of affinity purification relies upon the strong reversible association between biologically active proteins and their substrates and can be used to purify many classes of protein including antibodies (149). The crude serum containing the antibody to be purified is passed through a column of resin to which the corresponding antigen has been covalently bound. The antibody recognises and binds competitively to the antigen, whilst all other proteins without affinity for the antigen pass through. The bound antibody can then be eluted and collected from the column by interruption of the non-covalent interaction by a change in pH.

Another requirement of an antibody used to test expression in clinical material is that it recognises the protein of interest specifically and does not detect other

closely related proteins. As described in detail in 1.5.3, Rab25 is a member of the Rab11 family of GTPases, the members of which are closely homologous. Given the close homology between Rab25 and Rab11a and Rab11b, it was necessary to establish that the Rab25 antibody could effectively distinguish Rab25 from the closely-related Rab11a and/or Rab11b. To test this, a bank of cells lines was assembled all of which were known to express Rab11a or b to varying degrees and had previously been shown to express Rab25 at the level of mRNA.

This resource was used to assess the specificity of the affinity purified anti-Rab25 antibody in Western blots and in an immunohistochemistry "pseudotissue" model. The "pseudotissue" is comprised of cells which are suspended in a small volume of plasma to which thrombin is added to form a clot. This clot is then fixed and embedded in paraffin and can then be subjected to the same immunohistochemical processes as archival clinical tissue. This material provides a suitable substrate for optimisation of some of the experimental parameters for immunohistochemistry prior to investigation of Rab25 expression in whole tissues.

The specificity of the anti-Rab25 antibody was also assessed in paraffin-embedded mouse tissues with known expression levels of Rab25, and then by performing antibody pre-absorption experiments in tissues that expressed Rab25 abundantly.

3.2 Results

3.2.1 The use of GST-Rab25 for affinity chromatography of the anti-Rab25 antibody

A bacterial expression plasmid encoding a fusion protein of human Rab25 and GST under the control of a lac promoter was transformed into the BL21 stain of *E. Coli.* To induce expression of GST-Rab25 the transformed *E. Coli* were treated with IPTG and the bacteria were isolated by centrifugation and lysed. The expressed GST-Rab25 was the allowed to bind to a glutathione affinity resin and the bound protein eluted with glutathione and analysed by SDS-PAGE.

Coomassie blue staining and Western blotting of the column eluates indicated effective production of a protein corresponding to the predicted molecular weight for GST-Rab25, with an approximately equimolar concentration of a protein corresponding in molecular weight to GST. This band is presumed to indicate some cleavage of the GST-Rab25 protein during purification (Figure 3-1).

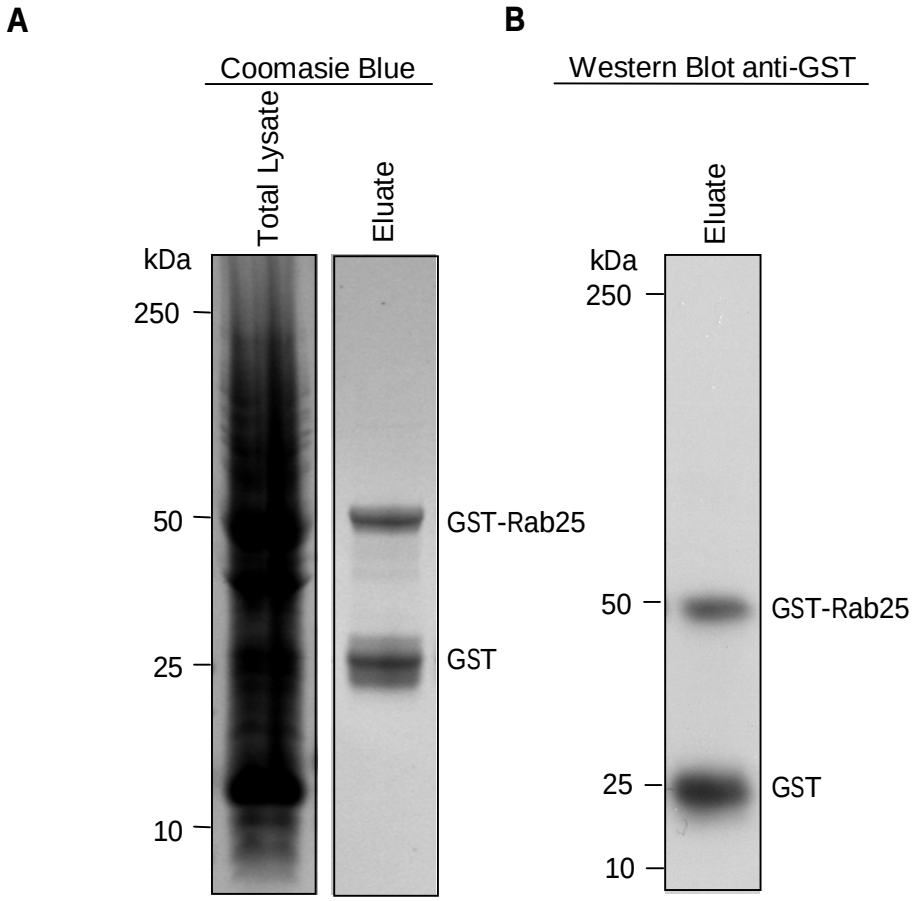


Figure 3-1 Expression and purification of GST-Rab25 protein.
A Coomassie blue staining of total lysate fraction and purified GST-Rab25 protein.
B Western blotting of purified GST-Rab25 protein (n=2).

The purified GST-Rab25 protein was covalently bound to an Affi-Gel column, incubated with the crude anti-Rab25 antiserum, and non-specifically attached material was removed by extensive washing. The anti-Rab25 antibody was then eluted from the GST-Rab25 resin using a low pH wash. Aliquots of crude serum, depleted serum and eluted antibody were kept for use in Western blotting. Western blotting of lysates of NIH 3T3, A2780-DNA3 and A2780-Rab25 cells was performed to validate the purification process. NIH3T3 cells are a mouse embryo

fibroblast line, used here as a non-epithelial cell line control. A2780-DNA3 and Rab25 cells are ovarian cancer cell lines which have been stably transfected with empty vector and HA-Rab25 respectively (Figure 3-2). The resulting purified antibody was then tested for its ability to recognise Rab25 using a number of different approaches.

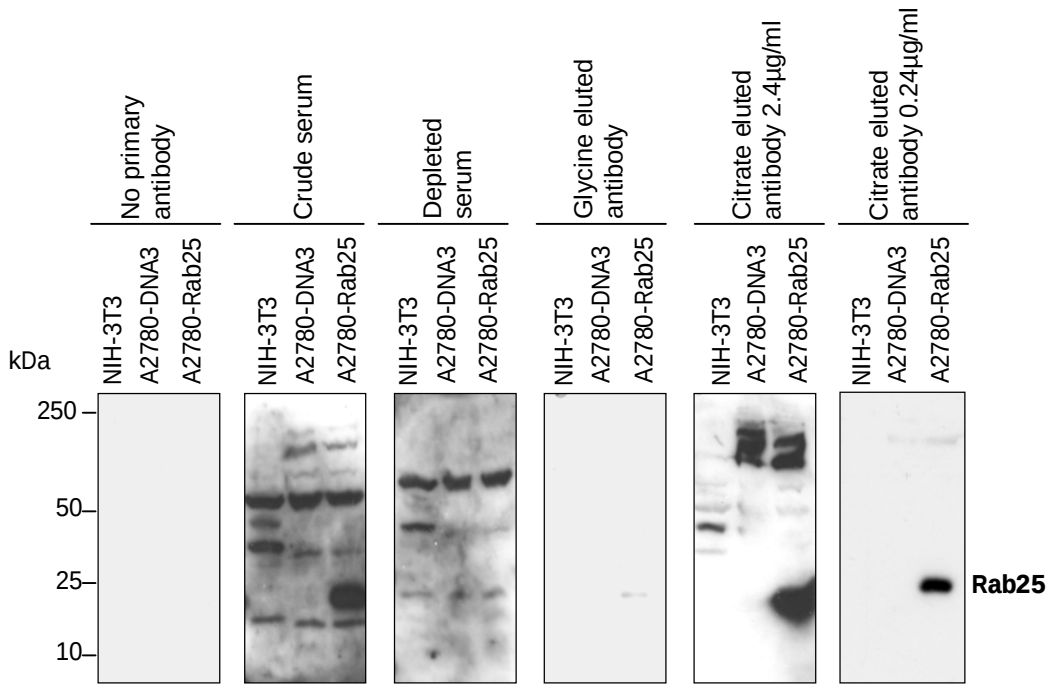


Figure 3-2 Assessment of anti-Rab25 antibody purity.
Western Blots were performed to assess the ability of affinity purified anti-Rab25 antibody to recognise Rab25 in NIH 3T3, A2780-DNA3 and A2780-Rab25 cell lysates. Blots were incubated with the indicated preparations of primary antibody (n=3).

3.2.2 Validation of anti-Rab25 antibody specificity

3.2.2.1 Use of purified GFP-tagged Rab25 and Rab11a proteins to evaluate anti-Rab25 antibody specificity

Purified protein preparations of GFP-tagged Rab25 and Rab11a were a gift from Patrick Caswell. Western blotting of these proteins using the anti-Rab25 antibody confirmed that the antibody recognised a protein of the appropriate molecular weight. Indeed, the predicted molecular weights of GFP-Rab25 and GFP-Rab11a are both approximately 50 kDa (27kDa for GFP plus 24kDa for the GTPases) and the antibody clearly recognises GFP-Rab25 but not GFP-Rab11a (Figure 3-3).

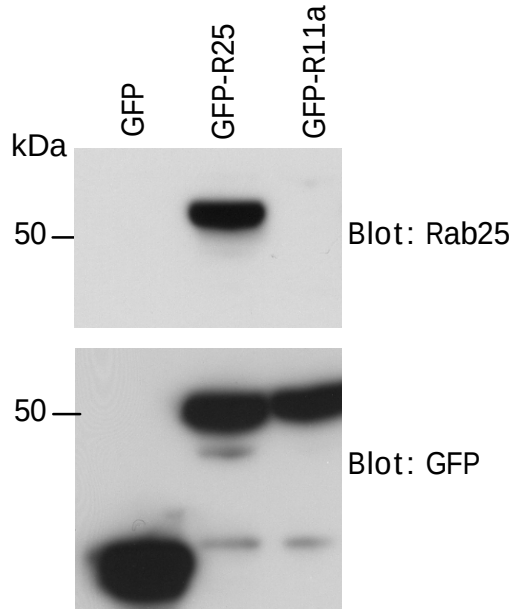


Figure 3-3 Assessment of anti-Rab25 antibody specificity.
Western blots of purified GFP, GFP-Rab25 and GFP-Rab11a protein were incubated with anti-Rab25 antibody (top panel) and anti-GFP antibody to assess gel loading (bottom panel) (n=3).

3.2.2.2 The use of Rab25 expressing and non-expressing cell lines to evaluate anti-Rab25 antibody specificity

Following confirmation that the anti-Rab25 antibody recognised a protein of the appropriate size, it was necessary to confirm that the antibody would specifically recognise Rab25 in cell lines. To do this, a panel of the following five human tumour cells lines was used:

i) A2780-DNA3 cells, an ovarian cancer cell line which has been stably transfected with an empty vector.

ii) A2780-Rab25 cells, an ovarian cancer cell line which has been stably transfected with an epitope-tagged Rab25, HA-Rab25.

iii) SNU-484 cells, a gastric cancer cell line which has been shown by RT-PCR not to express Rab25.

iv) SNU 601, a gastric cancer cell line which has been shown by RT-PCR to endogenously express Rab25.

v) MCF10ACA1, a breast cancer cell line which has been shown by RT-PCR to endogenously express Rab25.

To confirm the expression of Rab11a, Rab11b and Rab25 in A2780-Rab25 and A2780-DNA3 cells at the mRNA level, RNA was extracted from these cell lines and cDNA synthesised from this. RT-PCR was carried out using 1µl of each cDNA reaction. PCR products were run on 1% agarose gel and visualised using the Genesnap gel documentation system. Details, including primer sequences can be found in Section 2.6. In addition, Western blotting of the same cell lysates was performed, using a Rab11 antibody which does not distinguish between the Rab11 a and b subtype.

RT-PCR confirmed the presence of a band of the expected fragment size of Rab11a and Rab11b in both A2780-DNA3 and A2780-Rab25 cells, confirming the expression of Rab11a and Rab11b in these cells lines. Three potential candidate Rab25 RT-PCR primer pairs were designed and tested to determine the optimal

primers for further experimentation. Figure 3-4 A confirms that primer R25#2 is optimally designed to amplify Rab25 mRNA and that expression of Rab25 was limited to A2780-Rab25 cells. The primer pair R25#1 was minimally efficacious, and confirmed that the expression of Rab25 was limited to A2780-Rab25 cells, while the primer pair R25#3 did not amplify a product. The primer pairs R25#1 and R25#3 were not used in further analysis. Western blotting using a Rab11 antibody confirmed the presence of a band consistent with the expected molecular weight of Rab11 in both cell lines although, as already stated, this antibody cannot distinguish between Rab11a and Rab11b. Furthermore, incubation of blots from the same lysates with the anti-Rab25 antibody produced a 25kDa band only in the A2780-Rab25 cells (Figure 3-4).

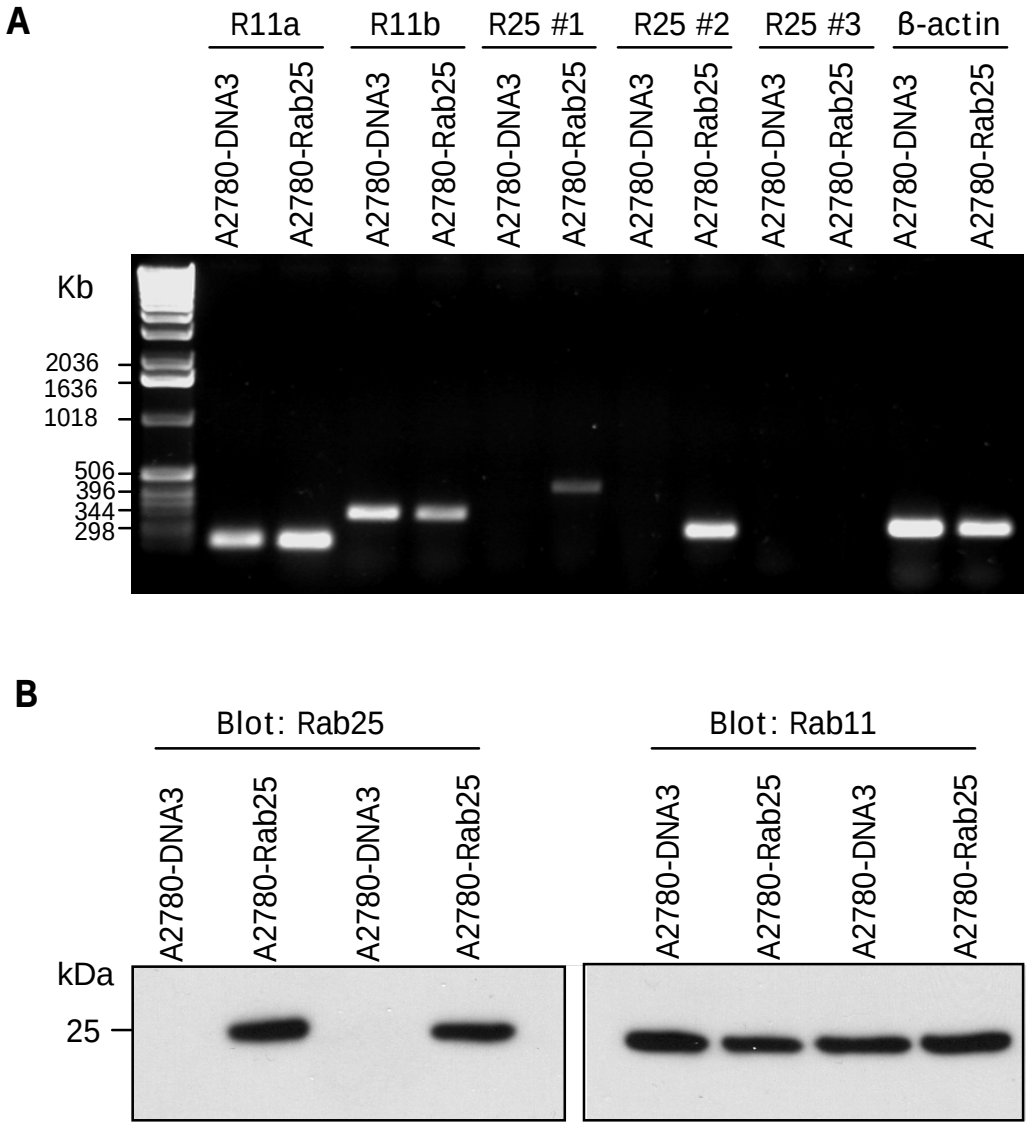


Figure 3-4 The anti-Rab25 antibody does not recognise Rab11
A Reverse transcriptase PCR reactions for Rab11a, Rab11b and Rab25 were performed on extracts of A2780-DNA-3 cells and A2780-Rab25 cells. Three sets of Rab25 primer pairs were used; R25#1, R25#2 and R25#3 (n=3).
B Western blots of lysates of A2780-DNA3 and A2780-Rab25 cells were incubated with anti-Rab25 and anti-Rab11 antibodies as indicated (n=3).

Given that expression of Rab25 in A2780-Rab25 cells is a result of stable transfection of a protein expression vector, it was necessary to assess the ability of the antibody to detect Rab25 by Western blotting in lysates of cells which express endogenous Rab25. In addition, it was necessary to establish the optimal concentration of antibody for routine use in Western blotting.

Western blotting of lysates from the panel of cell lines described above with the anti-Rab25 antibody, confirmed that a band of the appropriate molecular weight was evident in those cell lines previously shown by RT-PCR to express Rab25. Blots were incubated with serial dilutions of the anti-Rab25 antibody to determine the optimal conditions for Western blotting. Furthermore, the anti-Rab25 antibody, used at a concentration of 0.12µg/ml (1:5000 dilution), produced a number of non-specific bands in addition to a band at 25kDa. When diluted to 0.024 µg/ml (1:10,000 dilution), only one specific small molecular weight band was seen 25kDa band was seen in lysates of these cells, but a persistent high molecular weight band of greater than 200kDa remained, the nature of which was not clear. It is hypothesised that this band represents non-specific binding related to the presence of immunoglobulin within the lysate. However, potentially this represents non-specificity of the antibody, and further laboratory investigation using immunofluorescence and pre-absorbed immunohistochemical experiments were undertaken to assess the specificity of the antibody. (Figure 3-5).

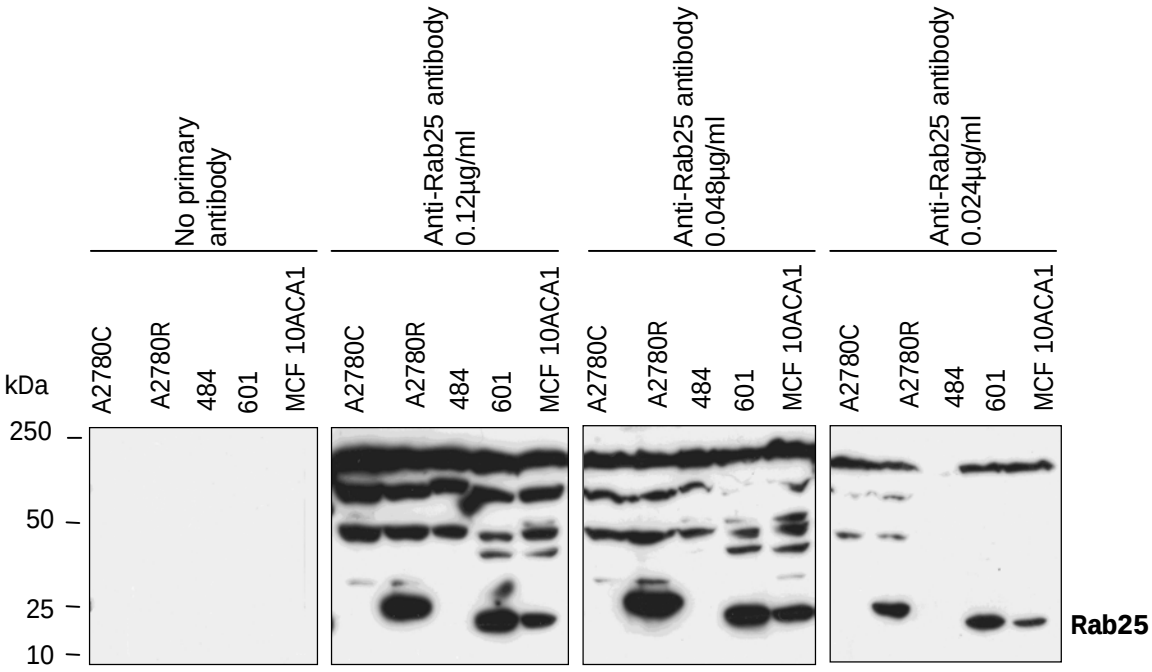


Figure 3-5 Titration of anti-Rab25 antibody to obtain the optimum concentration for Western Blot detection of Rab25 in lysates from a variety of cell lines.
Lysates from A2780-DNA3, A2780-Rab25, SNU-484, SNU-601 and MCF10a cells were subjected to Western blotting and the membranes incubated as indicated (n=3).

3.2.2.3 Use of the anti-Rab25 antibody in immunofluorescence

To validate the Rab25 antibody for use in immunofluorescence, A2780-Rab25 and A2780-DNA3 cells were grown on coverslips, fixed, permeabilised and incubated with primary and secondary antibody. Cells were then visualised on a confocal microscope and images captured.

A2780-DNA3 cells, when incubated with anti-Rab25 and anti-HA (recognising the epitope tag on the Rab25 expressed by these cells) antibodies, followed by the appropriate secondary antibody incorporating a fluorescent tag, exhibited no obvious signal when visualised on a confocal microscope. However, incubation of A2780-Rab25 cells under the same conditions revealed a clear signal with both the anti-Rab25 antibody and the anti-HA antibody. The merged image of these two signals confirmed that the position of the anti-Rab25 signal correlated with the signal produced by the anti-haemagglutinin antibody (Figure 3-6).

3.2.3 Validation of anti-Rab25 antibody for use in immunohistochemistry

3.2.3.1 Use of paraffin-embedded "pseudotissue"

With the aim of investigating the expression of Rab25 in paraffin-embedded breast cancer tissue samples, it was first necessary to optimise the conditions in which the Rab25 antibody would be used in immunohistochemistry. Prior to using the antibody in archival clinical material, thrombin clots incorporating suspensions of A2780-DNA3 cells, A2780-Rab25 cells, SNU-484 cells and SNU-601 cells were fixed in formalin and embedded in paraffin to create a "pseudotissue". The paraffin-embedded clots were then cut on a microtome in 2µm sections and immunohistochemistry performed. For comparison, Rab25 and ß-actin levels in the cells were also determined by Western blotting. An immunohistochemical signal was clearly seen in those cells confirmed by Western blotting to express Rab25. Minimal background signal was seen in those cells not expressing Rab25 (Figure 3-7).

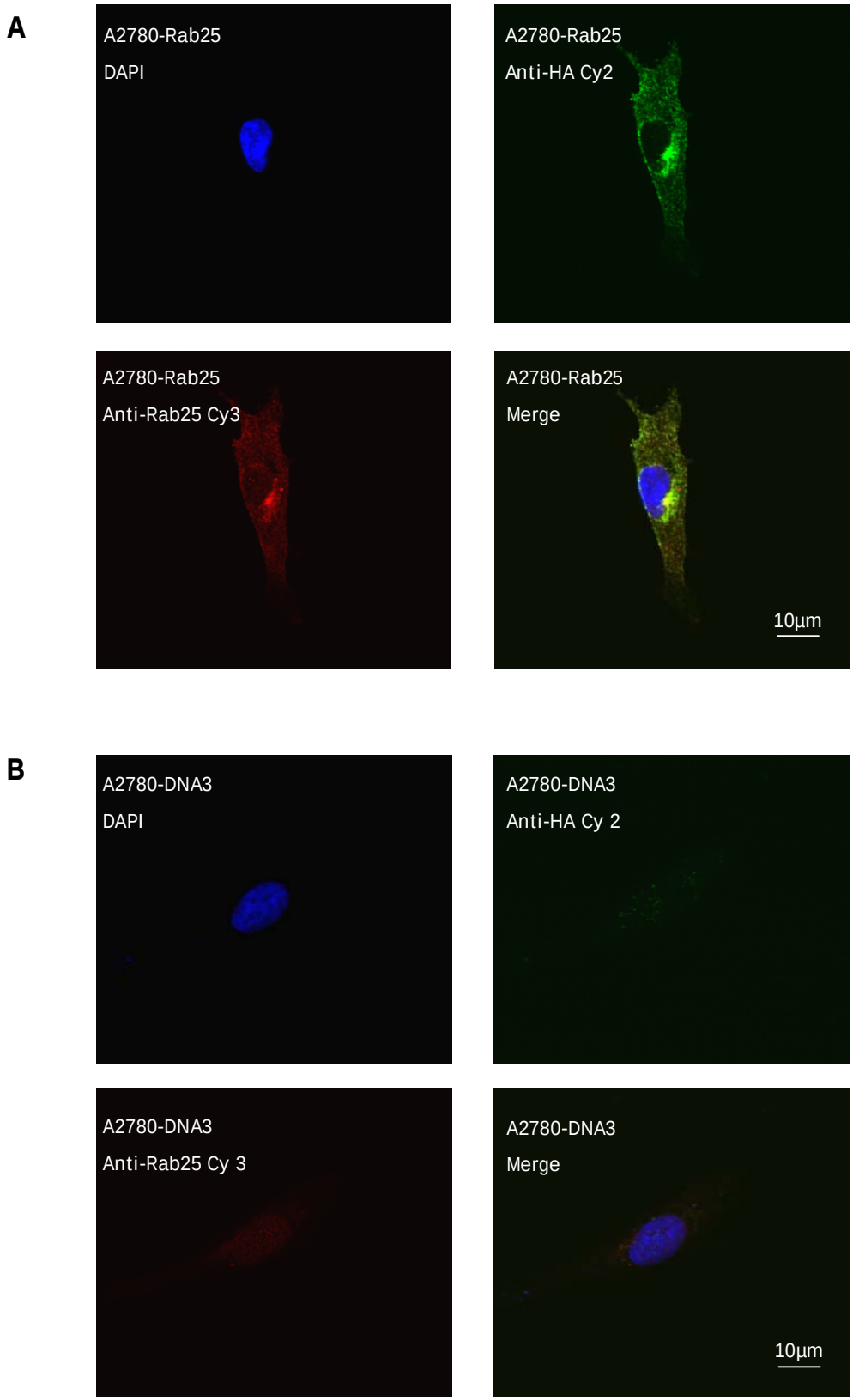


Figure 3-6 The anti-Rab25 specifically recognises Rab25 in an immunofluorescence platform.
A2780-Rab25 (A) and A2780-DNA3 (B) cells were fixed and incubated with antibodies recognising the HA epitope (green), Rab25 (red). The primary antibodies were then visualised using fluorescently-conjugated secondary antibodies as indicated. The cells were counterstained with DAPI (blue) to visualise the nucleus. Bar = 10um (n=3).

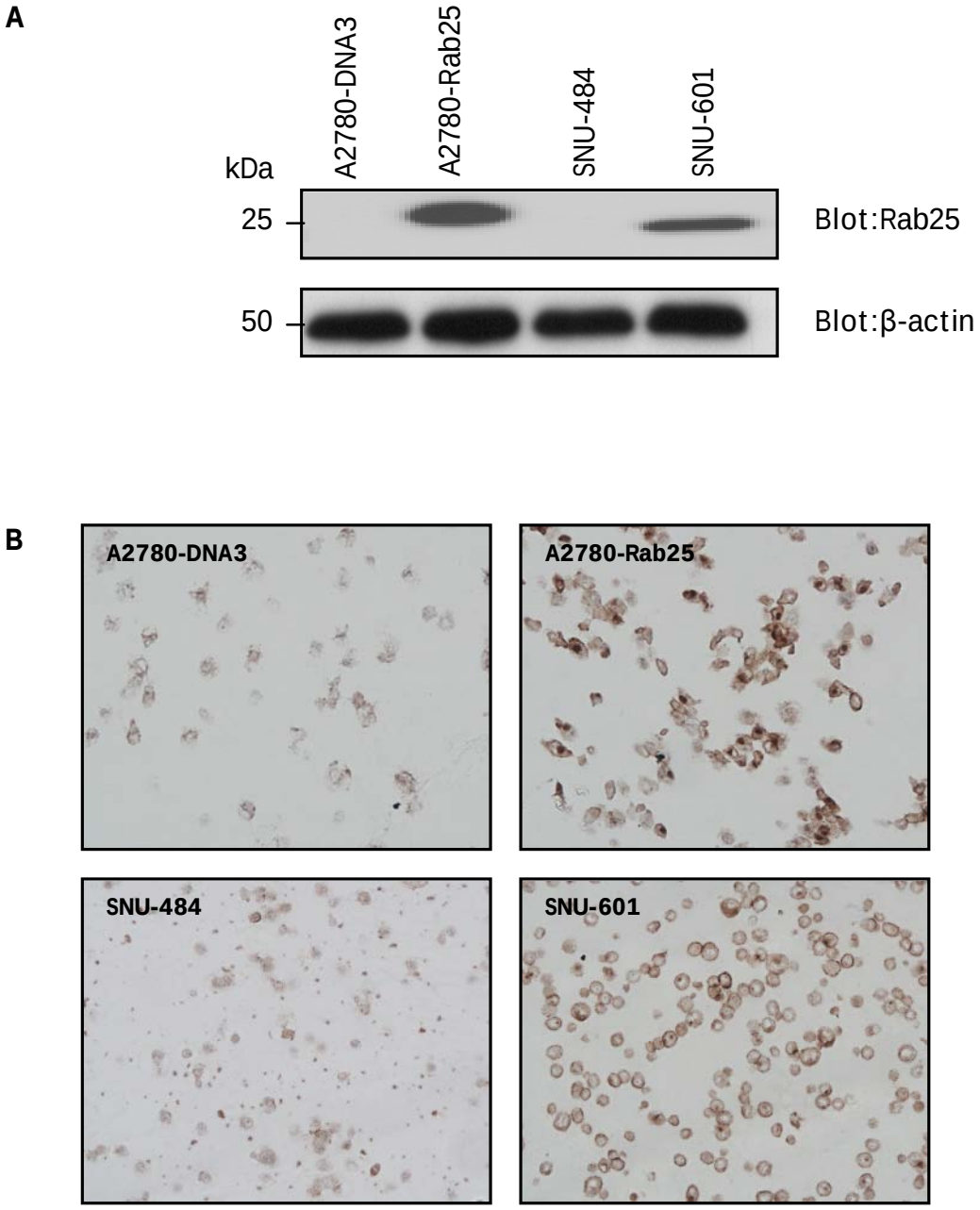


Figure 3-7 Detection of Rab25 expression by immunohistochemistry in pseudotissue pellets.
A Western blots of lysates from A2780-DNA3, A2780-Rab25, SNU-484 and SNU-601 cells were incubated with anti-Rab25 antibody (top panel) and anti-βactin antibody (lower panel).
B Immunohistochemical staining of A2780-DNA3, A2780-Rab25, SNU-484 and SNU-601 pseudotissue pellets was performed (n=3).

3.2.3.2 Optimisation of immunohistochemical conditions

Once it had been established that the anti-Rab25 antibody could detect Rab25 protein in pseudotissue pellets, further investigation was required to ascertain the optimal experimental conditions which would produce a reliable signal with minimal background staining. Serial 2µm sections from a block of a formalin-fixed paraffin embedded breast cancer specimen were a gift from Colin Nixon. Immunohistochemistry was simultaneously performed under different experimental conditions which included no antigen retrieval, antigen retrieval with EDTA, Citrate and Trypsin preparations and differing concentrations of the anti-Rab25 antibody.

The expected cellular distribution of Rab25 was cytoplasmic, with the potential for enhancement at the cellular membrane, given the observation that Rab25 is involved in apical recycling and co-locates with ß1 integrin at the cell surface. (119)

From this set of data the optimal conditions which were taken forward to subsequent experiments were antigen retrieval with EDTA buffer and an antibody concentration of 0.24µg/ml (Figure 3-8).

To further validate the antibody for use in immunohistochemistry it was necessary to ensure the specificity of the staining seen. 2µm sections of formalin fixed, paraffin-embedded mouse bladder and sections of the breast cancer tissue described above were antigen-retrieved using EDTA buffer and incubated with secondary antibody alone, anti-Rab25 antibody at a concentration of 0.24µg/ml and anti-Rab25 antibody at a concentration of 0.24µg/ml that had been pre-incubated with a 100 fold molar excess of purified GST-Rab25. A strong signal was detected when the antibody was incubated under optimal conditions, whilst secondary antibody alone and pre-incubated antibody produce minimal immunohistochemical signal (Figures 3-9 and 3-10). In addition, the images in Figure 3-10 confirm that the anti-Rab25 antibody can produce a reliable immunohistochemical signal in breast cancer tissue.

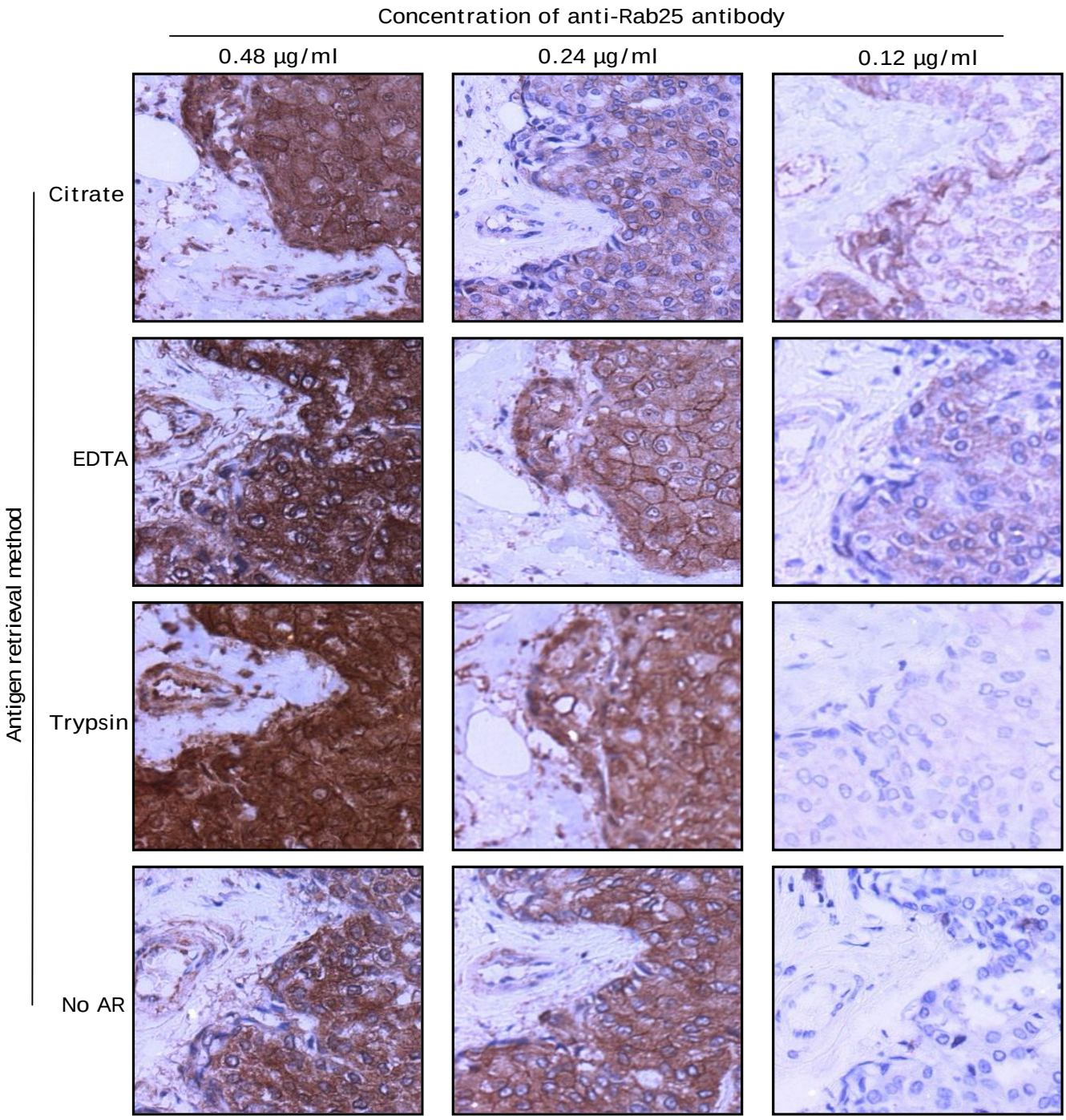


Figure 3-8 Optimisation of conditions for immunohistochemistry.
Sequential sections of a paraffin-embedded breast cancer specimen were prepared according to the indicated condition. The antigen retrieval method is indicated vertically and the concentration of anti-Rab25 antibody is indicated horizontally (n=3).

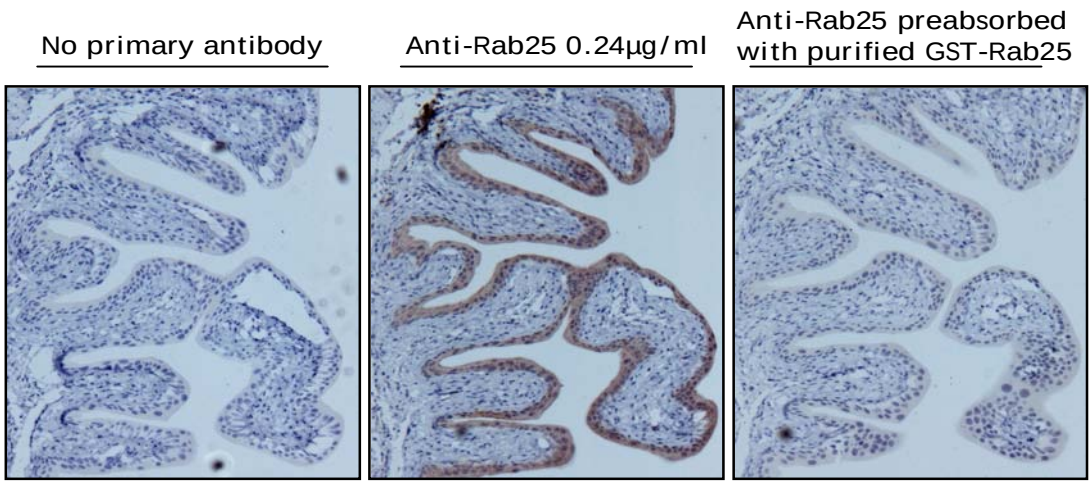


Figure 3-9 Immunohistochemical controls for anti-Rab25 antibody.
Sections of paraffin-embedded mouse bladder incubated with no primary antibody, anti-Rab25 antibody (2.4 µg/ml) and anti-Rab25 antibody (2.4 µg/ml) pre absorbed with an excess of purified GST-Rab25 protein (n=3).

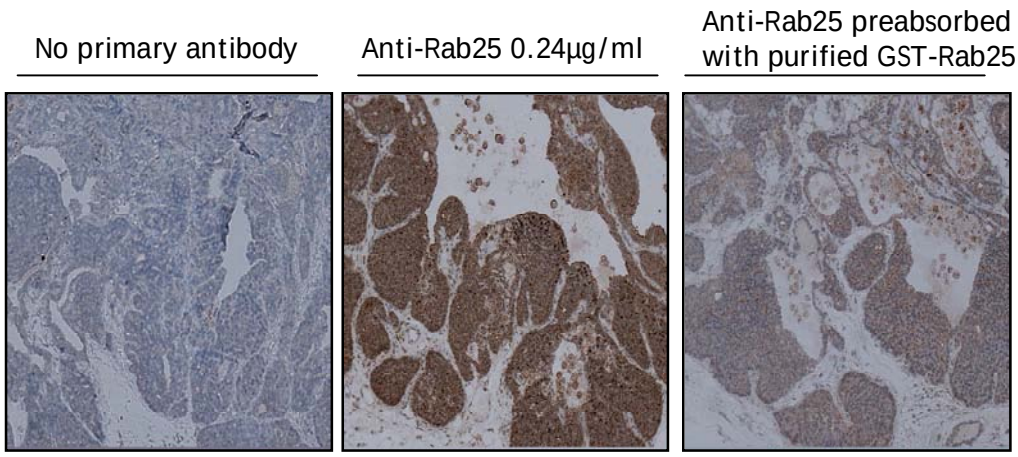


Figure 3-10 Immunohistochemistry with anti-Rab25 antibody in breast cancer tissue.
Sections of a paraffin-embedded breast cancer specimen incubated with no primary antibody**,** anti-Rab25 antibody (2.4 µg/ml) and anti-Rab25 antibody (2.4 µg/ml) pre-absorbed with an excess of Rab25 protein (n=3).

3.2.4 The investigation of Rab25 expression in whole mouse tissues

As an anti-Rab25 antibody had not previously been available, it had not hitherto been possible to determine the distribution of Rab25 protein expression in whole tissues. Once the optimal conditions for immunohistochemistry had been established, immunohistochemistry using the anti-Rab25 antibody was performed using whole mouse tissues from multiple organs. Human Rab25 shares 96% homology with mouse Rab25. In parallel with this, Western blotting of protein extracts of the same mouse tissues was performed to confirm the presence or absence of Rab25 in each tissue. Rab25 protein expression appeared to be localised to epithelial cells in the tissues investigated, and Western blotting of lysates of the same tissues with the anti-Rab25 antibody confirmed the presence of a 25 kDa band in those tissues which show strong staining for Rab25 by immunohistochemistry (Figure 3-11).

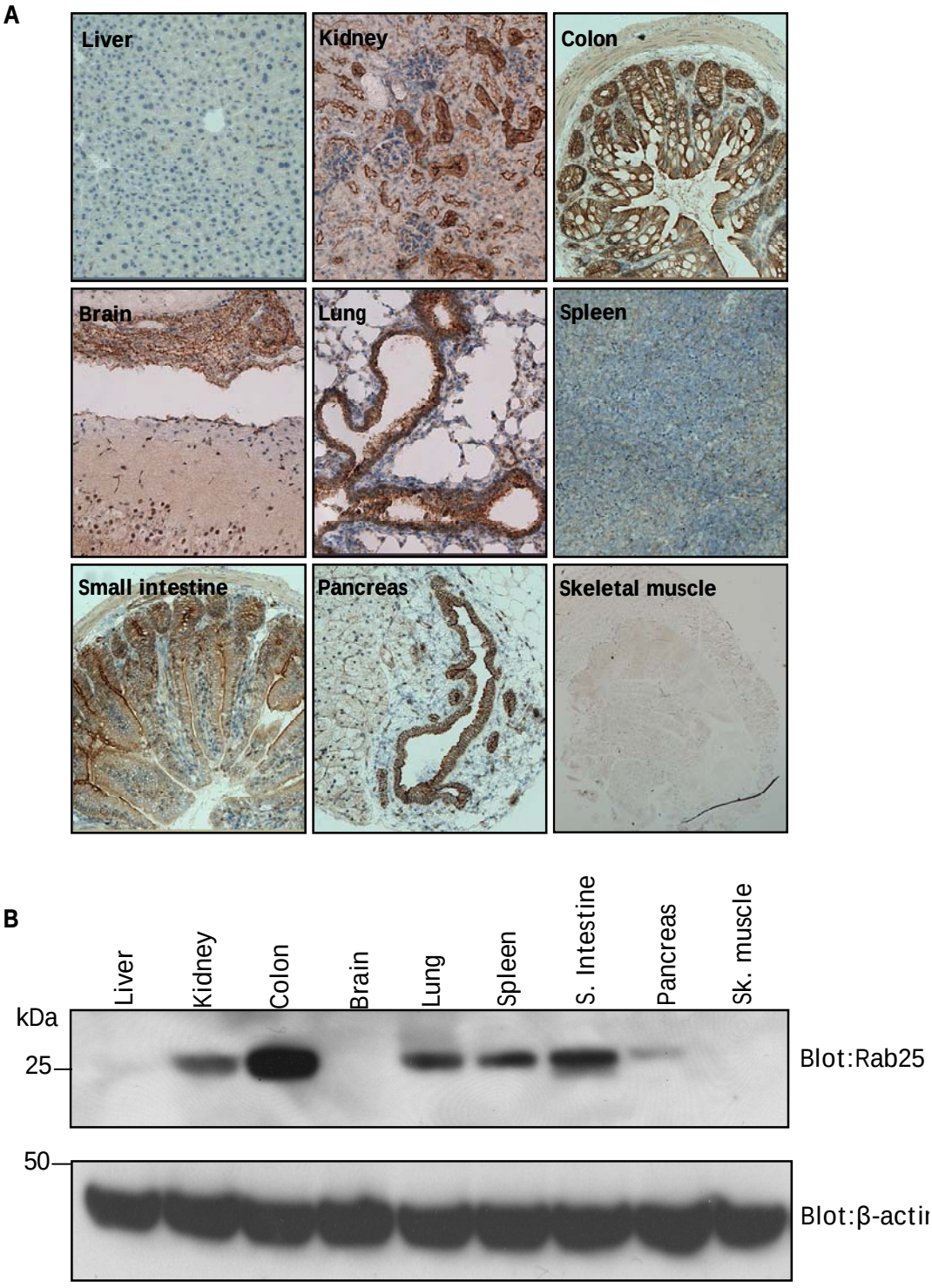


Figure 3-11 Use of immunohistochemistry to visualise Rab25 expression in normal mouse tissues.

A Immunohistochemical staining with anti-Rab25 antibody of a mouse tissues as shown.

B Lysates of the indicated mouse tissues were analysed by Western blotting and the membranes incubated with anti-Rab25 (upper panel) and anti-β-actin (lower panel) antibodies (n=2).

3.3 Discussion

Prior to proceeding to investigate Rab25 expression in breast cancer tissue samples, it was imperative that a specific and reliable antibody was available. These data confirm that the affinity purification process successfully yielded a polyclonal rabbit antibody against Rab25 which produced reliable and reproducible results when used in Western blotting, immunofluorescence and immunohistochemistry. Furthermore, the antibody did not recognise the very closely related family members, Rab11a and Rab11b.

Affinity purification is a well validated and robust methodology for small-scale laboratory production of antibodies (150). Figure 3-2 clearly shows a diminution in signal when lysate of A2780-Rab25 cells was incubated with depleted serum, when compared with the same lysate incubated with crude serum, indicating successful extraction of the antibody from the crude serum onto the Affigel column. The multiple bands present when cell lysates are incubated with crude serum and depleted serum are in keeping with the non-specific binding pattern often seen when crude serum is used in Western blotting. It was also confirmed that the anti-Rab25 antibody was successfully eluted from the column and when appropriately diluted, the anti-Rab25 antibody produced a single specific band of the predicted molecular weight in A2780-Rab25 cell lysate that is not present in A2780-DNA3 cells.

It was noted that, when used in Western blotting, there was a marginal difference between the molecular weight of the band recognised by the Rab25 antibody in the cell lines which endogenously express Rab25, when compared with the A2780-Rab25 stably expressing cell line (Figure 3-5.) As previously detailed, the Rab25 construct used a haemagglutinin tag which may account for the small observed difference in molecular mass.

For further study of Rab25 protein expression in tissues it was imperative that one could be confident that the affinity purified anti-Rab25 antibody did not also recognise the closely related family members Rab11a and Rab11b. Figure 3-3 confirms that the anti-Rab25 antibody does not recognise a purified protein preparation of Rab11a.

The position of the signal obtained when using the anti-Rab25 antibody in immunofluorescence preparations of A2780-Rab25 cells can be clearly seen to be in the perinuclear region of the cell and no such signal is seen in A2780-DNA3 cells (Figure 3-6.) This is entirely consistent with previous work localising Rab25 to this area in these cells and provides further evidence for the specificity of the antibody for Rab25 (119). The investigation of the anti-Rab25 antibody, as outlined above, yielded sufficient evidence to be confident in the specificity of the antibody, such that it was possible to move forward toward the ultimate goal of investigating Rab25 expression in breast cancer tissue samples.

Given that well-prepared human cancer tissue with associated clinical data is a precious resource, it was necessary to optimise the immunohistochemistry technique in non-human tissue prior to proceeding to human tissue. Furthermore, it is well recognised that non-specific immunohistochemical staining can be problematic, even with well-characterised antibodies (151). For these reasons the immunohistochemistry technique was first applied to "pseudotissues" of A2780-Rab25, A2780-DNA3 SNU-484 and SNU-601 cells. Figure 3-7 illustrates that that when these sections were incubated with the anti-Rab25 antibody, a strong immunohistochemical stain was achieved in those cell types known to express Rab25. Some background staining is seen in the control cells, and taking into account the additional high molecular weight bands seen in Western blotting in Figure 3-5, this raises the possibility of non-specificity of the antibody. However, the specificity of the staining seen in the immunofluorescence images in Figure 3-6, and the significant reduction in immunohistochemical staining with pre-absorption of the antibody seen in figures 3-9 ands 3-10, suggests that the antibody is producing real staining.

These important observations added further supporting evidence that the anti-Rab25 antibody would be sufficiently sensitive and specific to yield reliable results in the investigation of Rab25 expression in human breast cancer.

To further maximise the staining obtained by immunohistochemistry, it was necessary to optimise the experimental conditions. The fixation of fresh tissue in formalin causes significant chemical changes in the antigens of the tissue which leads to masking of the antigen from the antibody with which the tissue is incubated (152). The mechanisms by which this happens are essentially

unknown, but the changes have been shown to be reversible with high temperature heating (153). Manipulation of the composition of the solution in which the slides are heated can further enhance the extent to which antigens are retrieved. However, no single set of conditions has been shown to be optimal for all antibodies and, as such, it has become standard practice to determine the optimal conditions for a particular antibody using the "test battery" method. The antigen retrieval solutions used in the test battery for the anti-Rab25 antibody included citrate, EDTA and trypsin buffers and the results were compared with antigen retrieval with distilled water alone. In addition to the optimisation of the antigen retrieval method, it was necessary to ascertain the optimal concentration of anti-Rab25 antibody, which would provide a strong positive signal with minimal background staining. Figure 3-8 demonstrates the results obtained from the anti-Rab25 antibody "test battery," which incorporated both the method of antigen retrieval and concentration of antibody. From this experiment it can be seen that the optimal conditions for immunohistochemistry with the anti-Rab25 antibody were antigen retrieval with EDTA buffer and an antibody concentration of 2.4µg/ml and these parameters were adopted as the standard conditions for subsequent experiments.

Previous studies had indicated that Rab25 mRNA expression was most prevalent in the kidney, lung and mucosal layer of the stomach of rabbit and absent in other tissues such as brain, skeletal muscle and liver, leading these authors to hypothesise that Rab25 expression was limited to specific epithelial cells (99). More recent work, using steady state mRNA expression profiling and computational hierarchical clustering methods, has provided datasets which allow the expression profile of all Rab proteins in 79 human and 61 mouse tissues to be investigated (154). The expression profiles of individual rab proteins was generated using from the Human and Mouse Gene Atlases, which are publicly available datasets on normal human and mouse tissues. This "Membrome" dataset confirms that Rab25 mRNA expression is most prevalent in organs which have high proportion of epithelial tissue in relation to total organ mass, for example lung, tongue and skin. However, as the membrome has been developed from whole tissue samples, those tissues which have an epithelial component which is a much smaller component of the total organ mass, for example kidney and pancreas, show relatively lower Rab25 mRNA expression.

The data presented thus far confirm that the polyclonal anti-Rab25 antibody can reliably detect Rab25 in whole tissue samples. To confirm the findings of Goldenring (99) - that Rab25 protein expression was confined to epithelial cells - immunohistochemistry was performed on sections of whole mouse tissue, whilst Western blotting the same tissues concurrently. Figure 3-11 demonstrates clearly that Rab25 expression is present in the epithelial cell layer of small and large intestine, lung, kidney and pancreas of mouse tissue.

Of some interest is the observation that lysates from splenic tissue, when subjected to Western blotting, produced a band of predicted molecular weight when incubated with anti-Rab25 antibody, despite the spleen containing minimal epithelial elements. However, immunohistochemistry of the same tissue with anti-Rab25 antibody produced minimal signal. One explanation for this discrepancy is that dissection of the spleen can be technically difficult, particularly when performed by an inexperienced operator, and it is feasible that at the time of dissection the splenic tissue sample was contaminated with other intra-abdominal tissue, particularly small intestine or colon, both of which have now been shown to be high expressors of Rab25. However, the spleen was the only tissue of the lymphoreticular system to be investigated, and it is possible that elements of this system may express Rab25, but further investigation of this possibility was beyond the scope of this research.

In summary, the data presented here confirm that the affinity purification of a polyclonal anti-Rab25 antibody from rabbit serum was successful. Furthermore, the resulting antibody has been shown to produce reliable and consistent results when used in Western blotting, immunofluorescence and immunohistochemistry. For the first time it has been confirmed that Rab25 protein expression is confined to epithelial tissues and appears to be ubiquitous across a number of different, highly specialised epithelia.

4 Investigation of Rab25 protein expression in breast cancer.

4.1 Introduction

4.1.1 Laboratory identification of predictive markers in breast cancer

As discussed in Chapter 1, the importance of hormone receptor status in predicting response to treatment in breast cancer has been known for decades. Historically the assessment of hormone receptor expression in breast cancer specimens was by biochemical methods, the most widely used being the dextran - coated charcoal method which gave a quantitative measure described as fmol/mg of cytosolic protein. This method required fresh tissue and could be influenced by a number of factors including contamination by normal breast tissue and exposure to exogenous oestrogens such as the oral contraceptive pill.

Immunohistochemistry has been shown to be superior to biological methods of hormone receptor detection, having a greater sensitivity and specificity (155). However, as discussed in section 1.4.1, a high standard of quality assurance is required to produce consistently correct results. Despite the limitations and potential for variability of reporting which is inherent in immunohistochemistry, it is now the main technique used in identifying prognostic and predictive markers in clinical diagnostic laboratories and automated systems are well established.

While newer molecular techniques such as real time RT-PCR are in routine use in research laboratories, the resource implications and lack of automated systems means that at the present time such techniques are not feasible in clinical practice. In addition, any new test would have to be shown to be significantly superior to current techniques. As a result, for novel markers identified in a research setting which may have prognostic or predictive importance to be utilised in clinical practice, the techniques to identify them must be compatible

with current processes. In the main, this means the ability to detect the novel marker of interest with immunohistochemistry.

4.1.2 Immunohistochemical scoring systems in breast cancer

As previously described, there are many factors which can affect the eventual immunohistochemical result. Arguably the most important factor is that the assessment of the extent and intensity of the staining is subjective, and therefore the potential for error is moderate. In an attempt to standardise how immunohistochemical staining of breast cancer is reported, a number of scoring systems have been devised. The histo-score involves assessing the percentage of cells which stain on a 4 –point score of intensity, where 0 = no staining, 1 = weak, 2 = moderate and 3 = strong staining. The summation of each intensity score then produces the histo-score in the range 0-300 (156). The Quick score involves a score from 1-3 for intensity and a score from 1-4 based on the proportion of cells stained, which is then added together to give a maximal score of 7 (15). The Allred score is a modified version of the Quick score and expands the lower end of proportion of staining to give a score of 1-5 and an overall maximal score of 8 (157). Other systems give a percentage of cells stained regardless of intensity of staining.

In breast cancer diagnosis, an individual pathological specimen is routinely stained for ER, PR and HER2. ER staining is nuclear and different institutions use a variety of the scoring systems outlined. Assessment of HER2 status tends to be more uniform using the HerceptTest, which gives a score of 0, 1+, 2+ or 3+ depending on the intensity of the membrane staining. Borderline 2+/3+ cases may be further assessed using fluorescent in-situ hybridisation to confirm the HER2 status (158).

The recognition that scoring systems are central to robust and reproducible immunohistochemical assessment led the European Organisation for Research and Treatment of Cancer (EORTC) to issue a consensus statement on immunohistochemical scoring (159). This gave guidance on developing scoring systems for new and existing immunohistochemical tests which includes defining the relevant pattern of staining, defining a “cut-off” value for each category of

staining intensity and selecting areas to be assessed and reiterated the needs for robust quality assurance systems.

4.1.3 Tumour tissue microarrays

The high throughput immunohistochemistry technology described above has been made possible by the introduction of tissue microarray (TMA) technology. A TMA is created by embedding small cores of individual pathological specimens into a recipient paraffin block which can contain up to 1000 specimens. Simultaneous histological analysis can then be carried out on a large number of patient samples (160). This allows all samples included within the TMA to be subjected to identical processing techniques, reducing significantly the variability in staining conditions (161). A criticism of this methodology is that only a small section of the tumour is examined and this may be of importance in cases where antigen expression may not be homogeneous across the whole tumour. Despite this potential drawback, microarray technology is widely used in clinical and research laboratories to assess the expression of the protein of interest in a large cohort of patients.

4.1.4 Observer variability in immunohistochemical scoring

It is widely accepted that because of the subjective nature of immunohistochemical reporting, significant inter-observer variation may occur and published research papers invariably quote a measure of inter-observer variation. Historically, the most commonly quoted measure of variability is the kappa score, which corrects the proportion of agreement due to chance. The strength of agreement is measured on scale from 0-1,correlation being described as 0 – 0.2 = slight , 0.21 – 0.4 = fair , 0.41 – 0.6 moderate , 0.61 – 0.8 substantial and 0.81-1 almost perfect (162).

More recently, the interclass correlation coefficient has been shown to be a consistent method to calculate inter-observer variability in immunohistochemistry, when the scoring being analysed generates continuous, rather than discrete, data. The strength of agreement is measured as 0.7 = minimal acceptable standard, 0.8 = good and >/= 0.9 as excellent (163).

4.1.5 Investigation of Rab25 expression in breast cancer

As described in 1.6.2, there are significant discrepancies between the published data with regard to the patterns of expression of Rab25 in breast cancer. The studies published thus far have studied Rab25 expression at the RNA level using RT-PCR. Studies at the mRNA level are based on the assumption that mRNA levels are predictive of expressed protein levels, however only limited studies have been undertaken to explore this hypothesis. In general whilst an overall correlation between mRNA levels and protein levels has been shown, the strength of the correlation varies markedly between genes and between individuals (164). There is therefore merit in investigating the protein expression patterns of Rab25 and the relationship between Rab25 expression and clinical outcome in breast cancer in an attempt to further the understanding of the effect of this protein in this disease and to ascertain if Rab25 expression is a useful prognostic or predictive marker in breast cancer.

4.1.5.1 Development of a scoring system for immunohistochemical staining of breast cancer tissue with anti-Rab25 antibody

Given that the anti-Rab25 antibody had not previously been used for immunohistochemical applications, it was necessary to develop a scoring system to quantify the intensity of immunohistochemical signal produced. Prior to investigation of Rab25 expression in breast tissue, the properties of the antibody were characterised as described in Chapter 3. The development of the scoring system was made with reference to the EORTC consensus statement described above.

4.1.5.2 Investigation of Rab25 protein expression in archival clinical breast cancer tissue

To determine if there is a relationship between Rab25 protein expression and clinical outcome in breast cancer, immunohistochemistry was performed on a large cohort of breast cancer samples to give meaningful, statistically robust results.

Cores from breast cancer tissue from 566 primary breast cancers samples from patients who had been treated at Glasgow Royal Infirmary between 1984 and 1998 had previously been compiled in a tissue microarray – the West of Scotland breast cancer cohort. Pathological and clinical outcome data were available for all patients.

4.2 Results

4.2.1 Intensity of Rab25 immunohistochemical staining can be categorised by a simple numeric grading system

Using the commercially available BR804 breast tissue microarray which contains 35 cores of breast cancer tissue, each with a paired adjacent normal breast tissue core, immunohistochemistry was performed using the anti-Rab25 antibody. Figure 4-1 shows images which represent grade 1 = weak, grade 2 = moderate and grade 3 = strong staining of breast cancer samples. The percentage of cells stained in each core is approximately 100% and the intensity of staining was relatively homogeneous across each core. There were no cores in which staining was absent. This was performed by two independent observers, Dr Brendan Doyle and me, blinded to the available clinical data and was performed on two separate TMA slides. Positive and negative control slides of A2780-Rab25 and A2780-DNA3 tissue pellets were stained alongside each experiment.

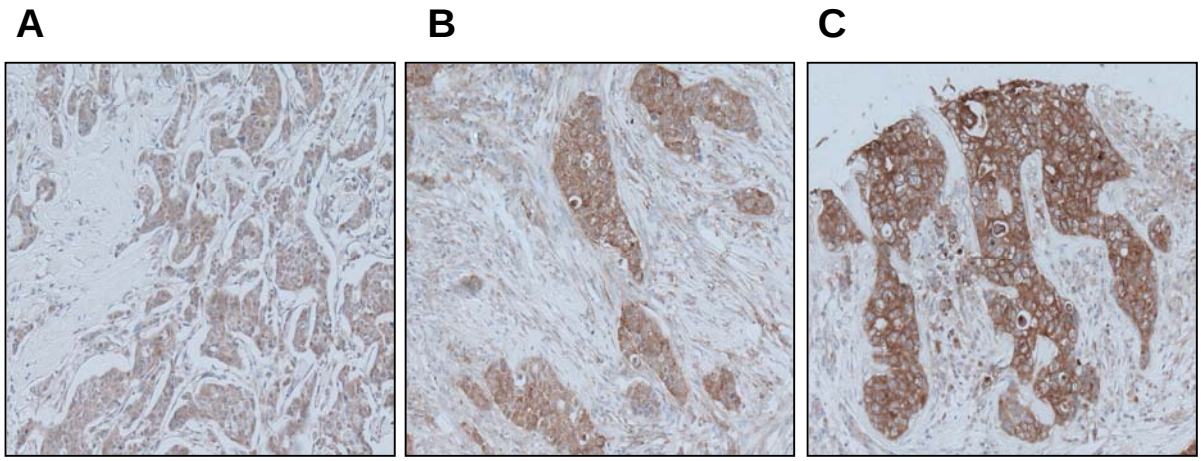


Figure 4-1 Immunohistochemical staining of breast cancer specimens with anti-Rab25 antibody.
Images demonstrate **A** Grade 1 weak staining, **B** Grade 2 moderate staining and **C** Grade 3 strong staining from a commercial TMA containing cores of breast cancer. All samples were processed simultaneously. Representative images shown.

It was observed that when staining was present, it was confined to the epithelial elements within the specimen and that stromal cells did not stain. Corresponding hematoxylin and eosin staining was performed on a sequential section for comparison. Staining was present at the same intensity across the whole specimen and the proportion of the sample stained approached 100% . It was therefore deemed impractical to develop a histoscore system based on both intensity and frequency of staining.

Figure 4-2 shows the frequency of each grade of staining of the non-malignant adjacent breast tissue (which is described as "normal" breast tissue by the manufacturer,) and breast cancer samples present on the BR804 tissue microarray slide and Table 4-1 shows the results of the Kolmogorov-Smirnov test of normality for these data which confirms that the expression of Rab25 observed in these samples is normally distributed.

To validate this scoring system further the process was repeated on a second commercial tissue microarray, BR1001, containing 40 cores of primary breast cancer tissue with paired samples of metastatic lymph node deposits from the same patient. Figure 4-3 shows the frequency of each grade of staining of the primary breast cancer and matched metastatic samples present on the BR1001 TMA. Table 4-2 shows the results of the Kolomogorov-Smirnov test for normality which confirms that the data in this case are not normally distributed. Despite the observation that the data are not normally distributed, there is still an even distribution of samples across the grading categories.

4.2.2 Level of Rab25 expression in normal breast tissue does not correlate with Rab25 expression level in matched cancer tissue

Statistical analysis of the BR804 dataset was carried out to ascertain if there was a correlation between Rab25 expression in normal breast cancer tissue and paired cancer tissue. Power calculations to determine sample size required to detect a correlation were performed using GPower 3.1 software. The number of paired cases to achieve a 95% power with a significance level of 0.005 is 13. The BR804 TMA contained 35 pairs of matched samples and the sample size was

therefore adequately powered to detect a significant difference if one was present.

As described above and shown in Table 4-1, the expression of Rab25 in the cores present on the BR804 TMA was normally distributed in both "normal" breast tissue and matched breast cancer tissue. However, there was no direct relationship between the expression level observed in "normal" tissue and the matched cancer sample. Table 4-3 shows the crosstabulation of Rab25 score between "normal" breast tissue and matched primary cancer tissue and also the number of pairs in which the "normal" tissue had a higher Rab25 score than the paired cancer, the number of pairs in which the "normal" tissue had a lower Rab25 score than the paired cancer and the number of pairs where the Rab25 score was identical in both "normal" tissue and cancer. To quantify the relationship between the Rab25 expression in "normal" and breast cancer tissue Pearson's coefficient was calculated as r = 0.293 (two tailed p-value 0.088) giving an $r^2 = 0.086$, confirming that there is no significant correlation between Rab25 expression levels in the "normal" breast tissue and paired cancer tissue in this dataset.

4.2.3 Level of Rab25 expression in primary breast cancer tissue does not correlate with Rab25 expression level in matched metastatic breast cancer tissue

Statistical analysis of the BR1001 dataset was carried out to investigate if there was a correlation between the Rab25 expression level in primary breast cancer and the level seen in matched locoregional lymph node deposits. The BR1001 TMA contained 40 pairs of matched samples and the sample size was therefore adequately powered to detect a significant difference if one was present.

Table 4-4 shows the crosstabulation of Rab25 score between primary breast cancer and matched metastatic cancer tissue and also the number of pairs in which the primary cancer tissue had a higher Rab25 score than the paired metastasis, the number of pairs in which the primary cancer tissue had a lower Rab25 score than the paired metastasis and the number of pairs where the Rab25 score was identical in both primary cancer tissue and metastasis. As the

data were not normally distributed the relationship between the Rab25 expression in primary cancer tissue and paired metastatic tissue was assessed using the non-parametric Wilcoxon Signed Ranks test, p = 0.400. Given that the sample size was adequately powered, this confirms that there is no correlation between the Rab25 expression level in primary breast cancer and paired metastatic deposits in this dataset.

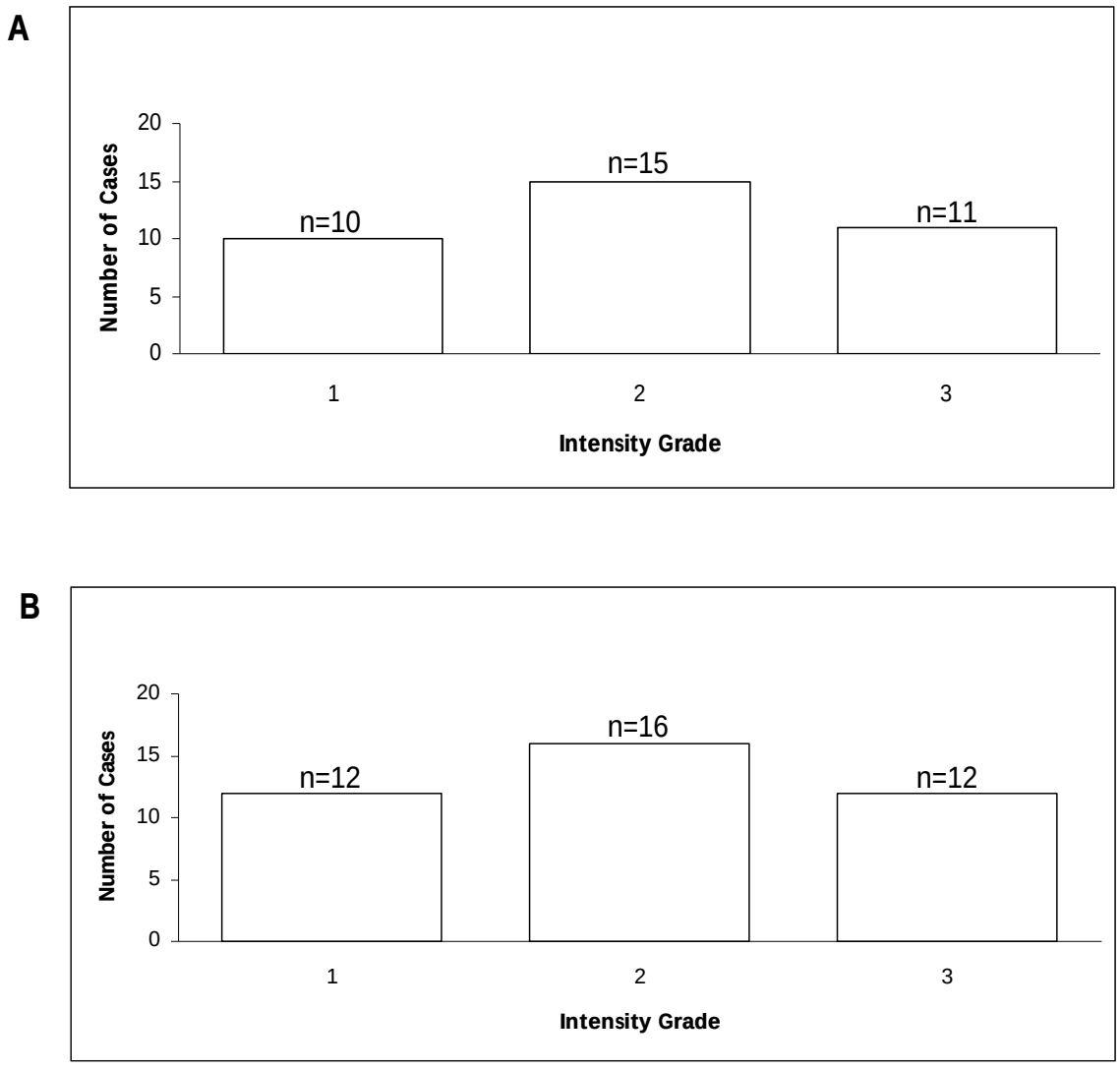


Figure 4-2 Distribution of the immunohistochemical intensity scores in the BR804 tissue microarray.
Frequency of Rab25 immunohistochemical staining characterised as Grade 1 (weak), Grade 2 (moderate) or Grade 3 (strong) in the BR804 tissue microarray in **A** normal breast tissue and **B** matched cancer tissue. Representative data shown (n=2) as both immunohistochemical runs produced identical results.

Table 4-1 Test of normality for Rab25 staining intensity results obtained from the BR804 breast cancer tissue microarray.

	Kolmogorov-Smirnov		
	Statistic	p value	passed normality test?
Rab25 Score Normal	.215	.082	yes
Rab25 Score Cancer	.223	.077	yes

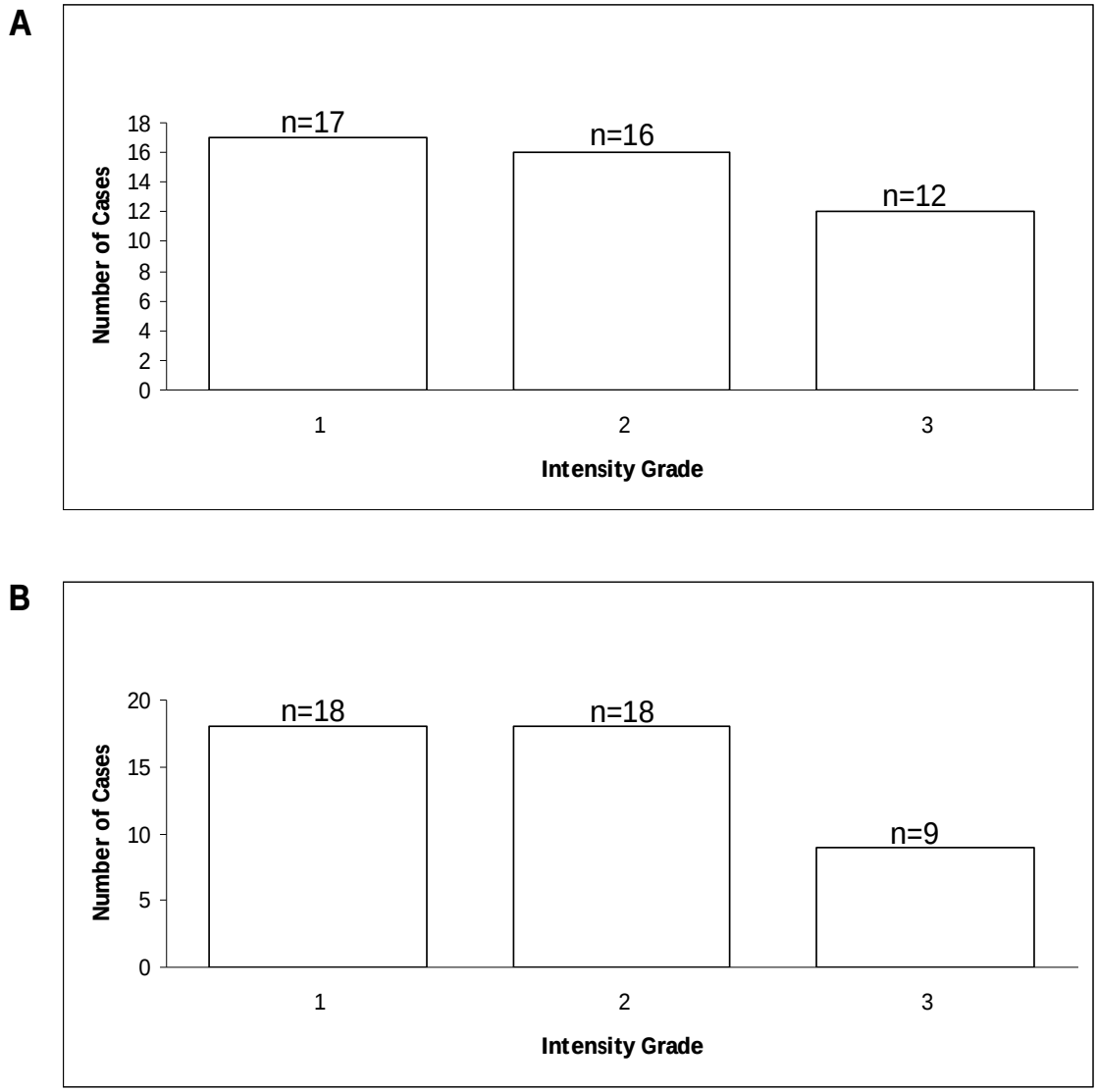


Figure 4-3 Distribution of the immunohistochemical intensity scores in the BR1001 tissue microarray.
Frequency of Rab25 immunohistochemical staining characterised as Grade 1 (weak), Grade 2 (moderate) or Grade 3 (strong) in the BR1001 tissue microarray in **A** primary breast cancer tissue and **B** matched metastatic lymph node deposits. Representative data shown (n=2) as both immunohistochemical runs produced identical results.

Table 4-2 Test of normality for Rab25 staining intensity results obtained from the BR1001 breast cancer tissue microarray.

	Kolmogorov-Smirnov		
	Statistic	p value	passed normality test?
Rab25 Score Primary	.255	0.0097	no
Rab25 Score Metastasis	.243	0.0058	no

Table 4-3 Cross tabulation of Rab25 score in matched normal and cancer tissue on the BR804 tissue microarray.

The number of pairs in which the score in normal tissue compared with primary cancer tissue is higher, lower and identical is shown.

		Normal tissue Rab25 score			
		1.00	2.00	3.00	Total
Cancer tissue Rab25 score	1.00	6	5	1	12
	2.00	2	3	8	13
	3.00	1	7	2	10
Total		9	15	11	35

Rab25 Score	Pairs
Normal < Cancer	10
Normal > Cancer	14
Ties	11

Table 4-4 Cross tabulation of Rab25 score in matched primary breast cancer and matched metastatic lymph node tissue on the BR1001 tissue microarray.
The number of pairs in which the score in metastatic tissue compared with primary cancer tissue is higher, lower and identical is shown.

		Primary Cancer Rab25 score			
		1.00	2.00	3.00	Total
Metastasis Rab25 score	1.00	6	8	3	17
	2.00	5	5	5	15
	3.00	3	2	4	9
Total		14	15	12	41

Rab25 Score	Pairs
Metastasis < Primary	16
Metastasis > Primary	10
Ties	15

4.2.4 Rab25 expression in a large clinical cohort

Sections from the West of Scotland breast cancer tissue microarray, and access to the clinical database, were gifts from Dr Joanne Edwards and were constructed as described in Section 2.1.5.2. Ideally TMA's should incorporate two or more cores from each sample, however, due to the large number of cases required to produce statistically robust results in studies involving breast cancer, a single core for each cancer was selected. 6 TMA's in total were constructed.

Corresponding clinical data were available and included age at diagnosis, tumour type, grade, size, nodal status, overall survival in days, oestrogen and HER2 receptor status. ER status was determined from the original pathological assessment at the time of surgery, and the Her2 status was determined using standard diagnostic immunohistochemical techniques on the constructed TMA. This was performed by Claire Orange, Department of Pathology, Glasgow University. A tumour was deemed to be ER positive if greater than 10% of cell stained positive for oestrogen receptor and HER2 positive if Hercept Test scoring was 3+. Details of post-operative treatment were not available. Table 4-5 gives

details of the baseline histopathological characteristics of the tumour samples included on the TMA.

Immunohistochemistry was performed on the TMA sections and slides were scored independently by two individuals, me and Dr Brendan Doyle, Specialist Registrar in Histopathology, using the grade 0-3 scoring system devised from the observations from the commercial TMAs. Inter-observer variability was assessed by calculation of Kappa score of 0.69. Those cores in which the observers disagreed were reassessed by both observers together and a consensus reached. Statistical analysis, including Kaplan-Meier life table analysis and Cox regression was performed using SPSS version 15 software. The terminal event used to assess survival was breast cancer related death.

In this breast cancer clinical cohort, 35.5% of tumours stained weakly, 46.7% stained moderately and 17.8% stained strongly for Rab25. Kaplan Meier survival curves were plotted for each of the levels of expression and test of equality of survival distributions of the different levels of Rab25 cytoplasmic scores was performed (Log Rank test.)

Table 4-5 Baseline characteristics of the tumour samples on the West of Scotland Breast Cancer tissue microarray.

Variable	Number of cases
Histopathological type	
Ductal	491
Lobular	29
Other	13
Missing	28
Histopathological Grade	
I	50
II	258
III	235
Missing	18
Size	
<20mm	178
20-49mm	231
>50mm	35
Missing	117
Oestrogen Receptor Status	
Negative	137
Positive	329
Missing	95
Nodal Status	
Negative	254
Positive	247
Missing	60
Her2 status	
Negative	451
Positive	48
Missing	62
Rab25 Score	
Grade 1	199
Grade 2	262
Grade 3	100

Univariate analyses and relative distribution of Rab25 cases relative to standard clinicopathological features are demonstrated in Table 4-6.

Table 4-6 Univariate analyses of Rab25 score in relation to known clinicopathological features in the West of Scotland Breast Cancer Cohort

	Rab25 Score			X^2 p-value
	1	2	3	
Age				
<50	45	73	26	0.387
>50	148	182	72	
Histopathological Grade				
1	24	23	3	0.40
2	81	121	56	
3	87	110	97	
Size				
<20 mm	64	88	26	0.772
21-49 mm	79	108	44	
>50 mm	14	15	6	
Lymph node status				
Positive	78	130	46	0.194
Negative	94	109	44	
Oestrogen Receptor Status				
Positive	118	152	59	0.447
Negative	52	67	18	
HER2 Status				
Positive	166	212	73	0.612
Negative	16	26		

The Kaplan-Meier survival curves for the total cohort is shown in Figure 4-4A and demonstrates that while the Kaplan Meier curves for survival cross, giving the log rank test low power to detect a true difference in breast cancer related survival between the three groups, there is a trend towards decreased survival with Grade 1 Rab25 staining, compared with Grade 2 and 3 staining. Further analysis confirmed no significant difference ($p = 0.679$, log rank test and hazard ratio 0.895, 95% CI 0.53-1.52) in the cumulative survival of patients whose tumours were scored as either moderate or strongly staining as shown in Figure 4-4B, and the data from these two groups were combined as "Rab25 positive" tumours, as distinct from the weakly staining "Rab25 negative" tumours, for further analysis. The Kaplan-Meier survival curves comparing the survival outcomes of patients with Rab25 positive tumours with those of patients with Rab25 negative tumours is shown in Figure 4-4C. Patients with Rab25 negative tumours showed a significantly reduced breast cancer specific survival when compared with patients with Rab25 positive tumours ($p = 0.001$, log rank test and hazard ratio 0.577, 95% CI 0.41-0.81.)

As the Kaplan Meier survival curves illustrated in Figure 4-4 converge or cross, the values given for log rank test and hazard ratio should be interpreted with caution. They are included here to illustrate the trends observed only as there is a clear departure from proportional hazard with increasing time.

4.2.5 Loss of Rab25 expression has a greater negative effect on survival of patients who have ER negative tumours compared with patients who have ER positive tumours

Of the 566 samples analysed, oestrogen receptor status was available for 466 cases; 329 were oestrogen receptor positive and 137 were oestrogen receptor negative. Subgroup analysis of the effect of Rab25 expression on the breast cancer related survival of those patients whose tumours have lost ER expression is shown in Figure 4-5B. These data demonstrate that patients with ER negative, Rab25 negative tumours had significantly reduced survival when compared with patients with ER negative Rab25 positive tumours, p = 0.015, log rank test, hazard ratio 0.51 (95% CI 0.30-0.87.) In patients who had tumours which had lost Rab25 expression, mean breast cancer related survival was 9.9 years (95% CI 7.5 – 12.3 years) compared with 13.3 years (95% CI 11.5 – 15.1 years) in patients with tumours which expressed Rab25. This represents an absolute reduction in 10 year survival of 3.4 years, which is approximately 22%, for those patients with an ER negative, Rab25 negative tumour, compared with those patients with an ER negative, Rab25 positive tumour.

The Kaplan-Meier curves illustrating the survival of patients with Rab25 negative or Rab25 positive tumours in patients whose tumours express the ER receptor are shown in Figure 4-5A. In this subgroup the effect of Rab25 loss is much less than in the ER negative cohort, p = 0.03, log rank test, hazard ratio 0.59 (95% CI 0.36-0.97) with mean breast cancer related survival in patients with weak Rab25 staining of 13.8 years (95% CI 11.9 - 15.8 years) compared with 15.1 years (95% CI 13.7-16.5 years) in patient with moderate or strong staining. However, as described previously, these curves cross at 10 years, suggesting that there is unlikely to be a statistically significant difference between these two groups.

These data reveal that loss of Rab25 has a significantly greater effect on the survival of patients who have tumours which have also lost expression of the oestrogen receptor.

4.2.6 Loss of Rab25 has maximal negative effect on the survival of patients with ER negative tumours that do not overexpress the HER2 receptor

Further analysis of the 137 patients with ER negative tumours revealed that the survival in those patients who had lost expression of Rab25 was further reduced in those patients whose tumours were deemed to be Her2 negative (score = 0,1+ or 2+) using the Hercept Test for HER2 expression. Of the 137 patients with ER negative tumours, 107 were also HER2 negative and 23 were Her 2 positive, described as 3+ using the Hercept Test.

Figure 4-6A demonstrates the breast cancer related survival of those patients whose tumours have lost ER expression and do not overexpress the HER2 receptor, as determined by immunohistochemical analysis. These data demonstrate that patients with ER negative, HER2 negative and Rab25 negative tumours had significantly reduced survival when compared with patients with Rab25 positive tumours, p = 0.005, log rank test, hazard ratio 0.43 (95% CI 0.23-0.79.) In patients with weak Rab25 staining, mean breast cancer related survival was 9.6 years (95% CI 7.0 – 12.2 years) compared with 13.8 years (95% CI 10.5 – 15.8 years) in patients with moderate or strong staining.

For comparison, the estimated survival plot showing the survival of patient with ER positive/HER2 negative cancer is shown in Figure 4-6B. In this subgroup the effect of Rab25 loss is much less than in the ER negative/HER2 negative cohort, p = 0.013, log rank test, hazard ratio 0.89 (95% CI 0.67-1.04). However, as described previously, these curves cross at 10 years, suggesting that there is unlikely to be a statistically significant difference between these two groups.

Estimated survival plots showing the effect of Rab25 status on patients with ER negative Her2 positive breast and ER positive Her2 positive cancer are not shown

as the number of patients with these tumour profiles was too small to yield meaningful results.

This represents an absolute reduction in 10 year survival of 4.2 years, which is approximately 26%, for those patients with an ER negative, Her2 negative Rab25 negative tumour, compared with those patients with an ER negative, Her2 negative Rab25 positive tumour.

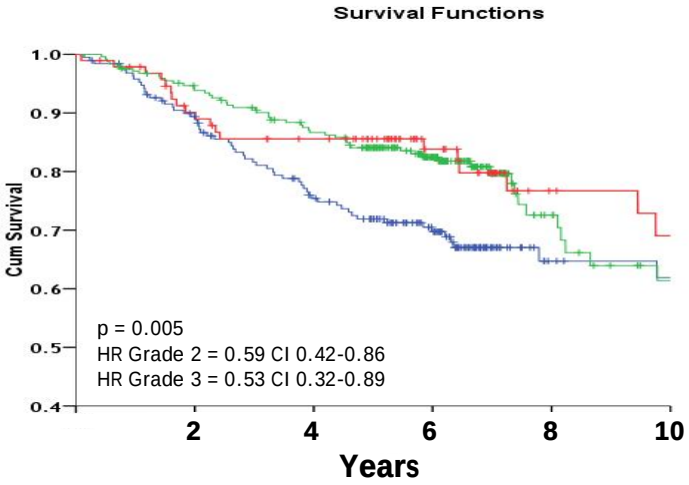
4.2.7 Loss of Rab25 is an independent prognostic indicator in breast cancer survival

In order to fully assess the effect of Rab25 on breast cancer related survival, Cox's multiple regression analysis was performed to control for the confounding variables of tumour size and grade, extent of lymph node involvement at the time of diagnosis and ER status. The Cox model provides an estimate of the effect of Rab25 loss after adjustment for the other known prognostic variables and gives an indication as to whether the effect of Rab25 loss is independent of these variables.

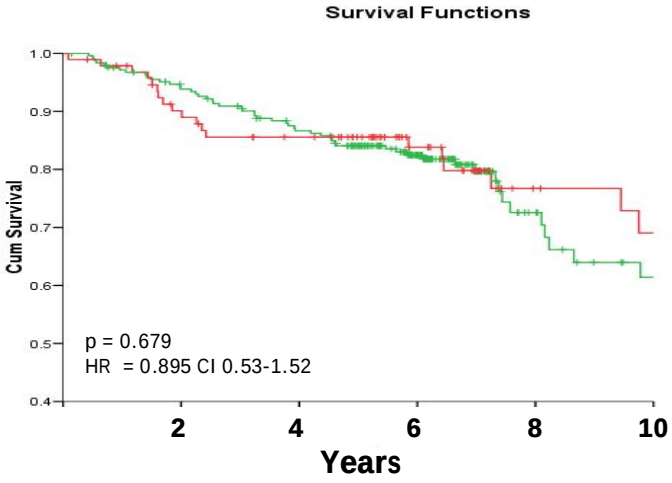
Table 4-7(A) shows the results of the Cox's multiple regression analysis to investigate the relationship between the prognostic factors described above. The data show that the hazard ratio for death in the Rab25 positive group is 0.59 of that of the Rab25 negative group, which represents a 40% decrease in the risk of death in the Rab25 positive group after adjustment for the other factors in the model. The significance of this result is demonstrated by the p value of 0.019.

Table 4-7(B) shows the manner in which categorical variables were handled in the model, which is necessary to interpret the individual regression coefficients given.

A



B



C

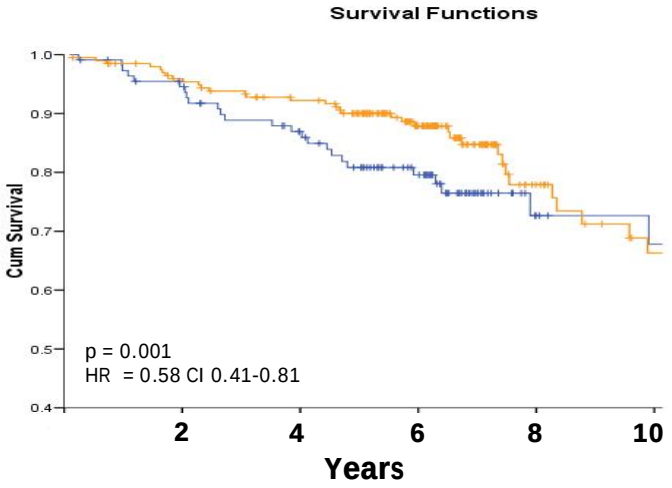


Figure 4-4 Kaplan Meier curves to estimate the overall breast cancer related survival of patients with low, medium or high expression of Rab25 in a cohort of 566 breast cancer cases. Legend continues on next page

Figure 4-4 Kaplan Meier curves to estimate the overall breast cancer related survival of patients with low, medium or high expression of Rab25 in a cohort of 566 breast cancer cases. Log rank test has been performed to compare survival between each group and hazard ratios are given. However, these results should be interpreted with caution as in this situation the log rank test has low power to detect a true difference and a statistically significant difference is unlikely to exist. Grade 1 is the reference indicator. 95% confidence intervals are shown.

Grade 1 – indicated in blue

Grade 2 – indicated in green

Grade 3 – indicated in red

Grade 2&3 combined - indicated in yellow

(A) Total cohort all grades
(B) Total cohort grade 2 and grade 3
(C) Total cohort grade 1 and grades 2&3 combined

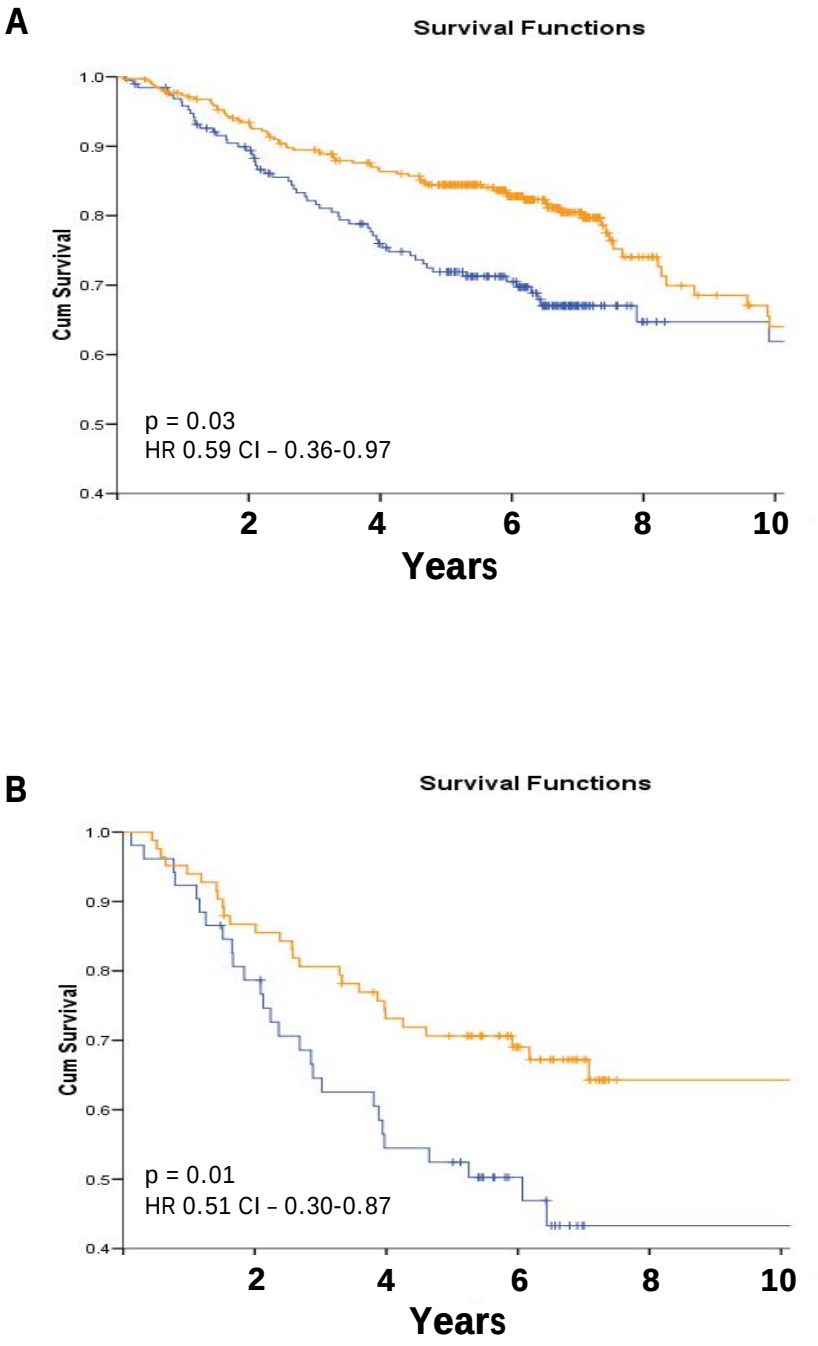


Figure 4-5 Kaplan Meier survival curves to estimate the overall survival of patients who have breast tumours which express, or have lost expression, of Rab25.
Log rank test was performed to compare survival between Rab25 positive and negative tumours (p-values shown.) Hazard ratio for death is also shown. Grade 1 is the reference indicator. Hazard ratio values should be interpreted with caution when survival curves cross as a statistically significant difference is unlikely to exist in this situation.
Grade 1 – indicated in blue
Grade 2&3 combined – indicated in yellow
(A) Oestrogen receptor positive tumours (n=329)
(B) Oestrogen receptor negative tumours (n=137)

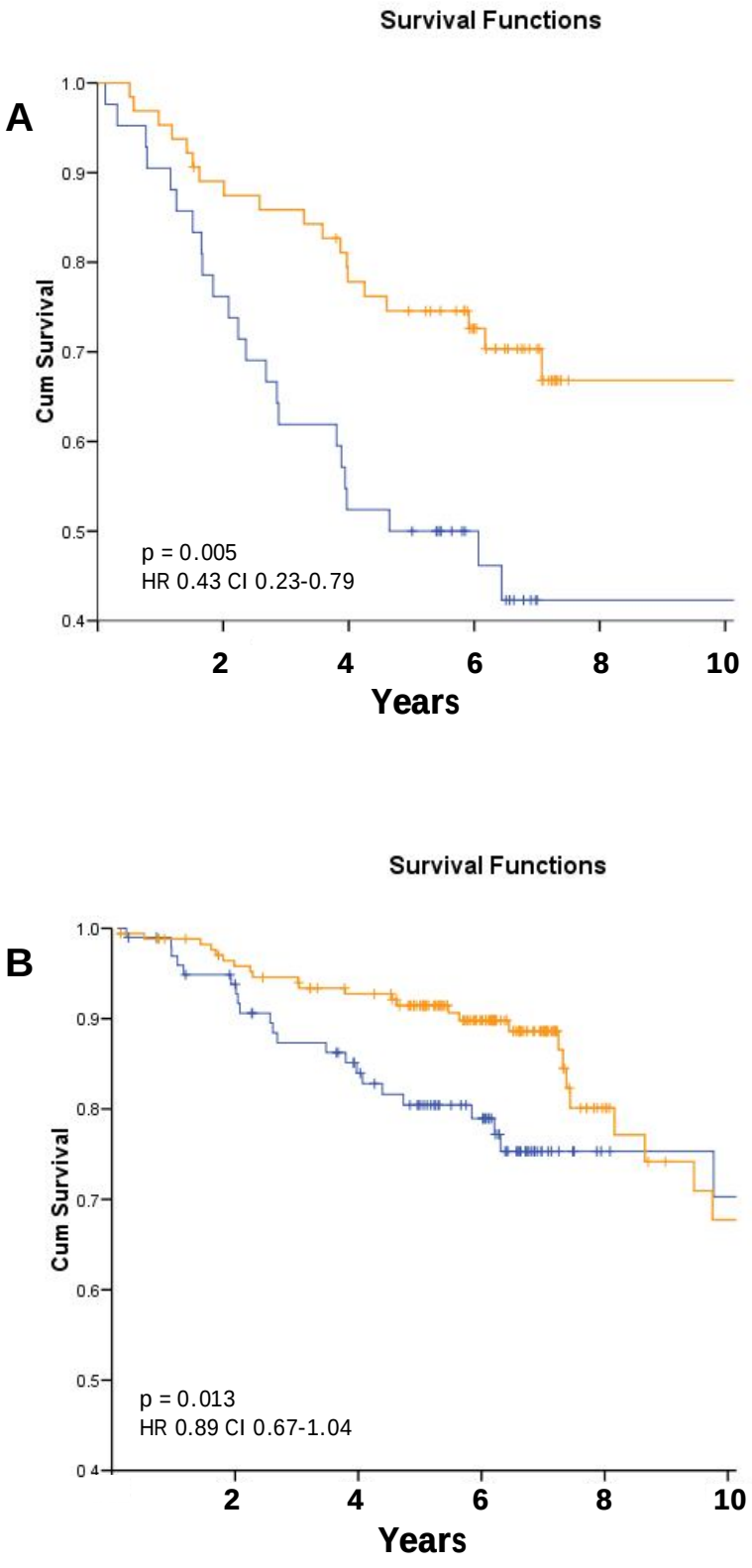


Figure 4-6 Kaplan Meier curves to estimate overall survival of patients who have breast tumours which express or have lost Rab25 expression in the ER-/Her2- and ER+/Her2- cohorts
(A) ER negative/Her2 negative tumours
(B) ER positive/Her2 negative tumours
Log rank test was performed to compare survival between Rab25 positive and negative tumours (p values shown.) Hazard ratio for death is also shown. Grade 1 is the reference indicator.
Grade 1 – indicated in blue
Grade 2&3 combined – indicated in yellow

Table 4-7 Cox's multiple regression model to assess the impact of breast cancer size, grade, nodal status, oestrogen receptor status, HER2 status and Rab25 status on survival.
A B – estimated coefficient, SE – standard error, Wald – ratio of B to SE squared, Sig – significance of Wald statistic, Exp(B) – Hazard ratio**. B** Categorical variables coding to demonstrate how categorical variables were treated during the analysis.

Cox's Multiple Regression model fitted to data from the West of Scotland Breast Cancer Cohort

	B	SE	Wald	df	Sig.	Exp(B)
sizecode			42.505	2	.000	
sizecode(1)	-2.035	.358	32.238	1	.000	.131
sizecode(2)	-1.782	.297	35.959	1	.000	.168
grade			4.095	2	.129	
grade(1)	-1.370	1.034	1.756	1	.185	.254
grade(2)	-.463	.273	2.890	1	.089	.629
Nodestatus	-.807	.255	10.025	1	.002	.446
ercode	.885	.256	11.946	1	.001	2.423
HER2bi3	.040	.336	.014	1	.906	1.041
Rab252and3comb	.594	.232	6.553	1	.010	1.812

Categorical Variable Codings

		Frequency	(1)[a]	(2)
grade[b]	1	24	1	0
	2	160	0	1
	3	170	0	0
sizecode[b]	1.00	141	1	0
	2.00	188	0	1
	3.00	25	0	0
Nodestatus[b]	1.00	180	1	
	2.00	174	0	
ercode[b]	0	114	1	
	1	240	0	
HER2bi3[b]	0	319	1	
	1	35	0	
Rab252and3comb[b]	1.00	125	1	
	2.00	229	0	

a. The (0,1) variable has been recoded, so its coefficients will not be the same as for indicator (0,1) coding.

b. Indicator Parameter Coding

c. Category variable: grade

d. Category variable: sizecode (<20=1, 20-49 =2, >50=3)

e. Category variable: Nodestatus (1=neg, 2=pos)

f. Category variable: ercode (ER>10 is classed as positive =1, negative =0)

g. Category variable: HER2bi3 (HER2 3+pos)

h. Category variable: Rab252and3comb (Rab252and3comb)

4.3 Discussion

The data reported here demonstrate for the first time that immunohistochemistry with an anti-Rab25 antibody produces staining patterns which can be graded by a semi-quantitative scoring system, and that application of this scoring system can produce clinically significant observations with regard to Rab25 expression in a large breast cancer population.

As there was no previous work using an anti-Rab25 antibody for immunohistochemistry, it was first necessary to ascertain if there was a spectrum of intensity of staining which would be amenable to a semi-quantitative scoring system. It was not possible to predict in advance if there would be any difference in the intensity of staining produced, particularly as immunohistochemistry using diaminobenzidine (DAB) is only stoichiometric at low levels of intensity (165), thus if the spectrum of Rab25 in breast tissue was beyond the stoichiometric threshold of DAB, it might not have been possible to visually detect any difference between samples. Similarly, it was possible that there would not be a detectable difference in Rab25 expression between breast cancer samples, and it was necessary to produce robust evidence to support the hypothesis that Rab25 protein expression levels did vary between samples, and that this variation could be quantified in a reproducible manner, before proceeding to further investigation using the clinical TMA.

Results obtained from the BR804 and BR1001 commercial TMA's confirmed that Rab25 expression could indeed be graded using a semi-quantitative scoring system comprising of Grade 1(weak), Grade 2 (moderate) or Grade 3 (strong) staining. The observation that individual cores stained with the same intensity across the whole core confirmed that a semi-quantitative scoring system which did not include the percentage of cells stained was sufficient to accurately represent the staining patters seen. Inter-observer variation was calculated to be within the acceptable range.

In addition, the distribution of this staining was spread across the three grades, suggesting that Rab25 protein levels did vary sufficiently between individual

breast cancer samples to allow the data generated from a large clinical cohort to be interrogated using standard statistical tests.

Investigation of the relationship between Rab25 protein expression in normal breast tissue and matched primary breast cancer, using the BR804 commercial TMA, was in response to the findings of Cheng et al. as outlined in section 1.6.2 (107). In the work presented here, demonstrable variation in Rab25 protein expression can be seen in normal breast tissue and in breast cancer tissue, but there is no obvious direct relationship between the level of Rab25 expression in the normal tissue and that observed in the matched cancer sample. In 14 out of 35 cases (40%) the cancer sample had less Rab25 expression than the matched primary, which is in keeping with the findings of Cheng, which demonstrated loss of Rab25 mRNA by RT-PCR in 44% of breast cancer samples when compared with matched normal tissue (111).

Investigation of the Rab25 expression in breast cancer and matched metastatic lymph nodes in the BR1001 commercial TMA demonstrated that there is no direct relationship between the Rab25 expression level in primary breast cancer and that seen in matched metastatic deposits. Work by Wang et al. (166) has shown that the Rab25 gene is upregulated in a population of cells which can migrate away from a primary xenograft and invade into Matrigel plug, when compared with the general population of xenograft tumour cells. Extrapolating the results from this work gave rise to the hypothesis that metastatic deposits of breast cancer may demonstrate increased Rab25 protein expression when compared with the primary tumour. The data shown in Table 4-4 show that this was the case in only 10 out of 41 (24%) cases and the majority of cases showed no change in Rab25 status or a decrease in Rab25 in the metastatic deposit when compared with the primary cancer.

These data suggest that Rab25 expression does not sequentially increase or decrease when moving along the spectrum of malignancy from normal tissue to primary cancer and metastatic disease. Furthermore, there is no direct relationship between the Rab25 expression found between matched sets of samples of normal and primary tumour, and primary tumour and metastatic deposit. Indeed, given that Rab25 has been shown to be expressed in normal breast tissue, it cannot be considered to be a cancer specific marker in the

sense that Rab25 expression is not a hallmark of cancer tissue when compared with normal tissue.

When interpreting these results, the small sample size should be borne in mind. However, the experiment contained sufficient numbers for the experiment to be adequately powered to detect a statistically significant difference if one was present. In addition, the clinical detail available was limited, for example no information was available on the clinical outcome or the oestrogen receptor status of the tumours. Even if this information had been available for these 35 samples, there would not have been sufficient numbers of ER-positive and ER-negative tumours for the experiment to be adequately powered for subgroup analysis. On this basis, ER staining was not undertaken on this cohort and therefore it was not possible to investigate further the findings of Cheng et al., who demonstrated that the loss of Rab25 in breast cancer when compared to matched normal tissue was seen predominantly in ER negative cancers, rather than ER positive cancers (112). In order to robustly investigate this hypothesis is was necessary to use a much larger cohort with associated good quality clinical data.

The West of Scotland Breast Cancer TMA was originally constructed to investigate the relationship between oestrogen receptor status and clinical outcome and a number of studies using the TMA have been published (167). Clinical data including tumour size, grade, ER status, Her2 status and lymph node status and breast cancer related survival time of the patients was available for investigation.

The observation that there was no significant difference in clinical outcome between patients with tumours with Grade 2 Rab25 staining and those with Grade 3 staining, and the subsequent combining of these two groups into a single "Rab25 positive" cohort, is in keeping with the method adopted for other clinically relevant prognostic indicators, such as HER2 where 0, 1+ and 2+ are deemed "HER2 negative", while those tumours which are 3+ are deemed positive for the purposes of treatment with trastuzumab (50).

When the importance of the oestrogen receptor status of breast cancer was first discovered, the percentage of the tumour sample which stained positive for the

oestrogen receptor was reported routinely in clinical practice as described in the scoring systems discussed above. Subsequent investigation demonstrated that, in terms of clinical relevance, tumours could be classified as either ER positive (greater than 10% of visualised cells stain positive for oestrogen receptor) or ER negative (less than 10% of visualised cells stain positive for oestrogen receptor) (168). The approach adopted for the data presented here is therefore not without precedent in the investigation of breast cancer.

The mechanisms by which Rab25 contributes to deceased breast cancer related survival in patients with ER negative breast cancer are not clear. One potential explanation is that Rab25 expression may be different between true luminal epithelial cancers and basal-like cancers. If Rab25 expression is lower in the myoepithelial cells from which basal-like cancers arise, it may be hypothesised that this would be reflected in lower expression in basal tumours. If the ER - negative/Rab25 group is composed of mainly basal-like cancers and the ER – negative/Rab25 group is composed of ER negative non-basal cancers, then the results presented here may simply reflect the well documented differential in survival between these two groups.

The clinical surrogate of basal-like breast cancer is the triple negative phenotype, as discussed in 1.3.2. While the ER and HER2 status of the tumours in this cohort are known, the PR status is not. However, as outlined in 1.4.2 only 1-5% of cancers are documented as having the ER-negative/PR – positive phenotype, and hence one can assume that only 1-5% of the cases include in the analysis presented here will be PR positive. Given the magnitude of difference in breast cancer related survival demonstrated, it would seem unlikely that knowledge of the PR status of these tumours would add any useful additional information.

Further work investigating expression profile of EGFR and cytokeratin expression in this cohort to identify the proportion of cancers which are truly basal is warranted, and then the effect of Rab25 status on survival may be clearer. In addition, further investigation of Rab25 expression in the different cellular components of the normal breast will be of value in determining whether loss of Rab25 expression is simply a reflection of the true tumour type or whether Rab25 is exerting a significant effect on survival.

Investigation of EGFR expression may reveal more information than simply the pathological subtype of the tumours. As discussed in 1.4.6, it has been hypothesised that Rab25 may be involved in an RCP/α5β1 integrin/EGFR complex and perturbation of this interaction by loss of Rab25 may affect trafficking of the EGFR receptor within cells. There is a significant inverse relationship between ER expression and EGFR expression (169), and further investigation of the interplay between EGFR, ER and Rab25 expression will be of value in determining if Rab25 plays a clinically significant role in the survival of patients with ER negative cancer and if Rab25 expression represents a potential therapeutic target.

The relationship between Rab25 expression and β1 integrin will be explored in more detail in Chapter 5.

5 The effect of siRNA knockdown of Rab25 expression in MCF7 breast cancer cells.

5.1 Introduction

5.1.1 MCF7 cells as a model for studying the cell biology of breast cancer

The MCF7 breast cancer cell line is one of the most commonly used cell lines in the laboratory investigation of breast cancer. The cell line was derived in 1970 from cells obtained from a pleural effusion of a patient with metastatic breast cancer (170). The cells express both oestrogen and progesterone receptors, but lack expression of HER2 (171), and the discovery of expression of steroid hormone receptors in this cell line has facilitated extensive investigation of cellular signalling in response to oestrogenic stimulation. Indeed, growth of MCF7 cells in tissue culture is greatly enhanced by the presence of oestrogenic compounds in the serum used in tissue culture medium (172) and *in vivo* xenograft work using MCF7 cells requires additional supplementation of exogenous oestrogen to promote xenograft growth (173).

MCF7 cells express surface ß1 integrin, but at lower levels than that seen in human breast epithelial cells (174), and have also been shown to express ß4 integrin (175) and the α2, α3 and α6 subunits (175, 176).

5.1.2 siRNA as a tool for the study of cell biology

In order to assess the effect of knockdown of Rab25 expression in MCF7 cells it was necessary to design an appropriate RNA interference (RNAi) strategy. The laboratory exploitation of the endogenous intracellular RNA interference system, also termed post-translational gene silencing, results in transient or stable knockdown of expression of the protein of interest. The endogenous RNA pathway is initiated by the Dicer enzyme, which cleaves long double-stranded RNA fragments into shorter sections approximately 20 nucleotides in length. The

structure of the resulting fragments is well defined, with a 3' overhang of 2 nucleotides on each strand (177).

Once the short RNA strands have been cleaved, one strand of each fragment, called the guide strand, associates with the RNA-inducing silencing complex (RISC) (178). This multi-protein complex uses the RNA strand as a template to identify complementary mRNA which, once identified, is degraded by the activation of the Argonaute proteins into 20-23 nucleotide sections, suggesting that the siRNA strand is targeting the specific cleavage of the mRNA molecule (179). The degradation of mRNA results in significantly reduced levels of mRNA template available for protein translation, in effect switching off the gene effect.

Exploitation of this process to investigate the in vitro effect of silencing a particular gene of interest can be achieved by the transfection of exogenous siRNA. Transfection with appropriately designed synthetic oligonucleotides mimicking the 21-23 nucleotide fragments produced by Dicer, results in a knock down of protein expression (180). However, this effect is transient and not transmitted to daughter cells.

For stable knockdown to be achieved, it is necessary to transfect a vector containing an expression sequence of a single RNA transcript which is designed as two palindromic 20-23 nucleotide sequences joined by a tightly structured hairpin loop, termed short hairpin RNA. Expression of this sequence in usually under the control of the H1 or U6 RNA polymerase III promoters, to ensure the sequence is always expressed. Once expressed, the hairpin is processed by Dicer and the resulting siRNA acts as described above (181). Such vectors are usually transmitted to daughter cells, resulting in a population of cells stably expressing the shRNA with consequent knockdown of protein expression.

5.2 Results

5.2.1 Rab25 protein expression is stably knocked down in MCF7 cells

To investigate the effect of Rab25 knockdown on MCF7 cells it was first necessary to confirm the expression of Rab25 in this cell line. Western blotting of lysate of the MCF7 breast cancer cell line using the anti-Rab25 antibody produced a band of the predicted molecular weight, confirming that this cell line expressed Rab25. Figure 5-1 confirms the presence of a band at 25kD in both A2780-Rab25 cells and MCF7 cells. As described in Chapter 3, the molecular weight of Rab25 in cells with endogenous expression of Rab25 is slightly smaller than the HA-tagged Rab25 produced by ectopic expression in A2780-Rab25 cells, and this is confirmed to be the case in MCF7 cells also.

5.2.2 Stable knock down of Rab25 in MCF7 cells

To investigate the effect of Rab25 knockdown in MCF7 cells it was necessary to design a short hairpin vector. Following confirmation of the hairpin sequence, MCF7 cells were transfected with either the pSUPERGFP-Neo Rab25 expression vector or a scramble control pSUPERGFP-NEO vector using Lipofectamine. The pSUPERGFP-Neo expression vector contained a GFP expression cassette and a cassette conferring neomycin resistance. Following transfection, cells were selected for the presence of the vector by growing the transfected cells in media to which neomycin had been added. When neomycin-resistant colonies had become established, 24 colonies of each of the pSUPERGFP-Neo Rab25 and pSUPERGFP-Neo control transfected cells were screened for the presence of the vector by fluorescence imaging. Those not exhibiting strong GFP expression were discarded, and the remaining colonies were screened for the expression of Rab25 by Western blotting. From the colonies with demonstrable knockdown of Rab25 expression by Western blotting, three colonies were selected for use in subsequent experiments, Rab25 KD clone 7, Rab25KD clone 10 and Rab25 KD clone 11. Of the scramble MCF7 control colonies, two were selected, MCF7 control clone 9 and MCF7 control clone 12.

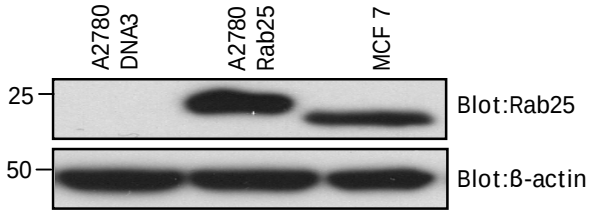
Western blotting of lysates from the selected clones MCF7 Rab25 KD clones 7, 10 and 11 and MCF7 Control clones 9 and 12, when incubated with anti-Rab25 antibody, confirmed the presence of a 25kD band consistent with the expression of Rab25. Figure 5-2 clearly shows a diminution of the signal in the clones stably expressing the pSUPERGFP-Neo vector when compared with the cells expressing the scramble control vector, confirming knockdown of Rab25 protein expression in the Rab25 KD clones.

5.2.3 Knockdown of Rab25 protein expression does not affect growth rate or colony formation in MCF7 cells

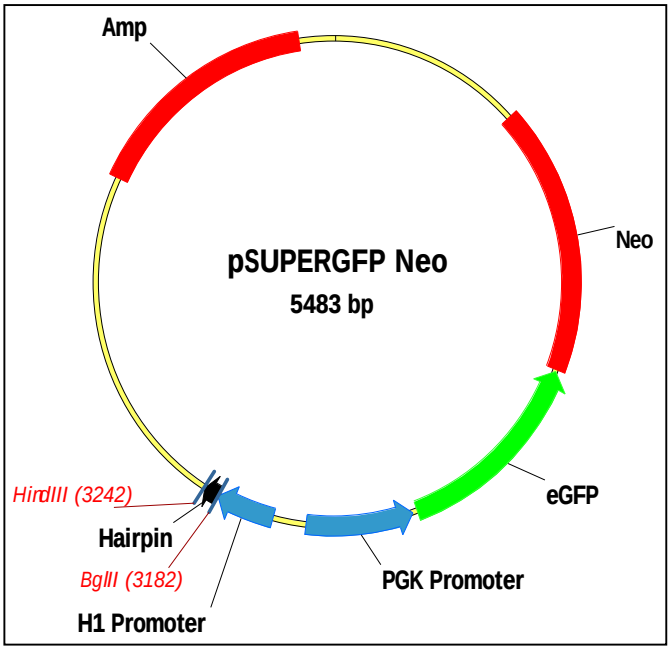
Prior to undertaking any experiments investigating the functional effect of knockdown of Rab25 protein expression, it was necessary to ascertain if Rab25 knockdown (Rab25KD) had any effect on the growth characteristics of MCF7 cells. Once it had been confirmed that the three colonies of MCF7 cells could maintain expression of the Rab25KD hairpin vector over time and in the absence of neomycin pressure, the effect of knockdown of Rab25 expression on the growth of these cells was investigated. Standard growth rate curves for the Rab25KD clones and MCF7 control clones were performed and the ability of the clones to form colonies in soft agar was investigated. Figure 5-3 illustrates the growth rates of MCF7 Rab25 KD and MCF7 control clones when grown in standard medium over a period of 8 days. All Rab25 KD clones and MCF7 control clones grew at a very similar rate, and hence the mean growth rate for each condition is shown for ease of presentation. There was no difference between the growth rate of MCF7 Rab25 KD cells and MCF7 control cells under these conditions.

The ability of MCF7 cells to form colonies when grown in soft agar was also unaffected by the knockdown of Rab25, when compared with MCF7 control cell clones. Figure 5-4 confirms that the mean total colony number identified was similar in MCF7 Rab25 KD and MCF7 control clones and in addition, the mean number of large colonies (colonies greater than 62.5 μm^2) formed was unaffected by the knockdown of Rab25 expression.

A



B



C

```
  1 ATGGGGAATG GAACTGAGGA AGATTATAAC TTTGTCTTCA AGGTGGTGCT GATCGGCGAA
 61 TCAGGTGTGG GGAAGACCAA TCTACTCTCC CGATTCACGC GCAATGAGTT CAGCCACGAC
121 AGCCGCACCA CCATCGGGGT TGAGTTCTCC ACCCGCACTG TGATGTTGGG CACCGCTGCT
181 GTCAAGGCTC AGATCTGGGA CACAGCTGGC CTGGAGCGGT ACCGAGCCAT CACCTCGGCG
241 TACTATCGTG GTGCAGTGGG GGCCCTCCTG GTGTTTGACC TAACCAAGCA CCAGACCTAT
301 GCTGTGGTGG AGCGATGGCT GAAGGAGCTC TATGACCATG CTGAAGCCAC GATCGTCGTC
361 ATGCTCGTGG GTAACAAAAG TGACCTCAGC CAGGCCCGGG AAGTGCCCAC TGAGGAGGCC
421 CGAATGTTCG CTGAAAACAA TGGACTGCTC TTCCTGGAGA CCTCAGCCCT GGACTCTACC
481 AATGTTGAGC TAGCCTTTGA GACTGTCCTG AAAGAAATCT TTGCGAAGGT GTCCAAGCAG
541 AGACAGAACA GCATCCGGAC CAATGCCATC ACTCTGGGCA GTGCCCAGGC TGGACAGGAG
601 CCTGGCCCTG GGGAGAAGAG GGCCTGTTGC ATCAGCCTCT GA
```

D

Name	Target Sequence
Scramble Control	GCACGCATGTCTACATCTA
Rab25	AACAATGGACTGCTCTTCC

Figure 5-1 Strategy for stable knockdown of Rab25 in MCF7 cells.
A Western blots of lysates of A2780-DNA3, A2780-Rab25 and MCF7 cells incubated with anti-Rab25 antibody (top panel) and anti- β-actin antibody (bottom panel.) **B** Schematic diagram of pSUPERGFP-Neo expression vector. **C** Rab25 DNA sequence – hairpin target sequence highlighted in red. **D** Target sequence of scramble control and Rab25 hairpin vector.

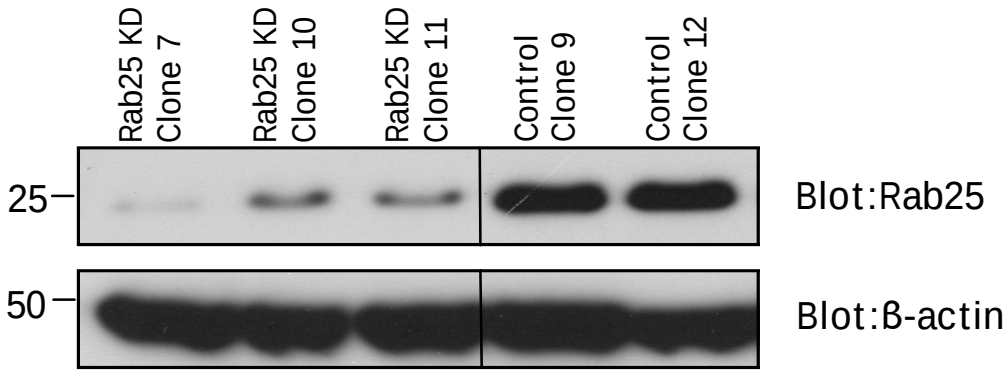


Figure 5-2 Assessment of Rab25 knockdown in MCF7 cells.
Lysates of MCF7 knockdown and control clones were Western blotted for Rab25 (top panel) β-actin (bottom panel)(n=3).

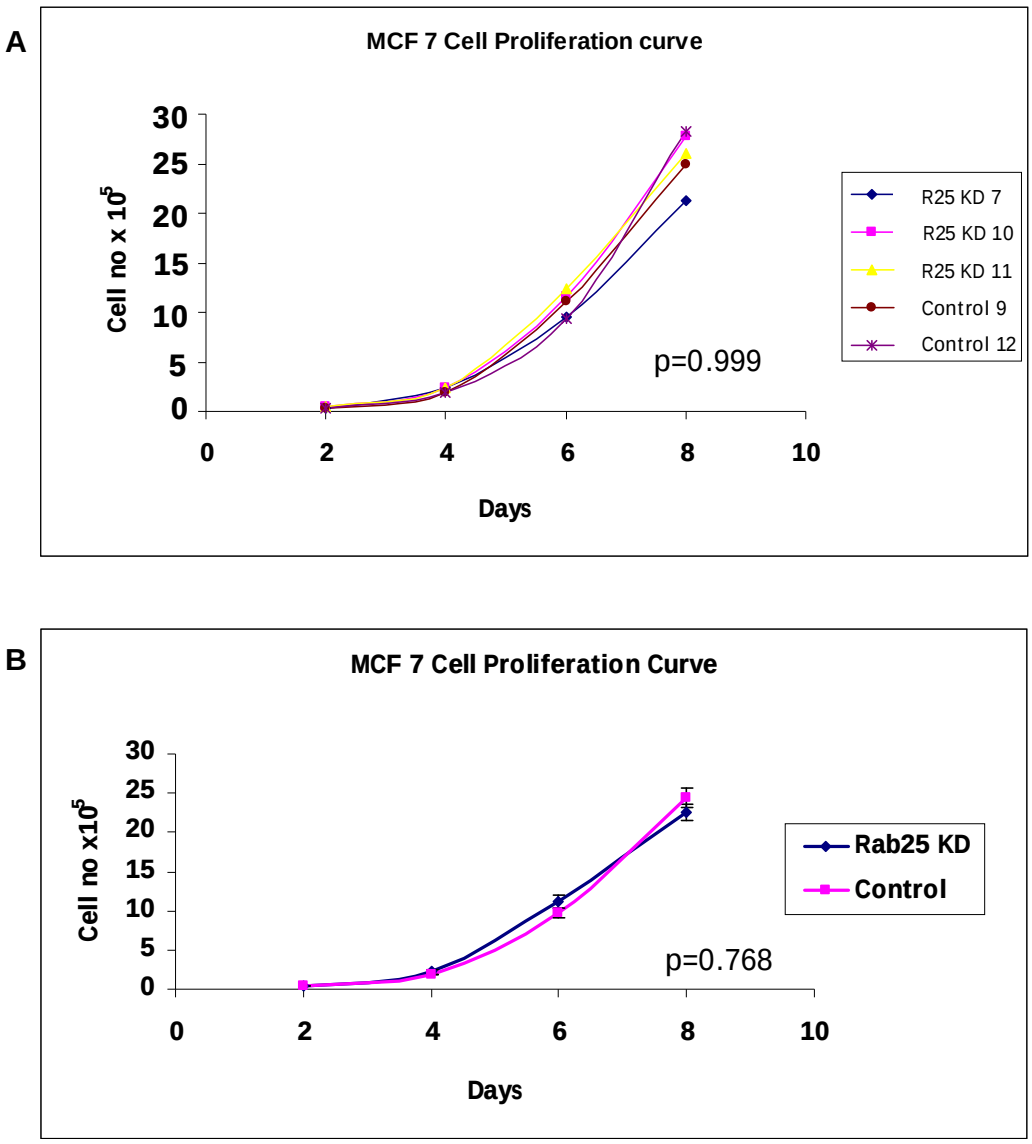


Figure 5-3 Effect of Rab25 knockdown on the proliferation rate of MCF7 cells. Cell proliferation curves for MCF7 Rab25 KD and Control cells
(A) Plot for Rab25KD clones 7, 10 and 11 and control clones 9 and 12. p value for one sided ANOVA shown **(B)** Rab25 and control clones grew at the same rate and the mean growth rate for each condition was calculated. Curves plotted are mean ±SEM of three colonies of KD and control cells. p value for students' t-test shown. Representative plot shown (n=3)

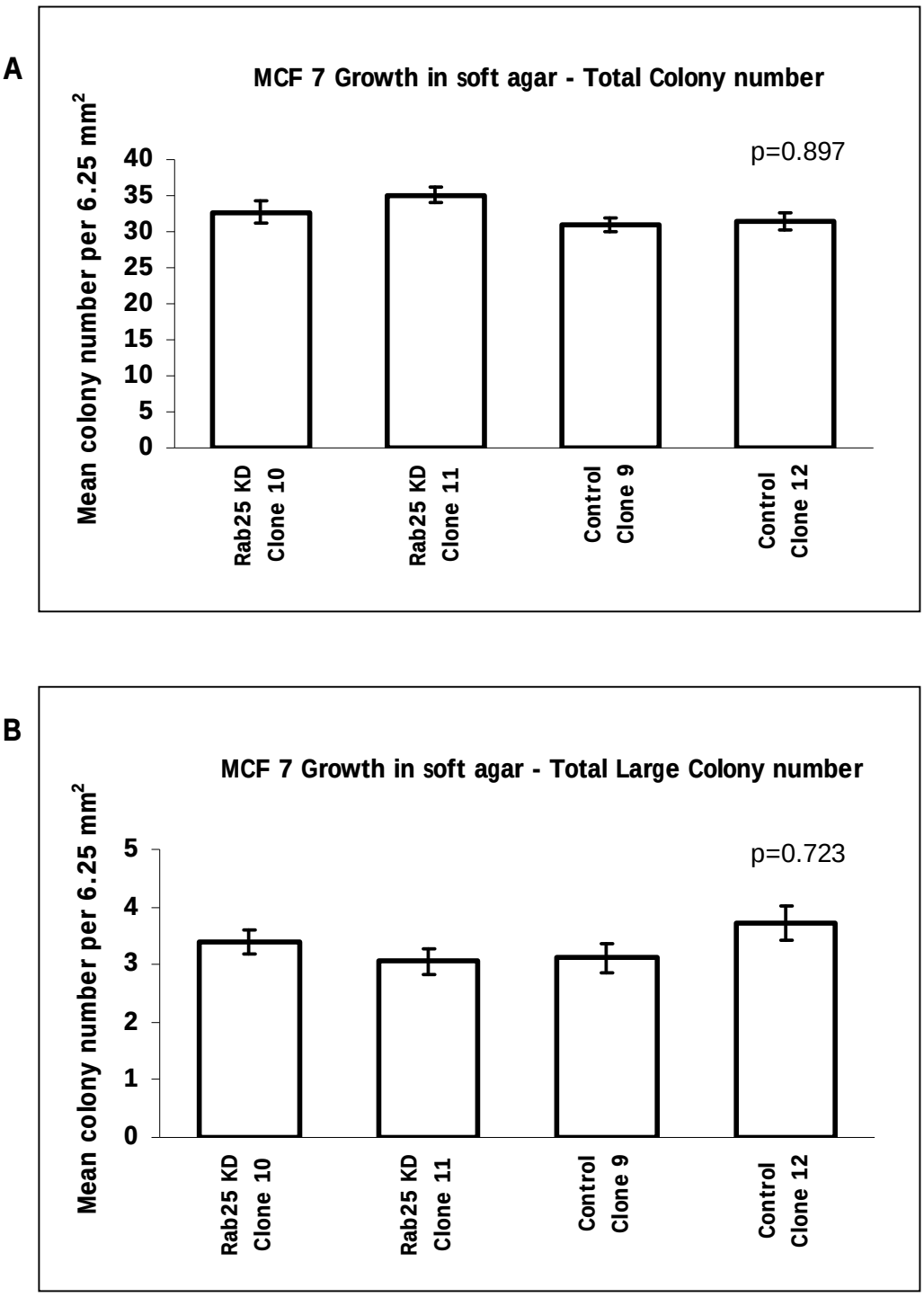


Figure 5-4 Effect of Rab25 knockdown on colony formation in MCF7 cells.
A Total number of colonies present per 6.25mm2 of soft agar. **B** Total number of colonies greater than 62.5µm2 present per 6.25mm2 of soft agar. Results are mean ± SEM of 3 experiments. p values for one way ANOVA shown.

5.2.4 Knockdown of Rab25 protein expression increases the rate of wound healing in MCF7 cells

Scratch wound assays were performed to assess the effect of the knockdown of Rab25 expression on migration of MCF7 cells on plastic. Figure 5-5 shows the mean net displacement of the wound edge over a 16 hour timeframe for MCF7 Rab25 KD and MCF7 control clones. These data indicate that the net displacement of the wound edge was significantly greater in MCF7 Rab25 KD clones when compared with MCF7 control clones.

5.2.5 Knockdown of Rab25 protein expression increases total surface and intracellular β1 integrin protein levels in MCF7 cells

Having demonstrated that MCF7 Rab25 KD cells migrate to a greater extent into a wound than MCF7 control cells, the hypothesis was generated that this observation may be related to integrin expression. To investigate the relationship between Rab25 and β1 integrin protein expression in the MCF7 Rab25KD and stable clones, Western blotting for total β1 integrin levels was performed. In addition, surface labelling of β1 integrin and subsequent immunoprecipitation of the β1 protein was performed to investigate the relative quantity of Rab25 protein at the surface of the cell. In brief, β1 integrin present at the cell surface was labelled with an NHS-*SS-Biotin* label. Following cell lysis, immunoprecipitation using β1 integrin antibody and Western blotting, the blot was incubated with streptavadin-HRP to produce a signal.

Figure 5-6 shows a Western blot of lysates of the MCF7 Rab25 KD and MCF7 control clones incubated with anti-β1 integrin antibody and anti-Rab25 antibody. This blot shows a significant increase in β1 integrin levels in the Rab25 KD cells compared with MCF7 control cells, suggesting an inverse relationship between Rab25 protein levels and total β1 integrin levels. Furthermore, the precursor form of β1 integrin (which is approximately 20kDa smaller than the mature, 120 kDa form of β1) is present in similar proportions to mature β1 protein

irrespective of Rab25 status, suggesting that knockdown of Rab25 does not enhance the conversion of proform ß1 integrin to mature ß1 integrin. Furthermore, Figure 5-6 demonstrates that, while there is a small increase in the surface levels of ß1 integrin in the MCF7 Rab25 KD cells when compared with MCF7 control cells, this increase is not in the same proportion as that seen in total ß1 protein.

5.2.6 Increased β1 integrin levels in MCF7 Rab25 KD cells are not a consequence of increased transcription

Quantitative RT-PCR was performed to investigate whether knockdown of Rab25 protein expression affected ß1 integrin mRNA levels. It was necessary to design and validate 4 sets of qRT-PCR primers; primers designed to amplify a product from ß1 integrin and Rab25 and the housekeeping genes ß-actin and GAPHD. Prior to performing qRT-PCR, it was necessary to ensure that each primer pair amplified a single product, that they amplified the correct product in a linear fashion over a range of concentrations of cDNA and did not amplify genomic DNA. Once the qRT-PCR primers had been validated and the optimal conditions for qRT-PCR had been confirmed, qRT-PCR to investigate the mRNA levels of Rab25 and ß1 integrin in the Rab25 KD and MCF7 control clones was performed. Details of how the primer properties were confirmed and qRT-PCR was performed are given in section 2.6.5.

Figure 5-7 confirms that the selected primers for Rab25, ß1 integrin, ß-actin and GAPDH amplified PCR products in a linear fashion over a wide range of concentration of cDNA and shows details of the efficiency of the primers. Confirmation that the primers amplified a single qRT-PCR product is shown in Figure 5-8.

Having validated the qRT-PCR primers sets, qRT-PCR for Rab25 and ß1 integrin and the housekeeping genes ß-actin and GAPDH was undertaken using RNA extracted from MCF7 Rab25 KD clone 10 and MCF7 Control clone 9. This confirmed that the Rab25 mRNA levels were lower in the MCF7 Rab25 KD cells compared with MCF7 control cells, in keeping with a functioning Rab25-targeted short hairpin vector. In contrast, there was no significant difference in the

mRNA levels for β1 integrin between MCF7 Rab25 KD and MCF7 control cells and these data are illustrated in Figure 5-9.

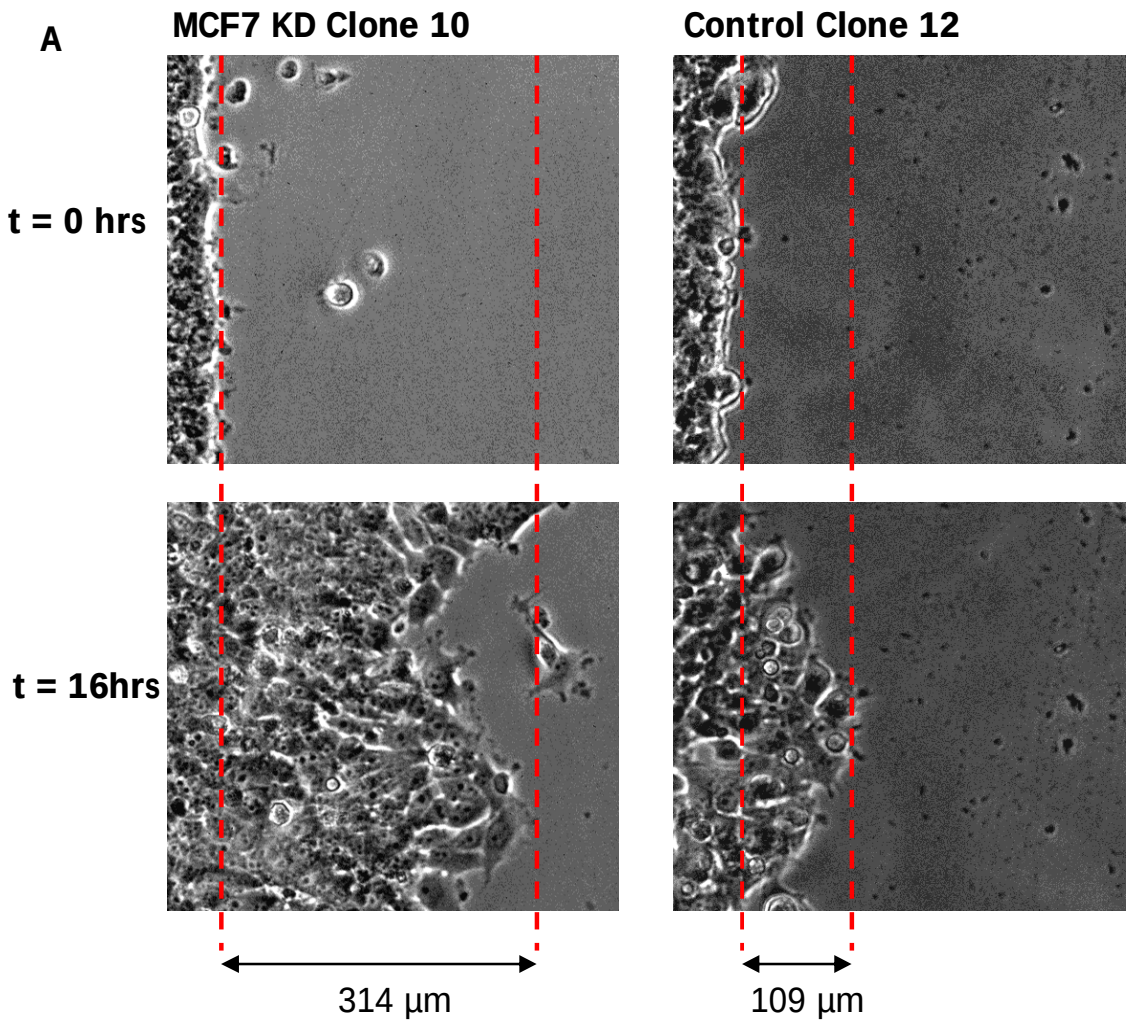


Figure 5-5 Effect of Rab25 knockdown on wound healing of MCF7 cells.
A Representative images of wound edge at time 0 and 16 hours. **B** Net displacement of wound edge over 16 hours. Results are mean ± SEM of three experiments. p value for one way ANOVA shown

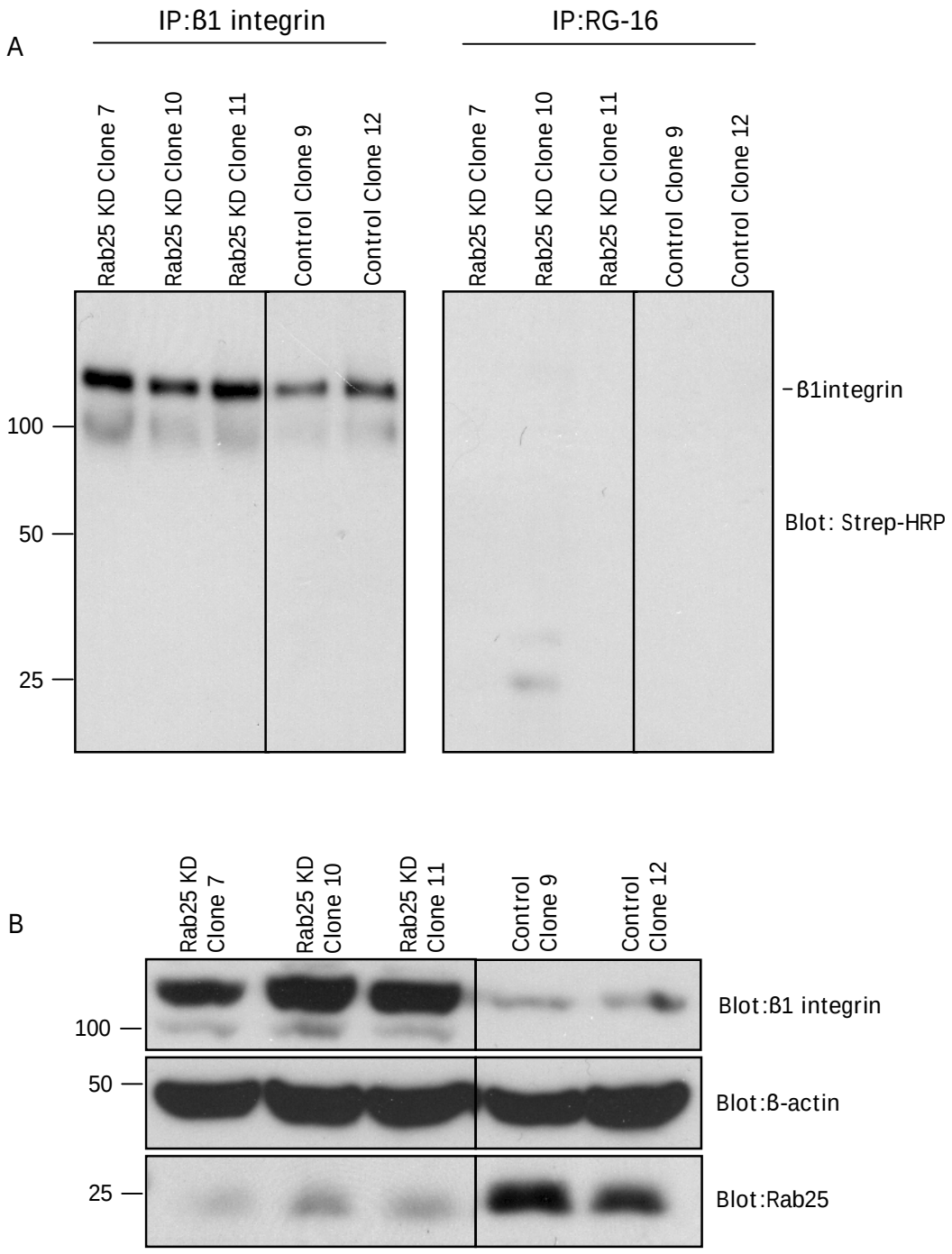
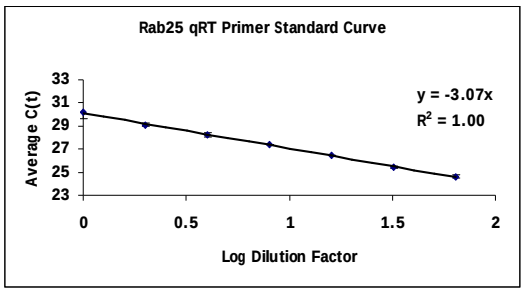
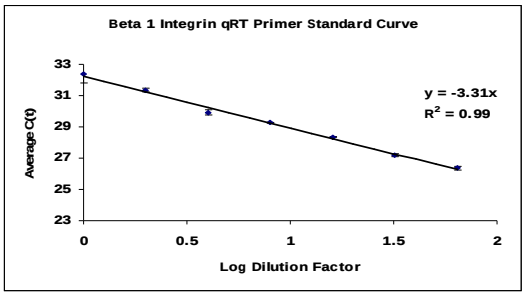


Figure 5-6 Effect of Rab25 knockdown on β1-integrin levels in MCF7 cells.
A Cell surface labelling of β1 integrin. Lysates of MCF7 Rab25 KD and Control clones surface labelled with –ss-biotin and immunoprecipitated with β1 integrin antibody (left panel) and RG-16 (right panel) were Western blotted and blots incubated with streptavidin-HRP (n=2). **B** Whole cell lysates of MCF7 Rab25 KD and Control clones prepared for immunoprecipitation above were Western blotted for β1 integrin (top panel), β-actin (middle panel) and Rab25 (bottom panel) (n=3).

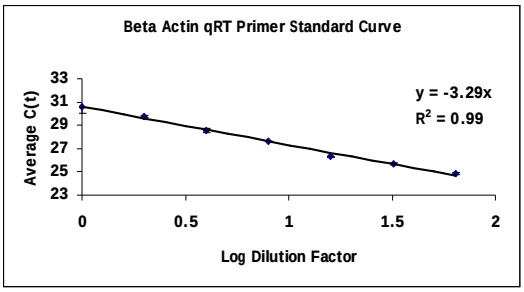
A



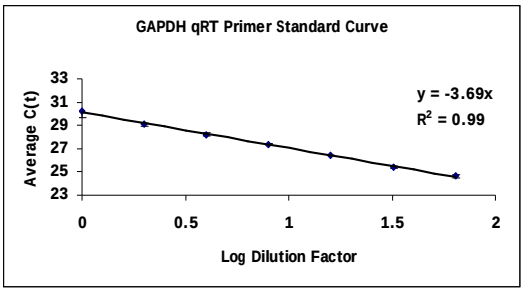
B



C



D



E

Primer	Efficiency
Rab25	111%
Beta 1 Integrin	104%
Beta-actin	101%
GAPDH	86%

Figure 5-7 Validation of quantitative RT-PCR primers.
A Standard concentration curve for Rab25 qRT-PCR primers. **B** Standard concentration curve for β1 integrin qRT-PCR primers. **C** Standard concentration curve for β-actin qRT-PCR primers. **D** Standard concentration curve for GAPDHqRT-PCR primers. **E** Primer efficiency.

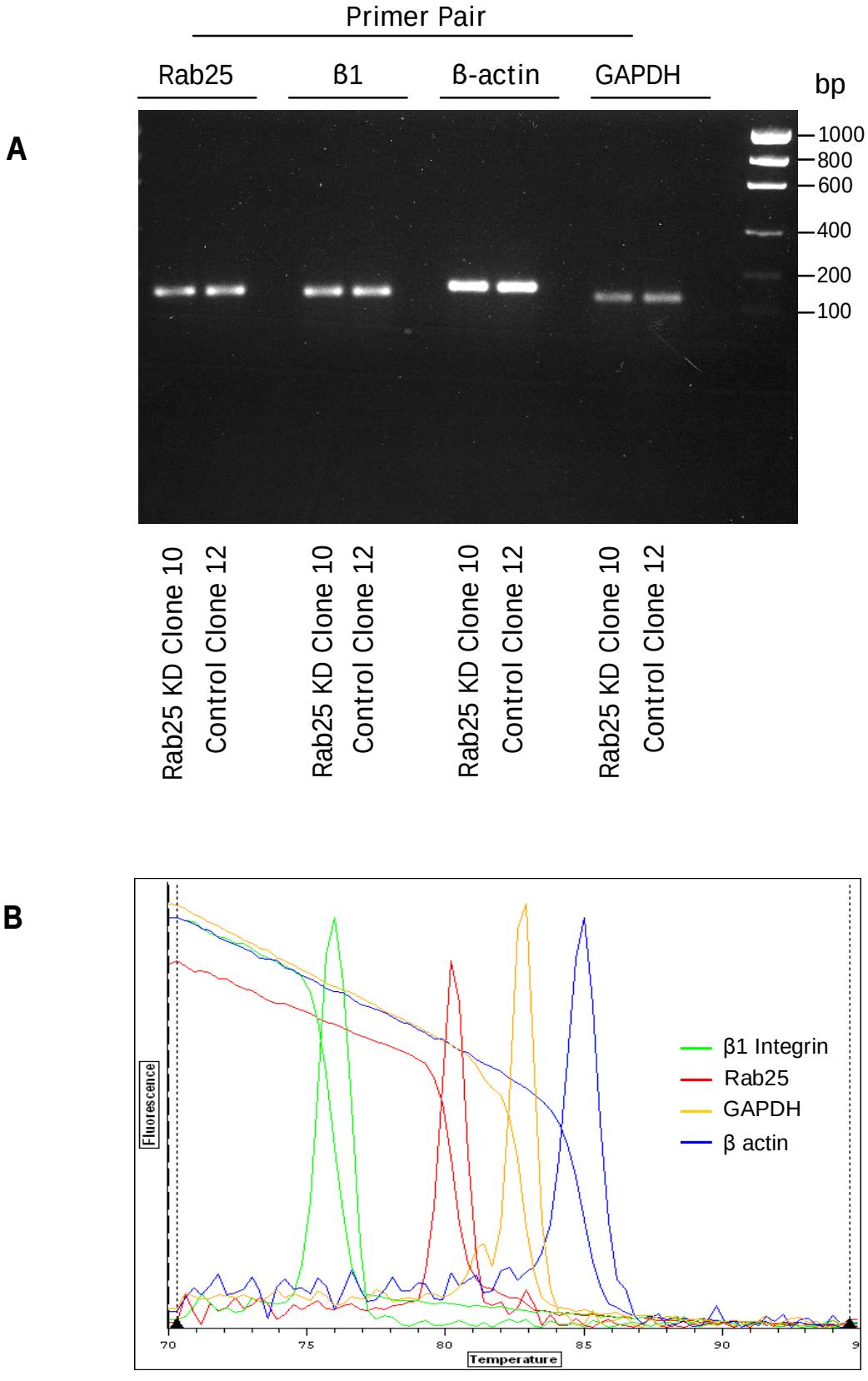
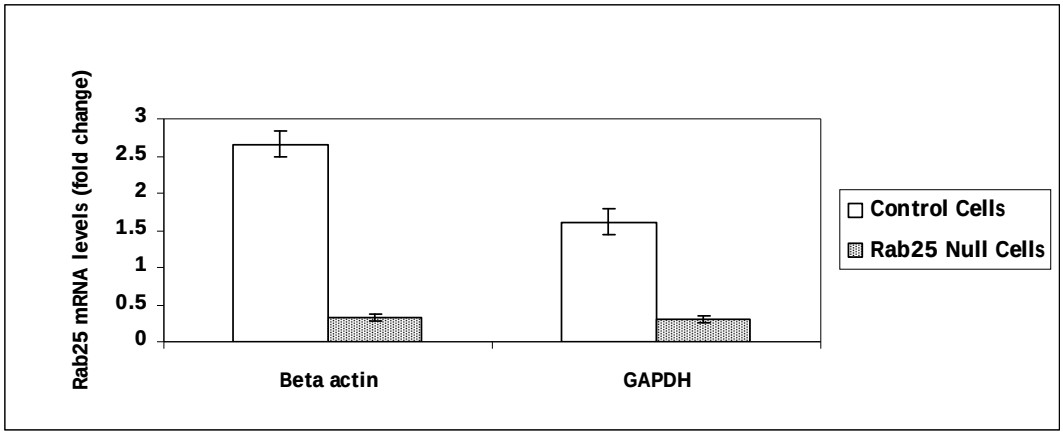


Figure 5-8 Quantitative RT-PCR primers produce a single product.
A RT-PCR product from MCF7-Rab25 KD and Control cells using Rab25, β1 integrin, β-actin and GAPDH primers. **B** Melting curves for Rab25, β1 integrin, β-actin and GAPDH primers.



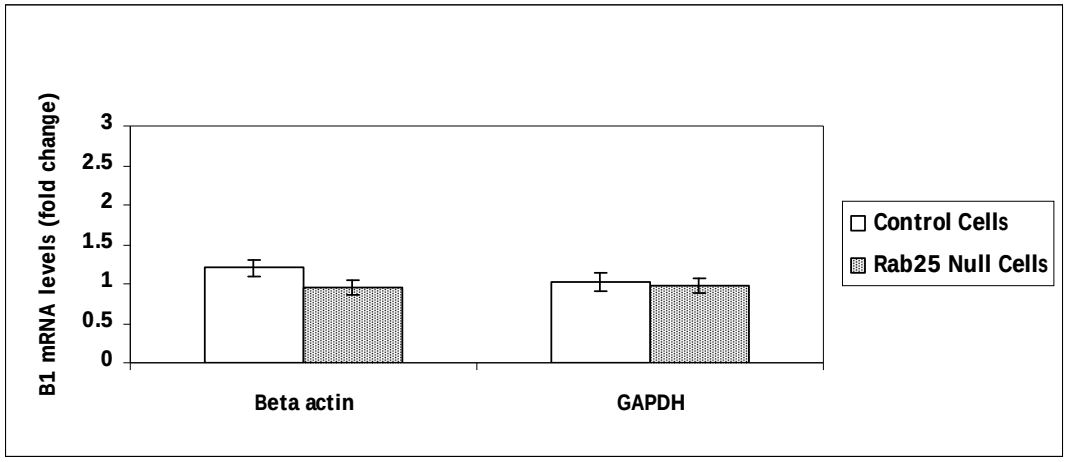


Figure 5-9 Quantitative RT-PCR of Rab25 and β1 integrin levels in Rab25 knockdown and control cells.
A Fold change in Rab25 mRNA levels in MCF7 Rab25 knockdown clone 10 and MCF7 Control clone 9 normalised for β-actin and GAPDH. **B** Fold change in β1 integrin levels in MCF7 Rab25 knockdown clone 10 and MCF7 Control clone 9 normalised for β-actin and GAPDH. Results are mean ± SEM of 9 experiments.

5.3 Discussion

At the commencement of this project, the majority of the published literature investigating the role of Rab25 in cancer focussed on the upregulation or overexpression of Rab25 in cancer tissue or cell culture systems. As discussed in sections 1.6 and 1.7, these data suggested that overexpression of Rab25 in breast and ovarian cancer tissue was associated with a poorer clinical outcome (107) and that forced expression of Rab25 in an *in-vitro* system promoted an invasive mode of migration in these cells (119). As previously discussed, the published studies which have investigated what effect the loss of Rab25 may have on cellular function are somewhat contradictory in nature (110, 111). The aim of the work presented here was to further inform the debate on the effect of the loss of Rab25 protein expression in cancer.

The data presented here confirm that Rab25 protein expression in the MCF7 breast cancer cell line was successfully abrogated by the stable transfection of the pSUPERGFP-Neo vector containing the short hairpin sequence targeted against Rab25. The level of Rab25 knockdown was maintained with increasing passage number of cells and following freezing and thawing of cells (data not shown)

Once the stable MCF7 Rab25 knockdown and control cell lines were established, it was possible to establish whether the growth characteristics of MCF7 cells were altered by the knockdown of Rab25 protein expression. The data presented here suggest that loss of Rab25 protein expression has no effect on the growth rate of MCF7 cells, nor on the ability of MCF7 cells to form colonies in soft agar.

These finding are in direct contrast with the findings of Fan et al, who describe a significantly decreased cellular proliferation rate, both *in vitro* and *in vivo*, of A2780 ovarian cancer cells stably transfected with a short hairpin expression vector targeted against Rab25 similar to the one described in this work (110). It is of note that the A2780-DNA3 cells used in this work, as described in Chapter 3, do not show evidence of Rab25 expression by either RT-PCR or Western blotting with an anti-Rab25 antibody. It therefore remains unclear whether, as a general

principle, proliferation of cells is dependent upon Rab25 function or whether these observations are cell type specific. Further work to investigate the effect of Rab25 loss on the proliferation of other cell lines is needed.

The observation, illustrated in Figure 5-5, that MCF7 Rab25 KD cells can migrate to heal a scratch wound at a faster rate than MCF7 control cells, suggests that the function of Rab25 in cancer progression may be related to the ability of these cells to migrate. Caswell et al. have demonstrated that Rab25 interacts directly and specifically with the β1 cytoplasmic tail of the α5β1 integrin, and overexpression of Rab25 in A2780 ovarian cancer cells promotes an invasive mode of cell migration in Matrigel inverse invasion assays (119). Again, the data presented here conflict with these published findings, as knockdown of Rab25 protein expression, rather than overexpression, resulted in an increased rate of migration in MCF7 cells. However, it should be noted that the effect described by Caswell et al was seen when cells were grown in Matrigel, but not apparent when the same cells were plated on plastic, giving rise to the suggestion that the effect of Rab25 expression may be dependent on the not only on the cell type, but also on the cell culture conditions. Multiple attempts to investigate the ability of the MCF7 Rab25 KD and control cells to invade into Matrigel or collagen plugs were made but were unsuccessful, as neither set of clones could invade the matrix to any demonstrable degree. Hence, no conclusions can be drawn with regard to the effect of loss of Rab25 on invasiveness of MCF7 cells from the data presented here.

The investigation into the β1 integrin status of the MCF7 Rab25 KD and control cells resulted directly from the observation that MCF7 Rab25 KD cells migrate more quickly than control cells to heal a scratch wound. Figure 5-6 clearly shows that total β1 integrin levels are significantly higher in MCF7 Rab25 KD cells when compared with controls. To further investigate the hypothesis that knockdown of Rab25 protein expression in MCF7 cells results directly in an increase in total β1 integrin levels, preliminary Rab25 rescue experiments were performed. A cherry-tagged Rab25 protein expression vector was transiently transfected into MCF7 Rab25 KD cells to restore the expression of Rab25. As shown in supplementary Figure S-1, Western blotting and incubation with anti-Rab25 antibody of lysates of these cells at 24, 48 and 72 hours confirmed the presence of a band at 50kD, in keeping with the predicted molecular weight of

Ch-Rab25, indicating successful rescue of Rab25 expression. Incubation of the same blot with anti-ß1 integrin antibody showed a reciprocal decrease in ß1 integrin expression with re-expression of Rab25. This experiment needs to be repeated to confirm this finding, but this early evidence intimates an inverse relationship between Rab25 and ß1 integrin expression in MCF7 cells.

While total ß1 integrin levels were significantly raised in MCF7 Rab25 KD cells compared with control cells, this magnitude of difference was not maintained at the cell surface, suggesting that the majority of the additional ß1 integrin in the MCF7 Rab25 KD cells was present intracellularly. However, although the difference in surface expression of ß1 integrin between MCF7 Rab25 KD and control cells was small, there was a demonstrable increase in ß1 integrin expression in the MCF7 Rab25 KD cells which may be sufficient to contribute to the enhanced wound healing ability observed.

To assess whether the increase in ß1 integrin observed in MCF7 Rab25 KD cells was as a result of transcriptional upregulation, qRT-PCR was performed to quantify the mRNA levels of Rab25 and ß1 integrin in both MCF7 Rab25 KD and control cells. This confirmed that, while the Rab25 mRNA levels were significantly reduced in MCF7 Rab25 KD cells compared with controls, as would be expected with in the presence of a short hairpin expression vector targeted against Rab25, there was no significant difference in ß1 integrin mRNA levels, despite the obvious difference in protein levels demonstrated on Western blotting.

In summary, the stable MCF7 Rab25 knockdown cell line did not exhibit any demonstrable difference in growth rate or ability to form colonies in soft agar when compared with MCF7 cells transfected with a scramble control vector. However, the MCF7 Rab25 KD cells did migrate to heal a scratch wound at a faster rate than controls. The observation that the Rab25 knockdown cells exhibit a greater total and surface level of ß1 integrin protein when compared with controls, may indicate a mechanism by which this increased migratory ability occurs, but more work is needed to investigate this hypothesis further.

6 General Discussion

The Rab GTPase family of proteins are the master regulators of intracellular transport, coordinating vesicular trafficking of both endogenous and exogenous pathways. Rab proteins are ubiquitously expressed in all mammalian cells, but some are expressed in specific cell types, such as Rab25 which is confined to epithelial cells.

Investigation into the role that Rab proteins play in cancer is relatively immature, and as such the data that are available are often contradictory. On commencing this project, there were published data to suggest that both over expression and loss of Rab25 in breast tumours may have a negative impact on the survival of patients. The aim of this work was to further inform the debate of the role of Rab25 in breast cancer.

The production and validation of a robust anti-Rab25 antibody was essential prior to undertaking the further work on human tissue and Rab25 knockdown cell lines. The results presented in Chapter 3 confirm, as far as possible, that the purified polyclonal antibody could reliably detect Rab25 in a number of laboratory applications. It was therefore possible to proceed to investigation of Rab25 in clinical samples and in cell culture work.

The results presented in Chapter 4 suggest that loss of Rab25 expression in patients with oestrogen receptor negative breast cancer is associated with significantly reduced breast cancer related survival, when compared with those patients who have cancers which express Rab25. Furthermore, survival of patients with ER negative and HER2 negative breast cancer was significantly poorer when the tumour had also lost expression of Rab25, compared with ER negative, HER2 negative cancers which had retained Rab25 expression.

As discussed in Chapter 4, it was not possible to ascertain if the tumour samples which were ER negative/HER2 negative represented cancers which were truly basal, and further investigation to confirm the status of these tumour is required. Confirming the PR status of these tumours is unlikely to add

significant further information, and immunohistochemical staining of the TMA for basal cytokertains and EGFR would be the most obvious next step.

If further investigation revealed that Rab25 loss was seen exclusively in basal cancers, it would then be necessary to investigate if the loss of Rab25 is purely a reflection of the Rab25 status of the distinct cells of origin of luminal and basal cancers. At the present time it is not known if Rab25 expression in normal breast luminal epithelial cells is similar to that in breast myoepithelial cells and immunohistochemistry may not be sufficiently sensitive to distinguish differences in Rab25 expression between these two cellular subtypes. Further work using micro-dissection of the cells of interest may be required.

The decision to investigate the effect of Rab25 loss in MCF7 cells was taken prior to the results from the TMA study being available. In retrospect, given the results obtained from the clinical study, that Rab25 loss appears to be associated with ER negative tumours, the choice of an ER negative cell line may have been advantageous. However, without the benefit of this knowledge, the MCF7 cell line was chosen as a well characterised cell line which expressed endogenous Rab25. Further work to investigate the effect of Rab25 knockdown in an ER negative breast cancer cell line, or indeed re-expression of Rab25 in an ER negative cell line which does not express Rab25, may be helpful in establishing if the effects seen here are widely applicable or simply cell line specific.

The data presented in Chapter 5 show that MCF7 Rab25 knockdown cells migrate to close a scratch wound at a faster rate, and that the Rab25 knockdown cells show increased total and surface β1 integrin protein levels, when compared with control MCF7 cells. It has been confirmed by qRT-PCR that the increase in β1 integrin protein levels seen in MCF7 Rab25 KD cells was not as a consequence of increased transcriptional activity.

One hypothesis to explain the rise in β1 integrin protein levels is that the increased intracellular levels of β1 integrin seen in MCF7 Rab25 KD cells may be as a result of impaired cellular degradation of the protein. As previously described, the work by Caswell et al (ref) has shown that Rab25 interacts with the β1 cytoplasmic tail of the α5β1 integrin dimer, promoting short loop recycling of the integrin to the cell surface. If Rab25 is necessary for the

recycling of the ß1 integrin, it can be hypothesised that in the absence of Rab25, the ß1 integrin is unable to transit thought the cell to the correct compartment for degradation, leading to increased total cellular protein levels. It is unlikely that Rab25 is the only pathway by which ß1 integrin is trafficked to the cell surface, and the increase in cellular protein levels of ß1 integrin will make more ß1 integrin available to other trafficking pathways and hence the small increase in surface ß1 integrin levels might be explained. Further work is required to examine this hypothesis more closely and to establish the mechanism by which knockdown of Rab25 expression results in the increase in intracellular ß1 integrin protein levels.

It would also be interesting to perform immunohistochemistry of the breast cancer TMA for ß1 integrin, to ascertain if the inverse relationship between Rab25 and ß1 integrin seen in the MCF7 KD cells is replicated in breast cancer tissue.

A further disadvantage of using the MCF7 cell line is that the cells do not invade in standard invasion assays and they do not form xenografts in nude mice in the absence of exogenous oestrogen supplementation. The animal licensing applications in place in the Beatson Institute for Cancer Research at the time of this work did not permit exogenous oestrogen supplementation of nude mice. Xenografts using the MCF7 Rab25 KD and control cell were attempted without oestrogen supplements, but were unsuccessful. Further work to investigate the behaviour of MCF7 Rab25 KD cells in xenograft models, would help to establish if the loss of Rab25 has any effect on cell growth *in vivo.* Knockdown of Rab25 in a cell line which can invade a matrix would also permit investigation of the effect of changes in growth conditions, such as the effect of exogenous factors such as EGF and ß1 integrin inhibitory antibodies, on the ability of cells to invade.

In conclusion, the work presented here suggests that the loss of Rab25 in patients with ER negative/Her2 negative breast cancers confers a negative effect on the survival of these patients. The mechanisms by which loss of Rab25 in these patients results in an absolute reduction in survival of 4.2 years when compared with patients with ER negative/Her2 negative cancers which have retained expression of Rab25, is not yet clear. One hypothesis is that the loss of

Rab25 results in the perturbation of ß1 integrin signalling within tumour cells, resulting in a more aggressive cancer.

Given that Rab25 is an intracellular protein without surface expression, it would seem difficult, with current pharmacological methods, to envisage Rab25 as a direct therapeutic target. However, if further work on the effect of Rab25 loss confirms a demonstrable effect on downstream effectors such as ß1 integrin or EGFR, then there may be indirect therapeutic targets which can be manipulated to improve the survival of this group of patients, for whom the outcome currently is poor.

7 References

1. www.info.cancerresearchuk.org/cancerstats. [cited; Available from:

2. Andre F, Slimane K, Bachelot T, Dunant A, Namer M, Barrelier A, et al. Breast cancer with synchronous metastases: trends in survival during a 14-year period. J Clin Oncol. 2004 Aug 15;22(16):3302-8.

3. D'Eredita G, Giardina C, Martellotta M, Natale T, Ferrarese F. Prognostic factors in breast cancer: the predictive value of the Nottingham Prognostic Index in patients with a long-term follow-up that were treated in a single institution. Eur J Cancer. 2001 Mar;37(5):591-6.

4. Olivotto IA, Bajdik CD, Ravdin PM, Speers CH, Coldman AJ, Norris BD, et al. Population-based validation of the prognostic model ADJUVANT! for early breast cancer. J Clin Oncol. 2005 Apr 20;23(12):2716-25.

5. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004 Dec 30;351(27):2817-26.

6. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol. 2006 Aug 10;24(23):3726-34.

7. Carter CL, Allen C, Henson DE. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. Cancer. 1989 Jan 1;63(1):181-7.

8. Bloom HJ, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer. 1957 Sep;11(3):359-77.

9. Molino A, Pedersini R, Micciolo R, Frisinghelli M, Giovannini M, Pavarana M, et al. Prognostic significance of laminin, laminin receptor, and bone marrow

micrometastases in breast cancer patients: are these markers of aggressive behavior and metastatic potential? Appl Immunohistochem Mol Morphol. 2003 Dec;11(4):311-8.

10. Giuliano AE, Kirgan DM, Guenther JM, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann Surg. 1994 Sep;220(3):391-8; discussion 8-401.

11. Ashikaga T, Krag DN, Land SR, Julian TB, Anderson SJ, Brown AM, et al. Morbidity results from the NSABP B-32 trial comparing sentinel lymph node dissection versus axillary dissection. J Surg Oncol. Aug 1;102(2):111-8.

12. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. Jun;17(6):1471-4.

13. Tan LK, Giri D, Hummer AJ, Panageas KS, Brogi E, Norton L, et al. Occult axillary node metastases in breast cancer are prognostically significant: results in 368 node-negative patients with 20-year follow-up. J Clin Oncol. 2008 Apr 10;26(11):1803-9.

14. Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, et al. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst. 2002 Oct 2;94(19):1494-503.

15. Leake R, Barnes D, Pinder S, Ellis I, Anderson L, Anderson T, et al. Immunohistochemical detection of steroid receptors in breast cancer: a working protocol. UK Receptor Group, UK NEQAS, The Scottish Breast Cancer Pathology Group, and The Receptor and Biomarker Study Group of the EORTC. J Clin Pathol. 2000 Aug;53(8):634-5.

16. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab. 1997 Dec;82(12):4258-65.

17. Klein-Hitpass L, Schorpp M, Wagner U, Ryffel GU. An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. Cell. 1986 Sep 26;46(7):1053-61.

18. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long term tissue culture. Nature. 1975 Aug 14;256(5518):592-3.

19. Pascual MR, Macias A, Moreno L, Lage A. Factors associated with prognosis in human breast cancer. V. The simultaneous use of estrogen and progesterone receptor measurements for prediction of short-term relapse. Neoplasma. 1985;32(2):247-56.

20. Kinsel LB, Szabo E, Greene GL, Konrath J, Leight GS, McCarty KS, Jr. Immunocytochemical analysis of estrogen receptors as a predictor of prognosis in breast cancer patients: comparison with quantitative biochemical methods. Cancer Res. 1989 Feb 15;49(4):1052-6.

21. Delozier T, Julien JP, Juret P, Veyret C, Couette JE, Graic Y, et al. Adjuvant tamoxifen in postmenopausal breast cancer: preliminary results of a randomized trial. Breast Cancer Res Treat. 1986;7(2):105-9.

22. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet. 1998 May 16;351(9114):1451-67.

23. Berry DA, Cirrincione C, Henderson IC, Citron ML, Budman DR, Goldstein LJ, et al. Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. Jama. 2006 Apr 12;295(14):1658-67.

24. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987 Jan 9;235(4785):177-82.

25. Winstanley J, Cooke T, Murray GD, Platt-Higgins A, George WD, Holt S, et al. The long term prognostic significance of c-erbB-2 in primary breast cancer. Br J Cancer. 1991 Mar;63(3):447-50.

26. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science. 1985 Dec 6;230(4730):1132-9.

27. Klapper LN, Glathe S, Vaisman N, Hynes NE, Andrews GC, Sela M, et al. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):4995-5000.

28. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol. 1996 Oct;16(10):5276-87.

29. Bates M KW, Fuchs E-M, Sperinde J, Leitzel K, Ali S, Weidler J, Wu YC, Chappey C, Huang W, Lipton A. Quantitative HER2 homodimer levels correlate with time to first recurrence in HER2-positive breast cancer patients who did not receive trastuzumab in the adjuvant setting. Abstract 1074 31st San Antonio Breast Cancer Symposium. 2008.

30. Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, et al. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. Mar 1;71(5):1871-82.

31. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell. 1990 Apr 20;61(2):203-12.

32. Bos JL. ras oncogenes in human cancer: a review. Cancer Res. 1989 Sep 1;49(17):4682-9.

33. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004 Apr;30(2):193-204.

34. Adjuvant systemic therapy for women with node-negative breast cancer. The Steering Committee on Clinical Practice Guidelines for the Care and Treatment of Breast Cancer. Cmaj. 1998 Feb 10;158 Suppl 3:S43-51.

35. Paik S, Bryant J, Park C, Fisher B, Tan-Chiu E, Hyams D, et al. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J Natl Cancer Inst. 1998 Sep 16;90(18):1361-70.

36. Paik S, Bryant J, Tan-Chiu E, Yothers G, Park C, Wickerham DL, et al. HER2 and choice of adjuvant chemotherapy for invasive breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-15. J Natl Cancer Inst. 2000 Dec 20;92(24):1991-8.

37. Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, et al. Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. J Clin Oncol. 1992 Jul;10(7):1049-56.

38. Miles DW, Harris WH, Gillett CE, Smith P, Barnes DM. Effect of c-erbB(2) and estrogen receptor status on survival of women with primary breast cancer treated with adjuvant cyclophosphamide/methotrexate/fluorouracil. Int J Cancer. 1999 Aug 20;84(4):354-9.

39. Menard S, Valagussa P, Pilotti S, Gianni L, Biganzoli E, Boracchi P, et al. Response to cyclophosphamide, methotrexate, and fluorouracil in lymph node-positive breast cancer according to HER2 overexpression and other tumor biologic variables. J Clin Oncol. 2001 Jan 15;19(2):329-35.

40. Henderson IC, Berry DA, Demetri GD, Cirrincione CT, Goldstein LJ, Martino S, et al. Improved outcomes from adding sequential Paclitaxel but not from escalating Doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. J Clin Oncol. 2003 Mar 15;21(6):976-83.

41. Hayes DF, Thor AD, Dressler LG, Weaver D, Edgerton S, Cowan D, et al. HER2 and response to paclitaxel in node-positive breast cancer. N Engl J Med. 2007 Oct 11;357(15):1496-506.

42. Konecny G, Fritz M, Untch M, Lebeau A, Felber M, Lude S, et al. HER-2/neu overexpression and in vitro chemosensitivity to CMF and FEC in primary breast cancer. Breast Cancer Res Treat. 2001 Sep;69(1):53-63.

43. Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. Pharmacol Ther. 2003 Aug;99(2):167-81.

44. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene. 1995 Jun 15;10(12):2435-46.

45. De Laurentiis M, Arpino G, Massarelli E, Ruggiero A, Carlomagno C, Ciardiello F, et al. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. Clin Cancer Res. 2005 Jul 1;11(13):4741-8.

46. Carlomagno C, Perrone F, Gallo C, De Laurentiis M, Lauria R, Morabito A, et al. c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J Clin Oncol. 1996 Oct;14(10):2702-8.

47. Borg A, Baldetorp B, Ferno M, Killander D, Olsson H, Ryden S, et al. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. Cancer Lett. 1994 Jun 30;81(2):137-44.

48. Dowsett M, Houghton J, Iden C, Salter J, Farndon J, A'Hern R, et al. Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. Ann Oncol. 2006 May;17(5):818-26.

49. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005 Oct 20;353(16):1659-72.

50. Seidman AD, Fornier MN, Esteva FJ, Tan L, Kaptain S, Bach A, et al. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. J Clin Oncol. 2001 May 15;19(10):2587-95.

51. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005 Oct 20;353(16):1673-84.

52. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, Alanko T, Kataja V, Asola R, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. N Engl J Med. 2006 Feb 23;354(8):809-20.

53. Slamon D EW, Robert N, et al. Phase III trial comparing AC-T with AC-TH and with TCH in the adjuvant treatment of HER2 positive early breast cancer: first interim efficacy analysis. Presented at the 28th Annual San Antonio Breast Cancer Symposium. December 8–11, 2005; San Antonio, Tex Abstract 1; 2005; 2005.

54. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res. 2005 Aug 15;11(16):5678-85.

55. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10869-74.

56. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000 Aug 17;406(6797):747-52.

57. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009 Mar 10;27(8):1160-7.

58. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A. 2003 Jul 8;100(14):8418-23.

59. Kaplan HG, Malmgren JA. Impact of triple negative phenotype on breast cancer prognosis. Breast J. 2008 Sep-Oct;14(5):456-63.

60. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. Jama. 2006 Jun 7;295(21):2492-502.

61. Kordon EC, Smith GH. An entire functional mammary gland may comprise the progeny from a single cell. Development. 1998 May;125(10):1921-30.

62. Stingl J, Eaves CJ, Zandieh I, Emerman JT. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat. 2001 May;67(2):93-109.

63. Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, et al. Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst. 2006 Jul 19;98(14):1011-4.

64. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007 Aug 1;13(15 Pt 1):4429-34.

65. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. Cancer. 2007 May 1;109(9):1721-8.

66. Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. Cancer. 2007 Jan 1;109(1):25-32.

67. Tan DS, Marchio C, Jones RL, Savage K, Smith IE, Dowsett M, et al. Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. Breast Cancer Res Treat. 2008 Sep;111(1):27-44.

68. Tischkowitz M, Brunet JS, Begin LR, Huntsman DG, Cheang MC, Akslen LA, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC Cancer. 2007;7:134.

69. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004 Aug 15;10(16):5367-74.

70. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res. 2008 Mar 1;14(5):1368-76.

71. Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. J Clin Oncol. 2004 Mar 1;22(5):777-84.

72. Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. J Clin Oncol. 2004 Mar 1;22(5):785-94.

73. Bell DW, Lynch TJ, Haserlat SM, Harris PL, Okimoto RA, Brannigan BW, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol. 2005 Nov 1;23(31):8081-92.

74. Milgrom E, Thi L, Atger M, Baulieu EE. Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. J Biol Chem. 1973 Sep 25;248(18):6366-74.

75. Horwitz KB, McGuire WL. Progesterone and progesterone receptors in experimental breast cancer. Cancer Res. 1977 Jun;37(6):1733-8.

76. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. Am J Clin Pathol. 2003 Jul;120(1):86-92.

77. Cheang MC, Treaba DO, Speers CH, Olivotto IA, Bajdik CD, Chia SK, et al. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. J Clin Oncol. 2006 Dec 20;24(36):5637-44.

78. Rhodes A, Jasani B, Barnes DM, Bobrow LG, Miller KD. Reliability of immunohistochemical demonstration of oestrogen receptors in routine practice: interlaboratory variance in the sensitivity of detection and evaluation of scoring systems. J Clin Pathol. 2000 Feb;53(2):125-30.

79. Bartlett JM, Ibrahim M, Jasani B, Morgan JM, Ellis I, Kay E, et al. External quality assurance of HER2 FISH and ISH testing: three years of the UK national external quality assurance scheme. Am J Clin Pathol. 2009 Jan;131(1):106-11.

80. Pichon MF, Milgrom E. Oestrogen receptor negative-progesterone receptor positive phenotype in 1,211 breast tumours. Br J Cancer. 1992 Jun;65(6):895-7.

81. Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast Cancer Res. 2007;9(1):R6.

82. Bernoux A, de Cremoux P, Laine-Bidron C, Martin EC, Asselain B, Magdelenat H. Estrogen receptor negative and progesterone receptor positive primary breast cancer: pathological characteristics and clinical outcome. Institut

Curie Breast Cancer Study Group. Breast Cancer Res Treat. 1998 Jun;49(3):219-25.

83. De Maeyer L, Van Limbergen E, De Nys K, Moerman P, Pochet N, Hendrickx W, et al. Does estrogen receptor negative/progesterone receptor positive breast carcinoma exist? J Clin Oncol. 2008 Jan 10;26(2):335-6; author reply 6-8.

84. Nuoffer C, Balch WE. GTPases: multifunctional molecular switches regulating vesicular traffic. Annu Rev Biochem. 1994;63:949-90.

85. Novick P, Zerial M. The diversity of Rab proteins in vesicle transport. Curr Opin Cell Biol. 1997 Aug;9(4):496-504.

86. Soldati T, Riederer MA, Pfeffer SR. Rab GDI: a solubilizing and recycling factor for rab9 protein. Mol Biol Cell. 1993 Apr;4(4):425-34.

87. Soldati T, Shapiro AD, Svejstrup AB, Pfeffer SR. Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. Nature. 1994 May 5;369(6475):76-8.

88. Ullrich O, Horiuchi H, Bucci C, Zerial M. Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature. 1994 Mar 10;368(6467):157-60.

89. Kishida S, Shirataki H, Sasaki T, Kato M, Kaibuchi K, Takai Y. Rab3A GTPase-activating protein-inhibiting activity of Rabphilin-3A, a putative Rab3A target protein. J Biol Chem. 1993 Oct 25;268(30):22259-61.

90. Plutner H, Cox AD, Pind S, Khosravi-Far R, Bourne JR, Schwaninger R, et al. Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. J Cell Biol. 1991 Oct;115(1):31-43.

91. Tisdale EJ, Bourne JR, Khosravi-Far R, Der CJ, Balch WE. GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the

endoplasmic reticulum to the Golgi complex. J Cell Biol. 1992 Nov;119(4):749-61.

92. Jones SM, Crosby JR, Salamero J, Howell KE. A cytosolic complex of p62 and rab6 associates with TGN38/41 and is involved in budding of exocytic vesicles from the trans-Golgi network. J Cell Biol. 1993 Aug;122(4):775-88.

93. Huber LA, Pimplikar S, Parton RG, Virta H, Zerial M, Simons K. Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J Cell Biol. 1993 Oct;123(1):35-45.

94. Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell. 1990 Jul 27;62(2):317-29.

95. Bielli A, Thornqvist PO, Hendrick AG, Finn R, Fitzgerald K, McCaffrey MW. The small GTPase Rab4A interacts with the central region of cytoplasmic dynein light intermediate chain-1. Biochem Biophys Res Commun. 2001 Mar;281(5):1141-53.

96. Sogaard M, Tani K, Ye RR, Geromanos S, Tempst P, Kirchhausen T, et al. A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell. 1994 Sep 23;78(6):937-48.

97. Cao X, Ballew N, Barlowe C. Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. Embo J. 1998 Apr 15;17(8):2156-65.

98. Simons K, Wandinger-Ness A. Polarized sorting in epithelia. Cell. 1990 Jul 27;62(2):207-10.

99. Goldenring JR, Shen KR, Vaughan HD, Modlin IM. Identification of a small GTP-binding protein, Rab25, expressed in the gastrointestinal mucosa, kidney, and lung. J Biol Chem. 1993 Sep 5;268(25):18419-22.

100. Lai F, Stubbs L, Artzt K. Molecular analysis of mouse Rab11b: a new type of mammalian YPT/Rab protein. Genomics. 1994 Aug;22(3):610-6.

101. Casanova JE, Wang X, Kumar R, Bhartur SG, Navarre J, Woodrum JE, et al. Association of Rab25 and Rab11a with the apical recycling system of polarized Madin-Darby canine kidney cells. Mol Biol Cell. 1999 Jan;10(1):47-61.

102. Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol. 1996 Nov;135(4):913-24.

103. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. Nature. 1991 Jan 10;349(6305):117-27.

104. Adari H, Lowy DR, Willumsen BM, Der CJ, McCormick F. Guanosine triphosphatase activating protein (GAP) interacts with the p21 ras effector binding domain. Science. 1988 Apr 22;240(4851):518-21.

105. Bhartur SG, Calhoun BC, Woodrum J, Kurkjian J, Iyer S, Lai F, et al. Genomic structure of murine Rab11 family members. Biochem Biophys Res Commun. 2000 Mar 16;269(2):611-7.

106. Kudoh K, Takano M, Koshikawa T, Hirai M, Yoshida S, Mano Y, et al. Gains of 1q21-q22 and 13q12-q14 are potential indicators for resistance to cisplatin-based chemotherapy in ovarian cancer patients. Clin Cancer Res. 1999 Sep;5(9):2526-31.

107. Cheng KW, Lahad JP, Kuo WL, Lapuk A, Yamada K, Auersperg N, et al. The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers. Nat Med. 2004 Nov;10(11):1251-6.

108. Louro R, Nakaya HI, Amaral PP, Festa F, Sogayar MC, da Silva AM, et al. Androgen responsive intronic non-coding RNAs. BMC Biol. 2007;5:4.

109. Davidson B, Zhang Z, Kleinberg L, Li M, Florenes VA, Wang TL, et al. Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from

diffuse malignant peritoneal mesothelioma. Clin Cancer Res. 2006 Oct 15;12(20 Pt 1):5944-50.

110. Fan Y, Xin XY, Chen BL, Ma X. Knockdown of RAB25 expression by RNAi inhibits growth of human epithelial ovarian cancer cells in vitro and in vivo. Pathology. 2006 Dec;38(6):561-7.

111. Cheng JM, Ding M, Aribi A, Shah P, Rao K. Loss of RAB25 expression in breast cancer. Int J Cancer. 2006 Jun 15;118(12):2957-64.

112. Cheng JM, Volk L, Janaki DK, Vyakaranam S, Ran S, Rao KA. Tumor suppressor function of Rab25 in triple-negative breast cancer. Int J Cancer. 2009 Sep 30.

113. Nam KT, Lee HJ, Smith JJ, Lapierre LA, Kamath VP, Chen X, et al. Loss of Rab25 promotes the development of intestinal neoplasia in mice and is associated with human colorectal adenocarcinomas. J Clin Invest. Mar 1;120(3):840-9.

114. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science. 1992 May 1;256(5057):668-70.

115. Korkola JE, Heck S, Olshen AB, Reuter VE, Bosl GJ, Houldsworth J, et al. In vivo differentiation and genomic evolution in adult male germ cell tumors. Genes Chromosomes Cancer. 2008 Jan;47(1):43-55.

116. Mor O, Nativ O, Stein A, Novak L, Lehavi D, Shiboleth Y, et al. Molecular analysis of transitional cell carcinoma using cDNA microarray. Oncogene. 2003 Oct 23;22(48):7702-10.

117. Watanabe T, Miura T, Degawa Y, Fujita Y, Inoue M, Kawaguchi M, et al. Comparison of lung cancer cell lines representing four histopathological subtypes with gene expression profiling using quantitative real-time PCR. Cancer Cell Int.10:2.

118. Goldenring JR, Ray GS, Lee JR. Rab11 in dysplasia of Barrett's epithelia. Yale J Biol Med. 1999 Mar-Jun;72(2-3):113-20.

119. Caswell PT, Spence HJ, Parsons M, White DP, Clark K, Cheng KW, et al. Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. Dev Cell. 2007 Oct;13(4):496-510.

120. Dazzi H, Hasleton PS, Thatcher N, Barnes DM, Wilkes S, Swindell R, et al. Expression of epidermal growth factor receptor (EGF-R) in non-small cell lung cancer. Use of archival tissue and correlation of EGF-R with histology, tumour size, node status and survival. Br J Cancer. 1989 May;59(5):746-9.

121. Hollstein MC, Smits AM, Galiana C, Yamasaki H, Bos JL, Mandard A, et al. Amplification of epidermal growth factor receptor gene but no evidence of ras mutations in primary human esophageal cancers. Cancer Res. 1988 Sep 15;48(18):5119-23.

122. Kameda T, Yasui W, Yoshida K, Tsujino T, Nakayama H, Ito M, et al. Expression of ERBB2 in human gastric carcinomas: relationship between p185ERBB2 expression and the gene amplification. Cancer Res. 1990 Dec 15;50(24):8002-9.

123. Mayer A, Takimoto M, Fritz E, Schellander G, Kofler K, Ludwig H. The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer. 1993 Apr 15;71(8):2454-60.

124. Yamanaka Y, Friess H, Kobrin MS, Buchler M, Beger HG, Korc M. Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. Anticancer Res. 1993 May-Jun;13(3):565-9.

125. Neal DE, Sharples L, Smith K, Fennelly J, Hall RR, Harris AL. The epidermal growth factor receptor and the prognosis of bladder cancer. Cancer. 1990 Apr 1;65(7):1619-25.

126. Agarwal R, Jurisica I, Mills GB, Cheng KW. The emerging role of the RAB25 small GTPase in cancer. Traffic. 2009 Nov;10(11):1561-8.

127. Caswell PT, Chan M, Lindsay AJ, McCaffrey MW, Boettiger D, Norman JC. Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. J Cell Biol. 2008 Oct 6;183(1):143-55.

128. Hales CM, Griner R, Hobdy-Henderson KC, Dorn MC, Hardy D, Kumar R, et al. Identification and characterization of a family of Rab11-interacting proteins. J Biol Chem. 2001 Oct 19;276(42):39067-75.

129. Zhang J, Liu X, Datta A, Govindarajan K, Tam WL, Han J, et al. RCP is a human breast cancer-promoting gene with Ras-activating function. J Clin Invest. 2009 Aug;119(8):2171-83.

130. Mills GB, Jurisica I, Yarden Y, Norman JC. Genomic amplicons target vesicle recycling in breast cancer. J Clin Invest. 2009 Aug;119(8):2123-7.

131. Subramani D, Alahari SK. Integrin-mediated function of Rab GTPases in cancer progression. Mol Cancer.9:312.

132. Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A. 1992 Oct 1;89(19):9064-8.

133. Schoenenberger CA, Zuk A, Zinkl GM, Kendall D, Matlin KS. Integrin expression and localization in normal MDCK cells and transformed MDCK cells lacking apical polarity. J Cell Sci. 1994 Feb;107 (Pt 2):527-41.

134. Ojakian GK, Schwimmer R. Regulation of epithelial cell surface polarity reversal by beta 1 integrins. J Cell Sci. 1994 Mar;107 (Pt 3):561-76.

135. Fassler R, Meyer M. Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev. 1995 Aug 1;9(15):1896-908.

136. White DE, Kurpios NA, Zuo D, Hassell JA, Blaess S, Mueller U, et al. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell. 2004 Aug;6(2):159-70.

137. Yao ES, Zhang H, Chen YY, Lee B, Chew K, Moore D, et al. Increased beta1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res. 2007 Jan 15;67(2):659-64.

138. Ioachim E, Charchanti A, Briasoulis E, Karavasilis V, Tsanou H, Arvanitis DL, et al. Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression. Eur J Cancer. 2002 Dec;38(18):2362-70.

139. Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med. 1999 Jun;5(6):662-8.

140. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol. 1997 Apr 7;137(1):231-45.

141. Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, et al. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res. 2006 Feb 1;66(3):1526-35.

142. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol. 1992 Mar;12(3):954-61.

143. Kim S, Bell K, Mousa SA, Varner JA. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol. 2000 Apr;156(4):1345-62.

144. Kim S, Bakre M, Yin H, Varner JA. Inhibition of endothelial cell survival and angiogenesis by protein kinase A. J Clin Invest. 2002 Oct;110(7):933-41.

145. J. W. Valle RKR, R. Glynne-Jones, A. Anthoney, J. Berlin, S. Yazji, T. Evans Phase II study of volociximab (M200), an α5ß1 anti-integrin antibody in metastatic adenocarcinoma of the pancreas (MPC). Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I Vol 24, No 18S (June 20 Supplement), 2006: 4111 2006.

146. S. Yazji RB, V. Kondagunta, R. Figlin. Final results from phase II study of volociximab, an α5ß1 anti-integrin antibody, in refractory or relapsed metastatic clear cell renal cell carcinoma (mCCRCC). Journal of Clinical Oncology, 2007 ASCO Annual Meeting Proceedings Part I Vol 25, No 18S (June 20 Supplement), 2007: 5094 2007.

147. Barton J. A multicenter phase II study of volociximab in patients with relapsed metastatic melanoma. J Clin Oncol 26: 2008 (May 20 suppl; abstr 9051). 2008.

148. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 May 1;29(9):e45.

149. Cuatrecasas P, Wilchek M, Anfinsen CB. Selective enzyme purification by affinity chromatography. Proc Natl Acad Sci U S A. 1968 Oct;61(2):636-43.

150. Moudgal NR, Porter RR. The use of antigen-cellulose suspensions for the isolation of specific antibodies. Biochim Biophys Acta. 1963 Apr 2;71:185-7.

151. Van der Sluis PJ, Boer GJ. The relevance of various tests for the study of specificity in immunocytochemical staining: a review. Cell Biochem Funct. 1986 Jan;4(1):1-17.

152. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. J Histochem Cytochem. 1997 Mar;45(3):327-43.

153. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991 Jun;39(6):741-8.

154. Gurkan C, Lapp H, Alory C, Su AI, Hogenesch JB, Balch WE. Large-scale profiling of Rab GTPase trafficking networks: the membrome. Mol Biol Cell. 2005 Aug;16(8):3847-64.

155. Barnes DM, Harris WH, Smith P, Millis RR, Rubens RD. Immunohistochemical determination of oestrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients. Br J Cancer. 1996 Nov;74(9):1445-51.

156. van Diest PJ, Weger DR, Lindholm J. Reproducibility of subjective immunoscoring of steroid receptors in breast cancer. Anal Quant Cytol Histol. 1996 Oct;18(5):351-4.

157. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol. 1999 May;17(5):1474-81.

158. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Specificity of HercepTest in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring system. J Clin Oncol. 1999 Jul;17(7):1983-7.

159. van Diest PJ, van Dam P, Henzen-Logmans SC, Berns E, van der Burg ME, Green J, et al. A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group. J Clin Pathol. 1997 Oct;50(10):801-4.

160. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med. 1998 Jul;4(7):844-7.

161. Rimm DL, Camp RL, Charette LA, Costa J, Olsen DA, Reiss M. Tissue microarray: a new technology for amplification of tissue resources. Cancer J. 2001 Jan-Feb;7(1):24-31.

162. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74.

163. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology. 2006 Jun;48(7):787-94.

164. Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, et al. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim Biophys Sin (Shanghai). 2008 May;40(5):426-36.

165. Fritz P, Wu X, Tuczek H, Multhaupt H, Schwarzmann P. Quantitation in immunohistochemistry. A research method or a diagnostic tool in surgical pathology? Pathologica. 1995 Jun;87(3):300-9.

166. Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E, et al. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res. 2004 Dec 1;64(23):8585-94.

167. Aubele M, Walch AK, Ludyga N, Braselmann H, Atkinson MJ, Luber B, et al. Prognostic value of protein tyrosine kinase 6 (PTK6) for long-term survival of breast cancer patients. Br J Cancer. 2008 Oct 7;99(7):1089-95.

168. Regan MM, Viale G, Mastropasqua MG, Maiorano E, Golouh R, Carbone A, et al. Re-evaluating adjuvant breast cancer trials: assessing hormone receptor status by immunohistochemical versus extraction assays. J Natl Cancer Inst. 2006 Nov 1;98(21):1571-81.

169. Ferrero JM, Ramaioli A, Largillier R, Formento JL, Francoual M, Ettore F, et al. Epidermal growth factor receptor expression in 780 breast cancer patients: a reappraisal of the prognostic value based on an eight-year median follow-up. Ann Oncol. 2001 Jun;12(6):841-6.

170. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst. 1973 Nov;51(5):1409-16.

171. Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem. 1973 Sep 10;248(17):6251-3.

172. Briand P, Lykkesfeldt AE. Effect of estrogen and antiestrogen on the human breast cancer cell line MCF-7 adapted to growth at low serum concentration. Cancer Res. 1984 Mar;44(3):1114-9.

173. Shafie SM, Grantham FH. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst. 1981 Jul;67(1):51-6.

174. Suzuki K, Takahashi K. Reduced substratum adhesion and decreased expressions of 1 and 4 integrins in human breast cancer cells with a property of anchorage-independent growth. Int J Oncol. 1999 May;14(5):897-904.

175. Bon G, Folgiero V, Bossi G, Felicioni L, Marchetti A, Sacchi A, et al. Loss of beta4 integrin subunit reduces the tumorigenicity of MCF7 mammary cells and causes apoptosis upon hormone deprivation. Clin Cancer Res. 2006 Jun 1;12(11 Pt 1):3280-7.

176. Vasaturo F, Malacrino C, Sallusti E, Coppotelli G, Birarelli P, Giuffrida A, et al. Role of extracellular matrix in regulation of staurosporine-induced apoptosis in breast cancer cells. Oncol Rep. 2005 Apr;13(4):745-50.

177. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001 Jan 18;409(6818):363-6.

178. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 2002 Sep 6;110(5):563-74.

179. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 2000 Mar 31;101(1):25-33.

180. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci U S A. 2001 Aug 14;98(17):9742-7.

181. Paul CP, Good PD, Winer I, Engelke DR. Effective expression of small interfering RNA in human cells. Nat Biotechnol. 2002 May;20(5):505-8.

Appendix

Supplementary figure

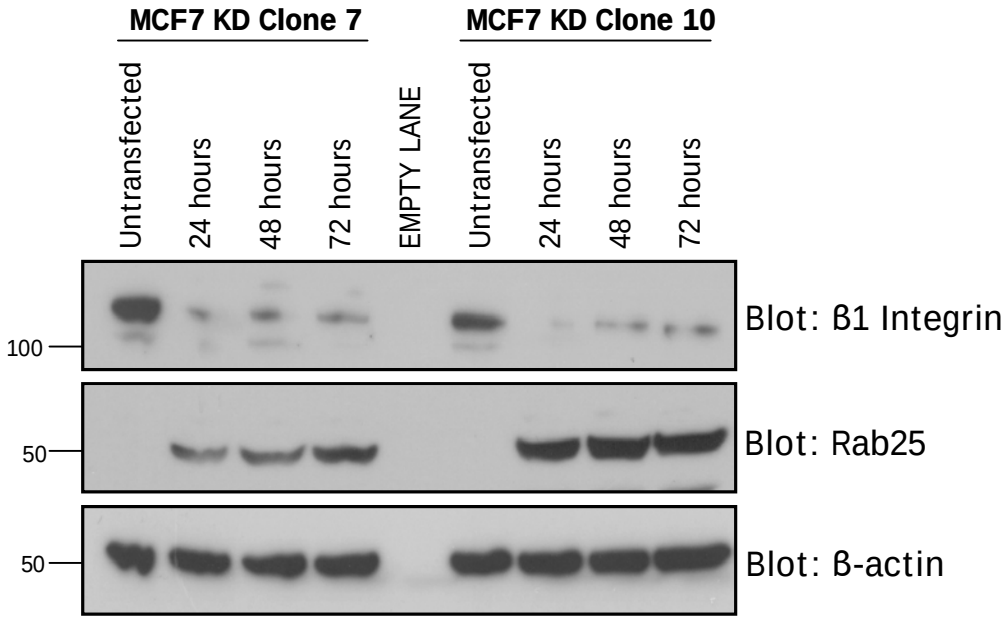


Figure S-1 Effect of exogenous expression of Cherry-Rab25 on β1 integrin levels in MCF7 cells.
Western blots of cell lysates of MCF7 KD clones 7 and 10 transfected with Cherry-Rab25, incubated with anti-β1 integrin antibody (top panel), anti-Rab25 antibody (middle panel) and anti- β-actin antibody (bottom panel).