

A new pathway in the induction of breast cancer

By

Rachel Jane Watkins

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Abstract

The association of breast cancer risk with menarche, menopause and first full-term pregnancy, as well as oral contraceptive use and hormone replacement therapy, suggests a role for hormones in the development of carcinomas. Pituitary tumor transforming gene Binding Factor (PBF) is a relatively uncharacterized gene implicated in endocrine neoplasia. Given the presence of putative oestrogen response elements (ERE) in its promoter, we assessed PBF regulation by oestrogen. PBF mRNA and protein expression were induced by both diethylstilbestrol and 17 β -estradiol in ER α -positive MCF-7 cells. Close analysis of the PBF promoter showed that the region -399 to -291 relative to the translational start site contains variable repeats of an 18bp sequence housing a putative ERE half-site (gccctc**GGTCA**gcctc). Sequencing the PBF promoter from 122 normal subjects revealed that individuals may be homozygous or heterozygous for between 1 and 6 repeats of the ERE. PBF expression was low or absent in normal breast tissue, but highly expressed in breast tumours. Individuals with greater numbers of repeats demonstrated higher PBF mRNA expression, and protein expression positively correlated with ER α status. Cell invasion assays revealed that PBF induces invasion through Matrigel, an action that could be abrogated both by siRNA treatment and specific mutation. Further, PBF is a secreted protein, and loss of secretion prevents PBF inducing cell invasion. Given that PBF is a potent transforming gene, these data suggest that oestrogen treatment in post-menopausal women may up-regulate PBF expression, leading to PBF secretion and increased cell invasion. Further, the number of ERE half sites in the PBF promoter may significantly alter the response to oestrogen treatment in individual subjects.

Dedication

To my parents

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Chapter 1

General Introduction

1.1 Breast cancer

Breast cancer remains the most common malignancy in women worldwide and is the leading cause of cancer-related mortality in females. More than 1.2 million cases are diagnosed each year, affecting 10-12 % of the female population and accounting for 500,000 deaths per year worldwide. Breast cancer is mainly a postmenopausal disease, with more than three-quarters of tumours being hormone responsive (Benson et al. 2009).

1.1.1 Pathology of breast cancer

1.1.1.1 Normal breast

The normal breast is composed of glandular structures that ensure milk production under hormonal control. The glandular tissue itself is composed of ducts, lobules and acini (Figure 1-1). Ducts function to transport milk from the acini to the nipple. There are approximately 15-20 large ducts per breast which divide into ever smaller ducts, this network constitutes the ductal tree. Histologically they are comprised of a single layer of epithelial cells lining the lumen and an external layer of myoepithelial cells around the basal membrane. Mammary lobules are found at the end of ducts and function as units of the breast ensuring the synthesis of milk. Each lobule consists of about twenty small glandular structures, called acini which open into the terminal duct. Like ducts, each acinus is double layered with the epithelial layer lining the lumen and the myoepithelial layer lining the basal membrane. Ducts and mammary lobules are surrounded by connective tissue composed of blood and lymphatic vessels, nerves, adipose and fibrous tissue (Guinebretiere et al. 2005).

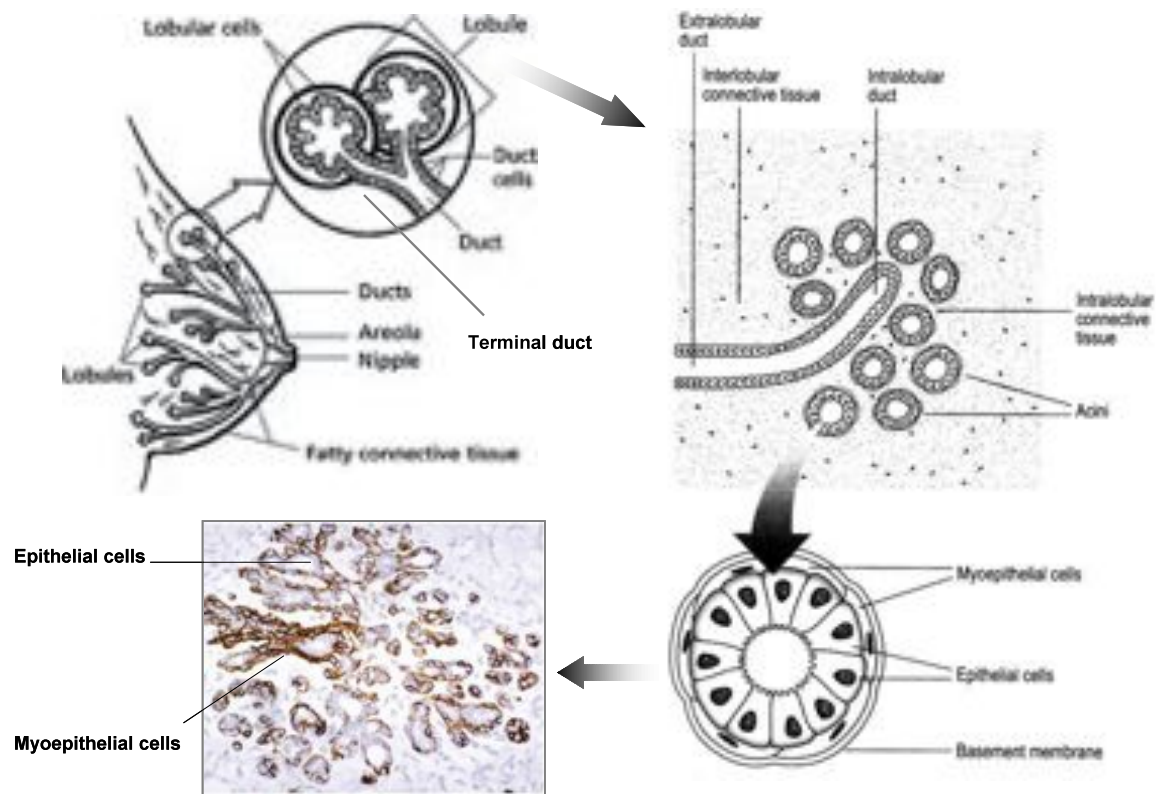


Figure 1-1. A schematic representation of the human breast, showing the organisation of ducts, lobules, acini and connective tissue. Taken and adapted from <http://freefrombreastcancer.blogspot.com>.

1.1.1.2 Non-invasive carcinomas

In non-invasive carcinomas (otherwise known as in situ carcinomas), malignant cells are enclosed in the normal structures, ducts or lobules, whose basal membrane persists. Two types are distinguishable according to the cytology and location of the proliferation. Ductal carcinomas in situ (DCIS), and lobular carcinomas in-situ (LCIS) (Guinebretiere et al. 2005).

A few decades ago, DCIS was considered rare and patients presented with a nipple discharge (Guinebretiere et al. 2005). Incidence rates of DCIS have risen dramatically in the United States and elsewhere since the early 1980's. This increase parallels an increase in the incidence of screening mammography which makes the detection of tumours more

likely than in the past (Ernster et al. 2002). DCIS can occur in both pre- and post-menopausal women. It is usually unifocal, being confined within one quadrant of the breast, although multicentricity can occur with larger lesions. DCIS is considered to be pre-malignant and over time may progress to invasive ductal carcinoma (Ogbagabriel et al. 2005; R.A.Walker 2004; William L.Mcguire M.D (ed) 1977).

LCIS occurs predominantly in pre-menopausal women and the number of women diagnosed with it has also increased in recent years (Afonso and Bouwman 2008). If it is found after the menopause it is usually associated with an invasive tumour. It is often multifocal within one breast and is frequently bilateral (R.A.Walker 2004; William L.Mcguire M.D (ed) 1977).

1.1.1.3 Invasive carcinomas

Invasive ductal carcinomas are the most common histological type of mammary carcinoma and represent more than 75 % of all invasive cases (Guinebretiere et al. 2005). They can occur in both pre and post menopausal women. The tumours are devoid of special features which are characteristic of other types of breast cancer (R.A.Walker 2004; William L.Mcguire M.D (ed) 1977).

The second most frequent type is invasive lobular carcinoma, which constitutes about 10 % of invasive breast carcinomas. Like invasive ductal carcinomas they can occur in pre- and post- menopausal women (Guinebretiere et al. 2005; Ogbagabriel et al. 2005). As its name suggests this tumour arises in the terminal parts of the lobule and is associated with a relatively poor prognosis (Ogbagabriel et al. 2005). While infiltrating ductal carcinomas usually originate at one focus in the breast, infiltrating lobular carcinoma can be multifocal throughout the breast. Histologically the cells are small and

uniform and are dispersed singly. A characteristic feature of these tumours is that the cells lack the cell adhesion molecule E-cadherin, which may account for them being dispersed singly (R.A.Walker 2004;William L.Mcguire M.D (ed) 1977).

1.1.2 Risk factors for the development of breast cancers

1.1.2.1 Female gender and age

Less than 1 % of all breast cancers occur in men, so female gender is an important risk factor (R.A.Walker 2004). As with most carcinomas, increasing age is another significant factor (Benson et al. 2009;R.A.Walker 2004). Up to the age of 40-45 years, the rate of increase is steep. Around the age of 50 years, corresponding to the average age of menopause, the rate of increase slows down and levels to a plateau after 80 years (Benson et al. 2009;R.A.Walker 2004). The incidences of oestrogen-receptor positive and negative tumours are similar up to the age of 50; however after this point the rate of increase of oestrogen-receptor negative tumours decreases significantly compared to oestrogen receptor positive tumours. Thus oestrogen-receptor negative tumours tend to occur earlier in life and oestrogen-receptor positive tumours are more common in older women (Figure 1-2) (Benson et al. 2009).

Figure 1-2 Age specific incidence rates (Surveillance, Epidemiology and End Results program of the National Cancer Institute(SEER) Database) in female patients with breast cancer. Taken from (Benson et al. 2009).

1.1.2.2 Age at menarche and menopause

There is significantly higher risk of developing breast cancer among women with an early age at menarche (R.A.Walker 2004). At the other end of the reproductive life, women whose natural menopause occurs before 45 years have only half the breast cancer risk of those who became menopausal after 55 years. Therefore women with 40 or more years of active menstruation have twice the breast cancer risk of those with less than 30 year of menstrual activity (R.A.Walker 2004).

1.1.2.3 Age at first full-term pregnancy

Nulliparous women (women who have never given birth to a child) have an increased risk of developing breast cancer. However, among parous women (women who

have given birth one or more times) protection is related to early age for the first full-term pregnancy. Women, in whom the first birth is delayed to the mid or late thirties, are at greater risk of developing breast cancer than nulliparous women (Ewertz et al. 1990; R.A. Walker 2004).

1.1.2.4 Weight, diet and breast cancer

Body mass index (BMI) [$\text{weight (kg)} / \text{height (m)}^2$] in the studies below was evaluated both as a continuous variable and/or was categorised according to the World Health Organisation guidelines. The definition in these guidelines are: underweight, $<18.5 \text{ kg/m}^2$, normal range between 18.5 and 24.0 kg/m^2 , grade 1 overweight between 25.0 and 29.9 kg/m^2 , grade 2 overweight between 30.0 and 39.9 kg/m^2 , and grade 3 overweight $>40.0 \text{ kg/m}^2$. In some studies there were too few patients in some of these groups so three levels of BMI were used to categorise women: ideal weight ($\text{BMI} < 24.9 \text{ kg/m}^2$), overweight ($\text{BMI } 25\text{-}29.9 \text{ kg/m}^2$) and obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) (Berclaz et al. 2004; Cleveland et al. 2007). For pre-menopausal women of above average weight ($\geq 30 \text{ kg/m}^2$) there is no increased risk of developing breast cancer when compared to women with a BMI between $20\text{-}25 \text{ kg/m}^2$ (Weiderpass et al. 2004). However, overweight ($25\text{-}29.9 \text{ kg/m}^2$) and obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) postmenopausal women are at higher risk of developing breast cancer (Montazeri et al. 2008). Obesity is also associated with greater tumour diameter ($\geq 2 \text{ cm}$), lymph node involvement and decreased survival in women diagnosed with breast cancer (Berclaz et al. 2004; Cleveland et al. 2007).

Diet is an obvious determinant of body weight. In rodents, a high fat diet increases the incidence of breast tumours (R.A. Walker 2004), and populations with high fat intakes generally have high rates of breast cancer (Key et al. 2003). Although these observations

suggest that a high fat diet may be a risk factor for breast cancer, the evidence is not as clear as it is for weight. Studies of individual women have not confirmed an association of high fat diets with breast cancer rates (Key et al. 2003). Insulin resistance which is characterised by hyperinsulinemia has been postulated as a risk factor for post-menopausal breast cancer (Stoll 1996).

1.1.2.5 Family history and genetic factors

Breast cancer is common, thus a history of a relative having breast cancer can be found in at least 10% of new cases. However, a large proportion of these will be sporadic cancers and not due to inherited genetic factors (R.A.Walker 2004). The risk of developing breast cancer is increased in first degree relatives of women with breast cancer, particularly if the index case is pre-menopausal. For example, the risk increases nine fold for first degree relatives of pre-menopausal women with bilateral breast cancer, although women with first-degree relatives with post-menopausal breast cancer have only a five fold increase in risk (R.A.Walker 2004).

Mutations within BRAC1 and BRAC2, genes involved in DNA repair mechanisms account for around three-quarters of hereditary breast cancer cases and confer a lifetime risk of between 80-85% by the age of 70 (Benson et al. 2009). There are also rare familial syndromes such as Li-Fraumeni, in which there is an association between sarcomas, brain tumours and breast cancer at a young age. This is linked in some families to abnormalities in the p53 gene (R.A.Walker 2004). Low penetrance genes such as CHEK mutations might collectively be responsible for up to 25% of familial cases; although they confer a reduced risk compared to the higher penetrance genes, they are more prevalent within the population (Benson et al. 2009)

1.1.2.6 Oral contraceptives/hormone replacement therapy

Contraceptive preparations with low oestrogen to progesterone ratios are currently considered safe and may even be protective for breast cancer (R.A.Walker 2004). However, women who recently used oral contraceptives containing more than 35 µg of ethinyl oestradiol per pill were found to be at a higher risk of breast cancer than users of lower dose preparations (≤ 35 µg of ethinyl oestradiol). This relationship was more marked among women below 35 years of age (Althuis et al. 2003)

In postmenopausal women the ovaries stop producing oestrogen and hormone replacement therapy (HRT) was introduced in the 1930s to reduce symptoms resulting from oestrogen depletion. These symptoms include hot flushes, sleeplessness, lethargy, depression, urogenital atrophy and vaginal dryness (Anon 2008). Early reports indicated that HRT was associated with significantly increased ratios of endometrial cancer and this led to the addition of progesterone to oestrogen containing preparations in order to control this problem (Cuzick 2008). The first reports of an increase in the risk of breast cancer amongst women on oestrogen only HRT came in 1976 from Hoover et al (Hoover et al. 1976). Subsequently the million women study showed that breast cancer risk is significantly increased for users of combined oestrogen/ progesterone preparations when compared with those having oestrogen only preparations (Cuzick 2008). A further increased risk with combined oestrogen-progesterone HRT compared to oestrogen alone indicates a potential significant role of progesterone signalling breast cancer development (Cork et al. 2008).

1.1.2.7 Hormones

The association of breast cancer risk with menarche, menopause and first full-term pregnancy as well as oral contraceptives and hormone replacement therapy indicates that hormones are likely to play a role in the development of carcinomas.

Early menarche and late menopause result in a higher number of menstrual cycles, with repeated surges of oestrogen and progesterone. This cyclical release of oestrogen and progesterone stimulates the primitive ducts of the immature breast to differentiate into terminal end buds, which branch into aveola buds and oestrogen and progesterone have a stimulatory effect on breast epithelium (Cork et al. 2008). The beneficial effects of early full-term pregnancy could be due to high concentrations of progesterone and/or prolactin protecting the breast cells from oestrogen in the long term (Benson et al. 2009;R.A.Walker 2004). The risks associated with obesity may be partly due to the ability of fat cells to synthesis oestrogens (Cleary and Grossmann 2009;R.A.Walker 2004)

1.1.2.7.1 Oestrogen

Oestrogens influence the growth, differentiation and function of tissues of the female reproductive system, i.e the uterus, ovary, and breast. The effects of oestrogens in tissues are mediated by oestrogen receptor alpha (ER α) and oestrogen receptor beta (ER β), which are expressed to varying extents in most organs (Fritz F.Parl 2000). This will be discussed in more detail in section 1.1.2.7.3. All naturally occurring oestrogens have an unsaturated (aromatic) A ring, a phenolic hydroxyl group at C-3 and a methyl group at position C-13 (Figure 1-3). 17 β -estradiol is the most potent oestrogen, followed by estrone and then estriol (Fritz F.Parl 2000).

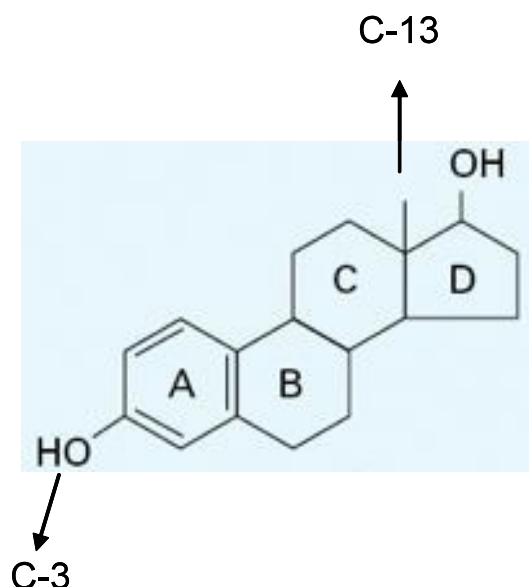


Figure 1-3. Structure of 17β -estradiol. Like all naturally occurring oestrogens it contains an unsaturated (aromatic) A ring, a phenolic hydroxyl group at C-3 and a methyl group at C-13.

The biological actions of oestrogens are regulated by their concentration in the circulation and their intracellular conversion into more or less active derivatives (Fritz F.Parl 2000). The source of circulating oestrogens in premenopausal women is predominantly the ovary and uptake of oestrogen from the circulation is the primary mechanism for maintenance of oestrogen concentrations in breast cancer tissue. The source of oestrogen production changes dramatically in postmenopausal women as most of the oestrogens are formed in peripheral tissues, especially in adipose tissue (Cleary and Grossmann 2009;Fritz F.Parl 2000).

1.1.2.7.2 Progesterone

Progesterone also known as P4 (pregn-4-ene3, 20-dione) is a C21 steroid hormone that is essential for normal breast development during puberty and in preparation for lactation and breast feeding. Progesterone is also involved in the female menstrual cycle

and embryogenesis (Lange and Yee 2008). The effects of progesterones are mediated by progesterone receptor A (PR-A), progesterone receptor B (PR-B) or progesterone receptor C (PR-C), and will be discussed in more detail in section 1.1.2.7.4 (Cork et al. 2008)

1.1.2.7.3 Oestrogen receptor

Hormones have an effect on cells only after interacting with specific receptors present on or in target cells. The ER is a member of the nuclear receptor superfamily for steroid and thyroid hormones, vitamin D, retinoids, and prostanoids. The ER α gene is located at 6q25.1 and extends over 140 kb. It contains eight exons and encodes a protein of 595 amino acids (Fritz F.Parl 2000). The ER, like other steroid hormone receptors, is a modular protein with distinct, largely autonomous domains having specific functions such as ligand binding, dimerisation, DNA binding and transactivation. In addition to a centrally located DNA binding domain, domain C, the ER contains two distinct transactivation domains. An N-terminal A/B domain containing activation function 1 (AF1) and a hormone dependant activation function (AF2) located in the E domain along with the ligand binding function of the ER. AF1 and AF2 work in a synergistic manner and are both required for full ER activity (Katzenellenbogen et al. 2001). The two remaining regions D and F are of variable size. D can be considered as a linker peptide between the DNA binding domain and the ligand binding domain, whereas F is a C-terminal extension region of the LBD (Ruff et al. 2000) (Figure 1-4).

In 1996 a second ER gene, ER β , was identified and localised to chromosome 14q22-24. Both ER genes show similar functional organisation and varying degrees of homology. The DNA binding Domain, domain C of ER β , is highly homologous to ER α , differing by only 2 amino acids. The ligand binding domain, domain E of ER β , shows 59 % identity to ER α ; however the A/B domain, the hinge region D and the F domain are not

conserved (Fritz F.Parl 2000;Ruff et al. 2000). Because of the differences in the ligand binding domain, ER α and ER β show different affinity for ligands. In addition the difference in the A/B domains, which houses AF-1 explains why the transcriptional activation of different oestrogen-responsive genes by ER α and β show distinctly different patterns. (Katzenellenbogen et al. 2001).

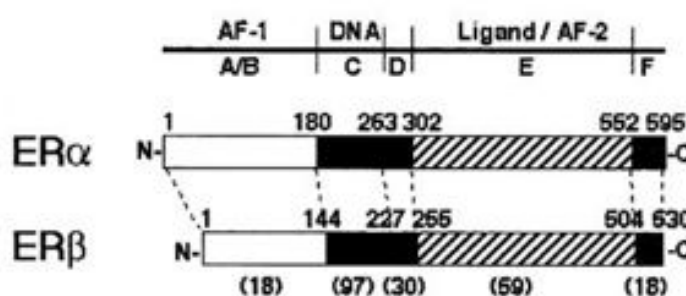


Figure 1-4 Schematics of the human oestrogen receptor alpha and beta. The structural domains of the receptors are shown. The A/B domain contains activation functional (AF1), domain C is a DNA binding domain, domain D is a hinge region which links the DNA binding domain and the ligand binding domain, domain E contains both the ligand binding region and activation function 2 (AF2). Domain F is a C terminal extension region of the ligand binding domain. Taken from (Katzenellenbogen et al. 2001).

The interaction of several domains is necessary for the oestrogen-dependent activation of gene transcription by the ER. In the classical pathway oestrogen initially binds to the ligand binding domain in region E and induces the migration of the receptor from the cytosol into the nucleus, the dissociation of heat shock proteins and the formation of stable homo (ER α/α or ER β/β) or heterodimers (ER α/β). Following ligand activation the ER dimer interacts via the DNA binding domain with regulatory DNA enhancer sequences termed oestrogen response elements (ERE). Full EREs are characterised as a 13-base pair palindrome with 5-bp stems separated by 3-bp spacer and a consensus sequence of GGTCAnnnTGACC. Half EREs (GGTCA) are also recognised by

the ER. Once bound, the oestrogen-ER-ERE complex promotes the dissociation or inactivation of co-repressor proteins and the recruitment of coactivator proteins. Corepressors and coactivators are involved in chromatin remodelling by virtue of their enzymatic activities as histone deacetylases and acetyltransferases, respectively (Fritz F.Parl 2000).

Most of the highly responsive oestrogen genes, including the vitellogenins and the progesterone receptor, have multiple variably spaced EREs within their regulator regions. The presence of multiple EREs results in synergistic activation of these genes. Synergism in the vitellogenin B1 gene was reported to result from the cooperative binding of the ER to two full-ERE sites whereas the ovalbumin gene is synergistically activated by a series of four half-palindromic sites (Sathya et al. 1997).

The non-classical pathway involves ER interactions with other proteins such as AP1 (Jakacka et al. 2001) and SP1 rather than direct binding to DNA. ER α is able to interact with SP1 to transactivate genes through binding SP1(N)_xERE half site motifs, in which there is considerable variability in the ERE site orientation and the number of nucleotides (N) found between the SP1 site and the ERE half site (Porter et al. 1997).

Oestrogens also have non-genomic actions. The rapid non genomic actions of oestrogens are mediated by receptors associated with the plasma membrane (Bishop and Stormshak 2008). These can be either ER located in or adjacent to the plasma membrane, or non-ER plasma membrane associated steroid binding proteins such as the G Protein coupled receptor 30 (GPR30) (Chen et al. 2008). The non-genomic action of oestrogen results in cellular responses such as increased levels of calcium or nitric oxide and the activation of multiple intracellular kinase cascades including mitogen activated protein

kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase A (PKA) and protein kinase C (PKC) (Chen et al. 2008).

The actions of oestrogens-ER complexes are antagonised by anti-oestrogens which bind with the ER in a manner which is competitive with oestrogen. Certain anti-oestrogens such as tamoxifen act as partial agonists/ antagonists, while others such as ICI 164,384 act more as complete antagonists (Fritz F.Parl 2000).

1.1.2.7.4 Progesterone receptor

The human PR gene consists of eight coding exons separated by seven non-coding introns. The two main nuclear isoforms, PR-A and PR-B, are independently regulated by defined promoter regions within the PR gene. PR-A is a truncated form of PR-B, lacking the amino terminal 164 amino acids that form the third transactivation domain (AF-3). In addition to the nuclear hormone receptors PR-A and PR-B, there is also a truncated and predominantly cytoplasmic PR protein PR-C. PR-C, a 60 Kda protein, is capable of forming heterodimers with PR-A and PR-B, and therefore is able to modulate their activity. The PR-C lacks a full DNA binding domain (domain C) as well as the first two transactivation domains (AF-3 and AF-1), so although it can potentially bind to hormones it cannot directly bind to progesterone response elements (PREs). PR-C can still influence transcriptional events as it is capable of interacting with nuclear co-factors (Cork et al. 2008) (Figure 1-5).

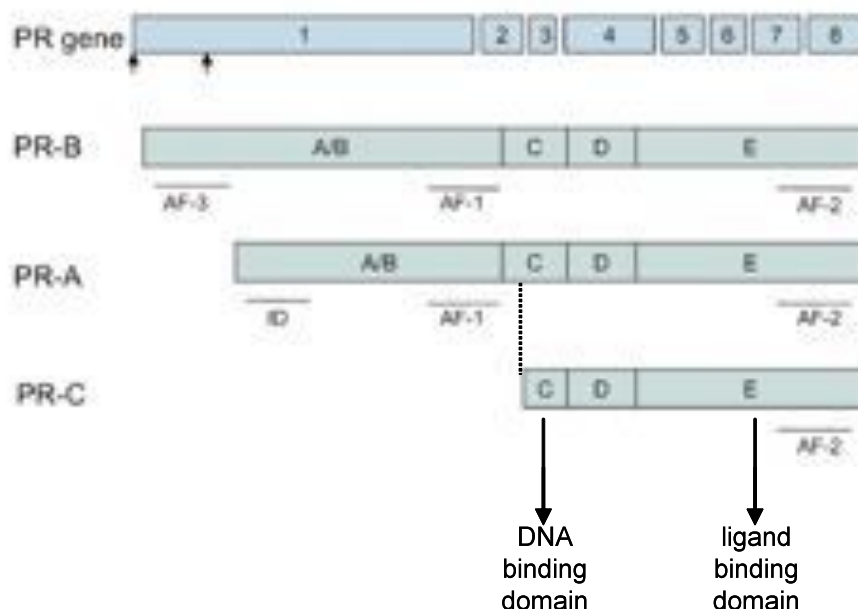


Figure 1-5 The progesterone receptor gene and main isoforms. Arrows indicate the PR-A and PR-B promoters. The A/B region contains the PR-B specific AF-3, AF-1 and the PR-A specific inhibitory domain (ID). The C region forms the DNA binding domain (DBD), whilst the D region forms the hinge region responsible for the nuclear localisation sequence (NLS). The E region contains (AF-2) and the hormone ligand binding domain (LBD). Taken from (Cork et al. 2008).

Like the ER, PR is a ligand activated nuclear transcription factor where progesterones exert their transcriptional activity by binding to the PR (Pasqualini 2007). Progesterone binds PR, inducing a conformational change which induces nuclear translocation, dimerisation and interaction with a specific PRE present in the promoter regions of target genes. However the presence of a PRE does not necessarily predict progesterone responsiveness as PR can also mediate its effects independent of PREs, through protein-protein interaction of PR with other sequence-specific transcription factors. Protein products from PR target genes are involved in a diverse range of cellular activities, including transcription, steroid and lipid metabolism, cell growth and apoptosis, protein and nucleic acid processing, and membrane associated signalling, indicating a broad range of potential effects (Cork et al. 2008).

Progesterone-PR complexes can also regulate genes independent of the transcriptional activity of the PR. Human PRs contain polyproline motifs in the amino-terminal which interact with the SH3 domain of Src, this then mediates the rapid activation of c-Src and downstream MAPK (ERK 1/2). Through this pathway progesterones regulate genes such as cyclin D1, which lack a PRE, and can mediate cell cycle progression. This pathway is thought to be mediated by PR-B rather than PR-A as PR-A is predominantly nuclear whilst PR-B is distributed between the nucleus and cytoplasm (Boonyaratanakornkit et al. 2008).

1.1.2.7.5 Oestrogen receptor expression in breast cancer

Normal breast epithelium is composed of stem cells which are capable of proliferation, and differentiated cells that are ER-positive or ER-negative. The non proliferating ER-negative cells comprise more than 80% of the total epithelial population. ER-positive cells, when stimulated by the presence of oestrogen, release paracrine growth factors which stimulate mitogenesis in the ER-negative stem cells. The organisation of the breast epithelium is disrupted in breast cancer as proliferating stem cells may express ER and the percentage of ER-positive cells is often much greater in tumour than in normal tissue. Some breast cancers contain 60 % ER-positive tumour cells (Fritz F.Parl 2000).

1.1.2.7.6 Progesterone receptor and breast cancer

Progesterones are commonly prescribed for contraception or as part of postmenopausal HRT in which progesterones are combined with oestrogen as a means to block oestrogen induced endometrial growth (Lange and Yee 2008). The effects of progesterone on breast cancer risk remain controversial. There have been some reports

suggesting a further increased risk of breast cancer with combined oestrogen-progesterone HRT compared to preparation containing oestrogen alone (Cork et al. 2008). On the other hand it has been demonstrated that progesterones can block the enzymes involved in estradiol bioformation in breast cancer cells and can stimulate the sulfotransferase activity which converts oestrogens into the biologically inactive sulfates, thus blocking oestrogen promotion of breast cancer (Pasqualini 2007). These controversial reports stem from the fact that there are a multitude of different progesterones which bind with differing affinity to PR-A or PR-B and these have different physiological functions due to differential gene regulation. Because of this, progesterones may have a synergistic (induced metabolic pathways) or antagonistic (ER downregulation, apoptosis) action on oestrogen induced mitotic pathways (Kenemans and Bosman 2003).

PR presence, along with ER status, is routinely measured in breast cancer specimens. Approximately 40% of breast tumours are ER positive and PR positive and these are more likely to respond to hormonal therapies, thus conferring a better prognosis. (Cork et al. 2008).

The balance of PR isoform expression is important in breast cancer management as overexpression of PR-A compared to PR-B changes the progesterone responsiveness of cells and signifies a poorer outcome of hormonal therapies (Cork et al. 2008).

1.1.3 The treatment of breast cancer

The management of patients with breast cancer involves surgery, radiotherapy, drug therapy or a combination of these (John Martin et al. 2007). After a diagnosis of breast cancer is confirmed, treatment depends upon the disease stage and pathological features such as receptor status and tumour grade. Disease stage is determined by tumour

size, the number and location of lymph nodes involved and the presence or absence of distant metastatic disease (Moulder and Hortobagyi 2008).

1.1.3.1 Surgery

For operable breast cancer, drug and chemotherapy treatment before surgery (neoadjuvant therapy) reduces the size of the tumour and facilitates breast conserving surgery; anti-oestrogen therapy and/or chemotherapy is chosen for steroid hormone receptor positive breast cancer and chemotherapy for steroid hormone receptor negative tumours or for younger women (John Martin et al. 2007). In the past, localised breast cancer was exclusively treated with radical mastectomy until several large randomised trials demonstrated equivalent rates of local recurrence and overall survival with lumpectomy followed by radiation therapy, also known as breast conserving surgery (Moulder and Hortobagyi 2008).

All women should be considered for adjuvant therapy following surgical removal of the tumour, as a significant number of patients with localised breast cancer harbour undetectable deposits of disease either locally or at distant sites (Moulder and Hortobagyi 2008). Adjuvant therapy is used to eradicate the micro-metastases that causes relapses and has been demonstrated to reduce the risk of breast cancer recurrence by 30-50% (John Martin et al. 2007). The choice of adjuvant therapy is determined by the risk of recurrence, steroid hormone receptor status of the primary tumour and menopausal status (John Martin et al. 2007). Adjuvant therapy comprises either cytotoxic chemotherapy or hormone antagonist therapy. Women with steroid hormone receptor positive breast cancer are considered for both hormone antagonist therapy as well as cytotoxic chemotherapy

whilst women with hormone receptor negative breast cancer are considered for cytotoxic chemotherapy (John Martin et al. 2007).

1.1.3.2 Hormonal therapy

Selective oestrogen response modulators (SERMs), such as tamoxifen and raloxifene, represent a group of chemical compounds that structurally resemble oestrogens, and upon binding to oestrogen receptors (ER), exert functionally different effects in normal tissue and cancer cells (Moulder and Hortobagyi 2008). For more than a quarter of a century, the SERM tamoxifen has been used to treat patients with hormone receptor positive breast cancer (Moulder and Hortobagyi 2008). Following surgery for operable breast cancer (radical mastectomy and breast conserving surgery), 5 years of therapy with tamoxifen when compared to no tamoxifen treatment halved the annual recurrence rates and reduced the breast cancer mortality rates by a third in women with ER positive breast cancer (Anon 2005). Treatment with tamoxifen along with radiation therapy after breast conserving surgery, compared to radiation therapy alone, has also demonstrated a significant reduction in the risk of developing invasive breast cancer in patients with ER positive DCIS (Fisher et al. 1999; Fisher et al. 2005)

Although SERMs are effective in premenopausal and postmenopausal women, other endocrine therapies are reserved for use in postmenopausal women alone. Selective aromatase inhibitors (AIs) such as anastrozole, letrozole and exemestane, which inhibit the enzyme which converts androgenic substrates into oestrogens have longer disease free survival, greater time to progression and longer times to recurrence when compared with SERMs in both the localised (early breast cancer) and advanced setting (metastatic) for postmenopausal patients with ER positive breast cancer (Buzdar 2003).

1.1.3.3 Chemotherapy

Chemotherapy is frequently used to reduce the risk of recurrence in patients with localised breast cancer and to palliate symptoms in patients with advanced breast cancer. Anthracyclines, such as doxorubicin and epirubicin, are the class of drugs most commonly used to treat breast cancer (Moreno-Aspitia and Perez 2009; Moulder and Hortobagyi 2008). Common chemotherapy regimes for breast cancer include AC (doxorubicin and cyclophosphamide) chemotherapy, AC + taxol (doxorubicin and cyclophosphamide plus paclitaxel) chemotherapy, and AT (doxorubicin and paclitaxel) chemotherapy. (<http://www.healthcentral.com/breast-cancer/chemo-regimen.html>). Advanced breast cancer can be treated using two different strategies of chemotherapy administration; the use of active agents sequentially or in combination regimes (Moulder and Hortobagyi 2008). In the adjuvant and neoadjuvant settings, combination chemotherapy regimes have maximised benefit by reducing tumour size and increasing disease free and overall survival (Anon 2005). The use of neoadjuvant chemotherapy was first introduced in the 1970s to downstage patients with inoperable locally advanced disease and led to the improvements in the number of patients able to undergo breast conserving surgery (Moulder and Hortobagyi 2008). Since the 1990s, the most prominent advances that have occurred with the use of chemotherapy have involved improving drug formulation and modification in routes or schedules of drug delivery (Moulder and Hortobagyi 2008). An example of a modification in the schedule of drug delivery is the administration of drugs with a shortened inter-treatment interval (dose dense schedule). In the INT C9741 trial, dose dense treatment (two week recycling of the drugs doxorubicin, paclitaxel and cyclophosphamide at their optimal doses (60, 175 and 600 mg/m² respectively) rather than

at the conventional 3 week intervals) improved disease free and overall survival compared to the conventional treatment (Citron et al. 2003).

1.1.3.4 Human epidermal growth factor (HER) family of receptors

The HER family of receptors consists of four receptor tyrosine kinases with similar homology: HER1 (also referred to as epidermal growth factor receptor/EGFR), HER2, HER3 and HER4. HER2 overexpression occurs in 20-30% of patients with breast cancer, and correlates with poor prognosis and decreased survival (Moulder and Hortobagyi 2008). Trastuzumab (Herceptin) is a recombinant, humanized monoclonal antibody against the extracellular domain of HER2 and has demonstrated significant activity when given in combination with chemotherapy. The addition of trastuzumab to paclitaxel after a regime of doxorubicin and cyclophosphamide reduced the rates of recurrence by half and the mortality rates by one third among women with HER-2 positive breast cancer (Romond et al. 2005). Lapatinib (GW572016) is a small-molecule inhibitor of the TK domain of both HER1 and HER2, which has also been shown to inhibit growth and induce apoptosis in EGFR/HER2 overexpressing breast cancer cells. Randomised trials investigating the use of lapatinib in the adjuvant setting are currently being designed (Moulder and Hortobagyi 2008).

1.1.3.5 New treatments for breast cancer are required

Although there are many treatment options for breast cancer, not all patients are cured and it remains a formidable health problem accounting for 500,000 deaths per year worldwide (Benson et al. 2009). As discussed earlier, anthracyclines are the most active and widely used chemotherapeutic agents used to treat breast cancer, but the increased use of these agents at an early stage of disease often renders tumours resistant to these drugs

by the time the disease recurs, thereby reducing the number of treatment options for metastatic disease (Moreno-Aspitia and Perez 2009). Moreover, even when these agents are used in the metastatic setting, treatment failure occurs in most cases (Moreno-Aspitia and Perez 2009). Although hormonal therapy can be used to treat ER positive breast cancer, it only provides a reduction in risk and cannot be used if the tumours are ER negative. In the same sense trastuzumab (herceptin) can only be used on those tumours (20-30 %) which over express HER2 (Moulder and Hortobagyi 2008). Additionally, as a result of the oestrogen agonist properties of tamoxifen, it has been associated with an increased risk of thromboembolic events and endometrial cancer (Moulder and Hortobagyi 2008). This together underpins the requirement for new effective treatments of breast cancer, especially in the metastatic setting.

1.2 Pituitar tumor transforming gene (PTTG)

Pituitary tumor transforming gene (PTTG) was initially isolated in 1997 as a gene overexpressed in rat pituitary tumour cells compared to normal pituitary tissue (Pei and Melmed 1997). Human PTTG was later isolated from a Jurkat cDNA library (Dominguez et al. 1998) and is a 202 amino acid protein, which shares 89% identity with rat PTTG. Fluorescent in situ hybridisation (FISH) identified that PTTG is localised to 5q33 (Zhang et al. 1999).

1.2.1 Structure of PTTG

Human PTTG is coded for by an open reading frame of 609 bp (Zhang et al. 1999). The protein can be divided into an amino-terminal basic domain (amino acids 1-101) and a carboxyl-terminal acidic domain (amino acids 102-202) (Dominguez et al. 1998).

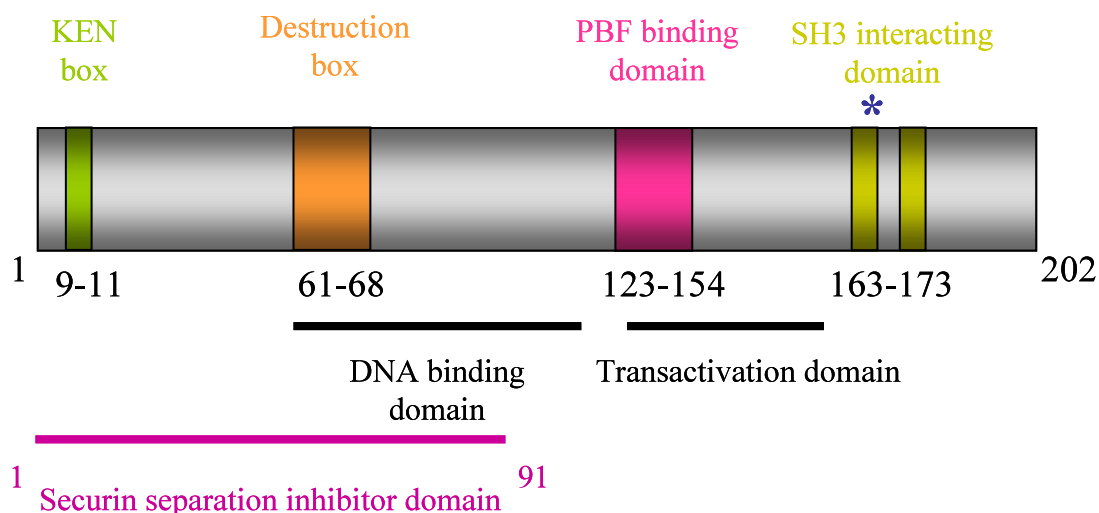


Figure 1-6 Schematic representation of human PTTG. The securin separation inhibitor domain, responsible for binding separase, is found between residues 1-91. The KEN box is located between residues 9-11 and the Destruction Box is found between residues 61-68. The PBF binding domain and SH3 interacting domain are located further towards the C-terminus between residues 123-154 and 163-173, respectively. The DNA binding domain is found between amino acids 61-118. The site of phosphorylation by Cdc2 at serine 165 is marked with an asterisk..

Detailed computational analysis of PTTG protein sequences revealed abundant Glu and Pro residues in the C-terminus, suggesting the presence of a transactivation domain (Wang and Melmed 2000b). Indeed the C-terminal region of PTTG was subsequently found to mediate transcriptional activation of c-Myc (Pei 2001). In addition, gel mobility shift assays revealed that PTTG can also bind DNA. Mutational studies uncovered that this DNA binding domain lies between amino acids 61 and 118 (Pei 2001) (Figure 1-6).

The subcellular localisation of PTTG has been investigated and initial studies suggested PTTG was predominantly a cytosolic protein with partial nuclear localisation (Dominguez et al. 1998) (Saez et al. 1999). Since PTTG can act as a gene transactivator its presence in the nucleus was not surprising; however it does not contain a nuclear localisation sequence (NLS). Nuclear translocation of PTTG is facilitated by the

activation of mitogen-activated protein kinase (MAPK) cascade or by its interaction with PTTG binding factor (PBF). Mutational studies show that this interaction occurs between amino acids 123-154 in PTTG and the C-terminal 30 amino acids of PBF (Chien and Pei 2000) (Figure 1-6).

The PROSITE Dictionary of Protein Sites and Patterns revealed that PTTG contains consensus motifs for cyclic AMP and cyclic GMP dependant protein kinase phosphorylation, casein kinase II phosphorylation and protein kinase C phosphorylation (Dominguez et al. 1998). However the sole confirmed phosphorylation site of human PTTG is on the serine 165 residue, which is phosphorylated by Cdc2 at the G2/M transition of mitosis in association with cyclins A and B (Ramos-Morales et al. 2000). The Cdc2 phosphorylation site in PTTG is located in the middle of a proline rich region which contains two PXXP motifs, allowing binding to SH3 domains (Zhang et al. 1999). Because of this, phosphorylation and dephosphorylation of Ser 165 may regulate the interaction of PTTG with SH3 containing proteins, interfering with SH3-mediated signal transduction pathways (Boelaert et al. 2004; Ramos-Morales et al. 2000).

As well as functioning as a gene transactivator (discussed in section 1.2.7), PTTG is the human securin (discussed in section 1.2.3). PTTG contains a KEN box located between amino acids 9-11 and a RXXL Destruction box (D-Box) found between residues 61-68, which allow it to carry out this function (Zur and Brandeis 2001) (Figure 1-6). It also contains a securin separation inhibitor domain between amino acids 1-91 which is responsible for the binding of separase (Ensemble, Protein ID ENSP00000377536 and ENSP00000344936). The KEN and D-box are both targets for ubiquitinylation, which signals the degradation of PTTG by the anaphase-promoting complex (APC), allowing the metaphase to anaphase transition and mitosis to occur.

1.2.2 PTTG tissue expression and gene family

The tissue expression pattern of rat PTTG mRNA was studied by Northern blot analysis. Among the adult tissues examined, the testis was the only tissue that expressed rat PTTG mRNA, although it was also found to be expressed at low levels in fetal liver (Pei and Melmed 1997). Northern blot analysis has also been used to determine the expression of human PTTG. In non-malignant adult tissues, high levels of expression were observed in testis, thymus and placenta and lower expression levels were observed in the colon, spleen, prostate, small intestine, ovary, heart, brain, liver, peripheral blood leukocytes, skeletal muscle, kidney and pancreas (Dominguez et al. 1998; Zhang et al. 1999). When the expression of human PTTG was examined in fetal tissue it was evident in the liver, testis and thymus (Zhang et al. 1999). PTTG has also been found to be highly expressed in all human cell lines derived from malignant tumours examined as well as in many primary tumours. This will be discussed in more detail in Section 1.2.5

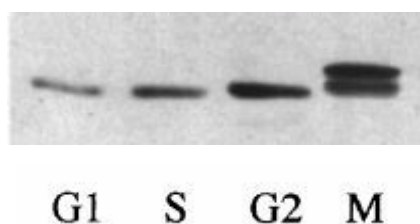
Two intronless genes, PTTG2 and PTTG3, which are homologous to PTTG1, have been reported. There exists 91% homology between PTTG1 and PTTG2 and 89% homology between PTTG1 and PTTG3, although PTTG2 and PTTG3 are only 84% identical at the amino acid level (Chen et al. 2000; Cho-Rok et al. 2006). Like PTTG1, both PTTG2 and PTTG3 contain two conserved proline rich motifs at the C-terminus (Chen et al. 2000). Using fluorescent in-situ hybridisation PTTG1 has been mapped to chromosome 5q35, whilst PTTG2 and 3 have been mapped to 4p15 and 8q13 respectively (Prezant et al. 1999) (Chen et al. 2000). Northern blot analysis of PTTG2 revealed it to be expressed at low levels in the spleen, prostate, testis ovary, small intestine and colon but not in the thymus or peripheral blood leukocytes; however PTTG 3 mRNA was largely

undetectable (Chen et al. 2000). As PTTG1 is the most abundantly expressed homologue the remainder of this thesis will be referring to PTTG1.

1.2.3 PTTG is the human securin

Among adult tissues examined PTTG mRNA is highly expressed in tissues that contain highly proliferative cells, suggesting a role for PTTG in proliferation. Indeed PTTG is expressed in a cell cycle dependant manner. PTTG levels are low in G1 and S phases but the amount of PTTG increases at the onset of S-phase and peaks in M phase. PTTG is then degraded during anaphase and daughter cells express little PTTG. Unlike in the G1, S and G2 phases of the cell cycle, during M phase PTTG protein migrates as a doublet on a Western blot, presumably due to phosphorylation during mitosis by Cdc2 (discussed in section 1.2.1) (Ramos-Morales et al. 2000) (Figure 1-7).

A.



B.

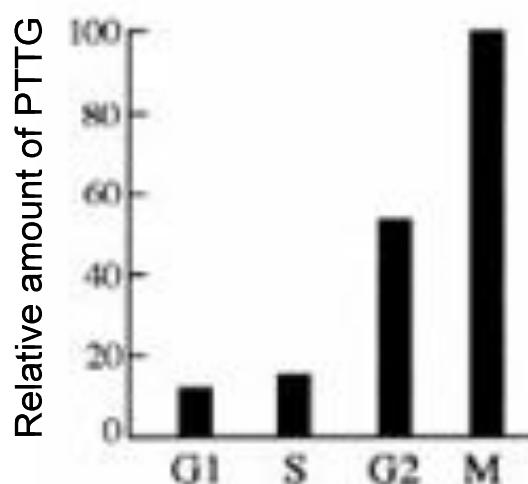


Figure 1-7. PTTG expression during the cell cycle. A. Western blot of extracts taken from HeLa cells arrested at phases of the cell cycle indicated. Probed with anti-PTTG antibody, the double band representing phosphorylated PTTG is observed during mitosis. B. Densitometry of the immunoblot demonstrating that PTTG expression is highest during mitosis. Adapted from (Ramos-Morales et al. 2000).

As discussed in section 1.2.1, PTTG is expressed both in the nucleus and cytoplasm. In accord with its role as a human securin, during early mitosis PTTG is localised to mitotic spindles (Yu et al. 2000b).

Eukaryotic cells proceed through the cell cycle in a strictly ordered fashion. Cells must precisely duplicate their chromosomal DNA during S phase, segregate their sister chromatids to opposite poles of the mitotic spindle during mitosis, assemble two nuclei and then divide their organelles, cytoplasm, plasma membrane and other cell contents into two daughter cells in a process known as cytokinesis (Ramos-Morales et al. 2000). Following description of its cell cycle-dependent expression with increased levels at mitosis, its phosphorylation by a cell cycle dependant kinase at the G2/M transition and its subcellular localisation, PTTG was discovered to be the human securin (Ramos-Morales et al. 2000; Yu et al. 2000b; Zou et al. 1999).

The first phases of mitosis are concerned with ensuring that all chromosomes attach to microtubules emanating from opposing poles, and the second with destroying the connections that link sister chromatids, to permit their migration to opposite poles of the cell during anaphase (Nasmyth 2005). Sister chromatid cohesion is mediated by a multi-subunit protein called cohesion. Cohesin's Scc1, Smc1 and Smc3 subunits form a tripartite ring structure, which hold sister DNA molecules together by trapping them inside their ring (Haering et al. 2008). Loss of cohesion is required for the separation of sister chromatids. The degradation of cohesins at the early stage of mitosis (during prophase) correlates with chromosome condensation and the visible appearance of

discrete chromatid arms. This process spares Scc1 at centromeric regions, resulting in sister chromatids still being attached. Removal of centromeric Scc1 occurs at the metaphase to anaphase transition and is carried out by the mitotic effector separase. This process is initiated by the anaphase promoting complex (APC) (Waizenegger et al. 2000), which mediates the ubiquitination of PTTG at its KEN and D-Box. The APC is controlled by Mad2, a component of the mitotic checkpoint which ensures that all kinetochores have become attached to microtubules (Chestukhin et al. 2003). As PTTG blocks the active site of separase (Waizenegger et al. 2002), degradation of PTTG releases separase from inhibition allowing it to cleave the Scc1 subunit of the cohesion complex. By acting as a negative regulator of separase, PTTG prevents the premature movement of chromosomes to the spindle poles during anaphase and ensures equal chromosome segregation and diploid cell division (Zou et al. 1999). In order to inhibit the function of separase before the onset of mitosis PTTG must be able to enter the nucleus. As it does not contain a nuclear localisation sequence (NLS), this process is facilitated by the activation of mitogen-activated protein kinase (MAPK) cascade or by its interaction with PTTG binding factor (PBF) (Figure 1-8).

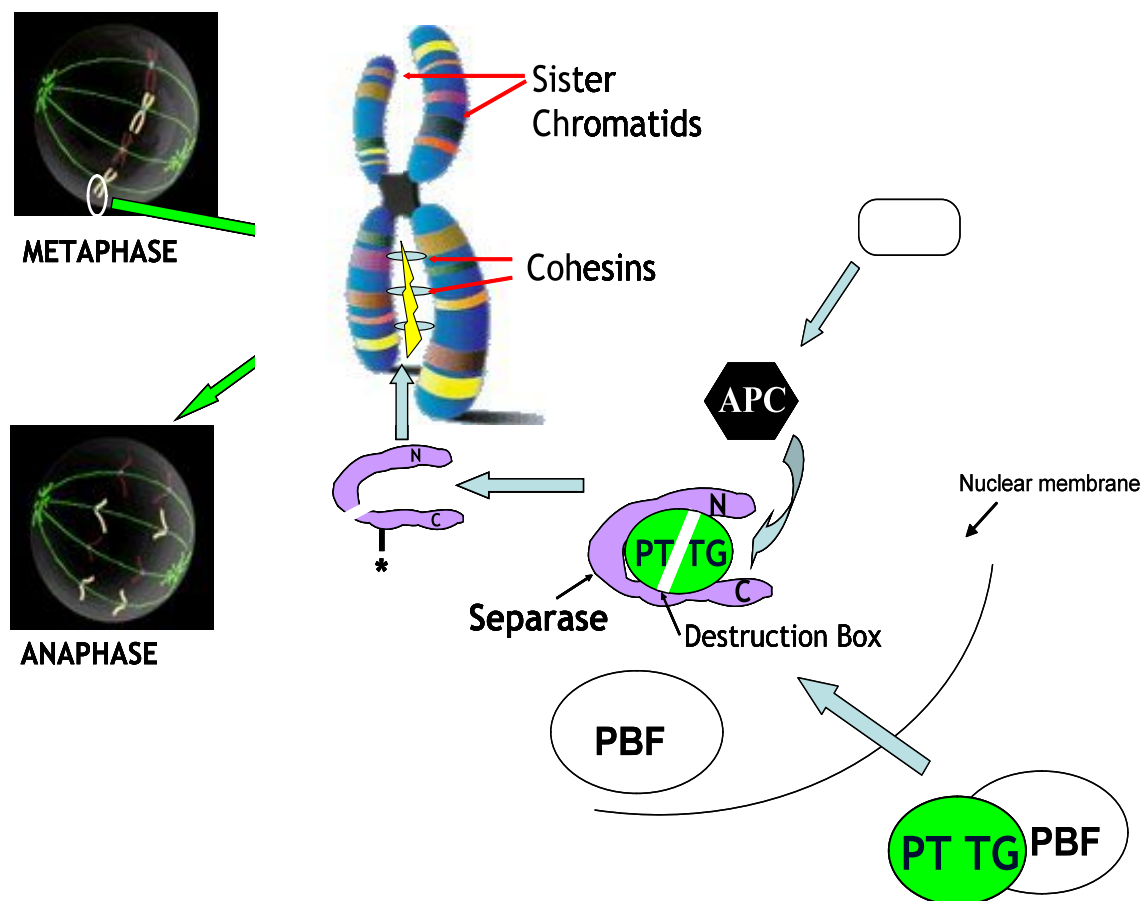


Figure 1-8: A schematic representation of the functional roles played by PTTG, PTTG binding factor (PBF) and separase during mitosis. PBF contains an NLS at its C-terminus, which facilitates the nuclear translocation of PTTG before mitosis. At the metaphase to anaphase transition, after the degradation of PTTG by APC, separase cleaves the SCC1 subunit of the cohesin complex allowing chromatid separation. PTTG's association with separase before this inhibits its activity, thus preventing premature chromatid separation.

1.2.4 Regulation of PTTG expression

The hPTTG gene consists of 5 exons and four introns, spanning more than 10kb (Chen et al. 2000). Exon 1 encodes the 5' untranslated sequence and nucleotides 1 to 91 of the coding sequence followed by a 310 bp intron. Exon 2 encodes nucleotides 92 to 276 and is followed by a 1.2 kb intron. Exon 3 encodes nucleotides 277 to 370 and is followed by a 2.4 kb intron. Exon 4 encodes nucleotides 371 to 529 and is followed by a 0.6 kb intron, and exon 5 encodes nucleotides 530 to 609 and the 3' untranslated

sequence. The transcriptional start site as determined by both primer extension and RNase protection analyses was initially mapped to adenine 37 (Kakar 1999). However, further studies identified an additional start site at -317 bp (Clem et al. 2003). Based on these results PTTG may also consist of six exons and five introns. This is consistent with the cDNA isolated from human fetal liver as this indicated the presence of a sixth exon (Zhang et al. 1999). Although there are two possible transcripts of PTTG, 5' primer extension analysis of the mRNA from human testis revealed the proximal transcription start site 37bp upstream of the translational start site as the major one (Clem et al. 2003) (Figure 1-9).

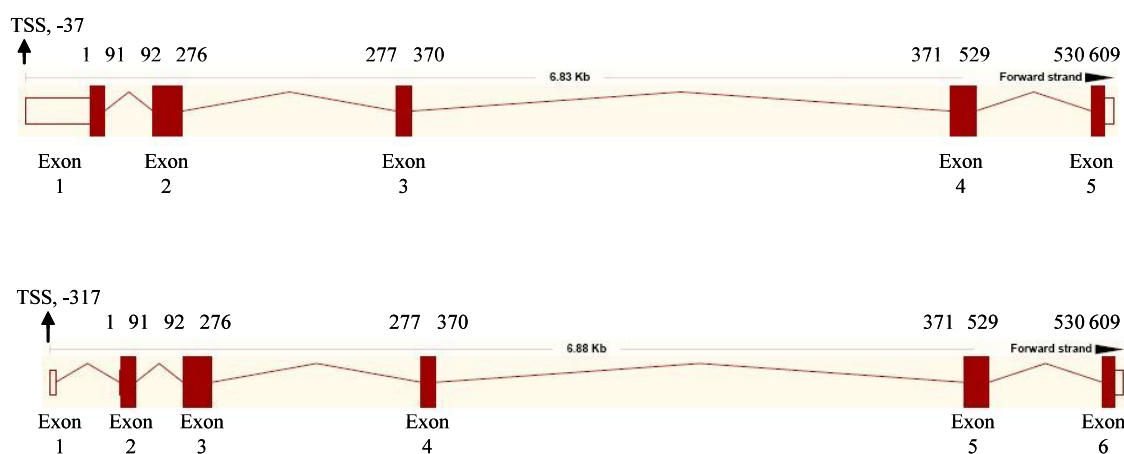


Figure 1-9 Human PTTG transcript structure. PTTG is comprised of 5 exons and 4 introns or 6 exons and 5 introns, depending on which of the transcriptional start sites (TSS) have been utilised (-37bp or -317bp upstream of the translational start site). Unfilled boxes represent untranslated regions, whilst the filled boxes represent translated regions. Taken from Ensembl.

5' deletion analysis of the human PTTG gene sequence suggested that the PTTG promoter resides between -161 and -3 (Kakar 1999). The PTTG promoter also contains an enhancer element which is found between -743 and -444. However, if the transcriptional

start site at 317bp upstream of the translation start site is utilised this would presumably become the proximal promoter. Within the enhancer element there are Sp1, AP1, AP2 and NF-Y binding sites. Both Sp1 and NF-Y have been shown to act co-operatively to play a crucial role in the transcriptional regulation of the human PTTG gene (Clem et al. 2003). Although Sp1 plays a dominant role, mutation of the NF-Y site caused a 25% reduction in activity (Clem et al. 2003). The transcription factor NF-Y has been functionally implicated in the regulation of PTTG by p53, because p53 is believed to bind to NF-Y preventing it from associating with the promoter, thus leading to suppression of PTTG expression (Zhou et al. 2003).

PTTG does not contain a TATA box sequence within 25-35 nucleotides upstream of the transcriptional start site. Promoters lacking a TATA box commonly have multiple GC boxes that bind the ubiquitous transcription factor SP1 to activate transcription (Pugh and Tjian 1991), consistent with the identification of multiple Sp1 sites in the PTTG promoter. The human PTTG promoter shows marked similarity in structural organisation to the rat and murine promoter. Both are composed of five exons and four introns and contain enhancer elements in similar locations (between -462 and -745 in rat PTTG and between -444 and -743 in human PTTG). In addition to this the rat and murine PTTG promoters also contain consensus Sp1 binding sites (Pei 1998) (Wang and Melmed 2000a).

In addition to the regulatory elements already mentioned, a number of other sites known to bind various transcriptional factors were identified in the human PTTG promoter. These include a Polyoma virus Enhancer Activator 3 (PEA-3) binding site, a cAMP responsive element (CRE), an insulin response element (IRE) and a nuclear factor 1 (NF1) binding sequence (Kakar 1999). In addition, a search for transcription factor

binding sites using TESS: Transcription Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) identified four putative oestrogen receptor α (ER α) binding sites at bases -525 to -531, -542 to -546, -773 to -777 and -1336 to -1340 relative to the transcriptional start site.

1.2.4.1 Regulation of PTTG by insulin

The PTTG gene sequence contains potential insulin response sequences in the 5' flanking region, suggesting that insulin may regulate PTTG expression. When examined by Northern and Western blots, insulin and insulin like growth factor-1 (IGF-1) was found to induce the mRNA and protein expression of PTTG in MCF7 breast cancer cells (Thompson, III and Kakar 2005). Both insulin and IGF-1 mediate their downstream effects via the two major signalling pathways PI3K/AKT and ras-MAPK (Thompson, III and Kakar 2005). The PI3K/AKT specific inhibitor LY294002 completely prevented the induction of PTTG mRNA by insulin and IGF-1 but the Ras-MAPK specific inhibitor PD98059 only partially blocked the stimulatory effect, suggesting insulin and IGF-1 regulate the expression of PTTG predominantly through the PI3K/AKT cascade (Thompson, III and Kakar 2005). Further studies employing actinomycin D, an inhibitor of transcription and PTTG promoter-luciferase constructs, indicated that rather than increasing the stability of PTTG mRNA and protein, insulin contributes to the synthesis of PTTG mRNA (Thompson, III and Kakar 2005).

Since hyperinsulineamia and insulin resistance have been classified as risk factors for breast cancer (Thompson, III and Kakar 2005) (Stoll 1996), and because hyperinsulineamia also serves as a marker for increased cancer risk in obese post-

menopausal women, insulin and IGF-1 may lead to induction of tumourigenesis through upregulation of PTTG (Thompson, III and Kakar 2005).

In addition to insulin, epidermal growth factor (EGF) transforming growth factor – α (TGF- α) and FGF-2 have also been shown to directly regulate the PTTG promoter and to increase the expression of PTTG (Heaney et al. 1999; Tfelt-Hansen et al. 2004).

1.2.4.2 Regulation of PTTG by oestrogen

Pituitary PTTG expression is regulated during the rat estrus cycle, with maximal expression occurring coincidentally with peak serum estradiol levels and proliferating cytoplasmic nuclear antigen (PCNA) expression, indicating that PTTG expression during rat estrus cycle is associated with oestrogen-mediated pituitary proliferation. As 2-hydroxy-estradiol (2-OHE₂) upregulated PTTG mRNA in GH3 cells, catechol-metabolites of estradiol may mediate pituitary oestrogenic actions (Heaney et al. 2002).

Induction of pituitary PTTG expression by oestrogen was found to be mediated through the oestrogen receptor since 2-OHE₂ and diethylstilbestrol induction of PTTG mRNA in GH3 cells was abrogated by the co-administration of the specific anti-oestrogen, ICI-182780. Selective anti-oestrogen treatment also blocked oestrogen-induced pituitary PTTG expression *in vivo* and inhibited PTTG expression in primary pituitary tumour cells *in vitro* (Heaney et al. 2002). Similar to anti-oestrogen, pre-treatment of animals with 2 mg progesterone for 48 hours before oestrogen treatment, abrogated pituitary PTTG expression *in vivo*. A possible mechanism for this involves the downregulation of the ER by progesterone (Heaney et al. 2002).

PTTG has also been found to be upregulated by oestrogen and progesterone treatment in mouse mammary epithelium cells. Other mitotic proteins p55CDC (also

known as cell division cycle 20 homolog, CDC20), cadherin 1 (Cdh1) and Mad2 were increased in post-translated modified forms as a consequence of oestrogen and progesterone stimulation. The effect of oestrogen and progesterone on proteins involved in cell cycle regulation in the mouse mammary epithelium appeared to be selective as expression of a number of other proteins such as Rad21, Cyclin B1, cell division cycle 2 (Cdc2), Rad51 and Cyclin E1 involved in various phases of the cell cycle remained unchanged (Pati et al. 2004).

1.2.5 PTTG and tumourigenesis

PTTG was found to be highly expressed in all human cell lines derived from malignant tumours examined (Zhang et al. 1999) and although its tissue expression is limited in normal tissues, it is highly expressed in many primary tumours including carcinomas of the lung (Kakar and Malik 2006), ovary (Puri et al. 2001), breast (Solbach et al. 2004), thyroid (Boelaert et al. 2003;Heaney et al. 2001), pituitary (McCabe et al. 2003;Pei and Melmed 1997), liver (Cho-Rok et al. 2006) colon (Heaney et al. 2000;Kim et al. 2007), adrenal gland, kidney, endometrium and uterus (Chen et al. 2000), haematopoietic neoplasms (Dominguez et al. 1998;Saez et al. 2002) and malignant astrocytes (Tfelt-Hansen et al. 2004). This implies that PTTG may play a role in the tumourgenic process. Indeed when NIH 3T3 cells stably overexpressing PTTG were injected subcutaneously into a thymic nude mice, all injected animals developed large tumours (Pei and Melmed 1997;Zhang et al. 1999).

When the PXXP motif found near the C-terminus of PTTG was mutated its transactivating ability and transforming ability was lost, suggesting that the transactivation and transforming abilities of PTTG are linked (Boelaert et al. 2004;Wang and Melmed 2000b;Zhang et al. 1999). A few of the genes regulated by PTTG include

fibroblast growth factor 2 (FGF-2) (Zhang et al. 1999), vascular endothelial growth factor (VEGF) (McCabe et al. 2002), c-Myc (Pei 2001) and metalloproteinase-2 (MMP-2) (Malik and Kakar 2006). By inducing FGF-2 and VEGF, PTTG has been shown to increase angiogenesis *in vitro* and *in vivo*, a process which is necessary for the expansion of the primary tumour as well as tumour metastasis (Kim et al. 2006; Zhang et al. 1999). Metalloproteinases (MMPs) are potent proteolytic enzymes known to play key roles in degradation of the extracellular matrix (ECM). In order to be able to invade and spread into surrounding normal tissue, tumour cells must be capable of degrading multiple elements of the ECM. By increasing the expression and secretion of MMP-2, PTTG regulates cell migration, invasion and tubule formation (Malik and Kakar 2006). c-Myc is a transcription factor involved in the regulation of many biological activities including cell growth and division, cell cycle progression, differentiation, metabolism, angiogenesis, cell adhesion and motility. De-regulation of c-Myc by PTTG may therefore result in apoptosis, genomic instability, uncontrolled cell proliferation, escape from immune surveillance and growth factor independence (Vita and Henriksson 2006).

PTTG, as well as being a transactivator, is also the human securin (See Section 1.2.3), and because of this its overexpression and underexpression lead to aneuploidy. PTTG was initially found to induce aneuploidy in MG-63 osteosarcoma cells as overexpression of PTTG-EGFP caused an increase in the severity and frequency of aneuploidy compared with cells transfected with plasmids encoding EGFP alone (Yu et al. 2000a). Later, MAD2 and PTTG levels have also been found to associate with aneuploidy in p53 null mammary cell following hormone stimulation (Ogbagabriel et al. 2005). In addition, members of our group have shown that PTTG induces genetic instability in thyroid cells (Kim et al. 2005) and in colorectal cancer (Kim et al. 2007). In colorectal cancer cells PTTG overexpression resulted in genetic instability through

inhibition of double stranded DNA repair activity (Kim et al. 2007). However in H1299 human cancer cells, failure of PTTG degradation or enhanced PTTG accumulation as a consequence of PTTG overexpression resulted in aneuploidy through inhibition of mitosis progression and chromosome segregation (Yu et al. 2003).

1.2.6 PTTG and breast cancer

PTTG has been shown to be over expressed in invasive and metastatic breast cancers compared with normal breast epithelium (Ogbagabriel et al. 2005) (Solbach et al. 2004) and when the expression levels of PTTG were investigated in MCF-7 breast cancer cells they were also found to be high (Zhang et al. 1999). Following analysis using immunohistochemistry, the highest levels of PTTG were found in tissue samples derived from metastatic breast cancer, and its expression was found to be a prognostic marker for lymph node invasion and tumour recurrence. Furthermore, PTTG levels correlated with breast tumour mitotic rates, degree of nuclear pleomorphism and ER α expression (Ogbagabriel et al. 2005) (Solbach et al. 2004).

PTTG has also been found to be over expressed in the peripheral blood collected from breast cancer patients compared to normal controls; this is because primary breast cancers begin shedding neoplastic cells into the circulation at an early stage (Chen et al. 2006). PTTG over-expression in the disseminated (metastatic) cancer cells also correlated with tumour size, lymph node metastasis and TNM (Tumour, Node, Metastasis) stage (Chen et al. 2006).

As PTTG expression was increased in cell lines which had been selected in the presence of tamoxifen (TAM), it may serve as a molecular marker associated with endocrine therapy resistance in breast cancer (Ghayad et al. 2009). Further evidence to

support this hypothesis comes from a well defined cohort of 48 relapsing or non-relapsing ER positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant TAM alone, as PTTG was also significantly over expressed in the group of patients who relapsed under TAM treatment compared to the patients who did not relapse. Univariate analysis showed that high expression levels of PTTG correlated with significantly shorter relapse free survival (Ghayad et al. 2009).

PTTG not only shows deregulated expression but also aberrant localisation in breast tumours. In normal breast, PTTG expression is localised to the cytoplasm of ductal epithelial cells, however in breast tumours its expression is found throughout the cytoplasm and in the nuclei (Ogbagabriel et al. 2005).

Unlike severe defects which would cause genomic instability and activate cell surveillance machinery, subtle defects such as SNPs in mitotic checkpoint genes could escape cell-cycle surveillance and lead to unrepaired DNA damage required for tumourgenesis. Indeed, in addition to being over expressed and differentially localised, a SNP in PTTG (C1892G) (rs2910203) has been associated with an increased risk of breast cancer, with the GG genotype occurring more frequently in cases (18.31%) compared to controls (16.00%) (Lo et al. 2007). When explored it was found that PTTG acted alone and in combination with reproductive risk factors, as the significance levels of the PTTG (C1892G) SNP association with breast cancer became more significant in women with a long period of oestrogen exposure before first full term pregnancy or with a lower number of full term pregnancies. This may be because oestrogen induced promotion of the proliferation of cells that have lost the mitotic checkpoint could lead to the accumulation of a greater number of genetic alterations (Lo et al. 2007).

1.3 Separase

Human separase, the binding partner of PTTG, is located on chromosome 12q13.13, encodes 31 exons spanning 25.34 Kb and was originally isolated from the human immature myeloid cell line KG-1 (Nagase et al. 1996). (Figure 1-10)

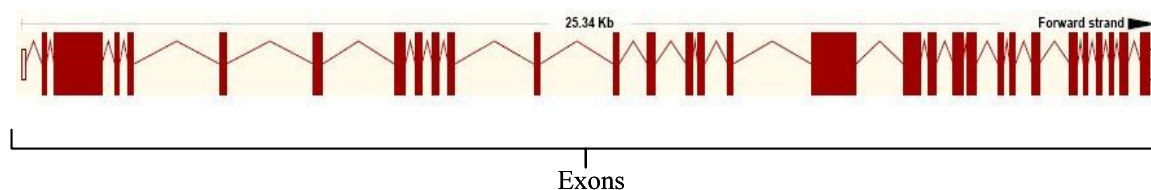


Figure 1-10 Human separase transcript structure. Separase is comprised of 31 exons, has a transcript length of 6623 base pairs and a translational length of 2120 residues. Taken from Ensembl.

1.3.1 Structure of separase

Between species, separases have a conserved function and share a general protein structure, although they do not show tight homology at the DNA level (Ross and Cohen-Fix 2002). Human separase consists of an unstructured central stretch of 280 residues that separates a superhelical N-terminal half comprising 26 armadillo (ARM) repeats from two C-terminal caspase domains, of which only the second one is active (Chestukhin et al. 2003). ARM repeats consist of about 40 amino acids which are composed of 3 alpha helices (Conti et al. 1998). All the phosphorylation and self-cleavage sites of separase are located either in the loop between ARM repeats 23 and 24 or in the central unstructured region. As well as two caspase domains, the C-terminus of separase contains a conserved cytosine residue at position 2029 in the active site and a Ca^{2+} binding motif. The cytosine residue is essential for the proteolytic activity of separase (Chestukhin et al. 2003). The N terminal of separase which contains the ARM repeats is responsible for binding to PTTG (Ross and Cohen-Fix 2002) (Viadiu et al. 2005). (Figure 1-11)

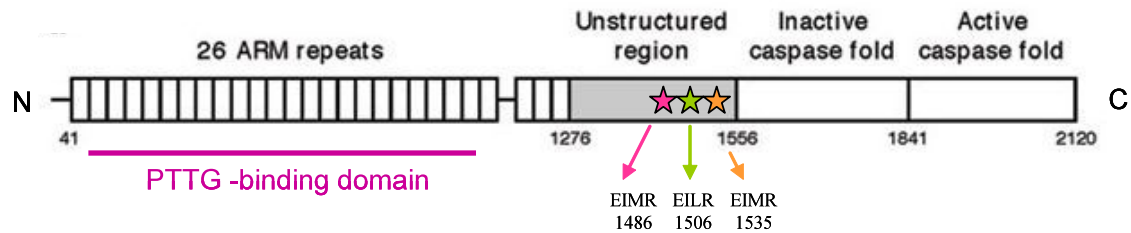


Figure 1-11 Domain structure of human separase. PTTG binds to separase at the N-terminus and can block its active site. Autocleavage of separase can occur at one of three amino acid sites, ¹⁴⁸³EIMR¹⁴⁸⁶ ¹⁵⁰³EILR¹⁵⁰⁶ and ¹⁵³²EIMR¹⁵³⁵. Taken and adapted from (Viadiu et al. 2005).

1.3.2 Separase is a cytosine endopeptidase

As discussed earlier in Section 1.2.3, removal of centromeric Scc1 occurs at the metaphase to anaphase transition and is carried out by separase. The cleavage of Scc1 appears to be both necessary and sufficient for initiation of anaphase and pole-ward movement of chromosomes. Separase belongs to the CD clan of cytosine proteases; during the majority of the cell cycle the endopeptidase activity of separase is suppressed, achieved either through the inhibitory binding of PTTG (see section 1.2.3) or by the cyclin dependant Kinase Cdc2. Cdc2 induces the phosphorylation of separase at Ser 1126 and Thr 1346, and this phosphorylation allows Cdc2 to bind to and inhibit separase activity (Waizenegger et al. 2002) (Chestukhin et al. 2003) (Gorr et al. 2005). Cdc2 and PTTG bind to separase at different regions, as mutation of separase in the region between positions 1126 and 1399 abolishes Cdc2 binding, but it does not interfere with the association of PTTG. Although they bind to separate regions of separase, PTTG and Cdc2 bind to separase in a mutually exclusive manner (Gorr et al. 2005). Cdc2 degradation is

mediated by the APC and occurs simultaneously with PTTG destruction. Dephosphorylation of separase as well as its release from PTTG is necessary for its activation (Nasmyth 2005).

Separase activation at the onset of anaphase coincides not only with securin destruction and cohesion cleavage, but also with the cleavage of separase itself. Autocleavage of separase can occur at one of three amino acid sites, ¹⁴⁸³EIMR¹⁴⁸⁶¹⁵⁰³EILR¹⁵⁰⁶ and ¹⁵³²EIMR¹⁵³⁵. This separates the conserved C-terminal catalytic domain of separase from the N-terminal portion and results in the formation of proteins with molecular masses of 65 kDa and 170 kDa (Waizenegger et al. 2002) (Chestukhin et al. 2003). All three cleavage sites contain the consensus ExxR, which is also found in the cleavage sites of known separase substrates. Separase self-cleavage was initially thought to occur in order to generate a form of the protease which is able to cleave Scc1. However, separase mutants that could not be cleaved were still active in cleaving Scc1 (Waizenegger et al. 2002). Subsequently separase autocleavage was found to prepare the enzyme for its own destruction, through generating forms that can be recognised by the N-end rule pathway (Waizenegger et al. 2002). This pathway recognises proteins depending on their N-terminal residue and targets proteins with certain destabilising N termini for degradation via ubiquitination. This is thought to deactivate separase following its activity at the metaphase to anaphase transition, allowing cohesion to form again (Waizenegger et al. 2002).

Not surprisingly given its role as an endopeptidase, human separase was found to localise to centrosomes/spindle poles before anaphase, but it was then abruptly lost at anaphase onset (Chestukhin et al. 2003).

1.3.3 Hormonal regulation of separase expression and aneuploidy

Separase expression is increased *in vivo* and *in vitro* following oestrogen and progesterone treatment (Pati et al. 2004). Although this could be explained by hormones inducing proliferation, BrdU-labelling experiments did not show any changes cell turnover (Pati et al. 2004). As oestrogen and progesterone response elements 203 bp and 93 bp 5' of the transcriptional start site have been identified in the mouse separase promoter, oestrogen and progesterone may elicit a direct effect on the separase promoter (Pati et al. 2004). Analysis of the human separase promoter also indicates the presence of progesterone responsive elements and oestrogen receptor binding elements (Pati et al. 2004).

Over-expression of separase in FSK-3 mouse mammary epithelial cells which have a p53 mutant background can induce aneuploidy (Zhang et al. 2008). Aneuploidy was found to be a result of premature separation of sister chromatids and formation of anaphase bridges. Further, when these cells conditionally over-expressing separase were injected into wild type mice they developed tumours within 3-4 weeks. Analysis of these tumours also indicated aneuploidy and chromosome instability (Zhang et al. 2008). These findings collectively provide evidence that overexpression of separase results in prematurely separated chromosomes and lagging chromosomes in anaphase (Zhang et al. 2008). Therefore in the absence of p53 function, aberrant separase activity may allow the accumulation of cells with aneuploidy (Pati et al. 2004).

1.3.4 Separase and breast cancer

In a panel of 30 human breast carcinomas, representing mostly infiltrating ductal carcinomas, separase was found to be overexpressed in 70% of matched tumour specimens, strengthening the significance of the oncogenic activity of separase in breast

carcinogenesis (Zhang et al. 2008). Further to this, separase expression correlated with a higher incidence of recurrence and a lower 5-year survival rate (Meyer et al. 2009)

As well as being over-expressed in breast cancer, separase is also mislocalised. In breast tumours, high separase expression is constitutively nuclear regardless of the proliferative status of the cells, whereas normal breast resting cells, which do not proliferate, show clear exclusion of separase from the nucleus in the majority of interphase cells (Meyer et al. 2009). High separase expression and nuclear localisation were found to be the strongest predictors of breast tumour status. The mechanistic significance of nuclear separase localisation is unclear, but there are several possible explanations. First, it is possible that the normal mechanism of active nuclear exclusion of separase may be overwhelmed by separase over-expression. Second, export of separase from the nucleus of proliferating tumour cells may be inefficient owing to separase over-expression. Third, high separase levels in the nucleus may poise the cells for division. Finally, it is known that cohesins are recruited to damaged sites along chromosomes during repair, and cohesin is removed by separase following DNA repair. Hence nuclear retention of separase in proliferating tumour cells could result in premature removal of cohesion, a process normally occurring only after the repair process is complete. Premature cohesion removal would enhance mutational defects in the tumour DNA-damage response (Meyer et al. 2009).

1.4 PBF

PTTG binding Factor (PBF), also known as PTTG1 interacting protein (PTTG1IP), was initially isolated due to its interaction with PTTG in a yeast two hybrid screen (Chien and Pei 2000). Mutational studies show that this interaction occurs between

amino acids 123-154 in PTTG and the C-terminal 30 amino acids of PBF (Chien and Pei 2000). PBF had previously been cloned and termed C21orf3. C21orf3 was classified as a putative type Ia plasma membrane protein as it contains a cleavable N-terminal signal and a single integral transmembrane region. Moreover a motif for rapid protein internalisation through coated pit-mediated endocytosis (YXRF), and two putative N-glycosylation sites, were identified. These features suggest C21orf3 could be a cell surface glycoprotein participating in ligand uptake (Yaspo et al. 1998).

PBF is located on chromosome 21q22.3 and has a predicted molecular mass of 22 KDa with an open reading frame of 180 amino acids (Chien and Pei 2000). Further analysis of PBF revealed that it also contains putative phosphorylation sites for cyclic AMP and GMP-dependant kinases, protein kinase C, and kinase II, an extracellular N-terminal cytosine-rich region, common to plexins, semaphorins and integrins (PSI domain) (Bork et al. 1999) and a bipartite nuclear localisation signal (NLS) (Chien and Pei 2000) (Figure 1-12).

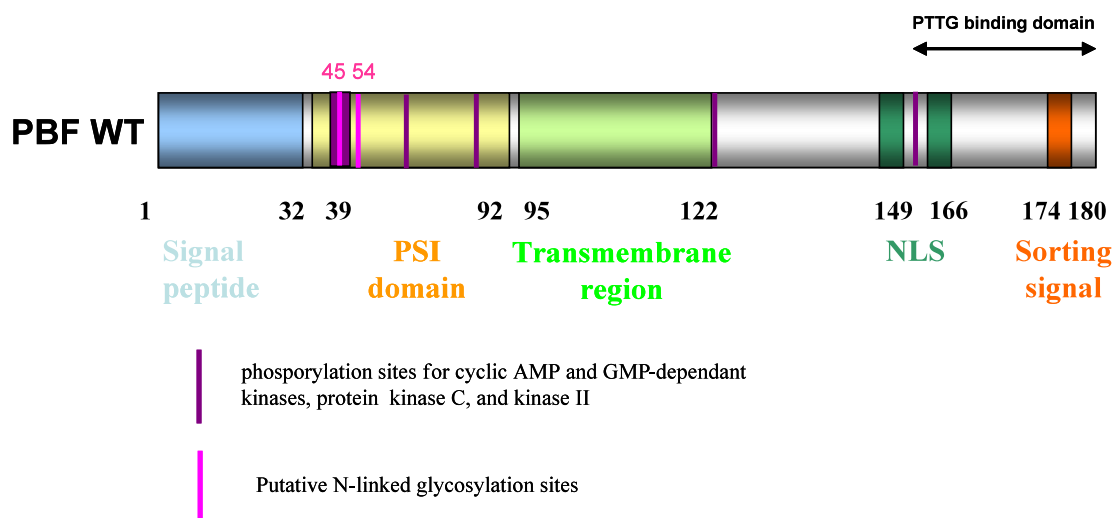


Figure 1-12 Schematic representation of the structure of PTTG binding factor (PBF). A putative, cleavable signal peptide is located at the N-terminus between amino acids 1- 32. A putative plexin, semaphrin and integrin (PSI) domain forms the remaining extracellular region between amino acids 39-92, which lies next to the putative transmembrane domain located between amino acids 95-122. At the C terminus between amino acids 149-166 there is a putative NLS and beginning at amino acid 174 there is a sorting signal for protein internalisation through endocytosis. The phosphorylation sites for protein kinase c, cAMP- and cGMP dependent protein kinases, and casein kinase II are shown in purple, and the two putative N-glycosylation sites at amino acids 45 and 54 are shown in pink.

1.4.1 PBF expression and localisation

PBF exhibits both nuclear and cytoplasmic expression (Stratford et al. 2005). To determine if the NLS of PBF is required for its nuclear localisation, COS-7 monkey kidney cells were transfected with HA-PBF containing a mutation in the NLS domain, and its localisation visualised by immunofluorescence. PBF expression as detected by immunofluorescence was shifted predominantly to a perinuclear and cytoplasmic location, suggesting that the NLS is required for the nuclear localisation of PBF (Chien and Pei 2000). In a manner similar to PBF, PTTG expression is also observed in the cytoplasm with partial nuclear staining. As PTTG does not contain a NLS, it is likely that PTTG requires shuttling into the nucleus. To determine if PBF can carry out this function, COS-7 monkey kidney cells were transfected with GFP-PTTG alone or in combination with

HA-PBF. An increased nuclear localisation of GFP-PTTG was observed when GFP-PTTG was co-transfected with HA-PBF compared to when it was transfected in isolation. This effect was abrogated when a PBF NLS mutant was used, indicating that the nuclear localisation domain of PBF facilitates the translocation of PTTG to the nucleus (Chien and Pei 2000).

As the transactivational function of PTTG would necessitate its presence in the nucleus, luciferase reporter assays were employed to assess the interaction of PTTG and PBF in FGF-2 regulation. When transfected alone, neither PTTG nor PBF significantly altered FGF-2 promoter activity. However when PTTG and PBF were co-transfected, luciferase activity was induced more than 3-fold. By transporting PTTG into the nucleus, PBF enables PTTG to carry out its transactivational control (Chien and Pei 2000).

Nucleotide sequence comparisons of PBF against the expressed sequence tag database identified more than 100 human ESTs derived from lung, uterus, heart, testis, bone marrow, pancreas, spleen, melanocytes, and neurons (Chien and Pei 2000). To determine the tissue distribution of PBF mRNA, a number of human tissues were investigated through Northern blot analysis. In all tissues examined PBF was expressed at high levels, suggesting PBF is widely expressed (Yaspo et al. 1998). This was later supported by a human RNA master blot which revealed that PBF mRNA is ubiquitously expressed (Chien and Pei 2000).

1.4.2 PBF and tumourigenesis

A potential role for PBF in tumourigenesis was proposed, as identity matches between PBF and expressed sequence tags corresponding to cDNAs over expressed in pancreatic cancer (Yaspo et al. 1998), colon carcinoma, Wilms tumour and parathyroid

tumours have been found (Chien and Pei 2000). In addition PBF expression was found to be increased in pituitary tumours (McCabe et al. 2003) and thyroid cancer (Stratford et al. 2005). In both pituitary tumours and thyroid cancer, PBF over-expression was correlated with PTTG over-expression (McCabe et al. 2003; Stratford et al. 2005). To investigate if PBF over-expression in these tumours could be due to stimulation by PTTG, primary human thyroid follicular cells as well as PTTG null HCT116 cells were transfected with wild type and mutated PTTG constructs. Wild type PTTG stimulated PBF mRNA expression but the mutant with a disrupted SH3-binding domain did not. This indicates the likely existence of a regulatory mechanism coordinating expression of the oncogene and its binding factor (Stratford et al. 2005).

To examine what influence increased PBF expression might have in cell transformation, NIH3T3 cell lines stably over expressing PBF, as well as wild type and mutated PTTG, were constructed and colony transformation assays conducted. PBF and PTTG over expression led to significant colony formation, although over-expression of mutant forms of PTTG which were unable to stimulate PBF mRNA expression did not. This study determined that PBF, when over-expressed, can induce cell transformation (Stratford et al. 2005). (Figure 1-13)

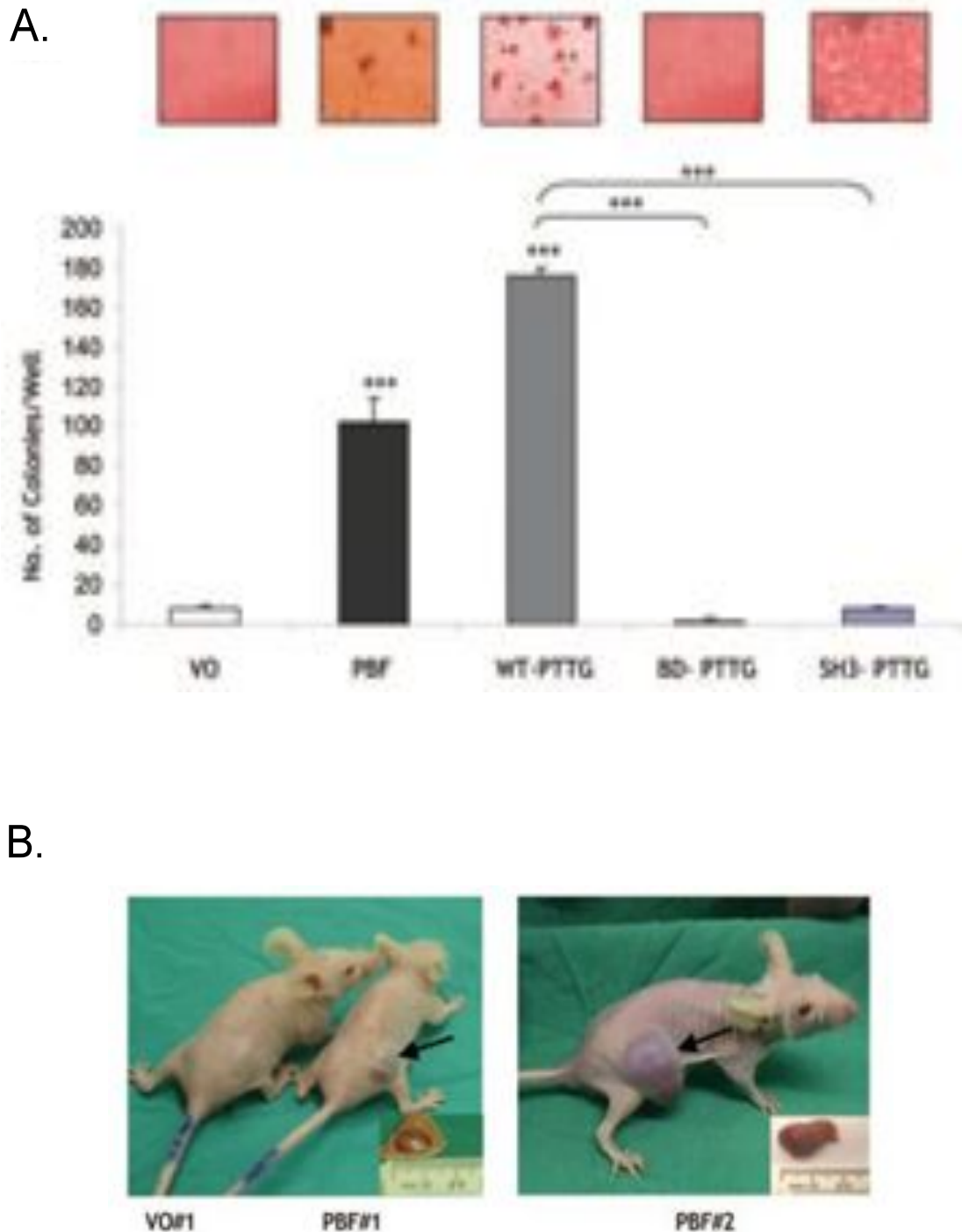


Figure 1-13. *A. In vitro cell transformation by PBF and PTTG as determined by soft agar using NIH3T3 cells stably transfected with PBF, wild type PTTG and mutated PTTG. Photomicrographs of cell colonies are shown above a graph quantifying the mean colony number per well. B. In vivo tumourigenesis by PBF as determined by tumour growth in nude mice injected with NIH3T3 cells overexpressing PBF, compared to vector only control. Taken from (Stratford et al. 2005).*

PBF expression is not only increased in thyroid cancer, it also correlates with early tumour recurrence (Stratford et al. 2005). Work from our group has demonstrated that PBF represses the expression of the sodium iodide transporter (NIS). Furthermore, we have shown that this is a direct promoter specific event occurring at the proximal NIS promoter and the human NIS upstream element (hNUE). PBF represses the hNUE by interfering with the binding of two transcriptional regulators PAX8 and USF1. In addition to this we have also shown that NIS mRNA and protein expression is reduced in thyroid cancer tissues compared to normals. NIS mediates active iodide (I^-) transport into thyroid cells, this function providing a unique and effective delivery system for detection and destruction of malignant thyroid cells with therapeutic doses of radioiodine. By down-regulating NIS, PBF fundamentally influences the prognosis of differentiated thyroid cancers (Boelaert et al. 2007). Despite facilitating PTTG nuclear entry, and presumably PTTG's interaction with separase, PTTG, PBF and Separase have not been examined together in breast cancer.

1.5 Hypothesis and aims

Breast cancer is mainly a postmenopausal disease, with more than three-quarters of tumours being hormone responsive (Benson et al. 2009). In post menopausal women, the ovaries stop producing oestrogen resulting in symptoms such as hot flushes, urogenital trophy, vaginal dryness and depression. Hormone replacement therapy (HRT) was introduced in the 1930s to reduce these symptoms (Anon 2008). Currently there are two different forms of HRT, oestrogen only or oestrogen plus progesterone. Both forms have been associated with an increased risk of breast cancer. However, some reports have shown that the risk is larger for oestrogen and progesterone preparations than oestrogen only preparations (Cuzick 2008; Hoover et al. 1976). We now hypothesise that

oestrogen/progesterone treatment in post-menopausal women may upregulate PBF, PTTG and separase expression. Subsequently, breast cells may show increased mitosis, and increased accumulation of genetic mistakes. In addition, we predict that by upregulating these genes oestrogen/progesterone will increase the invasiveness and motility of breast cells. This will be an important finding as the invasion of tumour cells into the surrounding host tissue is an important step in the metastatic process and metastasis of the primary tumour to distant sites is the main cause of death among breast cancer patients (Mangala et al. 2007).

The main aims set out for this body of work were 3 fold. Initially, I set out to determine if oestrogen and progesterone upregulated the expression of PBF, PTTG and separase in breast cancer cells *in vitro* and to determine whether these genes were overexpressed *in vivo* in breast cancer tissues. Secondly, I wanted to determine if oestrogen and progesterone influenced the activity of the PBF, PTTG and separase promoters. Finally, I sought to determine if the overexpression of these genes influenced the invasiveness of breast cancer cells.

Chapter 2

**Oestrogen and progesterone regulation of PBF, PTTG
and separase**

2.1 Introduction

Previously it has been reported that PTTG is overexpressed in pituitary tumours (Heaney et al. 2002) and that pituitary PTTG is induced by oestrogen, with maximum expression levels occurring coincidentally with peak serum estradiol levels, and nuclear antigen (PCNA) expression during the rat estrus cycle (Heaney et al. 2002). Naturally occurring catechol-metabolites of estradiol, 2-hydroxy-estradiol and diethylstilbestrol, also increased PTTG mRNA expression in rat GH3 cells, suggesting catechol metabolites of estradiol may mediate the oestrogen action (Heaney et al. 2002). Additionally, PTTG mRNA induction was abrogated *in vivo* and *in vitro* by coadministration of the specific anti-oestrogen, ICI-182780, implying that the oestrogen effects on PTTG are mediated through the oestrogen receptor (Heaney et al. 2002). PTTG mRNA and protein are also overexpressed in breast cancer, and expression levels correlate with mitotic rates, degree of nuclear pleomorphism and ER α expression (Ogbagabriel et al. 2005). Further, PTTG protein expression was induced in mice mammary epithelial cells after hormonal treatment with oestrogen and progesterone (Pati et al. 2004). This suggests that oestrogen may also regulate PTTG expression in breast tissue.

In addition to PTTG, PBF is upregulated in pituitary tumours and its expression correlates with PTTG, presumably because PTTG can stimulate PBF mRNA expression (Stratford et al. 2005). However, as examination of the PBF promoter in our laboratory revealed the presence of consensus EREs, oestrogen may also regulate the expression of PBF (Figure 2-1). PBF expression levels in breast cancer have not been investigated before.

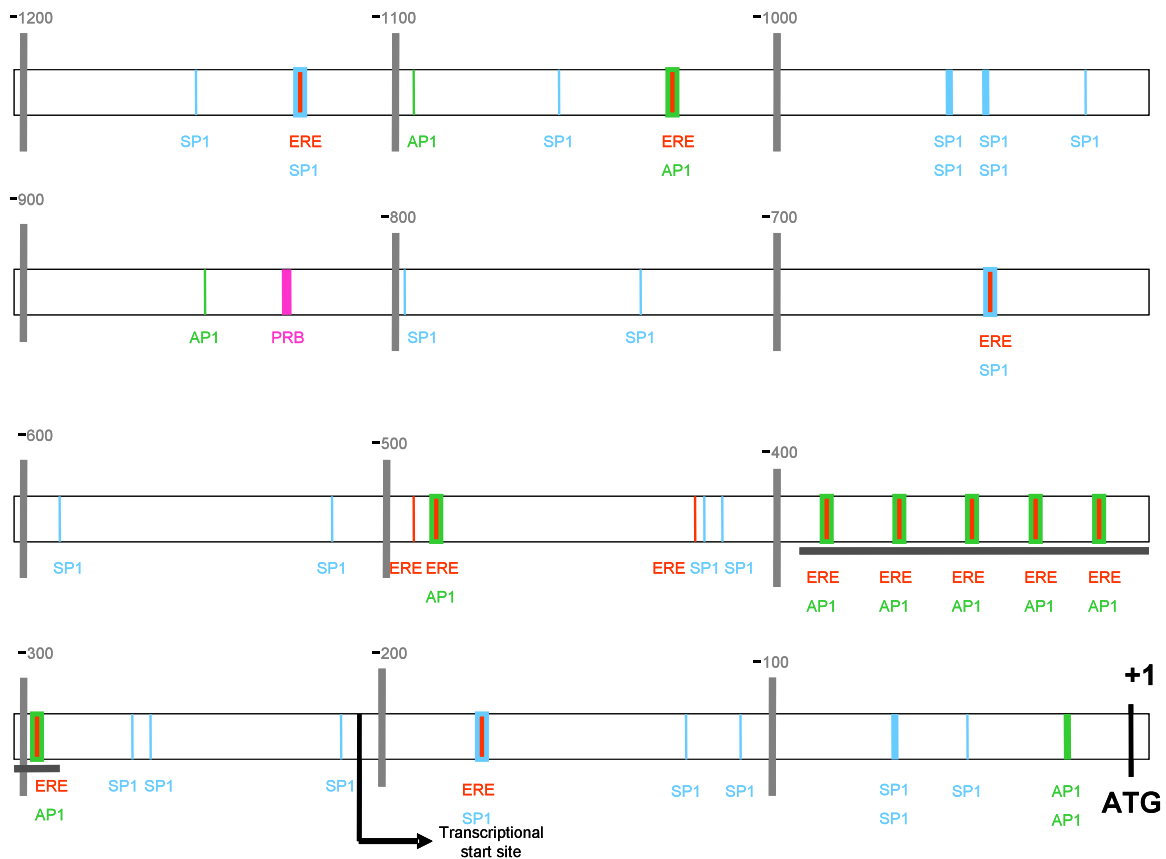


Figure 2-1: A detailed schematic representation of the first 1200 bp of the human PBF promoter upstream of the ATG start codon. Oestrogen response elements (ERE) are shown in red, SP1 sites are shown in blue, AP1 sites are shown in green and progesterone response elements are shown in pink. The transcriptional start site is located 211 bp upstream of the translational start site (ATG).

Like PTTG, separase protein expression levels have been shown to be significantly overexpressed in human breast tumours compared to matched normal specimens (Zhang et al. 2008) and separase mRNA and protein expression have been shown to be positively regulated by oestrogen and progesterone *in vivo*. The increase in separase expression observed after oestrogen and progesterone stimulation in mice is unlikely to be an indirect effect due to alveolar cell differentiation as oestrogen and progesterone also increased separase mRNA expression in a time-dependant manner in MCF-7 cells (Pati et al. 2004). In addition, analysis of the human and mouse separase promoter revealed the presence of consensus oestrogen and progesterone response elements (Pati et al. 2004). The aim of

this chapter was to determine whether PTTG and PBF are regulated by oestrogen and progesterone in MCF-7 breast cancer cells. We also wanted to investigate the finding that separase mRNA is upregulated by oestrogen and progesterone in this cell line, and to determine whether its protein expression levels were also induced.

2.2 Materials and Methods

Unless stated otherwise, all chemicals described in this thesis were sourced from Sigma-Aldrich (Poole, Dorset, UK).

2.2.1 Cell lines

MCF-7 human Caucasian breast adenocarcinoma cells were obtained from the European Collection of Cell Cultures (ECACC). They were established from the pleural effusion of a 69 year female caucasian suffering from a breast adenocarcinoma. MCF-7 cells exhibit some features of differentiated mammary epithelium including oestradiol synthesis and formation of “domes”. Domes correspond to a distinct stage of development of lobulo-alveolar present in the mammary gland of pregnant or lacting mammals (Zucchi et al. 2002). MCF-7 cells express the wild type and variant oestrogen receptors as well as the progesterone receptor. These cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10 % fetal bovine serum (FBS), L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100mg/ml). They were passaged twice weekly in T75 culture flasks and 0.25 % trypsin-EDTA (Gibco) was used to re-suspend the cells

2.2.2 Hormones

17 β -Estradiol (C₁₈H₂₄O₂) is the most potent form of mammalian estrogenic steroids. In humans it is produced primarily by the cyclic ovaries and the placenta but is also

produced by the adipose tissue of men and postmenopausal women. (PubChem compound CID5757).

Diethylstilbestrol ($C_{18}H_{20}O_2$) is a synthetic nonsteroidal oestrogen that was first synthesised in 1938 and frequently prescribed to women between the 1940s and the 1970s to prevent miscarriage and other pregnancy complications (Schrager and Potter 2004). In recent years it was found to be associated with an increased risk of breast cancer in women who took the drug during pregnancy and in women ≥ 40 years of age who were exposed to in utero (Palmer et al. 2006).

Progesterone ($C_{21}H_{30}O_2$) is primarily secreted by the corpus luteum and the placenta but can also be produced by the ovaries (pubChem compound CID5994). It has been discussed in more detail in Section 1.1.2.7.2

2.2.3 Hormonal treatment

MCF-7 breast cancer cells were maintained in RPMI 1640 phenol red free medium (Gibco) supplemented with 10 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml) for 7 days prior to hormone stimulation. Phenol red free medium was used because phenol red is a weak oestrogen mimic and can enhance the growth of cells which express the oestrogen receptor (Berthois et al. 1986).

Twenty-four hours prior to hormonal treatment of MCF-7 cells, T75 flasks were washed with PBS and cells resuspended using trypsin-EDTA. 12 and 6 well culture plates were seeded with 7.5×10^4 and 1.5×10^5 cells per well in 1 ml and 2 ml of complete phenol red-free medium respectively.

Cells were treated with diethylstilbestrol, progesterone and 17 β -estradiol either alone or in combination at final concentrations of 10 nM and 20 nM for 48 hours.

2.2.4 RNA extraction and reverse transcription

Total RNA was extracted using a single step acid guanidinium phenol-chloroform extraction method (TRI REAGENTTM; Sigma-Aldrich, St. Louis, MO, USA). Following the manufacturer's guidelines, cells were harvested from 12 well plates in 250 μ l TRI REAGENTTM. All samples were subjected to one freeze-thaw cycle at -80°C. Subsequently, 50 μ l chloroform (99+ %) was added to each reaction, and samples shaken vigorously for 15 seconds. After standing for 15 minutes at room temperature, samples were centrifuged at 12,000 g for 15 minutes at 4°C. This separates the mixture into 3 phases. The uppermost colourless aqueous phase containing RNA was transferred into a fresh tube and 125 μ l isopropanol (99+ %) was added. After mixing, the samples were left to stand at room temperature for 10 minutes before centrifugation at 12,000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed in 250 μ l 75% ethanol then centrifuged at 7500 g for 5 minutes at 4°C. The ethanol was discarded and the pellet left to air dry before being dissolved in 30 μ l of nuclease free water.

The concentration of RNA extracted was determined by absorbance of light at λ 260, and the ratio of λ 260: λ 280 used as an indication of sample purity, where RNA of good quality has a ratio between 1.8 and 2.0.

Synthesis of cDNA from 0.5 μ g RNA was carried out using the Reverse Transcription System (Promega). In a total volume of 20 μ l, each reaction consisted of 2.5 mM MgCl₂, 0.5 x Reverse transcriptase buffer (5 mM Tris-HCl pH 8.8, 25 mM KCl, 0.05 % Triton[®] X-100), 0.5 mM each dNTP, 0.5 U/ μ l Recombinant RNasin[®], 0.25 μ g Random

Primers and 6.9 U/μl AMV reverse transcriptase. Following the addition of RNA, which had been incubated at 70°C for 10 minutes, the whole reaction was incubated at room temperature for 10 minutes. Reverse transcription was performed on a Mastercycler gradient PCR machine (Eppendorf) under the following conditions: 42°C for 1 hour, 95°C for 5 minutes and 4°C for 5 minutes.

2.2.5 Quantitative real time PCR

Expression levels of mRNA in MCF-7 cells were assessed using TaqManTM chemistry. TaqManTM probes consist of a fluorophore FAM (6-carboxy-flourescien) or VIC (full chemical name patent protected) covalently attached to the 5' end, and a quencher, TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end. The quencher molecule suppresses the fluorescence emitted by the fluorophore when excited by the cyclor's light source via fluorescence resonance energy transfer (FRET). During FRET the high energy fluorophore acts as a donor by transferring energy to the low energy quencher. As the Taq polymerase extends the primer, the 5' to 3' exonuclease activity of the polymerase degrades the probe. Degradation of the probe releases the fluoropore from it and breaks the close proximity to the quencher, thereby increasing its fluorescent signal by relieving the quencher effect. Hence, fluorescence detected in the real-time PCR thermal cyclor is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

Quantitative primers and probes for the target genes PBF (Stratford et al. 2005), PTTG (McCabe et al. 2002) and separase (Pemberton et al. 2007) had all been validated previously by our group. The primer and probe sets designed for each target gene using the primer ExpressTM software (Applied Biosystems, Foster City, CA, USA), were tested for amplification efficiency and potential housekeeping gene interference and found to be

suitable for multiplex analysis (McCabe et al. 2002; Pemberton et al. 2007; Stratford et al. 2005). The primer and probe sequences are given in Table 1.

Gene	Forward Primer	Reverse Primer
PBF	CTCTTCTCAGTTTGTGAAACGCTAA	CTGCCCTGGGAGAATGACA
	Probe: AAGCCGCCTGACGGCACCCAGC	
PTTG	GAGAGAGCTTGAAAAGCTGTTTCAG	TCCAGGGTCGACAGAATGCT
	Probe: TGGGAATCCAATCTGTTGCAGTCTCCTTC	
Separase	CGGGATGATGGTGTGTATGG	CAAAGTCATAAACCAACACAGTGTAGA
	Probe: AGCAGTCTTTTCTTCAGATGTACTTTCAGGGACTTCA	

Table 1: Oligonucleotide sequences of PCR primers and TaqManTM probes used. Each probe possessed a 5' FAM reporter molecule and a 3' TAMRA quencher molecule.

PCR reactions were carried out in duplicate in 96-well optical plates (Applied Biosystems) in a total volume of 25 µl. This consisted of 900 nM each of forward and reverse PCR primers (Alta Biosciences), 150 nM FAM-labelled probe (Eurogentec), 1x qPCR Mastermix Plus QGS (Eurogentec), and 1.25 µl eukaryotic 18 s rRNA endogenous Control (Applied Biosystems). The 18s endogenous control was a pre-optimised primer and VIC-labelled probe mix, which was used as an internal reference to correct for variation in reverse transcription efficiency between samples. PCR was performed on the 7500 real-Time PCR system (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A threshold line was set in the exponential phase of the amplification curve for each gene and its corresponding 18s control data. The cycle threshold (Ct) value for

each reaction was then established by the cycle number at which each logarithmic PCR plot crossed the threshold line. The ΔCt value was determined by subtracting the 18s Ct value from the target gene Ct value. Finally, fold change was calculated using the equation $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ was the ΔCt value from the hormone treated group minus the ΔCt value for the vehicle treated group.

2.2.6 Protein extraction and quantification

Forty-eight hours after hormonal treatment, cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % v/v igeal CA-630, 6 mM sodium deoxycholate, 1 mM EDTA) containing 60 $\mu\text{l/ml}$ protease inhibitor cocktail (containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstainA, E-64, bestain, leupeptin, and aprotinin). Lysates were subjected to a freeze thaw cycle at -20°C to aid lysis.

Protein concentration was determined by the colourimetric Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA, USA). Protein standards of bovine serum albumin (BSA) were prepared at concentrations of 0, 0.25, 0.5, 0.75, 1 and 5 $\mu\text{g}/\mu\text{l}$. Reagent A (25 μl) and Reagent B (200 μl) were added to 5 μl of each BSA standard and protein standard in duplicate and incubated at room temperature in the dark for 15 minutes. Absorbance was measured at 690 nm in a Victor³ multilabel counter (Perkin Elmer, Waltham, MA, USA) and the protein concentrations calculated from the BSA standard curve produced.

2.2.7 Western blot analyses

Protein samples (30 µg) were incubated at 95°C for 5 minutes with an equal volume of Laemmli sample buffer (Bio-Rad) containing 43 mg/ml DL-Dithiothreitol (DTT), and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel consisted of 375 mM Tris-HCl pH 8.8, 10.6 % acrylamide from 30 % acrylamide: 0.8 % bis-acrylamide stock solution (Gene flow), 3.5 mM SDS, 0.1% (w/v) TEMED and 4.4 mM APS. A stacking gel was then prepared using 375 mM Tris-HCl pH 8.8, 3.8 % acrylamide from 30 % acrylamide/ 0.8 % bis-acrylamide stock solution, 7 mM SDS, 0.2 % (w/v) TEMED and 8.8 mM APS. The proteins were electrophoresed in a running buffer containing 25 mM Tris, 192 mM glycine and 3.5 mM SDS with 5 µl Precision Plus Protein Dual Color Standards as size markers.

The proteins were subsequently transferred to Hybond-P (Amersham Biosciences), a hydrophobic polyvinylidene difluoride (PVDF) membrane, which was pre-soaked briefly in 100 % methanol and then a transfer buffer consisting of 25 mM Tris, 192 mM glycine and 20 % methanol. Transfer of proteins from gel to membrane was via a current of 360 mA in transfer buffer for 1 hour. The membrane was then blocked in 5 % non-fat milk in Tris-buffered saline with Tween (TBS-T; 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.025 % Tween[®] 80). The membrane was probed with primary antibody, mouse monoclonal anti-pds-1 (PTTG) antibody (LabVision) at a concentration of 1:1,000, mouse monoclonal anti-separase antibody (Novus Biologicals) at a concentration of 1:500 or rabbit polyclonal anti-PBF antibody (made by Eurogentec (Seraing, Belgium) for our laboratory using the full length PBF protein as an epitope) (Smith et al. 2009) at a concentration of 1:1,000 in 5 % non-fat milk in TBS-T at 4°C for 16 hours.

Excess primary antibody was removed through 15 minute washes with TBS-T before incubation with secondary antibody, 650 ng/ml HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako) or 125 ng/ml HRP-conjugated polyclonal goat anti-rabbit immunoglobulins (Dako), in 5% non-fat milk in TBS-T at room temperature for 1 hour. The membrane was washed six times at 10 minute intervals in TBS-T, and subsequently the antigen-antibody complexes were detected using the ECL Plus chemiluminescent detection system (Amersham Biosciences) following the manufacturer's instructions.

To assess protein loading, following a TBS-T wash, the membrane was probed with mouse monoclonal anti- β actin antibody (clone AC-15; Sigma) at a concentration of 1:10,000 in 5 % non-fat milk in TBS-T. After three 10 minute washes in TBS-T, the membrane was incubated with secondary antibody, 650 ng/ml HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako), in 5 % non-fat milk in TBS-T at room temperature for 1 hour. Excess secondary antibody was removed with four 15 minute TBS-T washes and protein detected with ECL chemiluminescent detection system (Amersham Biosciences) following the manufacture's instructions.

2.2.8 Statistical analysis

Data were analysed using Sigma Stat (SPSS Science Software UK Ltd). The Kolmogorov-Smirnov test determined whether the data followed normal distribution. If the data set to be tested followed normal distribution, it was analysed using a two sample Student's t-test. However, if the data set was non-parametric, the Mann Whitney Rank Sum test was used for comparison between two groups of data. Significance was taken as $p < 0.05$. For quantitative real time PCR, all statistics were performed on the ΔC_t values to avoid potential bias through transformation of the data with the equation $2^{-\Delta\Delta C_t}$.

2.3 Results

2.3.1 Oestrogen and progesterone regulate the mRNA and protein expression levels of PBF

To determine whether oestrogen or progesterone regulate the mRNA and protein expression levels of PBF, MCF-7 cells were treated with diethylstilbestrol or progesterone alone or in combination. Compared to vehicle, PBF mRNA was induced maximally by 20 nM diethylstilbestrol (2.1 ± 0.1 fold, $p < 0.05$, $N=3$), 10 nM progesterone (1.8 ± 0.04 -fold, $N=3$) and by diethylstilbestrol plus progesterone (10nM of each, 1.9 ± 0.3 -fold, $N=3$) (Figure 2-2). PBF protein levels were significantly upregulated by 10 nM diethylstilbestrol (2.5 ± 0.4 -fold, $p < 0.001$, $N=3$), 20 nM diethylstilbestrol (2.3 ± 0.5 -fold, $p < 0.05$, $N=3$), 10 nM progesterone (1.4 ± 0.1 -fold, $p < 0.05$, $N=4$), 10 nM diethylstilbestrol plus 10 nM progesterone (2.9 ± 0.6 -fold, $p < 0.05$, $N=3$) and 20 nM diethylstilbestrol plus 20 nM progesterone (2.4 ± 0.4 -fold, $p < 0.05$, $N=3$), (Figure 2-3).

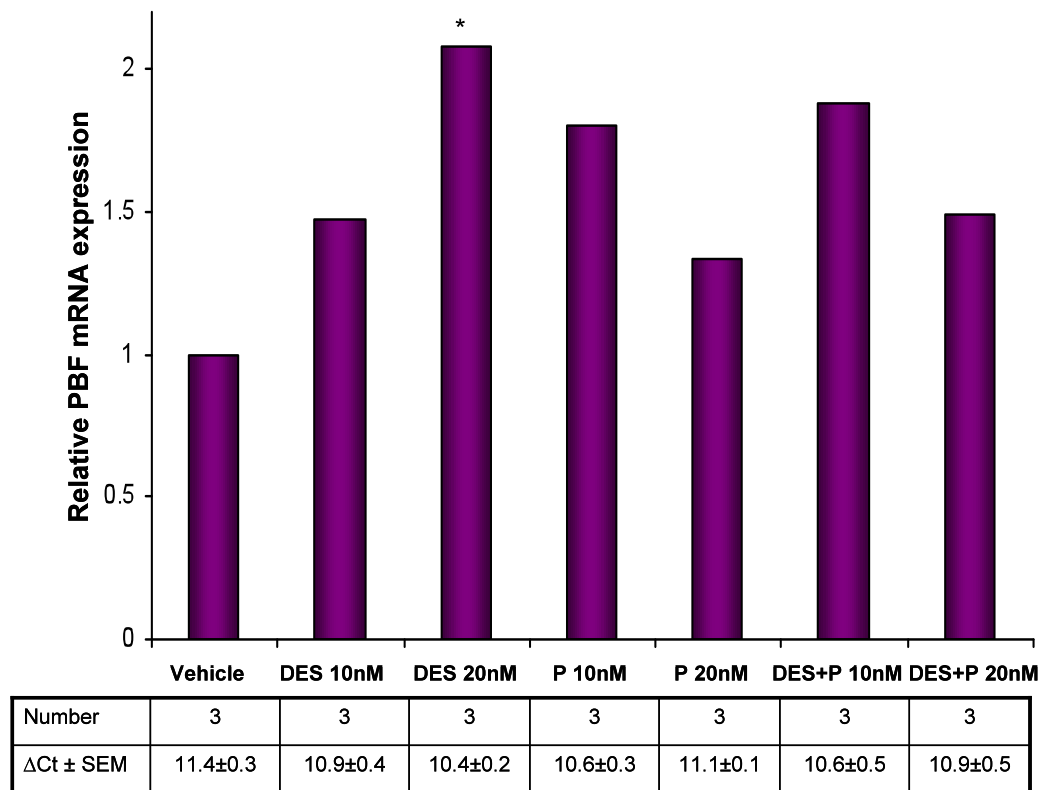


Figure 2-2: Effect of diethylstilbestrol and progesterone on PBF mRNA expression levels in MCF-7 cells. Gene expression is displayed as a fold change relative to the vehicle treatment given the arbitrary value of 1.0. The number of samples used and the mean ΔCt values \pm the standard error of the mean for each group, obtained using quantitative RT-PCR, are given in the corresponding columns in the table underneath the graph ($P < 0.05^*$, $N = 3$). DES = diethylstilbestrol, P = progesterone.

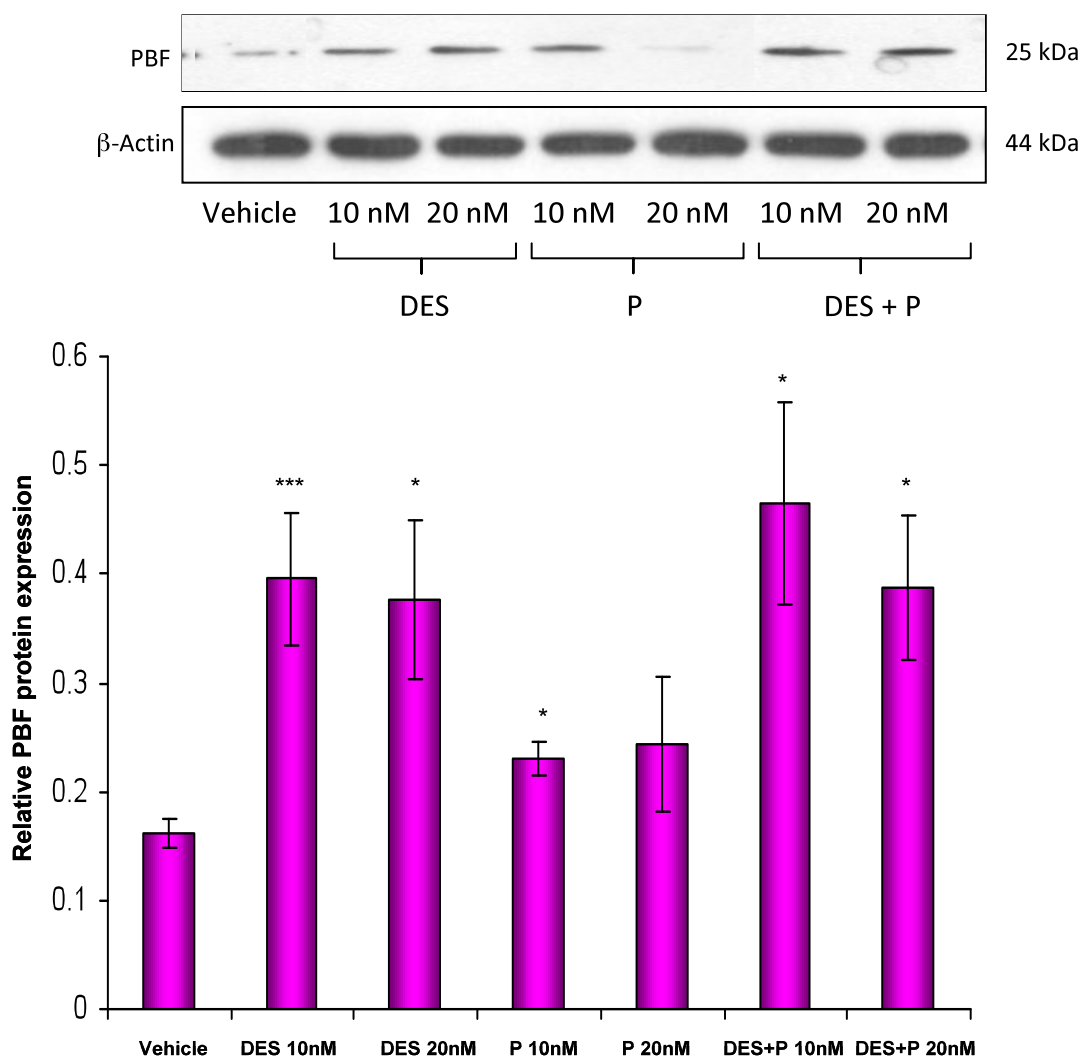


Figure 2-3: Effect of diethylstilbestrol and progesterone on PBF protein expression as determined by scanning densitometry from Western blotting. Expression of PBF was normalised to the intensity of β -actin in parallel blots ($N=3$, $p < 0.05^*$, $p < 0.001^{***}$). DES = diethylstilbestrol, P = progesterone.

2.3.2 Oestrogen and progesterone regulate the protein expression levels of PTTG

In the same samples that were used to analyse PBF mRNA and protein expression levels, PTTG expression levels were also examined. mRNA levels were determined by real time PCR using PTTG primers and probes and PTTG protein expression was detected through Western blotting using a PTTG antibody. Compared to vehicle, PTTG mRNA

was not significantly altered by diethylstilbestrol or progesterone alone or in combination (Figure 2-4). PTTG protein levels were significantly upregulated by 10 nM diethylstilbestrol (2.7 ± 0.4 -fold, $p < 0.001$, $N=3$), and 20 nM diethylstilbestrol plus 20 nM progesterone (2.1 ± 0.1 -fold, $p < 0.05$, $N=3$), (Figure 2-5).

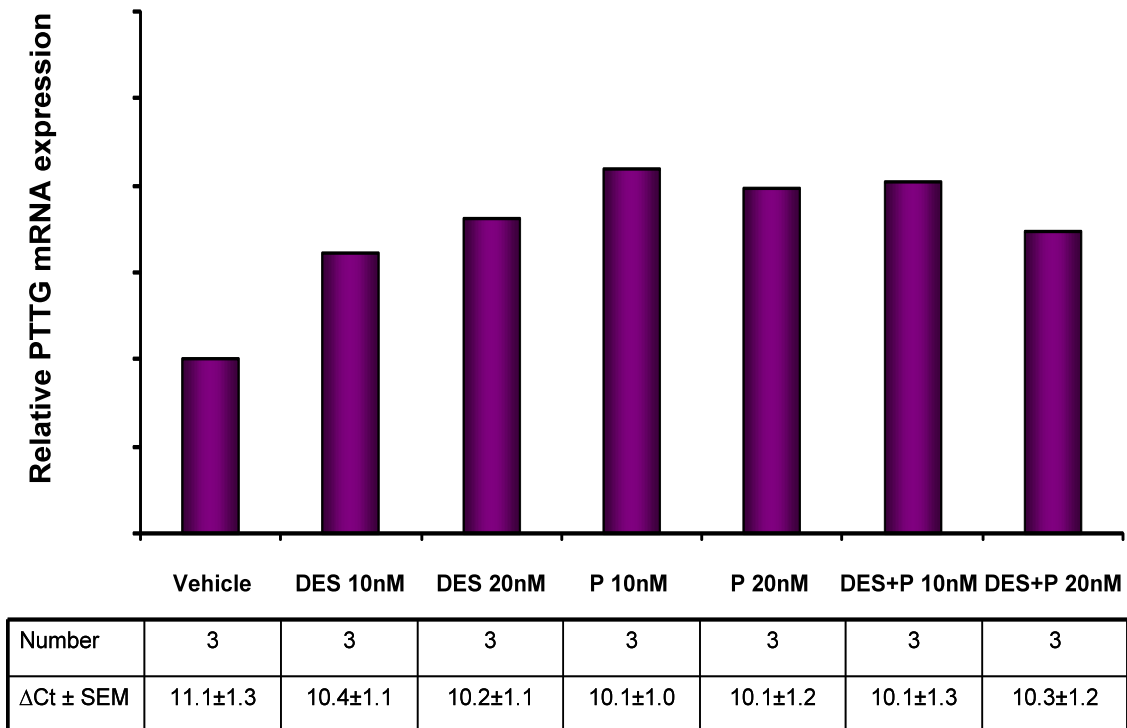


Figure 2-4: Effect of diethylstilbestrol and progesterone on PTTG mRNA expression levels in MCF-7 cells. Gene expression is displayed as a fold change relative to the vehicle treatment given the arbitrary value of 1.0. ($N=3$). DES = diethylstilbestrol, P = progesterone.

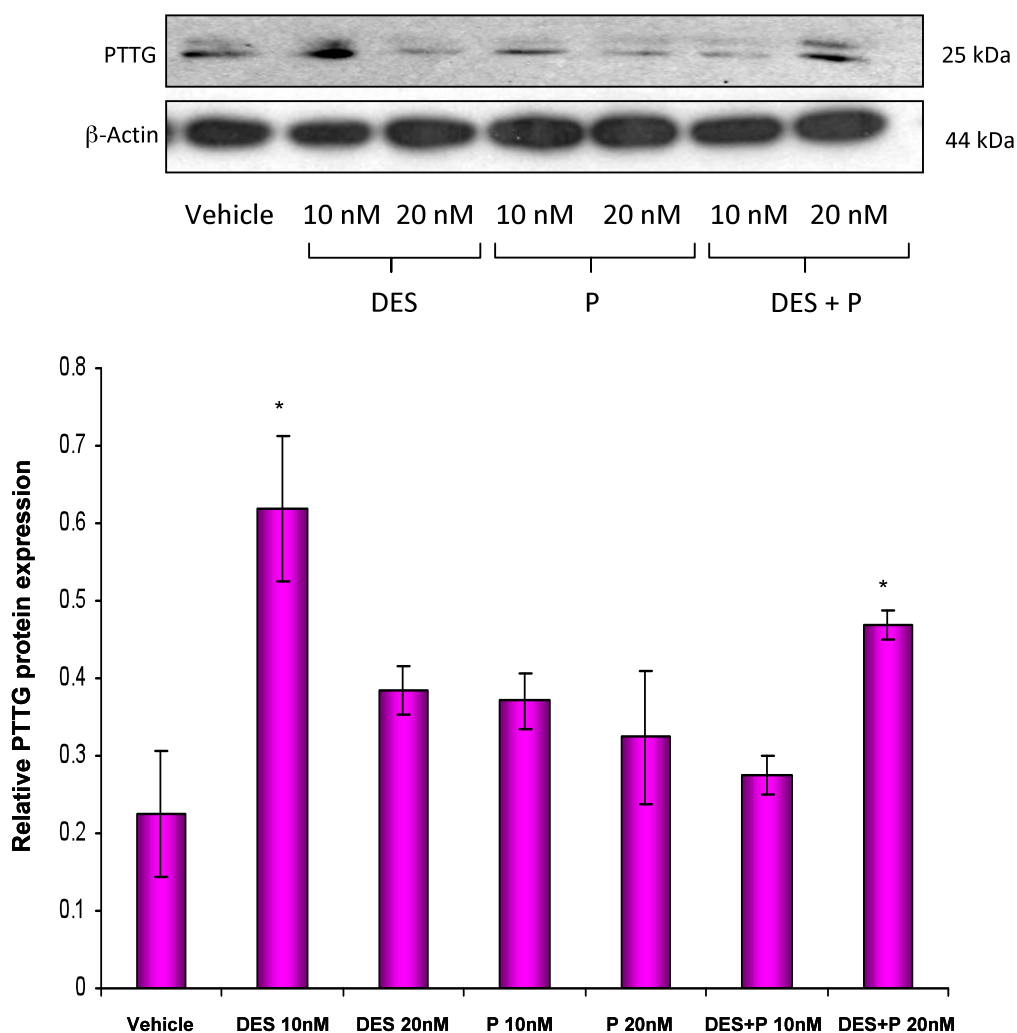


Figure 2-5: Effect of diethylstilbestrol and progesterone on PTTG protein expression as determined by scanning densitometry from Western blotting. Expression of PTTG was normalised to the intensity of β -actin in parallel blots ($p < 0.05^*$, $N=3$). DES = diethylstilbestrol, P = progesterone. PTTG runs as a doublet at 25 to 29 kDa, both bands were assessed and combined in scanning densitometry.

2.3.3 Oestrogen and progesterone do not regulate the mRNA or protein expression levels of separase

Similarly to PBF and PTTG, the alteration in separase mRNA and protein expression was examined in response to hormones. Separase mRNA (Figure 2-6) and protein (Figure 2-7) expression levels were not significantly altered by oestrogen or progesterone treatment either alone or in combination.

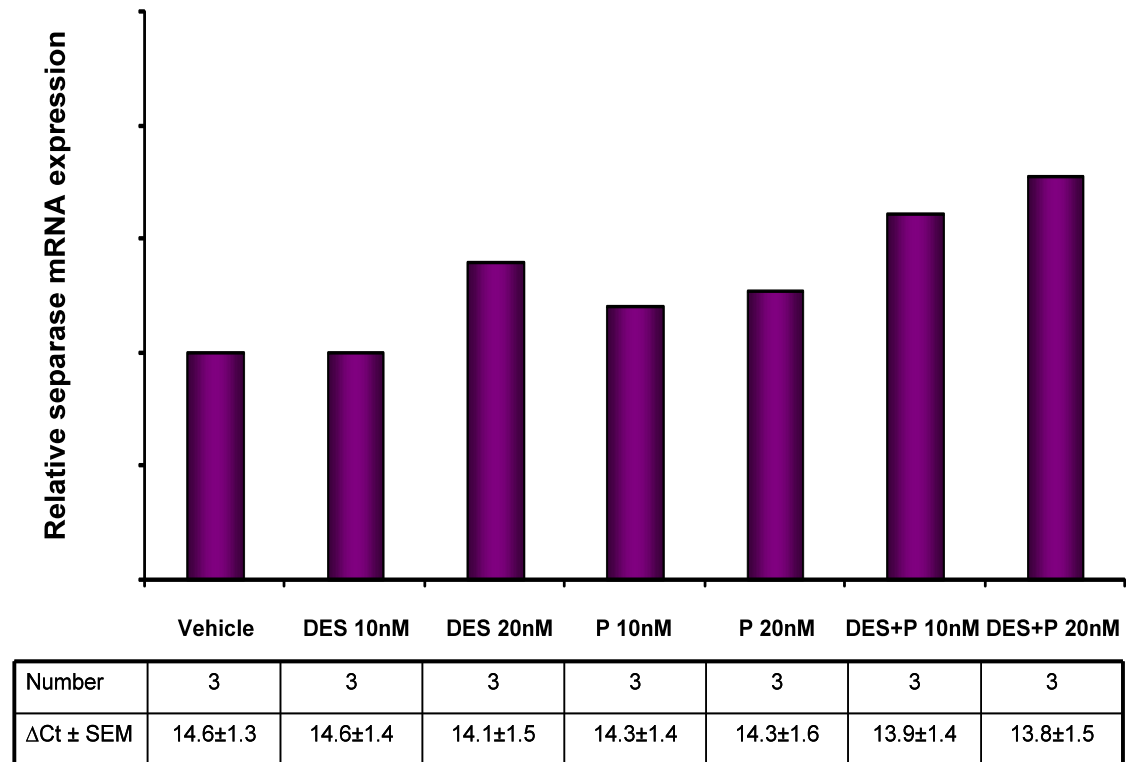


Figure 2-6: Effect of diethylstilbestrol and progesterone on separase mRNA expression levels in MCF-7 cells. Gene expression is displayed as a fold change relative to the vehicle treatment given the arbitrary value of 1.0. (N=3). DES = diethylstilbestrol, P = progesterone.

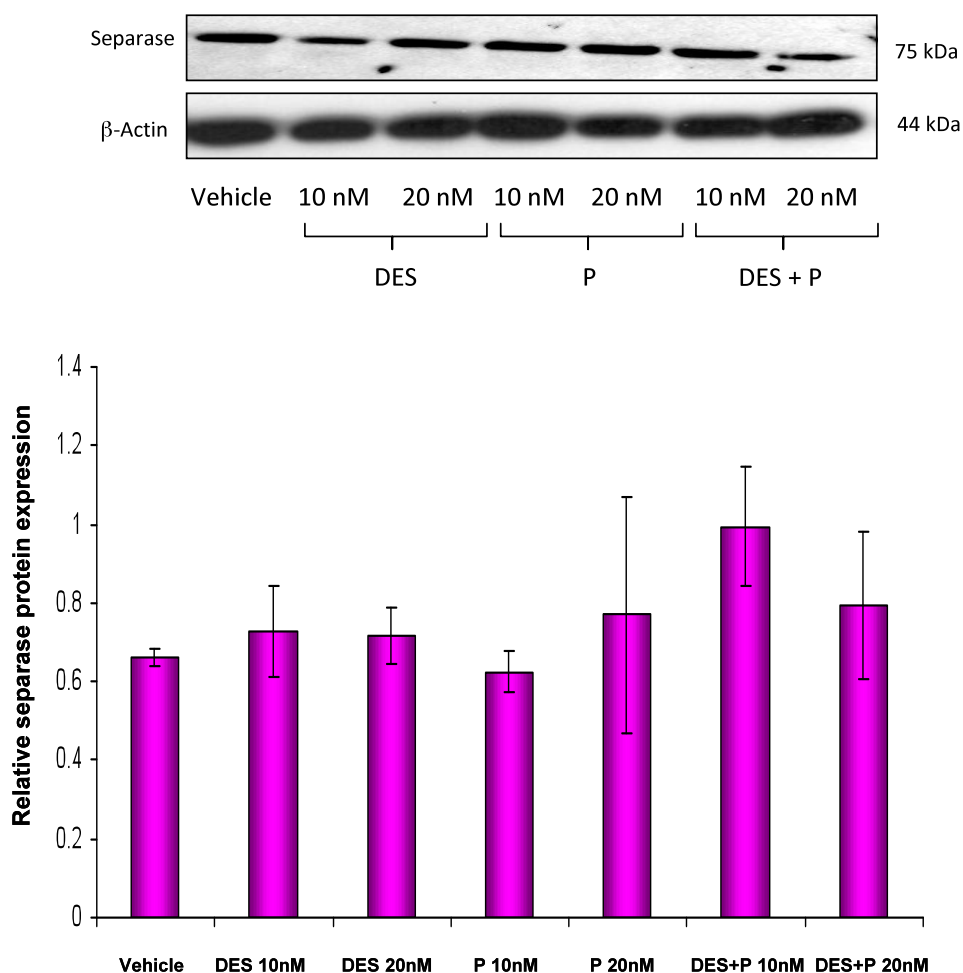


Figure 2-7: Effect of diethylstilbestrol and progesterone on separase protein expression as determined by scanning densitometry from Western blotting. Expression of separase was normalised to the intensity of β -actin in parallel blots ($N=3$). DES = diethylstilbestrol, P = progesterone.

2.3.4 17β -Estradiol has the same effect as diethylstilbestrol on PBF mRNA and protein expression levels

In this study, diethylstilbestrol has been used to investigate the role of oestrogen on the regulation of PBF, PTTG and separase mRNA and protein expression, given that it previously elicited a stronger oestrogenic response on PTTG mRNA expression compared to 2-hydroxy-estradiol (Heaney et al. 2002). Whilst diethylstilbestrol is commonly used to investigate the effects of oestrogen, it is a synthetic oestrogen. The major *in vivo*

oestrogen secreted by the premenopausal ovary is 17β -estradiol. Because of this, MCF-7 cells were treated with 17β -estradiol to determine whether it was also able to regulate the mRNA and protein expression levels of PBF, the gene which gave the most robust response to oestrogen treatment. Compared to vehicle, PBF mRNA was significantly upregulated by 10 nM 17β -estradiol (2.2 ± 0.4 -fold, $p < 0.05$, $N=3$) and by 20 nM 17β -estradiol (3.3 ± 0.9 -fold, $p < 0.05$, $N=3$) (Figure 2-8). PBF protein levels were also significantly induced by 17β -estradiol at final concentrations of 10 nM (3.1 ± 0.1 -fold, $p < 0.001$, $N=3$) and 20 nM (3.0 ± 0.2 -fold, $p=0.001$, $N=3$) (Figure 2-9).

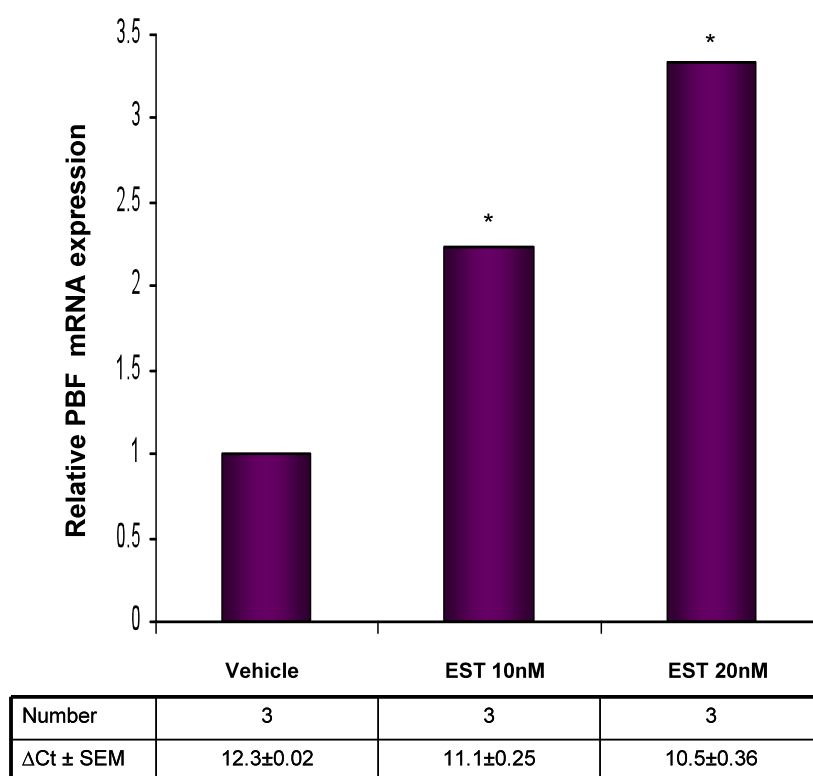


Figure 2-8: Effect of 17β -estradiol on PBF mRNA expression levels in MCF-7 cells. Gene expression is displayed as a fold change relative to the vehicle treatment given the arbitrary value of 1.0. ($P < 0.05^*$, $N=3$). EST = 17β -estradiol.

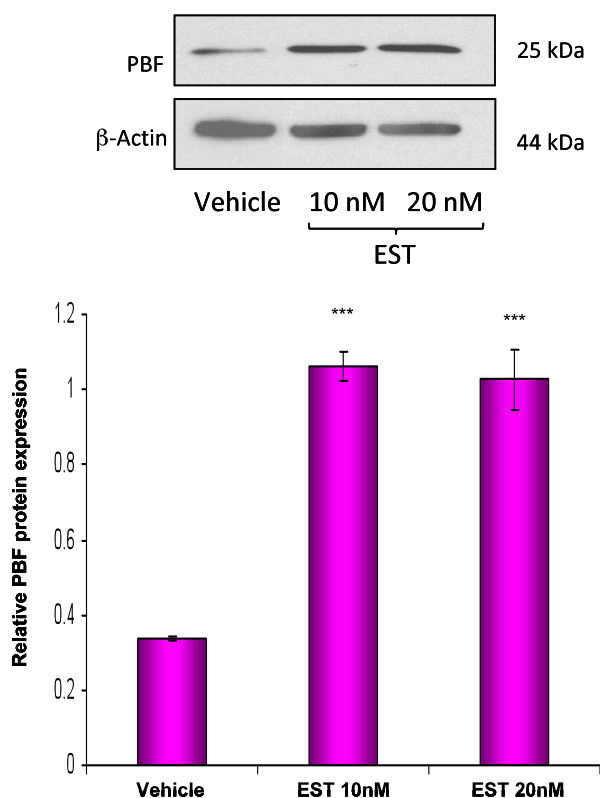


Figure 2-9: Effect of 17 β -estradiol on PBF protein expression as determined by scanning densitometry from Western blotting. Expression of PBF was normalised to the intensity of β -actin in parallel blots ($p < 0.001^{***}$, $N=3$). EST = 17 β -estradiol.

2.4 Discussion

Previous studies have implicated oestrogen and progesterone in the regulation of PTTG and separase (Heaney et al. 2002; Pati et al. 2004). In keeping with this, as breast tissue is responsive to hormones, increased expression of PTTG and separase has been observed in breast cancer when compared to normal breast tissue (Ogbagabriel et al. 2005; Zhang et al. 2008). PBF expression in breast cancer however, has never been investigated before.

To determine whether oestrogen and progesterone could regulate PTTG, PBF and separase mRNA and protein in human breast cells, ER α and progesterone receptor

positive MCF-7 cells were treated with diethylstilbestrol and progesterone at final concentrations of 10 nM and 20 nM either alone or in combination. Alterations in PBF, PTTG and separase mRNA and protein expression levels were subsequently measured by TaqMan™ PCR and Western blotting.

PBF is a poorly characterised protein in terms of function and it is not known what regulates its expression. The data obtained show that PBF mRNA and protein expression are upregulated in response to diethylstilbestrol. 17 β -estradiol, unlike diethylstilbestrol, is a naturally occurring oestrogen and is the main oestrogen secreted by the pre-menopausal ovary. I also confirmed that PBF mRNA and protein expression are upregulated in response to 17 β -estradiol. In addition PBF protein was upregulated by progesterone alone and in combination with diethylstilbestrol, although non-significant these treatments also upregulated PBF mRNA. As both the mRNA and protein levels are upregulated this suggests a direct effect on the PBF promoter. Indeed after close examination of the human PBF promoter, multiple putative consensus oestrogen and progesterone response elements were identified (Figure 2-1). One caveat of these experiments is that I did not examine the effects of 17 β -estradiol in combination with progesterone on PBF mRNA and protein expression. The reason for this is that the effects of 17 β -estradiol were examined retrospectively after it was realised that it would be important to investigate the actions of a naturally occurring oestrogen in addition to the synthetic diethylstilbestrol. Subsequently, given the larger magnitude of effect, we concentrated predominantly on the actions of oestrogen rather than progesterone.

In keeping with previous reports, PTTG protein expression was upregulated in response to diethylstilbestrol alone and in combination with progesterone (Pati et al. 2004). However, despite the fact that PTTG mRNA expression in pituitary GH₃ cells has

been shown to be upregulated by oestrogen (Heaney et al. 2002), PTTG mRNA expression levels in MCF-7 cells were not significantly altered by any of the hormonal treatments examined. As the same oestrogen (diethylstilbestrol) was employed the difference observed is most likely due to the different cell types used. Because PTTG mRNA expression was not regulated by oestrogen or progesterone in MCF-7 cells, the upregulation of PTTG protein expression levels observed is unlikely to be due to promoter specific events. The upregulation of PTTG protein expression could conceivably be due to post translational effects, leading to stabilisation of the protein. However, such mechanisms lie beyond the scope of the current investigation.

The human and murine separase promoter contains consensus oestrogen and progesterone response elements (ERE and PRE), and one previous study has indicated that separase mRNA is up regulated in MCF-7 cells in response to the combined treatment of oestrogen and progesterone (Pati et al. 2004). Despite this previous report we did not detect any response to any of the hormonal treatments examined. As the precise oestrogens and progestones used were not reported, the discrepancies in our findings may be because different hormonal compounds were employed. However, our experiments were carried out multiple times with thorough controls, and we were unable to replicate the findings of Pati et al (2004).

In summary, I have shown that PBF mRNA and protein are upregulated by oestrogen and progesterone, and that PTTG protein is upregulated by oestrogen and progesterone in MCF-7 cells. These data suggest that the PBF promoter may be regulated by oestrogen and progesterone. To investigate this further, I set out to clone the PBF promoter into a luciferase expression vector and to determine hormonal effects on the PBF promoter in dual luciferase assays.

Chapter 3

Investigation into the regulation of PBF transcription.

3.1 Introduction

In Chapter 2, diethylstilbestrol, 17 β -estradiol and progesterone were shown to increase the expression of PBF mRNA and protein; however the mechanism through which this is achieved has not been identified.

The classical mechanism of oestrogen receptor alpha (ER α) action is associated with oestrogen-induced formation of a nuclear ER α homodimer which can bind oestrogen response elements (ERE) in the 5' regulatory regions of target genes. Once bound to the ERE, the homodimers recruit coactivators and general transcription factors to activate gene transcription. The coactivators may possess chromatin remodelling activities or recruit additional proteins (Jakacka et al. 2001) (Safe 2001). The non-classical pathway involves ER interactions with other proteins such as AP1 (Jakacka et al. 2001) and SP1 rather than direct binding to DNA. ER α is able to interact with SP1 to transactivate genes through binding SP1(N)_xERE half site motifs, in which there is considerable variability in the ERE site orientation and the number of intervening nucleotides (N) (Porter et al. 1997). The co-operative interaction of SP1 and ER proteins plays an important role in the transcriptional activation of multiple growth regulatory genes in breast cancer cells (Safe 2001).

Similarly to oestrogen, progesterone activates the progesterone receptor (PR) by dimerisation and phosphorylation of the receptor, resulting in its binding to cis-acting progesterone response elements (PRE) on DNA. Two isoforms of the PR have been identified; PRA and PRB (94 and 114 kDa respectively) (An et al. 2005). The PRA isoform lacks 164 amino acids found at the N-terminus of PRB. Although both isoforms

are ligand activated transcription factors, in general PRB is transcriptionally more active than PRA (An et al. 2005)

In contrast to PBF, PTTG and separase mRNA expression levels remained unaltered after hormonal treatment. Diethylstilbestrol, 17 β -estradiol and progesterone may have direct effects on the PBF promoter. Further evidence to support this hypothesis is provided by the TESS: Transcriptional Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>), as examination of the human PBF promoter using this programme revealed consensus ERE, PRE, SP1 and AP1 sites within the first 1200 bp upstream of the ATG start codon, see Figure 2-1. The aim of this study was therefore to determine whether the induction of PBF expression by oestrogen and progesterone is a direct promoter-specific effect. Promoter studies using luciferase reporter constructs containing the human PBF promoter were employed in MCF-7 human breast adenocarcinoma cells.

3.2 Materials and methods

3.2.1 Cell lines

MCF-7 Human Caucasian breast adenocarcinoma cells were maintained as described in section 2.2.1

3.2.2 Cloning the PBF promoter

Primers were designed to amplify the first 1kb of the human PBF promoter upstream of the transcriptional start site. The forward primer contained a Kpn1 restriction enzyme site whilst the reverse primer contained a HindIII restriction enzyme site (Table 2).

Forward primer	5' GGG-GTA-CCT-CGG-GCA-GGC-AGA-GGC-GG 3'
Reverse primer	5' CCC- AAG- CTT- TGC- GCC-TGC-GCG-ATA-GAG 3'

Table 2: Sequences of the PCR primers designed to amplify the first 1kb of the human PBF promoter upstream of the transcriptional start site. The KpnI restriction enzyme site is shown in pink and the HindIII restriction enzyme site is shown in blue.

3.2.2.1 PCR amplification of the human PBF promoter

The PCR reaction consisted of 5 µl of human genomic DNA obtained from a *ex-vivo* human normal thyroid specimen, 600 nM each of forward and reverse primer (Alta Biosciences), 1 mM dNTPs, 5 % glycerol, 3 mM MgCl₂ (Bioline, London, UK), 1 × NH₂ reaction buffer (Bioline) and 2.5 U Biotaq™ DNA polymerase (Bioline), which was made up to a final volume of 50 µl with nuclease free water.

The PCR cycling conditions used were 95°C for 5 minutes for the initial denaturation of DNA template, 40 cycles of 95°C for 30 seconds (denaturation), 67.1°C for 30 seconds (primer annealing) and 72°C for 2 minutes (extension), followed by a final step of 72°C for 7 minutes. This was performed on a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany). The PCR product was subsequently electrophoresed on a 2 % agarose (Bioline) gel in 1× TAE (Tris-Acetate-EDTA) buffer.

3.2.2.2 Gel extraction of PCR product

The 1 kb PCR product was excised from the gel using a scalpel and extracted from the agarose using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions, the microfuge extraction protocol was followed. Briefly, the gel fragment was dissolved in 3 volumes of Buffer QG to 1 volume of gel

(100 mg-100 ml) at 50°C. One gel volume of isopropanol was added and the solution was centrifuged through a QIAquick column. After discarding the flow-through, the column was washed with buffer QG and then with buffer PE, which contained ethanol. A final centrifugation step minus wash solution ensured maximal removal of ethanol before elution in 30 µl nuclease-free water.

3.2.2.3 Restriction enzyme digestion and ligation of pGL3 enhancer and PCR product.

The pGL3 Enhancer vector (Promega) was selected to house the first 1kb of the human PBF promoter upstream of the transcriptional start site, between the Kpn1 and HindIII restriction sites within the multiple cloning region (Figure 3-1).

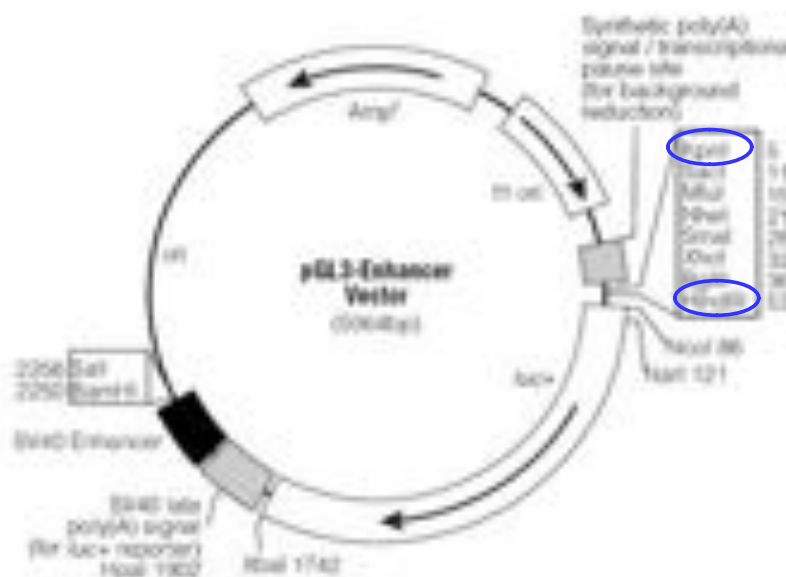


Figure 3-1: Plasmid map of the pGL3 Enhancer vector. The Kpn1 and HindIII restriction sites into which the PBF promoter was cloned are circled in blue. Diagram obtained from <http://www.promega.com/>.

In a total volume of 20 µl, 1 µg pGL3 enhancer and 13 µl gel extracted PCR product were digested using 10 U Kpn1 (Fermentas, Burlington, Ontario, Canada), 40 U

HindIII (Fermentas) and 1 × KpnI buffer (Fermentas) for 2 hours at 37°C. Digested vector and PCR product were electrophoresed on a 1.5 % agarose gel and gel extracted using QIAquick gel extraction kit as described in 3.2.2.2.

The ligation reaction was carried out at room temperature for 3 hours and consisted of 3 µl digested vector, 14 µl digested PCR product, 1 × T4 DNA ligase buffer (Promega) and 3 U T4 DNA ligase (Promega) in a total volume of 20 µl.

3.2.2.4 Transformation into DH5α competent cells

According to the manufacturer's instructions, 5 µl of the ligation reaction were transformed into Subcloning Efficiency DH5TM Competent Cells (Invitrogen). The ligation reaction was added to 50 µl bacterial cells and incubated on ice for 20 minutes. The cells were heat-shocked for 45 seconds at 37°C and returned to ice for a further 2 minutes. Luria Broth (LB) was added to a total volume of 1 ml and the cells incubated at 37°C for 1 hour with shaking at 200 rpm. The cells were subsequently pelleted by centrifugation at 13 000 rpm for 3 minutes and, after the supernatant was decanted off, resuspended in the remaining LB. The cell suspension was then spread on an LB-agar (1 % agar) plate with 100 µg/ml carbenicillin (Melford, Ipswich, Suffolk, UK) and incubated at 37°C for 16 hours.

3.2.2.5 Plasmid DNA purification and sequence verification

Bacterial colonies in which the PBF promoter was successfully ligated into the plasmid vector were used to inoculate 5 ml LB containing carbenicillin (100 µg/ml) and incubated at 37°C overnight with shaking at 200 rpm. Plasmid DNA was purified from these cultures using the Wizard[®] Plus SV minipreps DNA purification system (Promega)

according to manufacturer's instructions. Briefly, the cells were pelleted by centrifugation and the supernatant discarded. The pellet was resuspended and cells lysed. The addition of alkaline protease solution was followed by incubation at room temperature for 5 minutes to inactivate endonucleases. Cell lysis was terminated with neutralisation solution and centrifugation produced a cleared lysate, which was transferred to a spin column. The DNA bound to the column was washed twice with an ethanol-based wash solution and then centrifuged without wash solution to ensure maximal removal of ethanol. Finally, plasmid DNA was eluted from the column with 50 µl nuclease-free water.

The sequence of the plasmid was confirmed using the pGL3 Enhancer primer RV3. From this point onwards the plasmid pGL3enhancer + 1kb of the human PBF promoter will be referred to as pGL3enhancer_PBFpromoter (Figure 3-3).

Sequencing the first 1kb of the human PBF promoter upstream of the transcriptional start site, once it had been cloned into the pGL3 enhancer plasmid, exposed the presence of an 18bp repeated sequence (gccctcggtcagcctc) which varied in length between individuals in the population. Although the published sequence of this region of the PBF promoter contains 6 direct repeats of the 18bp sequence (Ensemble/ENSG00000183255), the fragment amplified and cloned in here contained only 3 repeats. Further investigation revealed that 3 repeats is the most common length, and this will be discussed in more detail in Chapter 4.

3.2.2.6 Maxiprep of pGL3enhancer_PBFpromoter

Once the cDNA sequence had been confirmed, larger quantities of DNA were purified by maxiprep. Originating from the same colonies that were selected initially for plasmid DNA purification and sequencing, 100 ml bacterial cultures containing

carbenicillin (100 µg/ml) were incubated at 37°C for 16 hours. Plasmid DNA was subsequently isolated using the GenElute™ HP plasmid Maxi-Prep kit (Sigma) following the manufacturer's instructions. To pellet the bacterial cells, 100 ml culture was centrifuged at 5,000 g for 10 minutes at 4°C. Following resuspension, the cells were lysed with an alkaline-SDS solution, in which chromosomal DNA was denatured. When the lysate was neutralised by acidic sodium acetate, chromosomal DNA renatured and formed aggregates, which were co-precipitated along with protein-SDS complexes and higher molecular weight RNA. This material was removed by passing through a filter syringe. The filtered lysate was centrifuged at 3,000 g for 2 minutes to bind the plasmid DNA to a column and again to wash the DNA with an ethanol based solution. Another 5 minute wash step was performed before the DNA was eluted in 3 mls of nuclease free water by centrifugation at 3,000 g for 5 minutes. The quantity of plasmid DNA obtained was determined by measuring absorbance at a wavelength of 260 nm on a Genova UV spectrometer (Jenway, Dunmow, Essex, UK).

3.2.3 Subcloning of the PBF promoter into pGL3 basic vector

In addition to the pGL3 enhancer vector, the pGL3 basic vector (Promega) was selected to house the first 1kb of the human PBF promoter upstream of the transcriptional start site between Kpn1 and HindIII restriction sites within the multiple cloning region (Figure 3-2). The basic vector was chosen in addition to the enhancer vector because high constitutive luciferase measurements were recorded on the Centro LB 960 Microplate Luminometer with all hormonal conditions examined (Berthold Technologies, Bad Wildbad, Germany). As the pGL3 basic vector lacks eukaryotic promoter and enhancer sequences, the expression of luciferase activity in cells transfected with this plasmid depends on the insertion and proper orientation of a functional promoter upstream from

luciferase gene. In contrast, the pGL3 Enhancer vector contains an SV40 enhancer downstream of the luciferase gene and poly (A) signal, which could mean subtle regulation of the PBF promoter by oestrogen, would be missed.

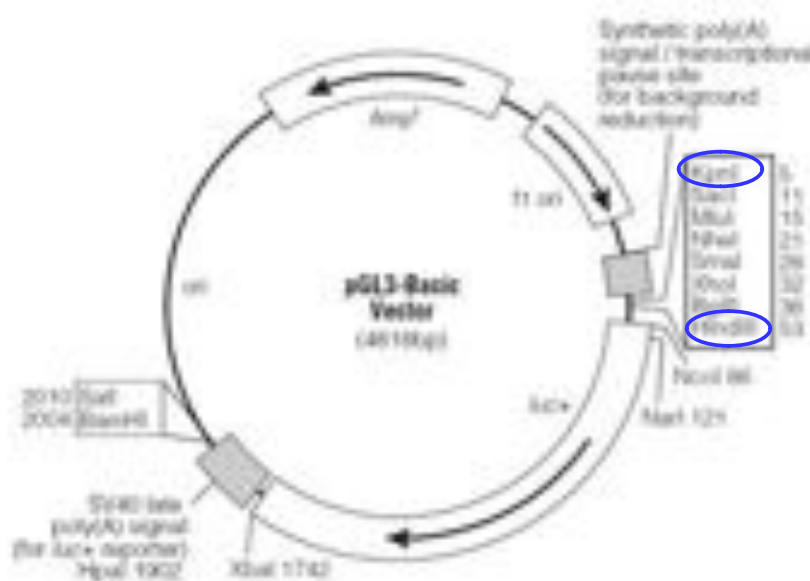


Figure 3-2: Plasmid map of the pGL3 basic vector. The *KpnI* and *HindIII* restriction sites into which the PBF promoter was cloned are circled in blue. Diagram obtained from <http://www.promega.com>.

3.2.3.1 Restriction enzyme digestion of pGL3enhancer_PBFpromoter and pGL3 basic

In a total volume of 40 μ l, 4 μ g pGL3 basic or pGL3enhancer_PBFpromoter were digested using 40 U *KpnI* (Fermentas, Burlington, Ontario, Canada) and 1 \times *KpnI* buffer (Fermentas) for 2 hours at 37 $^{\circ}$ C. 4 μ l of the digested vectors were electrophoresed on a 1.5 % agarose gel whilst the remaining 36 μ l were cleaned up using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) (see section 3.2.2.2.).

In a total volume of 50 μ l, 24 μ l of the cleaned singularly digested pGL3 basic and pGL3enhancer_PBFpromoter were digested using 50 U *HindIII* (Fermentas) and 1 \times

buffer R for 2 hours at 37⁰C. Digested vectors were electrophoresed on a 1.5 % agarose gel. The pGL3 basic vector and PBF promoter insert from the pGL3enhancer_PBF promoter vector were gel extracted using QIAquick gel extraction kit as described in Section 3.2.2.2.

3.2.3.2 Ligation of the restriction enzyme digested pGL3 basic vector and PBF promoter and transformation into DH5α competent cells

The ligation reaction consisted of 0.064 pmoles digested vector, 0.064 or 0.192 pmoles of the PBF promoter insert, 1 × T4 DNA ligase buffer (Promega) and 3 U T4 DNA ligase (Promega) in a total volume of 20 µl. This was incubated for 16 hours at 4°C following which 3 U T4 DNA ligase was added and incubated for a further 3 hours at 22°C. 5 µl ligation reaction were transformed into Subcloning EfficiencyTM DH5TM Competent Cells (invitrogen) as described in Section 3.2.2.4

3.2.3.3 Plasmid DNA purification, sequence verification and maxiprep of pGL3basic_PBFpromoter

Bacterial colonies in which the PBF promoter was successfully ligated into the plasmid vector were used to inoculate 5 ml LB containing carbencillin (100 µg/ml) and incubated at 37°C for 1 hour with shaking at 200 rpm. Plasmid DNA was purified from these cultures using the Wizard[®] Plus SV minipreps DNA purification system (Promega) as described in 3.2.2.5. Sequencing was carried out using the pGL3 basic primer RV3. For simplicity from this point onwards the plasmid pGL3basic + 1kb of the human PBF promoter will be referred to as pGL3basic_PBFpromoter (Figure 3-3).

Once the cDNA sequence had been confirmed, larger quantities of DNA were purified by maxiprep. Originating from the same colonies that were selected initially for plasmid DNA purification and sequencing, 100 ml bacterial cultures containing carbencillin (100 µg/ml) were incubated at 37°C for 16 hours. Plasmid DNA was subsequently isolated using the GenElute™ HP plasmid Maxi-Prep kit (Sigma) as described in 3.2.2.6.

3.2.4 Mutagenesis of pGL3basic_PBFpromter

As discussed earlier in Section 3.2.2.5, when we sequenced the pGL3enhancer_PBFpromoter construct we identified that the PBF promoter contained an 18 bp (gccctc**ggtcac**gcctc) repeated region which varied in length between individuals in the population. This repeat sequence houses an ERE half site (ggtca), and we therefore wanted to determine if this region of the PBF promoter was responsive to oestrogen. To achieve this, a third luciferase construct was created. This construct contained a deletion of the PBF promoter between the bases -1181 and -510 relative to the translational start site, so that only the EREs between bases -510 and -211 remained. This section of the PBF promoter between -510 and -211 contains the 18 bp hypervariable region which houses an ERE half site (Figure 3-3).

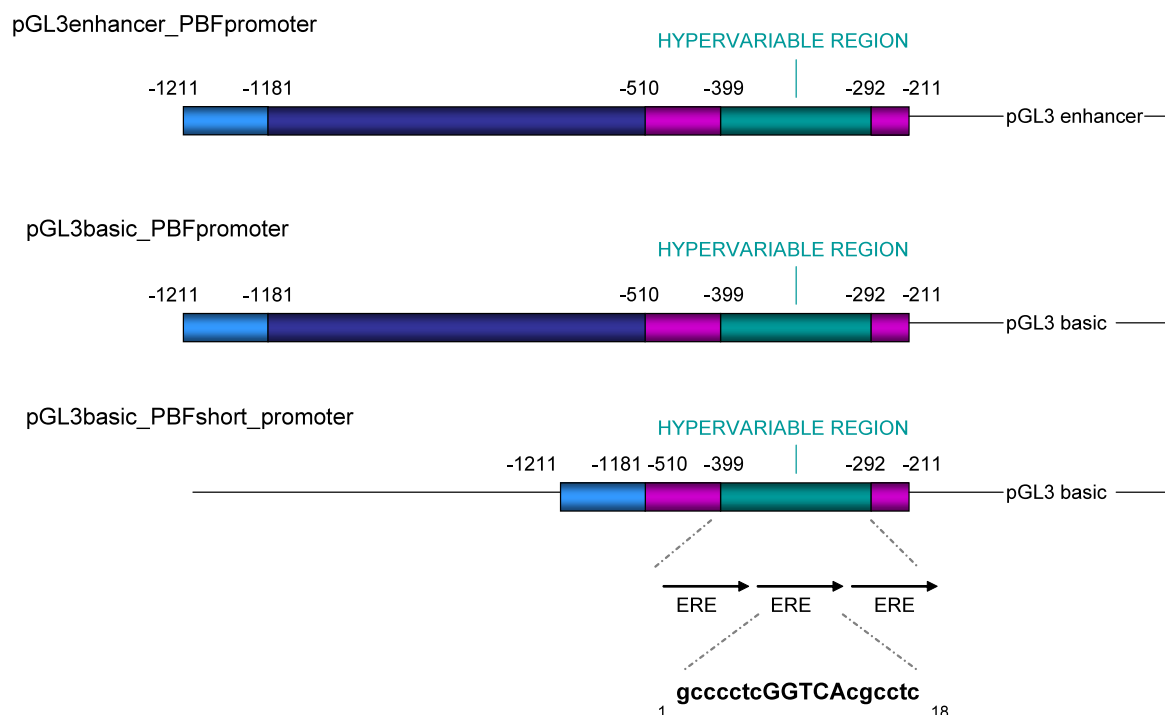


Figure 3-3 Schematic representation of the *pGL3enhancer_PBFpromoter*, *pGL3basic_PBF promoter* and the *pGL3basic_PBFshort_promoter* luciferase constructs. Numbering is relative to the translational start site and is based on the Ensemble sequence (ENSG00000183255, Aug 2009) which contains 6 repeats of the 18bp hypervariable region, however as explained in section 3.2.2.5 our construct contains only 3 repeats.

The *pGL3basic_PBFpromoter* plasmid was mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK). 50 ng of the *pGL3basic_PBFpromoter* plasmid template was combined with 125 ng of each of the complementary mutagenic primers (Table 3), 1 µl dNTP mix, 2.5 U *Pfu Turbo* DNA polymerase, 5 µl 10 x reaction buffer and distilled water to a final volume of 50 µl. Following the initial denaturation of the dsDNA template for 30 seconds at 95°C, 18 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 1 minute and primer extension at 68°C for 6 min.

Dpn1 (10 U), a restriction enzyme which specifically cleaves methylated and hemimethylated DNA, was added directly to each reaction and incubated at 37°C for 1

hour to digest the original template dsDNA. The *DpnI*-treated DNA (1 μ l) was then transformed into 50 μ l XL1-Blue supercompetent cells, which repair the nicks in the mutated plasmid, and grown on LB-ampicillin (100 ng/ml) agar plates at 37°C overnight.

Bacterial colonies in which the PBF promoter was successfully mutated were used to inoculate 5 ml LB containing carbencillin (100 μ g/ml) and incubated at 37°C for 1 hour with shaking at 200rpm. Plasmid DNA was purified from these cultures using the Wizard[®] Plus SV minipreps DNA purification system (Promega) as described in 3.2.2.5. Sequencing was performed using the pGL3 basic primer RV3. For simplicity from this point onwards the pGL3basic_PBFpromoter plasmid containing a deletion between -1181 and -510 will be referred to as pGL3basic_PBFshort_promoter (Figure 3-3).

Once the cDNA sequence had been confirmed, larger quantities of DNA were purified by maxiprep using the GenElute[™] HP plasmid Maxi-Prep kit (Sigma) as described in 3.2.1.6.

Forward_PBF promoter	5' CGG AGG CTT TTA GCA GGA CAG CCC TTT AGG ATG GAG 3'
Reverse_PBF promoter	5' CTC CAT CCT AAA GGG CTG TCC TGC TAA AAG CCT CCG 3'

Table 3. Sequences of the PCR primers designed to delete the human PBF promoter between bases -1181 and -510 relative to the translational start site.

3.2.5 Transient transfections

MCF-7 cells were seeded into 12 well plates at densities of 4×10^4 and 6×10^4 cells per well and incubated for ≥ 16 hours at 37°C, 5 % CO₂. Cells were then transfected

with a total of 1 ug plasmid DNA/well. This was made up from a combination of the vectors phRL-TK, pGL4.75, pGL3enhancer_PBF promoter, pGL3 basic_PBF promoter, pGL3basic_PBFshort_promoter and pSG5-HEGO (a wild type human ER α expression vector) to enhance ER α expression. ER α was transfected rather than ER β as the expression of oestrogen regulated genes is mediated predominantly by ER α in both breast epithelial cells and in breast cancer (Carroll et al. 2005). Following optimisation as per the manufacturer's instructions, FUGENE 6 transfection reagent (Roche, Indianapolis, IN, USA) was used at a ratio of 3 μ l to 1 μ g DNA.

Transfection efficiency was determined by co-transfection with phRL-TK (Promega), a construct containing a synthetic *Renilla* luciferase cDNA under the control of the herpes simplex virus thymidine kinase (HSV-TK) promoter. The *Renilla* cDNA has been modified for use in mammalian cells by replacing codons not frequently used in mammalian cells to increase expression efficiency, and removing a number of transcription factor consensus sequences that may interfere with promoter studies

3.2.6 Hormonal treatment

MCF-7 cells were maintained in RPMI 1640 phenol red free medium (Gibco) supplemented with 10 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml) for 7 days prior to hormone stimulation.

If cells were not transfected with the pSG5-HEGO vector, cells were treated with diethylstilbestrol, 17 β -estradiol and progesterone at final concentrations of 10 nM and 20 nM, simultaneously with transfection. However if they were transfected with the pSG5-HEGO vector they were treated with hormones 24 hours later. This was done to give the ER time to be synthesised before cells were treated with hormones.

3.2.7 Luciferase assays

MCF-7 cells were exposed to hormonal treatments for 24 and 48 hours before being washed briefly in PBS and harvested by the addition of 200 μ l (12 well plates) of 1 \times Passive Lysis Buffer (PLB: Promega, provided as part of the Dual Luciferase Reporter assay kit). Plates were placed on a rocker platform for 15 minutes at room temperature before transfer of lysates to storage at -80°C until ready to assay.

Luciferase assays were carried out using the Promega Dual Luciferase Reporter Assay System according to manufacturer's guidelines. This assay measures the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferases sequentially in a single reaction. The firefly luciferase reporter activity (in this case, obtained from activity of the pGL3 basic or pGL3 enhancer plasmid, where luciferase expression is driven by the PBF promoter), is measured first by adding luciferase assay reagent II (LAR II) to generate a stabilized luminescent signal. Once the luminescent signal has been quantified, the reaction is quenched and the *Renilla* luciferase reaction initiated simultaneously by the addition of a second reagent, 'stop and glo', to the sample. The corresponding luminescence for *Renilla* can then be measured.

Reagents were prepared immediately prior to the assay. LAR II substrate was resuspended in the buffer provided, and 50 \times 'stop and glo' solution was diluted to 1 \times stock in the relevant buffer, also provided in the kit. 20 μ l of each lysate were added to wells of 96-well microplate, and background luminescence was measured in each sample prior to the addition of reagents. Once 100 μ l of LARII reagent was added to each sample and mixed gently, a second reading was taken. Finally 100 μ l 1 \times 'stop and glo' was added on top of the existing mixture and a third reading recorded.

Luciferase activity in each cell lysate was measured in duplicate in Relative Light Units (RLU) on the Centro LB 960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity in the pGL3 constructs was normalised to *Renilla* activity in each experiment to correct for transfection efficiency.

3.2.8 Statistical analysis

Data were analysed using Sigma Stat (SPSS Science Software UK Ltd). The Kolmogorov-Smirnov test determined whether the data followed normal distribution. If the data set to be tested followed normal distribution, it was analysed using a two sample Student's t-test. However, if the data set was non-parametric, the Mann Whitney Rank Sum test was used for comparison between two groups of data.

3.3 Results

3.3.1 Effects of diethylstilbestrol and progesterone on the human PBF promoter, using pGL3enhancer_PBFpromoter as the reporter construct

To determine if diethylstilbestrol and progesterone have a direct effect on the human PBF promoter, MCF-7 cells were transfected with the pGL3enhancer_PBFpromoter luciferase construct and treated with oestrogen and progesterone for 48 hours. There was no significant effect on luciferase activity by any of the hormonal treatments examined (Figure 3-4).

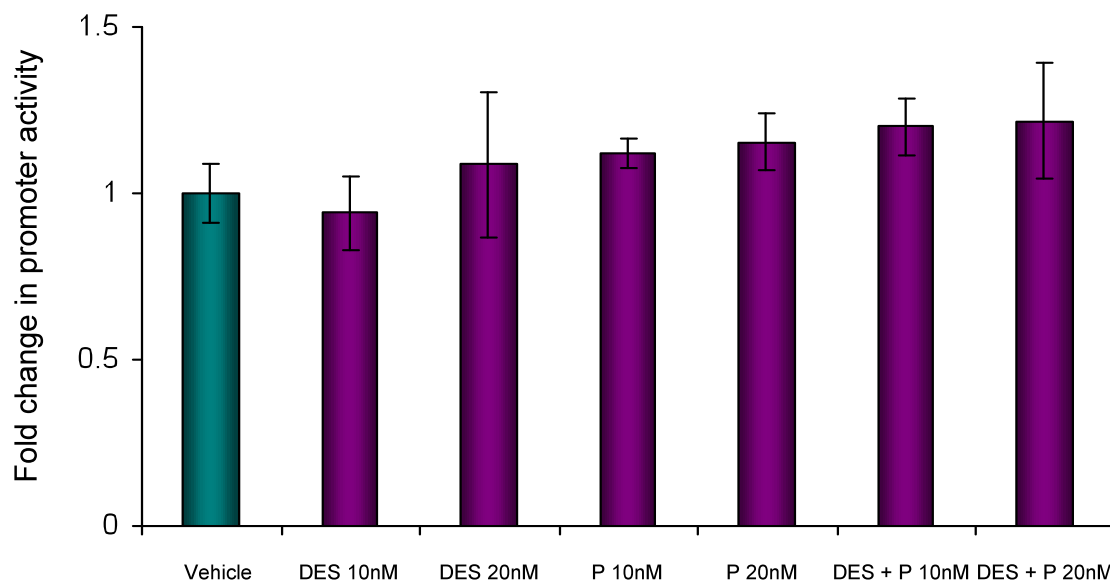


Figure 3-4: Effect of diethylstilbestrol and progesterone on the activity of the first 1kb of the human PBF promoter upstream of the transcriptional start site in MCF-7 cells using the pGL3enhancer_PBF promoter plasmid as the reporter construct. Luciferase activity was corrected for transfection efficiency using Renilla expression and normalised to vehicle (taken as a value of 1). The mean fold change \pm the standard error of the mean have been plotted (N=3 experiments). DES = diethylstilbestrol, P = Progesterone.

3.3.2 Effect of diethylstilbestrol and progesterone on the PBF promoter using pGL3basic_PBFpromoter as the reporter construct

Initially the first 1kb of the human PBF promoter upstream of the translational start site was cloned into the pGL3 enhancer vector, which contains an SV40 enhancer downstream of the luciferase gene and the poly (A) signal. The presence of an enhancer resulted in high constitutive levels of luciferase activity (Figure 3-5).

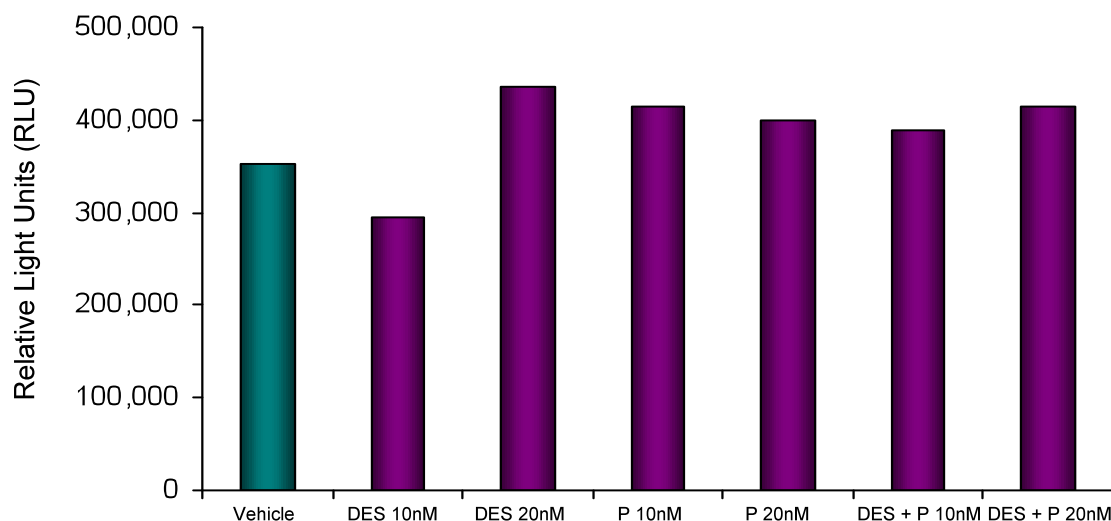


Figure 3-5: An example of the effect of diethylstilbestrol and progesterone on the activity of the first 1kb of the human PBF promoter upstream of the transcriptional start site. The luciferase data have been left in the form of RLU to show the high constitutive levels of luciferase activity obtained using the pGL3 enhancer vector. (N=1). DES = diethylstilbestrol, P = Progesterone.

The pGL3basic vector lacks eukaryotic promoter and enhancer sequences, therefore the expression of luciferase activity in cells transfected with this plasmid depends more empirically on the insertion and proper orientation of a functional promoter upstream from luciferase gene. To see if the regulation of the PBF promoter by diethylstilbestrol and progesterone was being missed due to the presence of the strong SV40 enhancer, it was subcloned from the pGL3enhancer vector into the pGL3basic vector.

After subcloning, MCF-7 cells were transfected with the pGL3basic_PBFpromoter luciferase construct and treated with oestrogen and progesterone for 48 hours. However, there was still no significant effect on luciferase activity by any of the hormonal treatments examined (Figure 3-6).

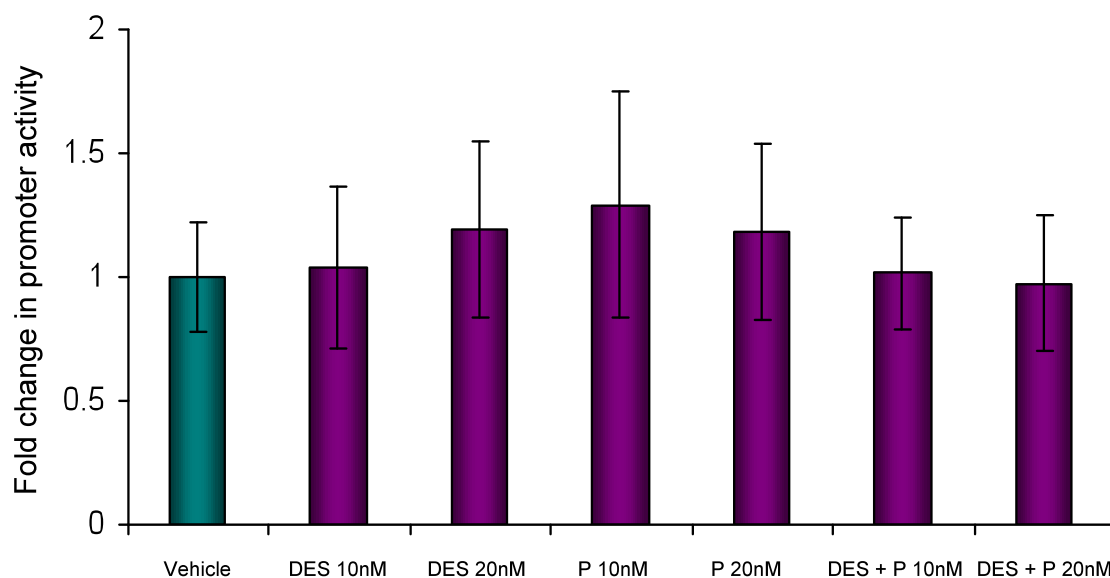


Figure 3-6: Effect of diethylstilbestrol and progesterone on the activity of the first 1kb of the human PBF promoter upstream of the transcriptional start site in MCF-7 cells using the pGL3basic_PBF promoter plasmid as the reporter construct. Luciferase activity was corrected for transfection efficiency using Renilla expression and normalised to vehicle (taken as a value of 1) (N=6). DES = diethylstilbestrol, P = progesterone.

3.3.3 Effect of diethylstilbestrol and progesterone on the PBF promoter after transfection of oestrogen receptor alpha (ER α)

In the absence of hormone, oestrogen receptors are largely located in the cytosol. Hormone binding to the receptor triggers a number of events starting with migration of the receptor from the cytosol into the nucleus, dimerisation of the receptor and subsequent binding of the dimer receptor to ERE. The DNA receptor complex can then recruit other proteins which are responsible for transcriptional regulation (Chen et al. 2008). As large quantities of vector DNA were being transfected into MCF7 cells, the amount of oestrogen receptor present may have become a limiting factor, preventing an oestrogen response being detected.

To determine if the amount of oestrogen receptor present was a limiting factor, MCF-7 cells were co-transfected with pSG5-HEGO, a wild type human ER α expression vector, and the pGL3basic_PBFpromoter luciferase construct. 24 hours post transfection; cells were treated with oestrogen with and without progesterone for 48 hours. There was no significant effect on luciferase activity by any of the hormonal treatments examined (Figure 3-7).

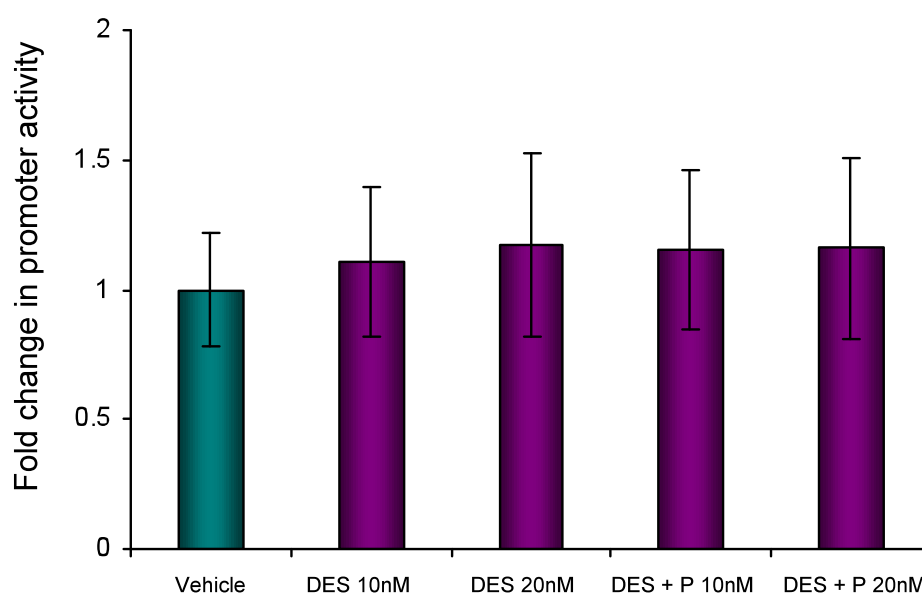


Figure 3-7: Effect of diethylstilbestrol with and without progesterone on the activity of the first 1kb of the human PBF promoter upstream of the transcriptional start site in MCF-7 cells transfected with the human ER α expression vector HEGO. Luciferase activity was corrected for transfection efficiency using Renilla expression and normalised to vehicle (taken as a value of 1) (N=6). DES = diethylstilbestrol, P = Progesterone.

3.3.4 Effect of 17 β -estradiol on the PBF promoter after transfection of oestrogen receptor alpha (ER α)

17 β -Estradiol (C₁₈H₂₄O₂) occurs naturally and is the major oestrogen secreted by the premenopausal ovary (PubChem compound CID5757). The oestrogen used so far in this study is the synthetic oestrogen diethylstilbestrol (C₁₈H₂₀O₂). Although both

oestrogens induced the levels of PBF mRNA and protein expression levels (see Chapter 2), 17 β -estradiol and diethylstilbestrol may work via different pathways to achieve this effect, given that small changes in ligand structure can result in major changes in the biological character of the receptor (Katzenellenbogen et al. 2000).

To determine if 17 β -estradiol influenced the activity of the PBF promoter, MCF-7 cells were co-transfected with pSG5-HEGO and the pGL3basic_PBFpromoter luciferase construct. 24 hours after transfection, cells were treated with 17 β -estradiol for 48 hours. There was no significant effect on luciferase activity by 17 β -estradiol (Figure 3-8).

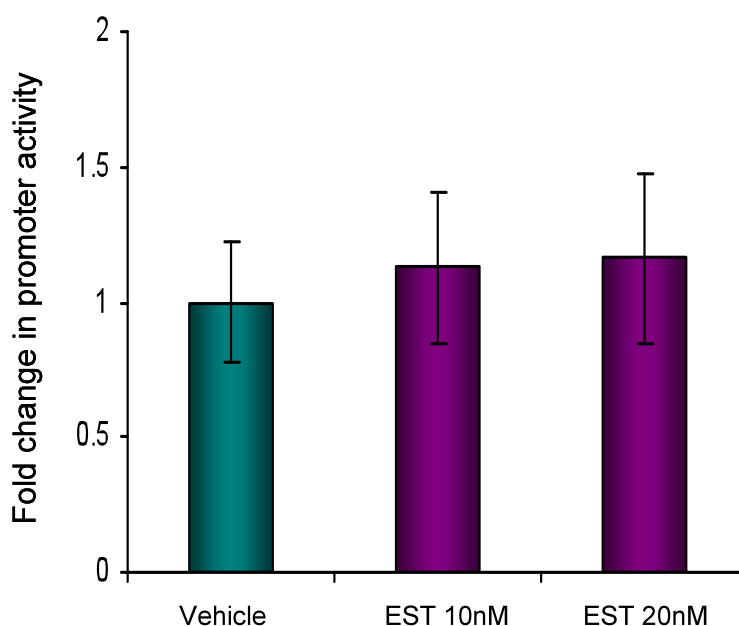


Figure 3-8 : Effect of 17 β -estradiol on the activity of the first 1kb of the human PBF promoter upstream of the transcriptional start site in MCF-7 cells transfected with HEGO. Luciferase activity was corrected for transfection efficiency using Renilla expression and normalised to vehicle (taken as a value of 1) (N=6). EST = 17 β -estradiol.

3.3.5 Effects of diethylstilbestrol and 17 β -estradiol on the PBF promoter using pGL3basic_PBFshort_promoter as the reporter construct

The PBF promoter contains an 18bp (gccctc**ggtc**acgcctc) repeated region which varies in length between individuals in the population, a phenomenon which will be discussed in more detail in Chapter 4. This repeat sequence houses a ERE half site (ggcca), and to investigate the possibility that the 18bp repeat region of the PBF promoter is regulated by oestrogen, 671 bp of the PBF promoter was deleted, creating the pGL3basic_PBFshort_promoter plasmid. This removed most of the ERE, AP1 and SP1 sites and all the PREs; however the 3 half EREs found within the 18 bp region were retained (Figure 3-3). At the time of transfection with pGL3basic_PBFshort_promoter MCF-7 cells were treated with 20 nM diethylstilbestrol and 20 nM 17 β -estradiol for 24 and 48 hours. The effects of progesterone on the PBF promoter were no longer investigated as the consensus PRE motif had been removed. After 24 hours, 20 nM diethylstilbestrol (1.5 ± 0.1 fold, $p < 0.01$, N=5) and 20 nM 17 β -estradiol (1.3 ± 0.1 fold, $p < 0.05$, N=5) significantly induced the PBF promoter activity. Similarly after 48 hours diethylstilbestrol (1.8 ± 0.2 fold, $p < 0.01$, N=5) and 20 nM 17 β -estradiol (1.7 ± 0.3 fold, $p < 0.05$, N=5) significantly increased the activity of the PBF promoter.

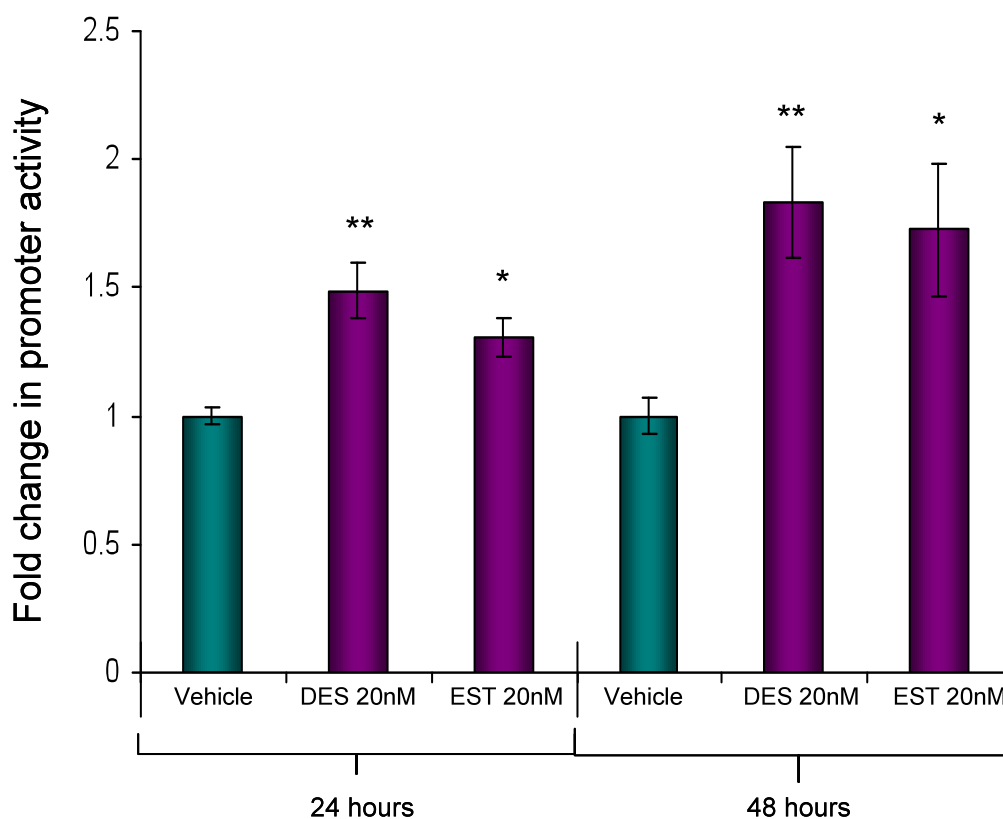


Figure 3-9 Effect of diethylstilbestrol and 17β -estradiol on the activity of the short PBF promoter region -510 to -211 in MCF-7 cells. Luciferase activity was corrected for transfection efficiency using Renilla expression and normalised to vehicle (taken as a value of 1) ($P < 0.01^{**}$, $P < 0.05^{*}$, $N=3$). DES = diethylstilbestrol, EST = 17β -estradiol.

3.4 Discussion

Chapter 2 demonstrated that human PBF mRNA and protein expression in MCF-7 cells is regulated by oestrogen and progesterone. However, the mechanism by which this is achieved remains to be discovered. In addition to its mRNA levels being increased by oestrogen and progesterone the PBF promoter contains consensus EREs and PREs (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>). Together this suggests that the PBF promoter could be directly regulated by oestrogen and progesterone. To investigate this possibility, the first 1kb of the human PBF promoter upstream of the transcriptional start site was cloned into the pGL3 enhancer vector. This vector contains an SV40 enhancer

down-stream of the luciferase gene and the poly (A) signal, and when present this enhancer often results in the transcription of the luciferase genes at higher levels. Indeed when this vector was transfected into MCF-7 cells, even in the absence of hormonal treatment, very high luciferase measurements were recorded. When we subsequently examined the effect of oestrogen and progesterone on the PBF promoter using this plasmid, no response was detected.

To address the possibility that a potential hormonal response was being masked by SV40 promoter activity, the first 1kb of the human PBF promoter upstream of the transcriptional start site was subcloned from the pGL3enhancer vector into the pGL3 basic vector. As the pGL3 basic vector lacks eukaryotic promoter and enhancer sequences, the expression of the luciferase gene depends on the insertion and proper orientation of a functional promoter upstream. When the effects of hormones on this construct were examined there were still no significant changes in activity. This implies that this region of the PBF promoter is not responsive to oestrogen and progesterone. However very high luciferase measurements were still recorded even in the absence of hormones, indicating that the effects of oestrogen and progesterone may still be being masked by the high constitutive activity of this region of the PBF promoter.

As large quantities of vector DNA were being transfected into MCF-7 cells, we were concerned that the amount of oestrogen receptor present had become a limiting factor. To determine if this was the case, MCF-7 cells were co-transfected with a wild type human ER α expression vector (pSG5-HEGO) and the pGL3basic_PBF promoter construct. No significant alterations in luciferase activity were recorded, indicating that this concern was misplaced.

Although both diethylstilbestrol and 17 β -estradiol upregulated the expression of PBF mRNA and protein expression, we had not investigated the effects of 17 β -estradiol on the activity of the PBF promoter. Because 17 β -estradiol is a naturally occurring oestrogen whilst diethylstilbestrol is a synthetic oestrogen, 17 β -estradiol and diethylstilbestrol may mediate their effect via different pathways. To determine if 17 β -estradiol could influence the activity of the PBF promoter, MCF-7 cells were cotransfected with the pSG5-HEGO and pGL3basic_PBF promoter constructs. Like diethylstilbestrol, 17 β -estradiol did not influence the activity of the PBF promoter.

In addition to examining the responsiveness of 1kb of the human PBF promoter upstream of the transcriptional start site, I also investigated the responsiveness of a smaller region of the PBF promoter which contained an 18bp repeated region (gccctc**ggtcac**gcctc) within which there is a consensus ERE half site (ggtca) (Figure 3-3). When this plasmid was transfected into MCF-7 cells both diethylstilbestrol and 17 β -estradiol significantly induced the activity of the PBF promoter. This effect was evident after 24 and 48 hours, indicating that this region of the promoter is responsive to both diethylstilbestrol and 17 β -estradiol. As the first 1kb of the human PBF promoter contains a high number of regulatory regions (Figure 2-1), these could have been driving the activity of the pGL3enhancer_PBFpromoter and the pGL3basic_PBFpromoter construct to such an extent that the activity of the smaller region of the PBF promoter containing the variable 18bp repeated region housing a consensus ERE was being missed. This hypothesis is supported by the high readings obtained on the luminometer, using both the pGL3enhancer_PBFpromoter and pGL3basic_PBFpromoter even in the absence of hormones. Additionally work from our group demonstrates that PBF is an abundant protein (Smith et al. 2009), suggesting strong constitutive activation of the promoter.

3.4.1 Concluding remarks

In this Chapter it has been shown that a small region of the PBF promoter which contains 1 full ERE and 4 half EREs is responsive to both diethylstilbestrol and 17 β -estradiol (Figure 3-3). As the activity of the PBF promoter was induced 1.3 to 1.8-fold and PBF mRNA was induced 1.5 to 3-fold at identical timepoints and at identical doses of oestrogen, these data indicate that the short promoter region -510 to -211 confers most, but not all, of PBFs responsiveness to DES and EST. These promoter assays have therefore uncovered some of the functional EREs in the PBF promoter, but others which lie further upstream in the PBF promoter may still be undiscovered. This is because EREs located far away from the promoter are capable of interacting with the promoter by a looping mechanism (Sathya et al. 1997), and when the association of the ER with the complete nonrepetitive sequence of human chromosomes 21 and 22 was mapped by combining chromatin immunoprecipitation with tiled microarrays, a few of the ER binding sites were found directly adjacent to ER target genes, but most were found at significant distances including several >100 kb removed from transcriptional start sites (Carroll et al. 2005). To find out whether there are additional functional EREs in the PBF promoter, a larger region will need to be investigated. I attempted to undertake this work by cloning 2, 3 and 4 kb of the human PBF promoter upstream of the transcriptional start site into the pGL3basic vector, but technical difficulties in the PCR amplification prevented me from completing this line of investigations.

Chapter 4

Identification of a polymorphic region in the PBF promoter

4.1 Introduction

The published sequence of the PBF promoter (Ensemble/ENS00000183255) contains 6 direct repeats of an 18bp sequence containing a putative consensus oestrogen response element (ERE) half site (gcccttcGGTCAGcctc) (Figure 4-1). During cloning of this promoter region into the pGL3 enhancer vector, I noted that this 18bp repeat region varied in number between individuals. The presence of multiple EREs results in synergistic activation of genes (Sathya et al. 1997), and this may relate to tumour behaviour. In addition this may confer an increased sensitivity to oestrogen therapy which may ultimately affect prognosis.

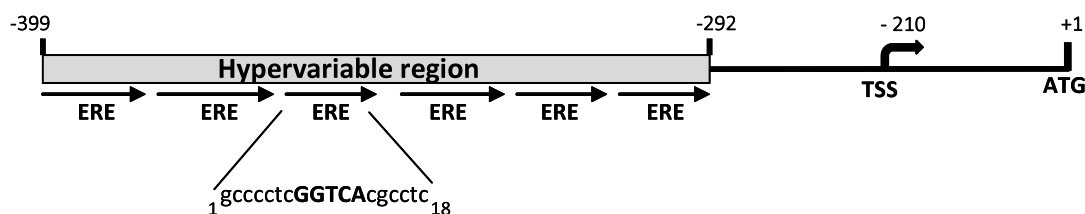


Figure 4-1: Schematic representation of the region of the PBF promoter which contains an 18 bp repeated sequence (gcccttcGGTCAGcctc) housing a consensus oestrogen response element (ERE) half site (GGTCA). The transcriptional start site (TSS) is located 210 bp upstream of the translational start site (ATG).

The aim of this study was to determine whether individuals have different numbers of EREs in the PBF promoter. PCR amplification of the region of the PBF promoter containing the 18 bp repeated sequence housing putative ERE half sites was carried out using three sets of primers. The resulting cDNA products were then sequenced to determine the number of repeats and hence ERE half sites they contained.

4.2 Materials and methods

4.2.1 DNA

Genomic DNA from normal and tumourous colorectal samples and normal thyroid specimens had previously been extracted from snap frozen tissue by Dr Dae Kim (University of Birmingham, Birmingham UK) (Kim et al. 2005; Kim et al. 2007). DNA from patients with normal thyroid function was also obtained from whole blood and was a kind gift from Dr Steve Gough (University of Birmingham, Birmingham, UK) (Simmonds et al. 2006).

4.2.1.1 Genomic DNA extraction from breast tumour and normal tissue.

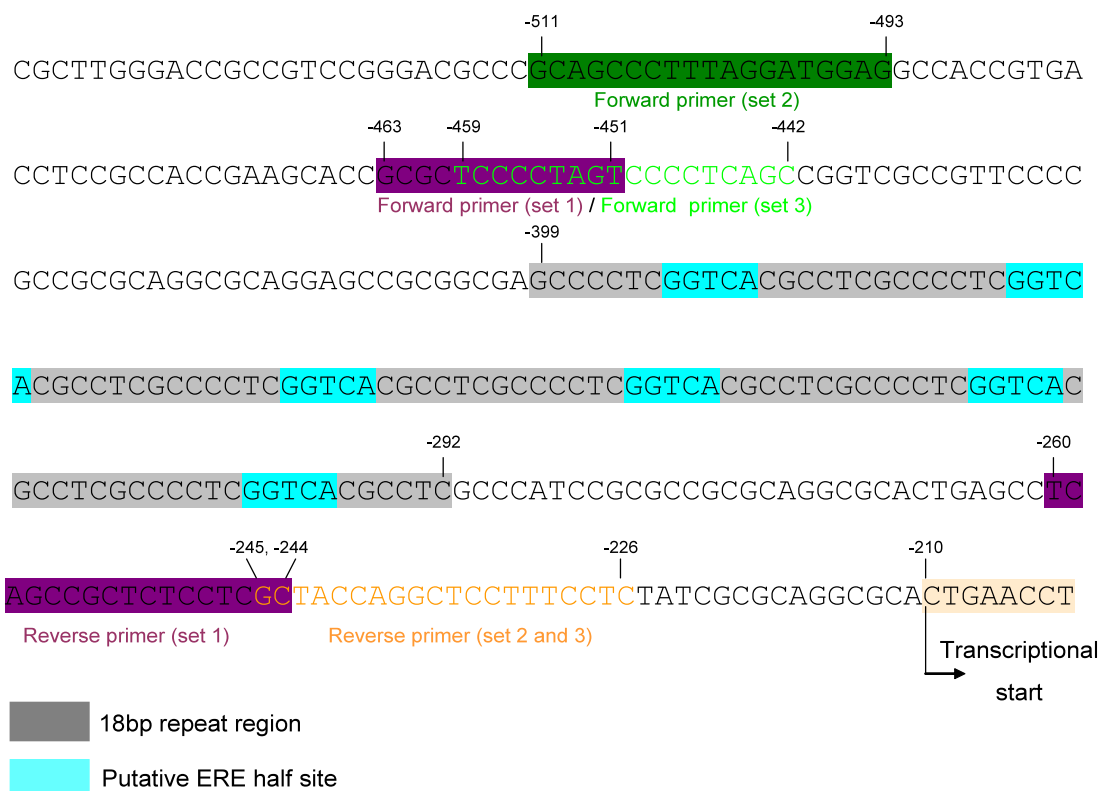
Formalin fixed, paraffin embedded tissues sections were a kind gift from Dr Gary Reynolds (Liver Research laboratories, University of Birmingham, Birmingham, UK) or were purchased from US Biomax (PO Box 1854, Rockville, MD20849). Breast tumour paraffin-embedded samples arranged on tissue microarrays (TMAs) were available from 27 patients who underwent surgery at the Queen Elizabeth hospital, Birmingham. Breast normal paraffin-embedded samples taken at breast reduction surgery were available from 1 patient and 2 were brought in from US biomax (PO Box 1854, Rockville).

Genomic DNA was isolated from formalin fixed and paraffin embedded breast tumour and normal tissue using the Pinpoint Slide DNA Isolation System™ kit (Zymo Research, USA). Paraffin was removed from the slides by incubating them in xylene at room temperature for 1 hour, during which the xylene was changed once. The slides were then hydrated by washing progressively for 2 minutes in 100 %, 70 %, 50 % ethanol and then pure water. Tissue samples were then removed from the slide by applying 0.5 µl of Pinpoint™ solution per mm². DNA was subsequently extracted in 50 µl of extraction buffer and 5 µl of proteinase K, for 4 hours at 55°C and for 10 minutes at 98°C. The final

10 minute incubation at 98°C serves to inactivate the proteinase K. Following extraction the DNA was purified, given that fixing and staining can inadvertently add PCR inhibitors to the tissue. DNA was purified by passing it through a Zymo-spin I column after the addition of 100 µl of DNA binding solution. Before elution of the DNA in 10 µl of nuclease free water, the column was washed twice in 150 µl of PP wash buffer.

4.2.2 Sequencing the 18 bp repeat region of the PBF promoter

Three primer sets were designed to amplify the region of the PBF promoter which contains a repeated 18 bp sequence (Figure 4-2).



	Forward	Reverse
Primer set 1	5'GCG-CTC-CCC-TAG-TCC-CCT	5' GCG-AGG-AGA-GCG-GCT-GA
Primer set 2	5'GCA-GCC-CTT-TAG-GAT-GGA-G	5'GAG-GAA-AGG-AGC-CTG-GTA-GC
Primer set 3	5'TCC-CCT-AGT-CCC-CTC-AGC	5'GAG-GAA-AGG-AGC-CTG-GTA-GC

Figure 4-2: The sequence of the region of the PBF promoter which contains an 18 bp repeated sequence housing a putative consensus oestrogen response element (ERE) half site. The sequences of the 3 primer sets are also given in the Table below. After PCR amplification, primer set 1 produced a 220 bp product, primer set 2 produced a 286 bp product and primer set 3 produced a 234 bp product (All sizes are based on individuals who contain 6 direct repeats of the 18 bp sequence housing a putative ERE half site).

4.2.2.1 PCR amplification of the human PBF promoter using primer set 1

The PCR reaction consisted of 5 µl of human genomic DNA, 600 nM each of forward and reverse primer (Alta Biosciences), 1mM dNTPs, 5 % glycerol, 3 mM MgCl₂ (Bioline, London, UK), 1 × NH₂ reaction buffer (Bioline), 2.5 U Biotaq™ DNA polymerase (Bioline), made up to a final volume of 50 µl with nuclease-free water.

The PCR cycling conditions used were 95°C for 5 minutes for the initial denaturation of DNA template, 40 cycles of 95°C for 30 seconds (denaturation), 69.6°C for 30 seconds (primer annealing) and 72°C for 30 seconds (extension), followed by a final step of 72°C for 7 minutes. This was performed on a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany). The PCR product was subsequently electrophoresed on a 2 % agarose (Bioline) gel in 1× TAE (Tris-Acetate-EDTA) buffer (Eppendorf).

4.2.2.2 PCR amplification of the PBF promoter using primer sets 2 and 3

PCR reactions consisted of 5 µl of human genomic DNA which had been extracted from snap frozen tissue and whole blood or 10 µl of human genomic DNA which had been extracted from paraffin embedded tissue slides, 600 nM each of forward and reverse primer (Alta Biosciences), 0.8 mM dNTPs, 5 % glycerol, 1.5 mM MgCl₂ (Bioline, London, UK), 1 × NH₂ reaction buffer (Bioline) and 2.5 U Biotaq™ DNA polymerase (Bioline), made up to a final volume of 50 µl with nuclease free water.

The PCR cycling conditions used were 98°C for 5 minutes for the initial denaturation of DNA template, 40 cycles of 94°C for 30 seconds (denaturation), 61.6°C for 40 seconds (primer annealing) and 72°C for 30 seconds (extension). This was performed on a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany). The PCR product was subsequently electrophoresed as described in section 4.2.2.

4.2.3 Gel extraction of the PCR product and sequence verification

PCR products were excised from the gel using a scalpel and extracted from the agarose using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) (See Section 3.2.2.2). After gel extraction samples were sent for sequencing (Cogenics, Hope End, Takely, Essex, CM22 6TA, UK) to determine the numbers of ERE half sites found at each allele. Sequencing was carried out using the forward primer of primer set 1 or 2.

4.2.4 Statistical analysis

Statistical analysis were performed using MINTAB version 14.0 (Minitab Ltd, Coventry, UK) as the statistical package. If the data set to be tested was large enough so that no field had a value below 1, it was analysed using the Chi-squared test.

4.3 Results

4.3.1 Confirmation of repeat lengths and checking the reliability of PCR

Amplification of DNA containing repeat regions by PCR can be problematic; this is because of the Streisinger Slippage Mechanism (Streisinger et al. 1966). When repeated sequences are present, one strand (template or daughter) can skip and reanneal to the complementary strand, offset by an integral number of repeats. Replication from such a slipped intermediate would result in gain or loss of an integral number of repeats depending on which strand of the duplex slips.

To eliminate the possibility that the differences in repeat length observed could have been due to PCR errors, the amplification of the DNA samples was repeated with the

original primer set. In addition, 2 additional sets of primers were designed and employed. All primer sets revealed the same number of repeats (Figure 4-3).

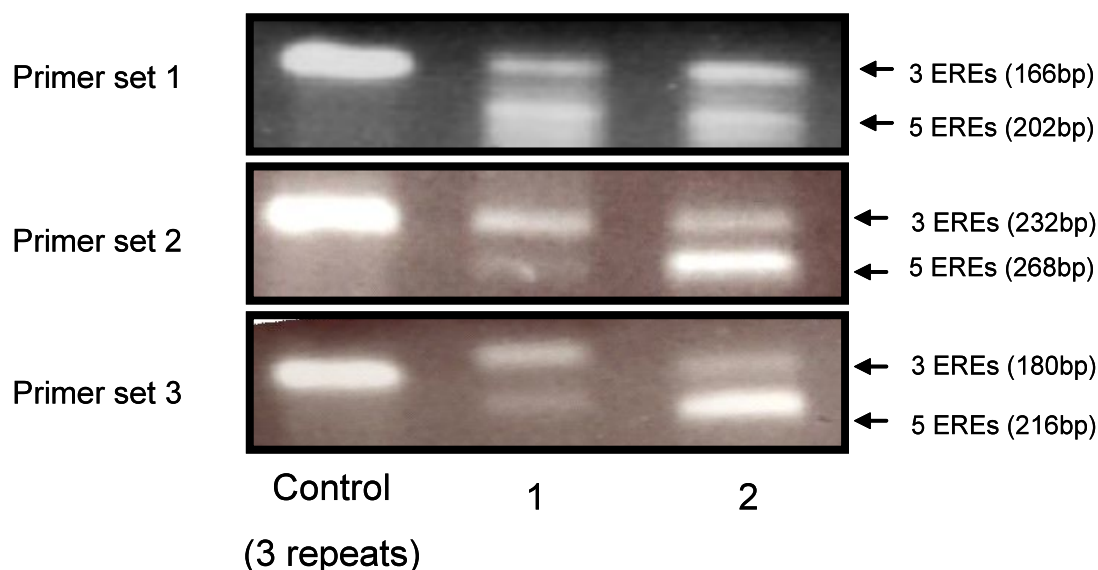
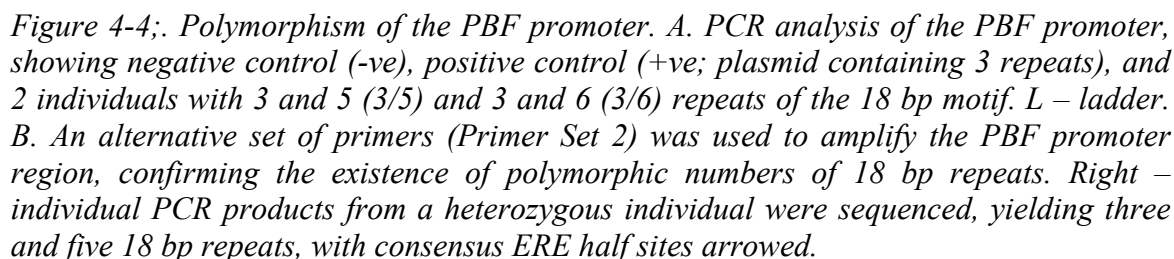


Figure 4-3 An agarose gel image showing the products of the PCR amplification of the 18bp repeat region found within the human PBF promoter using primer sets 1,2 and 3. DNA samples 1 and 2 which were both heterozyous for three and five repeats originated from snap frozen colorectal normal tissue. The positive control was amplified from the pGl3basic-PBFpromoter luciferase construct, which had previously been sequence and found to contain three 18 bp repeats (See Sections 3.2.2.5 and 3.2.3) .

4.3.2 Allele frequencies in various normal and disease tissue

To confirm our initial findings that the 18 bp repeat region containing a putative consensus ERE (gccctccGGTCacgcctc) varied in length between individuals, the PBF promoter was amplified from DNA samples originating from normal or tumour breast tissue, normal or tumour colorectal tissues, normal thyroid tissue or normal thyroid whole blood samples. After gel extraction, these samples were sent for sequencing (Cogenics, Hope End, Takeley, Essex, CM22 6TA, UK) to determine the number of repeats found at each allele (Figure 4-4).



123

Number of ERE half sites		1	2	3	4	5	6
Breast normals (N=3)	Number of alleles	0	0	6	0	0	0
	Frequency (%)	0	0	100	0	0	0
Breast tumours (N=27)	Number of alleles	0	0	40	0	14	0
	Frequency (%)	0	0	74	0	25.9	0
Colorectal normals (N=24)	Number of alleles	0	0	40	1	6	1
	Frequency (%)	0	0	83.3	2.1	12.5	2.1
Colorectal tumours (N=23)	Number of alleles	0	0	28	5	13	0
	Frequency (%)	0	0	60.9	10.9	28.3	0
Thyroid normals (N=45)	Number of alleles	2	0	76	0	8	4
	Frequency (%)	2.2	0	84.4	0	8.9	4.4
Overall (122)	Number of alleles	2	0	190	6	41	5
	Frequency (%)	0.8	0	77.9	2.5	16.8	2

Table 4 Allele number and frequencies of the number of 18 bp repeats (gccctccGGTCacgctc) containing a putative consensus oestrogen response element (ERE) half site (GGTCA). DNA samples originated from normal or tumour breast tissues, normal or tumour colorectal tissues thyroid normal tissues or thyroid normal whole blood samples. In total 122 different DNA samples were analysed.

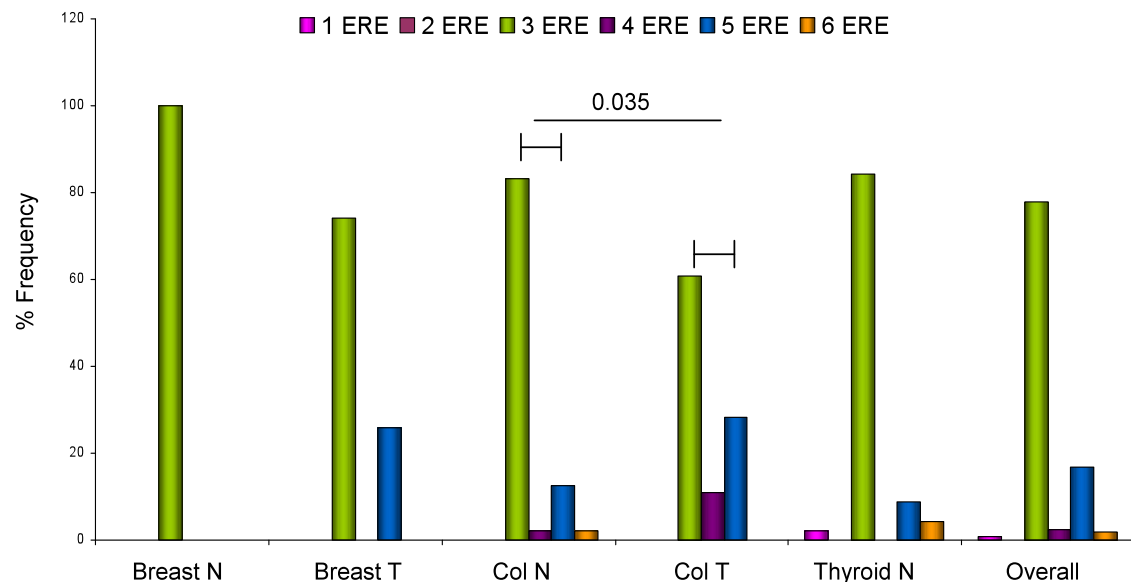


Figure 4-5 Allele frequencies of the number of 18 bp repeats (gccctccGGTCacgctc) containing a putative consensus oestrogen response element (ERE) half site (GGTCA). In total 122 different DNA samples were analysed. This consisted of 3 normal breast samples, 27 breast tumour samples, 24 colorectal normal samples, 23 colorectal tumour samples, and 45 thyroid normal samples.

4.4 Discussion

In this chapter I have shown that the PBF promoter contains an 18 bp repeat region housing a consensus ERE half site (gccctccGGTCAcgcctc) which varies in length between individuals in the population (Figure 4-1). The most common number of repeats found is 3, however alleles consisting of 1, 4, 5 and 6 repeats are also apparent. The published sequence of this region of the PBF promoter contains 6 direct repeats of the 18 bp sequence (Ensemble/ENSG00000183255). However, the studies undertaken in this chapter suggest that this is misleading as the most common repeat length is 3.

During this study DNA from 3 normal breast tissues and 27 tumour samples were analysed. Normal samples were homozygous for 3 repeats whilst the majority of the tumour samples were heterozygous for 3 and 5 repeats. Additionally when the repeat frequencies between colorectal normal and tumour samples were compared there was a statistically significant increase in the number of 5 alleles in the tumours compared to the normals. Together these data suggest that PBF in tumour cells may be regulated by a higher number of tandemly repeated ERE-half sites. However, the allele frequency data comparing normal and tumourous breast specimens must be treated with caution, because the normal frequency data is only derived from 3 different individuals. It would have been preferable to increase this number, but these resources were not available to me.

An important precedent to this work has been reported. A polymorphic 23 bp sequence housing an ERE half site and a oestrogen related receptor alpha ($ERR\alpha$) binding site (ERRE) is present in the promoter of the human oestrogen related receptor α gene (ESSRA), with one to four copies of the 23 bp sequence mediating copy number-dependant transcriptional response to $ERR\alpha$, PGC-1 α and oestrogen (Laganiere et al. 2004). To determine if the 18 bp repeats housing an ERE half site found in the PBF promoter are capable of acting in synergy, the pGL3basic_PBFshort_promoter construct

described in the previous chapter could be mutated to contain a variable number of repeats, and luciferase assays conducted before and after oestrogen treatment. I attempted to undertake these experiments, but because of the highly repetitive nature of this region of the promoter, mutagenesis experiments consistently failed to work

Expansion and reduction of repeat lengths in DNA can occur as result of slippage during DNA replication (Streisinger et al. 1966) and homologous recombination. Homologous recombination serves to create genetic diversity in both mitotic and meiotic cells (Sung et al. 2003). During meiosis two homologous sister chromatids align side by side. When cuts occur in one strand of both DNAs, the single stranded DNA (ssDNA) molecules can cross and join the homologous strand forming a chisma. The two homologues exchange DNA segments from the chiasma to the end of the chromosome (Figure 4-6).

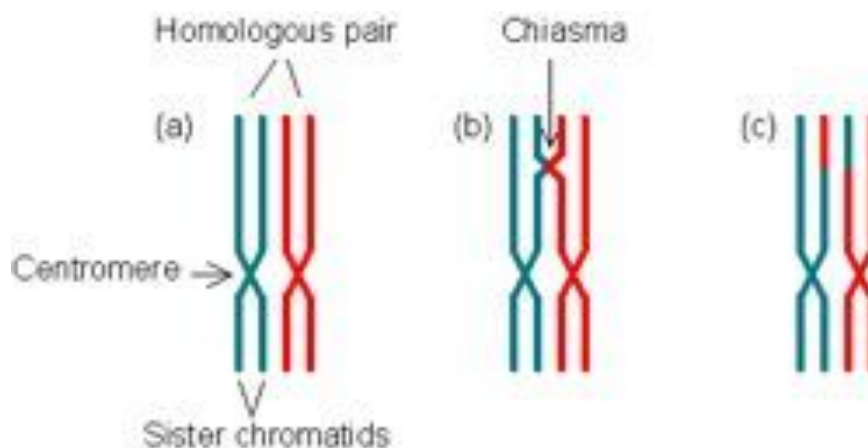


Figure 4-6 DNA cross over during meiosis. (a). Two homologous pairs of sister chromatids align side by side. (b). The two homologs are connected at a certain point called chiasma. (c). The two homologs exchange the DNA segment from the chiasma to the end of chromosomes. Taken from <http://www.web-books.com/MoBio/Free/Ch8D2.htm>

Homologous recombination is also an important mechanism for eliminating double strand breaks. Furthermore homologous recombination is involved in restarting stalled replication forks (Sung et al. 2003), by using the ability of single stranded DNA (ssDNA) molecules derived from the processing of double strand breaks or stalled replication forks to invade a homologous duplex. Once the ssDNA molecule has invaded the homologous duplex and migrated, a joint molecule is formed. Resolution of the joint molecule yields recombinants that either entail a reciprocal exchange of genetic information flanking the initiation site (cross over recombinants) or not (non cross over recombinants). This depends on whether the DNA strands are cut along the vertical or horizontal line (Sung et al. 2003) (Figure 4-7).

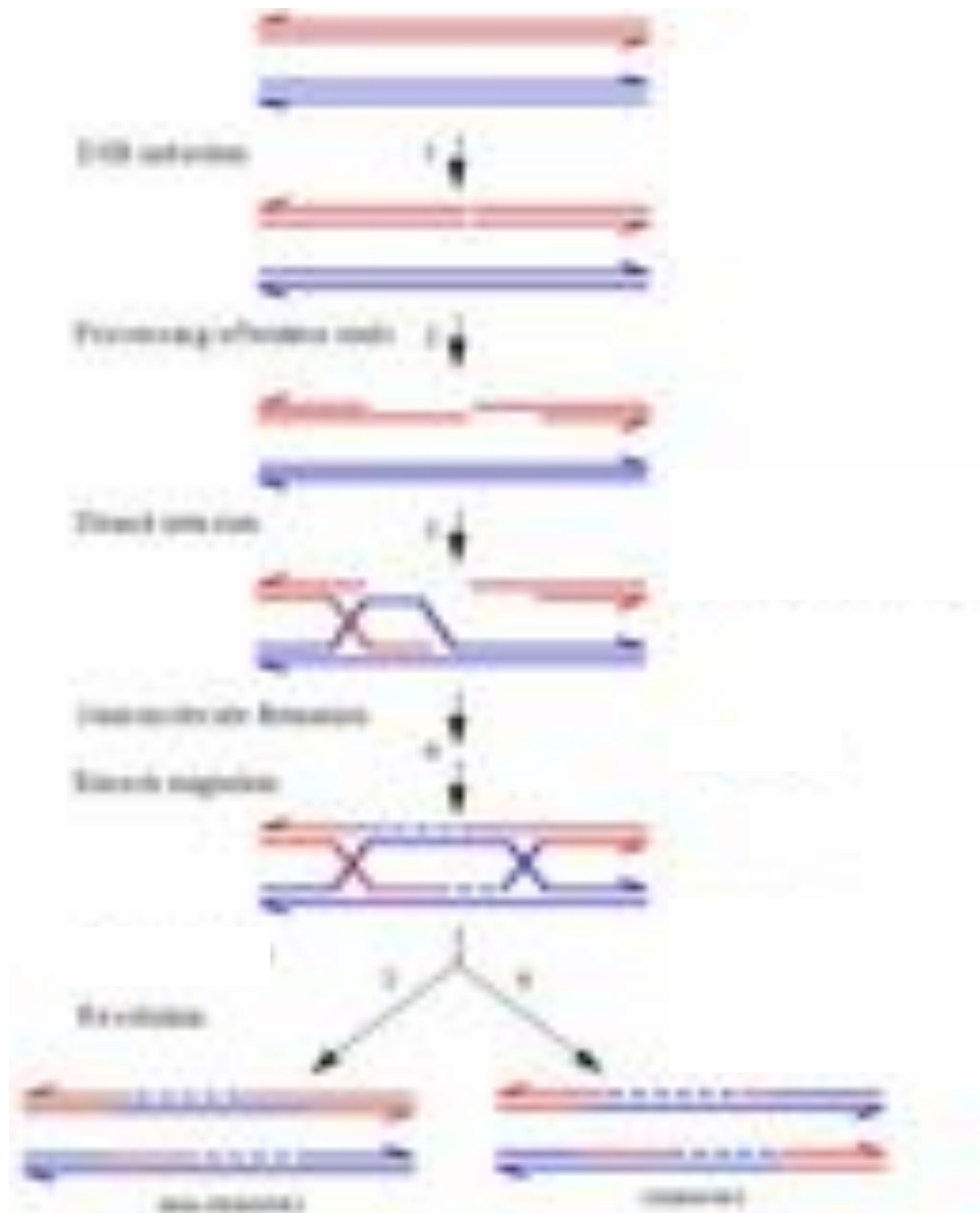


Figure 4-7. Repair of double strand breaks using homologous recombination. 1. Both strands of a DNA molecule are cut. 2. The broken ends are processed, forming single stranded DNA (ssDNA). 3. ssDNA invades the homologous duplex. 4. Branch migration allows the formation of a joint molecule. Resolution of the joint molecule can occur by cutting the DNA strands along either the vertical line or horizontal line. 5. The horizontal cut results in non crossover recombinants. 6. The vertical cut will generate crossover recombinants. Taken and adapted from <http://atlasgeneticsoncology.org/Deep/Images/DoubleStrandBreak2.gif>

Slip mispairing can occur when DNA is in a single stranded form during repair and recombination. Due to significant sequence homology the repetitive sequence region of a chromatid may not line up exactly with its corresponding region in the identical sister chromatid resulting in unequal crossover. Slip mispairing either during replication, recombination or repair is thought to be the major mechanism underlying the variable number of tandem repeats observed in the human genome (Veeraraghavan et al. 2003). This may provide a mechanism explaining the variable number of repeats found in the PBF promoter amongst individuals in the population. Those individuals who have a higher number of ERE half sites in the PBF promoter may be more susceptible to the overexpression of PBF after oestrogen exposure. As work from our group has previously demonstrated that overexpression of PBF in NIH3T3 cells leads to significant colony transformation, overexpression of PBF may subsequently initiate tumour development.

Cancer susceptibility gene mutations fall into two general categories: gatekeepers and caretakers. Gatekeepers are genes whose mutation or altered expression relieves normal controls on cell division, death or lifespan, promoting the outgrowth of cancer cells. Caretakers are genes whose disruption cause genetic instability, increasing the frequency of alterations in gatekeeper genes (Venkitaraman 2002). The explanation why colon tumour samples have more ERE half sites in the PBF promoter than their corresponding matched normal samples may be that tumour cells have acquired mutations in caretakers and gatekeeper genes which make them susceptible to double strand breaks or stalled replication forks. Additionally they may have gained mutations increasing their rate of proliferation. Because the amount of homologous recombination, DNA synthesis and mitosis will increase in tumour cells compared to normal cells, the repeat region in the PBF promoter may be more prone to expansion. Increasing the number of ERE half sites in the PBF promoter within tumour cells could in turn drive progression of the

disease, as PBF, a known transforming gene may be more susceptible to overexpression as a result of oestrogen exposure.

Chapter 5

PBF expression in normal and breast tumour tissue.

5.1 Introduction

Breast cancer is the most frequent malignancy in the female population. Many of the risk factors are well known; these include longer exposure to endogenous hormones (early menarche, late menopause, nulliparity), and exposure to exogenous hormones (oral contraceptives, hormone replacement therapy) (Althuis et al. 2003; R.A. Walker 2004) (Hoover et al. 1976) (Cuzick 2008) (Cork et al. 2008). One explanation for these observations is that oestrogens promote the growth and progression of tumour cells (Biglia et al. 2004; Braendle 2000).

In previous chapters, we showed that the activity of the PBF promoter is modulated by oestrogen, and when MCF-7 cells were exposed to oestrogen treatments, PBF mRNA and protein expression levels were upregulated. In contrast PTTG and separase mRNA expression remained unaltered after oestrogen treatment. As breast cells are responsive to oestrogen, and because oestrogen promotes the growth and progression of ER positive tumour cells, we wanted to determine if PBF mRNA and protein expression levels were raised in breast cancers compared to normal specimens. To this end RNA was extracted from formalin fixed, paraffin embedded tissue sections for quantitative real time PCR assays. In addition PBF protein expression was determined through immunohistochemistry assays.

5.2 Materials and methods

5.2.1 Paraffin embedded tissue sections

Formalin fixed, paraffin embedded tissues sections were a kind gift from Dr Gary Reynolds (Liver Research laboratories, University of Birmingham, Birmingham, UK) or were purchased from US Biomax (PO Box 1854, Rockville, MD20849). Breast tumour paraffin-embedded samples arranged on tissue microarrays (TMAs) were available from 146 patients who underwent surgery at the Queen Elizabeth hospital, Birmingham (101 Cases of no special type, 6 cases of ductal carcinoma in situ, 4 cases of medullary carcinoma, 1 atypical medullary carcinoma, 7 cases of mucinous type, 2 cases of tubular, 22 cases of lobular, 1 lobular-papillary and 1 benign tumour) (Murray et al. 2003). The TMAs contained between two and four representative samples of tumour per patient (Murray et al. 2003). Complete clinical follow-up data including tumour type, tumour grade, vascular invasion, lymph node stage (LNS), Nottingham Prognostic Index (NPI), and ER α status was available for the breast cancer series. Breast normal paraffin-embedded samples taken at breast reduction surgery were available from 3 patients and 5 normal breast specimens were brought in from US biomax (PO Box 1854, Rockville).

5.2.2 RNA extraction and reverse transcription.

RNA was isolated from paraffin-embedded tissues on a microscope slide using the PinpointTM Slide RNA isolation System II kit (Zymo Research, USA). Initially, paraffin was removed from the slides by submerging them in xylene for 1 hour, changing the xylene once. The samples were then hydrated by washing progressively for 2 minutes in 100 %, 70 %, 50 % ethanol and then in pure water. To remove the desired tissue area

from the slide, 0.5 μl of PinpointTM Solution per mm^2 was applied. RNA was subsequently extracted in 20 μl of RNA Digestion Buffer and 5 μl of Proteinase K for 4 hours at 55°C. Following the 55°C incubation, 2 volumes of RNA Extraction Buffer and 1 volume of 100% ethanol were added before transferring the solution into a Zymo-Spin ITM column. Before elution of the RNA in 10 μl of pre-warmed RNA Elution Buffer, the column was washed twice with 200 μl of RNA Wash Buffer.

The concentration of RNA extracted was determined by absorbance of light at $\lambda 260$, and the ratio of $\lambda 260:\lambda 280$ used as an indication of sample purity. RNA with a $\lambda 260:\lambda 280$ ratio between 1.8 and 2.0 was deemed of good quality.

Synthesis of cDNA from 35 ng RNA was carried out using the Reverse Transcription System (Promega) as described in section 2.2.4.

5.2.3 Quantitative real time PCR

Expression levels of PBF mRNA in normal and tumour tissue were assessed using TaqManTM chemistry as described in section 2.2.5.

Quantitative primers and probes for PBF (Stratford et al. 2005) had been validated previously and their sequences are given in Table 1.

PCR reactions were carried out in duplicate in 96-well optical plates (Applied Biosystems) in a total volume of 25 μl . To confirm that amplification was not an artefact of genomic contamination, reverse transcriptase negative (RT-) controls, RNA negative (RNA-) and water only controls were carried out.

5.2.4 Avidin-Biotin Complex immunohistochemistry

Paraffin was removed from the slides using HistoClear (Raymond Lamb Limited, Eastbourne, UK) and the slides rehydrated using 100 %, 90 %, 70 % and 50 % ethanol sequentially. Endogenous peroxidase activity was then inhibited by incubating with 0.3 % hydrogen peroxide (H_2O_2) for 30 minutes at room temperature. Prior to the addition of the primary antibody, non-specific binding was blocked with 5 % normal serum in dilution buffer (1 % IgG-free BSA and 0.1 % Tween-20 in PBS) for 60 minutes at room temperature. The primary antibody, rabbit polyclonal anti-PBF (Smith et al. 2009), was used at a final concentration of 1:200. Primary antibody was diluted in 2 % normal serum in dilution buffer and incubated in a humidified chamber for approximately 16 hours at 4°C. Specificity of staining was confirmed by carrying out controls in which no primary antibody was used.

Excess primary antibody was removed with 5 minute washes in PBS before incubation with the biotinylated secondary antibody (0.5% biotinylated secondary antibody and 1.5% normal serum in dilution buffer) for 50 minutes at 4°C, followed by 10 minutes at room temperature in a humidified chamber.

Excess secondary antibody was removed with 5 minute washes in PBS and slides were incubated with the avidin-biotin complex (ABC) (2 % reagent A, 2 % reagent B in PBS) supplied by Vector Laboratories (Ignold Road, Burlingame, CA 94010) for 30 minutes at room temperature in a humidified chamber. ABC contains biotinylated peroxidase molecules and therefore allows the detection of the biotinylated secondary antibody with 3-3'-diaminobenzidine (DAB). Excess ABC was removed with 5 minute washes in PBS and slides were developed using the DAB substrate kit (Vector

Laboritries, Burlingame) for peroxidase (5 ml H₂O, 2 drops buffer mix, 4 drops DAB mix and 2 drops H₂O₂), for 2 minutes at room temperature. Nuclei were counterstained using Mayers Haematoxylin for 15 seconds. Subsequently, slides were dehydrated using 50 %, 70 %, 90 % and 100 % ethanol sequentially and cleared in Histoclear. Finally, tissue sections were covered with coverslips using Vectamount (Vector Laboritries, Burlingame).

Evaluation of immunohistochemistry was performed by Dr Gary Reynolds, (Liver Research laboratories, University of Birmingham, Birmingham UK). The whole of each section was assessed under light microscopy. Individual sections were scored according to the intensity (0 = not present, +1= least intense, +3 = most intense) and percentage (<25/ 25-50/ 50-75/ >75) of PBF staining (Figure 5-1). Each section was stained and scored at least twice (Figure 5-1).

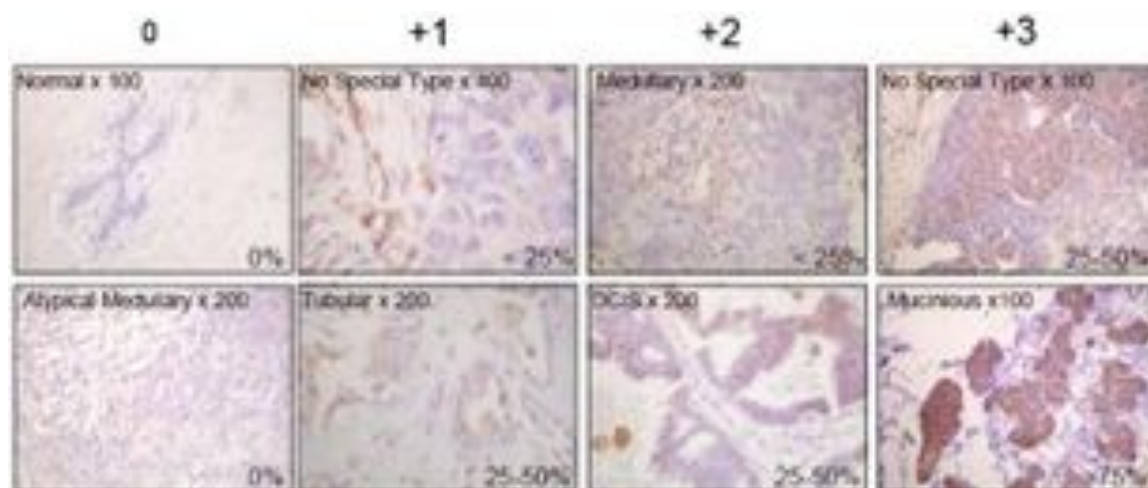


Figure 5-1. Representative immunohistochemical examination of PBF staining (brown) in 1 normal breast sample (US BioMax, Rockville, MD, USA) and 7 tumour samples from TMA sections. Columns 1-4 represent the different staining intensities observed, 0= negative, 1= least intense, 3= most intense. The percentages given in the bottom right hand corners indicate the percentage of PBF expression observed in the whole of each section.

5.2.5 Statistical analysis

Statistical analyses were performed using MINTAB version 14.0 (Minitab Ltd, Coventry, UK) as the statistical package. Associations between PBF mRNA expression data and the number of ERE-half sites found in the PBF promoter were analysed using the Chi Squared test. Complete clinical follow-up encompassing tumour type, tumour grade, vascular invasion, lymph node stage (LNS), Nottingham Prognostic Index (NPI) and ER α status was available for the breast cancer series. Correlations between clinical follow up data, PBF intensity and percentage were also explored using Chi-squared tests. Statistical analysis was not conducted on tumour type as the majority of patients were classified as having no special type, resulting in categories in which the numbers were too small to analyse. Significance was taken as $p < 0.05$.

5.3 Results

5.3.1 PBF mRNA is overexpressed in breast tumours when compared to normals

To determine if PBF mRNA was upregulated in breast tumours compared to normal breast tissues RNA was extracted from formalin fixed, paraffin embedded microscopic slides, and after reverse transcription, PBF mRNA levels were measured using quantitative real time PCR. Because our normal breast tissue samples did not express detectable levels of PBF, data could not be plotted as fold changes. The data has therefore been plotted as Δ CT values from TaqMan RT PCR. These data show that PBF was undetectable in all of the normal breast samples (N=6); however the majority of the tumour samples had Δ CT values above 6.5 (N=20) (Figure 5-2). To rule out the chance of missing significant regions of epithelial cells on the relatively large sections of normal

breast tissue, 3 regions of each normal breast were examined, but all 3 sampled regions remained negative.

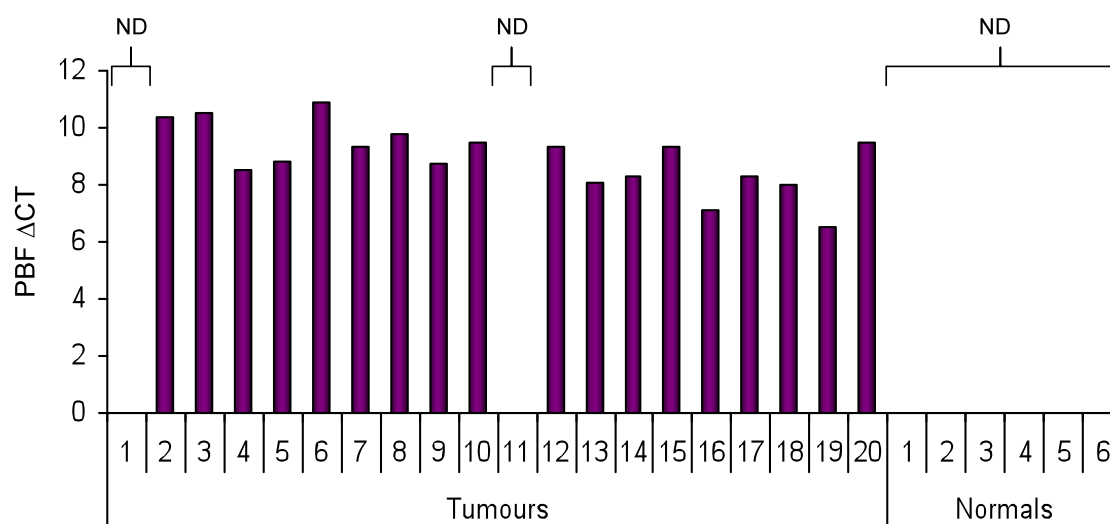


Figure 5-2: Expression of PBF mRNA in breast tumours compared to normals. Gene expression is displayed as a Δ CT value, using 18s as the internal control. ND = not detected.

5.3.2 PBF mRNA levels correlate with the number of putative, consensus half EREs found in the PBF promoter

In Chapter 4 it was shown that the PBF promoter contains an 18 bp repeat region housing a consensus half ERE (gccctccGGTCAgcctc) which varies in length between individuals in the population. To determine if the number of half-EREs in the PBF promoter of breast tumour and normal samples relates to PBF mRNA expression, the repeat length data from Chapter 4 and the expression data from section 5.3.1 were compared. It was found that the majority (75%) of the homozygotes (3/3 EREs) had undetectable levels of PBF expression. This proportion was much lower (14%) in heterozygotes (3/5 EREs) ($p=0.044$). Although numbers were too low to draw any real conclusions these observations suggest that larger scale association studies would be a

useful future target to determine if this is a predisposing site for instability in the initiation or progression of ER α positive breast cancer. By contrast, when the repeat length data from Chapter 4 and the PBF protein expression data were compared no significant association was identified.

5.3.3 PBF protein is overexpressed in breast tumours compared to normal breast.

To determine whether PBF protein expression varied between breast cancers and normal breast specimens, immunohistochemistry was performed on 8 normal breast tissues and 146 tumour tissues which had been paraffin embedded and formalin fixed onto glass slides. To facilitate the screening of multiple tissue blocks, tissue microarrays (TMAs) had previously been prepared using the breast tumour tissue (Murray et al. 2003). PBF was low or absent in normal breast samples, whereas it was strongly expressed in epithelial cells of all grades of breast tumour (Figure 5-3).

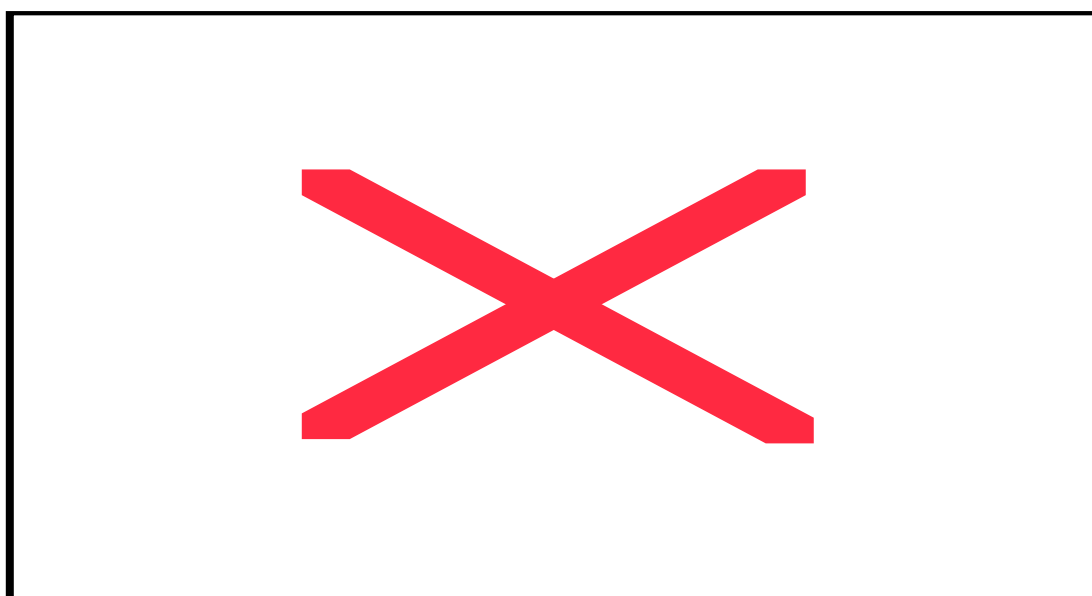


Figure 5-3 Representative immunohistochemical examination of PBF staining in 3 normal breast samples (N1-N3; US BioMax, Rockville, MD, USA; 40x magnification) and 3 tumour samples from TMA sections. T1 - Grade I; T2 – Grade II; T3 – Grade III; all 40x magnification. PBF expression was predominantly apparent in epithelial cells of tumours, and not in normal breast cells.

When the intensity of PBF staining and the percentage of tumour cells expressing PBF were uni-variantly correlated with the clinical data available on the breast cancers it was found that ER status positively correlated with PBF percentage expression ($p < 0.001$). 59 % of ER positive patients had PBF expression levels above 50%, whereby 18% of ER negative patients had expression levels above 50 %. However, ER status did not correlate with intensity of PBF staining. The remaining categories (tumour grade, vascular invasion, lymph node stage and the Nottingham Prognostic Index) did not correlate with PBF staining intensity or percentage expression (Table 5).

	PBF intensity (0,1 vs 2,3)	PBF percentage (< 50 % vs > 50 %)
Tumour grade (1,2 vs 3)	0.33	0.76
VI (Present vs Not seen)	0.45	0.52
Lymph node stage (0,1 vs 2,3)	0.12	0.62
NPI (< 3.4 vs ≥ 3.4)	0.23	0.10
ER status (Positive vs Negative)	0.10	<0.001

Table 5. The p values obtained when the intensity of PBF staining and the percentage of tumour cells which expressed PBF were correlated to tumour type, tumour grade, vascular invasion (VI), lymph node stage, Nottingham Prognostic Index (NPI) and ER status. Correlations between intensity of PBF staining and percentage of PBF positive tumour cells and the demographic information were compared using Chi-squared.

5.4 Discussion

In previous chapters I have shown that PBF mRNA and protein expression levels are upregulated in response to oestrogen, and that a small region of the PBF promoter housing multiple half-ERE sites is regulated by oestrogen. Exposure to oestrogen is a well known risk factor for the development of breast cancer (Biglia et al. 2004; Braendle 2000) and the growth of approximately 40 % of all human breast cancers is dependent on the presence of oestrogen-ER complexes (Lazennec et al. 1999). In light of this information we wanted to determine if PBF mRNA and protein were overexpressed in breast tumours compared to normals.

PBF mRNA and protein were overexpressed in breast tumour samples compared to normal breast specimens. In the cohort of breast tumours analysed by immunohistochemistry there was 1 benign tumour in which, similar to normal breast tissues no PBF staining was present. Despite a relatively small number of formalin fixed paraffin embedded samples yielding both mRNA and promoter sequencing data, when the 18 bp repeat length data from Chapter 4 and the expression data from section 5.3.1 were compared, 14 % of heterozygous (3/5) and 75 % of homozygotes (3/3) samples had undetectable levels of PBF expression implying that a greater number of EREs results in higher levels of PBF expression. Low N numbers are attributed to the difficulty in successfully extracting RNA and DNA from the same small fixed section of tissue. Although numbers were too low to draw any real conclusions these observations suggest

that larger scale association studies would be a useful future target to determine if this is a predisposing site for instability in the initiation or progression of ER α positive breast cancer. This is because a greater number of EREs in a breast tumour would potentially mean a greater response to circulating oestrogen and hence higher expression of a known transforming gene. When the repeat length data from Chapter 4 and the PBF protein expression data were compared, no significant association was identified. As mentioned above, sequencing a polymorphic region of DNA from FFPE TMAs is technically difficult, which necessarily meant that our sample size was low. Coupled with the fact that expression measured through immunohistochemistry is semi-quantitative, these drawbacks may have hampered our power of statistics.

As we had detailed demographic patient information encompassing tumour grade, vascular invasion, lymph node stage, Nottingham Prognostic Index and ER status, this information was correlated to PBF protein staining intensity and percentage expression. A positive association was identified with PBF expression and ER status ($P < 0.001$), whereby 59 % of ER positive patients and 18% of ER negative patients had PBF expression levels above 50 %, supporting our data that PBF is upregulated by oestrogen. The intensity of PBF staining and the percentage of PBF expression did not correlate with tumour grade, vascular invasion, lymph node stage or the Nottingham Prognostic Index. Similarly, the intensity of PBF staining did not correlate with ER status. The lack of associations between clinical outcome and PBF expression was surprising given that work from our group has previously demonstrated an association between PBF expression and thyroid tumour recurrence (Stratford et al. 2005). A matched normal: tumour cohort would allow a much more detailed interrogation of the association between promoter polymorphism and clinical outcome, as we could determine the fold induction of PBF

expression in tumours compared to normals, thus removing the natural variation between individuals in the population

Although this chapter shows that both PBF mRNA and protein expression levels are upregulated in breast tumour samples compared to normals, N numbers for the normals were low. Ideally we would have liked additional normal breast specimens both for the mRNA and protein data, but unfortunately we did not have this many slides available to us. However, the data presented in this chapter demonstrate that PBF is abundantly expressed in breast tumours, but not in normal breast.

Chapter 6

**The effect of oestrogen and PBF on the invasiveness of
MCF-7 cells**

6.1 Introduction

In Chapter 2, diethylstilbestrol and 17 β -estradiol were shown to upregulate the expression of PBF mRNA and protein. In Chapter 3, we went on to show that a small region of the PBF promoter which contains one full ERE and multiple half EREs found within an 18bp (gccctccGGTCAcgcctc) repeated sequence was responsive to oestrogen. Further to this we have shown that PBF mRNA and protein expression levels are upregulated in breast tumours compared to normals. However, the function of PBF remains relatively undefined.

Distant metastases rather than the primary tumour are the main cause of death in patients with breast cancer (Mangala et al. 2007). While women with early (localised) disease can now expect to survive for the rest of their lives disease free, metastatic disease remains incurable (Cufer 2007). An important step in the metastatic process is the invasion of tumour cells into surrounding host tissue (Mangala et al. 2007). So far there is no clear understanding of the molecular alterations that govern the process causing primary tumour cells to metastasise. The elucidation of novel proteins and pathways which contribute to this process are therefore now required.

To determine if the overexpression of PBF could alter the invasive capacity of MCF-7 cells, BD-matrigel based invasion assays were conducted.

6.2 Materials and methods

6.2.1 Cell lines and plasmids

MCF-7 cells were maintained as described in section 2.2.1.

The full length PBF cDNA, previously sub-cloned into pCI-neo, was tagged at the 3' end with the sequence TACCATACGACGTCCCAGACTACGCT, which encodes the human influenza virus haemagglutinin (HA) epitope (YPYDVPDYA), by Dr Anna Stratford (Stratford et al. 2005).

HA-tagged PBF mutants were created by Dr Martin Read (Division of Clinical and Experimental sciences, University of Birmingham, Birmingham, UK). The PBF HA plasmid was mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK). Mutagenesis primer sequences are shown in (Table 6).

Mutagenesis Primer	Sequence (5'– 3')
Mutant 1_forward	GAGAGGCGGATACGGCAGGAGGAATACCCATACGACGTCC CAGACTAC
Mutant 1_reverse	GTAGTCTGGGACGTCGTATGGGTATTCCTCCTGCCGTATCC GCCTCTC
Mutant 2_forward	CTCCTGCTGCTCATCCCGAACTTTGAGGCGCTGATC
Mutant 2_reverse	GATCAGCGCCTCAAAGTTCGGGATGAGCAGCAGGAG

Table 6 Mutagenesis primer sequences used to create PBF mutant plasmids.

Mutant 1 consisted of amino acids 1-149 only and hence, lacked the NLS and the tyrosine based sorting signal. Mutant 2 had a deletion of amino acids 29-93 and, therefore, lacked a complete signal peptide and PSI domain (Figure 6-1).

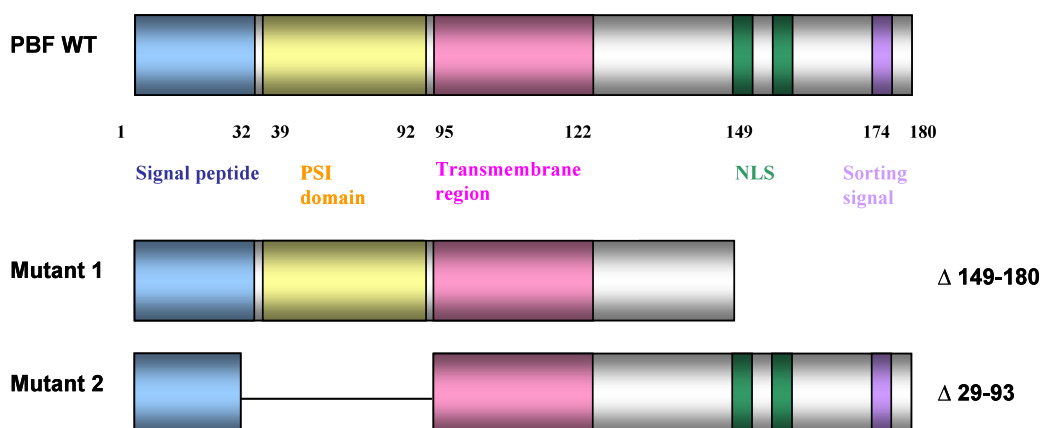


Figure 6-1 A schematic diagram of wild type PBF, PBF Mutant 1 and PBF Mutant 2. All mutants were created using the Quickchange site-directed mutagenesis kit. Wild type and mutant PBF were HA tagged at the C- terminus.

6.2.2 Transient transfection

Cells were seeded into T25 culture flasks at a density of 8×10^5 cells per flask in 5 ml of complete RPMI 1640 (Gibco). Twenty-four hours later, transient transfections were performed using 15 μ l FUGENE 6 (Roche) with 5 μ g DNA (pCI-neo (VO), HA-PBF, HA-PBF Mutant 1 or HA-PBF mutant 2) per flask, following the manufacturer's instructions.

Approximately 6 hours post-transfection, medium was changed to 5 ml of RPMI 1640 (Gibco) supplemented with 2 % FBS, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml). After a further 16 hours, cells were trypsinised and resuspended to a final concentration of 5×10^5 or 1×10^6 cells per ml if invasion assays were to be harvested after 24 or 48 hours respectively. Cells were resuspended in RPMI 1640 (Gibco) supplemented with 2 % FBS, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml). Invasion assays were then conducted.

6.2.3 Oestrogen treatment

MCF-7 cells which had been maintained in complete phenol red free RPMI 1640 (Gibco) for 6 days were seeded into T25 culture flasks at a density of 8×10^5 cells per flask in 5 ml of complete phenol red free RPMI 1640 (Gibco). Twenty-four hours later, cells were treated with vehicle (100% ethanol) or 10 nM diethylstilbestrol.

Approximately 6 hours post oestrogen treatment, medium was changed to 5 ml of RPMI 1640 supplemented with 2 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l), streptomycin (100 mg/ml) and 10 nM diethylstilbestrol or vehicle. After a further 16 hours, cells were trypsinised and resuspended to a final concentration of 5×10^5 or 1×10^6 cells per ml if invasion assays were to be harvested after 24 or 48

hours respectively. Cells were resuspended in phenol red free RPMI 1640 supplemented with 2 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l) streptomycin (100 mg/ml) and 10 nM diethylstilbestrol or vehicle. Invasion assays were then conducted.

6.2.4 Establishing the efficiency of the PBF specific siRNA, and determining the time period of its transient effect

MCF-7 cells were seeded in T25 cell culture flasks at a density of 3.45×10^5 cells per flask or in 6 well plates at a density of 1.38×10^5 cells per well and incubated with 50 nM of Scrambled (negative control #1, Ambion) or PBF specific siRNA (#14399 and #147350 (mixed in equal quantities; Ambion). siRNA was delivered into the cells using siPORT (Ambion) as per manufacturer's instructions. Briefly, 20 μ l of siPORT was diluted to a final volume of 250 μ l for T25 flasks or 8 μ L of siPORT was diluted to a final volume of 100 μ l for 6 well plates in phenol red free RPMI 1640, and was allowed to stand at room temperature for 10 minutes. 2 μ M Scrambled or 2 μ M PBF siRNA were diluted to 1.25 nM in a final volume of 250 μ l for T25 flasks or 100 μ l for 6 well plates of phenol red free RPMI 1640. Dilute siPORT and siRNA were then mixed and incubated for a further 10 minutes at room temperature before being dispensed into T25 cell culture flasks or 6 well plates. 5.75 ml or 2.3 ml of MCF-7 cells at a density of 6×10^4 cells per ml were then overlaid in T25 flasks or 6 well plates respectively; this gives a final concentration of 50 nM for both the Scrambled and PBF siRNA. 72 hours later, protein was harvested from the MCF-7 cells plated into the 6 well plates, as described in section 2.2.6. Alternatively, MCF-7 cells plated into T25 flasks were trypsinised and re-seeded into 6 well plates at a density of 1.5×10^5 cells per well, and protein harvested after 24 and 48 hours as described in section 2.2.6.

6.2.5 Incubation with PBF siRNA and simultaneous oestrogen treatment

48 hours prior to treatment with 10 nM diethylstilbestrol, MCF-7 cells which had been maintained in complete phenol red free RPMI 1640 (Gibco) for 6 days were seeded in T25 cell culture flasks at a density of 3.45×10^5 cells per flask and incubated with 50 nM of Scrambled (negative control #1, Ambion) or PBF specific siRNA (#14399 and #147350 (mixed in equal quantities; Ambion). siRNA was delivered into the cells using 20 μ l of siPORT (Ambion) as per section 6.2.4.

Approximately 6 hours post oestrogen treatment, medium was changed to 5 ml of phenol red-free RPMI 1640 medium (Gibco) supplemented with 2 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l), streptomycin (100 mg/ml) and 10 nM diethylstilbestrol or vehicle. After a further 16 hours, cells were trypsinised and resuspended to a final concentration of 5×10^5 cells per ml in RPMI 1640 phenol red free medium (Gibco) supplemented with 2 % charcoal stripped serum, L-Glutamine (2 mM), penicillin (10^5 U/l), streptomycin (100 mg/ml) and 10 nM diethylstilbestrol or vehicle. Invasion assays were then conducted.

Alternatively, 3.45×10^5 cells were seeded into 6 well plates and treated with 10 nM diethylstilbestrol or vehicle. 24 hours later, at the completion of the invasion assay, protein was extracted from these cells, as described in section 2.2.6. This allowed the efficiency of the PBF knockdown to be visualised by Western blotting.

6.2.6 Invasion assay

BD falcon™ cell culture inserts (8 μ M pore size) were coated with 40 μ l of growth factor reduced BD Matrigel™ per cm² of growth area. 1×10^5 or 2×10^5 MCF-7 cells were then seeded into the upper compartments of the inserts in a total volume of 200 μ l RPMI 1640 medium or the phenol red free alternative (Gibco) supplemented with 2 %

FBS or charcoal stripped serum respectively, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml). Subsequently 800 μ l of RPMI 1640 medium or the phenol red free alternative (Gibco) supplemented with 20% FBS, or charcoal stripped serum respectively, L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml) was added to the well below the BD falcon™ cell culture inserts. The choice of normal or phenol red free RPMI 1640 depended on whether the cells had previously been transiently transfected or treated with oestrogen. Phenol red free RPMI 1640 was chosen if the affects of oestrogen were going to be investigated. After 24 or 48, hours cells were fixed and stained using Mayers Haemoxilyn (Sigma) and eosin (Sigma). Cells which had migrated through the filter were counted using a light microscope at 20 x magnification.

6.2.7 Western blot analyses

Protein samples (30 μ g) were incubated at 95°C for 5 minutes with an equal volume of Laemmli sample buffer (Bio-Rad) containing 43 mg/ml DL-Dithiotheriol (DTT), and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described in section 2.2.7.

The membrane was probed with primary antibody, rabbit polyclonal anti-PBF (Smith et al. 2009) at a concentration of 1: 1, 000 in 5 % non-fat milk in TBS-T at 4°C for 16 hours. After six 10 minute washes in TBS-T, the membrane was incubated with secondary antibody, 125 ng/ml HRP-conjugated polyclonal goat anti-rabbit immunoglobulins (Dako), in 5 % non-fat milk in TBS-T at room temperature for 1 hour.

To assess protein loading, following a TBS-T wash, the membrane was probed with mouse monoclonal anti- β actin antibody (clone AC-15; Sigma) at a concentration of 1:10, 000 in 5 % non-fat milk in TBS-T for 1 hour at room temperature. After three 10

minute washes in TBS-T, the membrane was incubated with the secondary antibody, 650 ng/ml HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako), in 5 % non-fat milk in TBS-T at room temperature for 1 hour.

6.2.8 MTT assays

The MTT assay is a quantitative colorimetric assay for cell survival and proliferation. Metabolically active cells can take up the yellow tetrazolium salt MTT (3-(4,5 dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide), where it is reduced by active mitochondria to purple formazan crystals (Mosmann 1983).

6.2.8.1 Transient transfection

Cells were seeded into 96 well plates at a density of 5×10^3 cells per well in 200 μ l of complete RPMI 1640. Twenty-four hours later, transient transfections were performed using 0.6 μ l Fugene 6 (Roche) with 200 ng DNA (pCI-neo or HA-PBF) per well, following the manufacturer's instructions.

24 and 48 hours post-PBF transfection, 100 μ g of MTT was added to each well and the cells incubated for 3 hours at 37°C, 5 % CO₂. Cell medium was removed and formazan crystals produced by active mitochondria were dissolved in 100 μ l of DMSO. The absorbance of the DMSO was then read on the Victor³ multilabel counter at 560 nm to estimate cell number.

6.2.8.2 Oestrogen treatment

MCF-7 breast cancer cells were maintained in complete phenol red free RPMI 1640 (Gibco) for 6 days prior to seeding them into a 96 well plate at a density of 5×10^3 cells per well in a total volume of 200 μ l of complete phenol red free RPMI 1640 medium. Twenty-four hours after seeding, cells were treated with diethylstilbestrol and 17β -estradiol at final concentrations of 10 nM and 20 nM.

48 hours post-oestrogen treatment, MTT assays were conducted as per section 6.2.8.1.

6.2.9 Statistical analysis

Data were analysed using Sigma Stat (SPSS Science Software UK Ltd). The Kolmogorov-Smirnov test determined whether data followed a normal distribution. If the data set to be tested followed a normal distribution, it was analysed using a two sample Student's t-test. However, if the data set was non-parametric, the Mann Whitney Rank Sum test was used for comparison between two groups of data. Significance was taken as $p < 0.05$.

6.3 Results

6.3.1 Overexpression of PBF increases the invasiveness of MCF-7 cells

To ascertain whether PBF overexpression could alter cellular invasion, MCF-7 cells were transiently transfected with VO or PBF and invasion assays were conducted. As a starting point 1×10^5 and 2×10^5 MCF-7 cells were left to invade through growth

factor reduced BD Matrigel™ basement membranes for 24 or 48 hours. The cells which had migrated through to the underside of the filter were then stained and counted.

Over a time period of 24 hours when seeded at a density of 1×10^5 cells per insert, PBF overexpression significantly increased the invasiveness of MCF-7 cells through matrigel (107 ± 39 cells compared to VO (69cells), N=6, $p < 0.01$). PBF overexpression also increased the invasiveness of MCF-7 cells after 48 hours when seeded at a density of 1×10^5 or 2×10^5 cells per insert (349 ± 189 cells compared to VO (67 cells), N=6, $p < 0.01$ and 67 ± 14 cells compared to VO (45 cells), N=6, $p < 0.01$). The later density of 2×10^5 gave a much smaller increase in invasion when compared to 1×10^5 . At this density the cells began to clump together and this may have impaired their ability to invade.

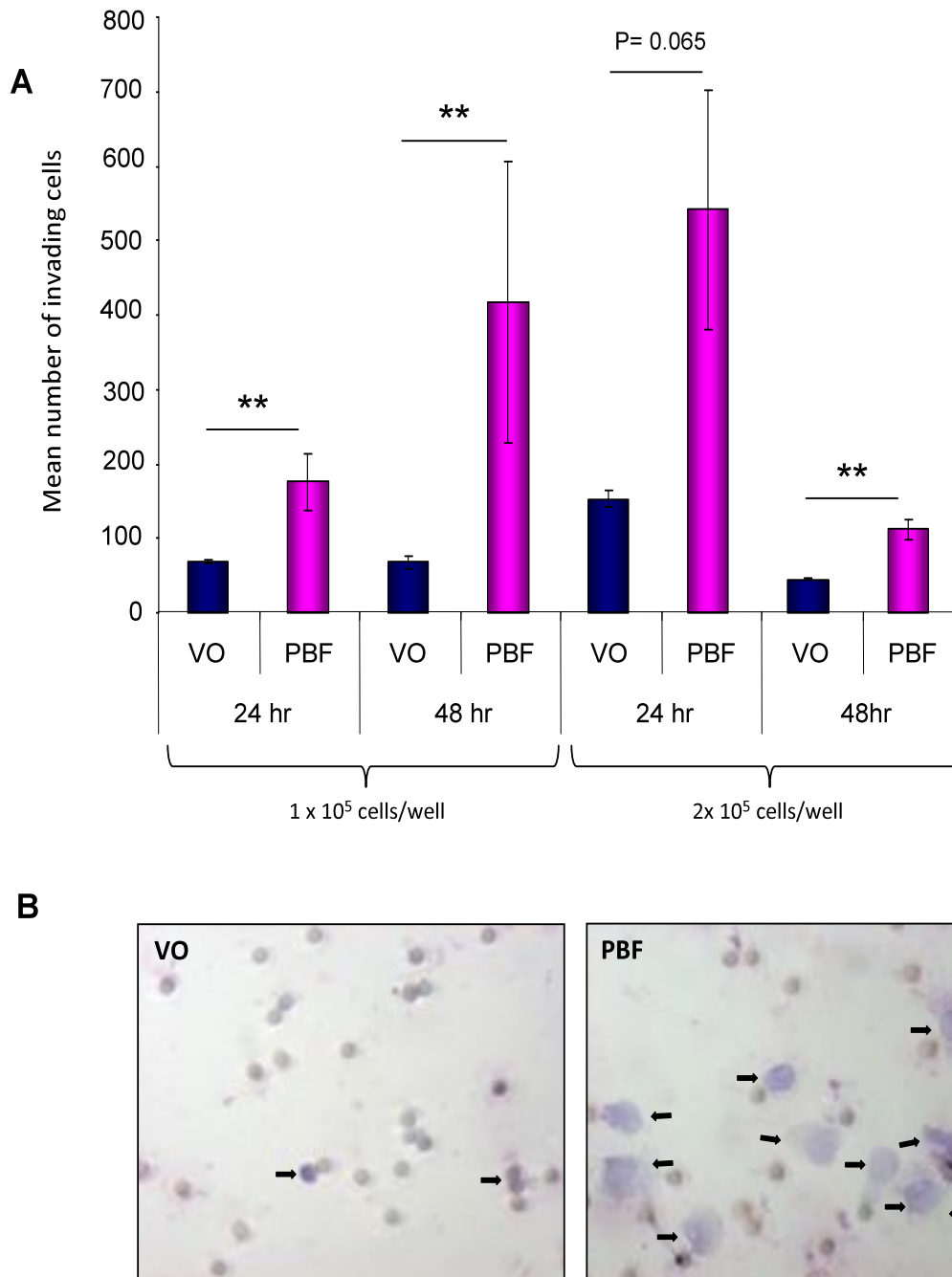
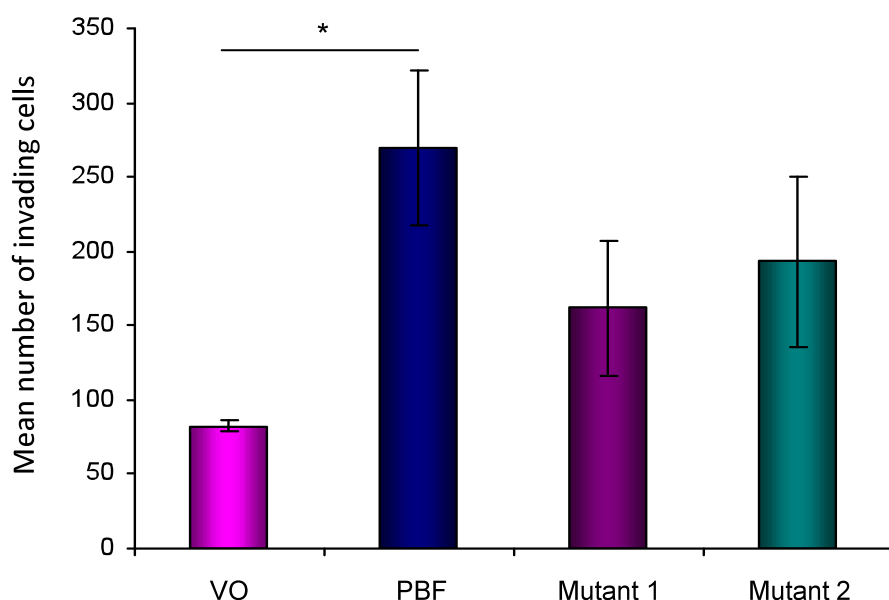


Figure 6-2: Effect of PBF overexpression on the invasiveness of MCF-7 cells. A. 1×10^5 or 2×10^5 MCF-7 cells were seeded into the upper compartments of BD falcon™ cell culture inserts coated with growth factor reduced BD matrigel™. After 24 or 48 hours the cells which were able to migrate through to the underside of the filter were fixed, stained and counted using a light microscope at 20 x magnification. (N=6, ** $p < 0.01$). B. Images of the 8μM filters after invasion. MCF-7 cells were stained with Mayers Haemoxylin (nuclear, blue) and eosin (cytoplasm, pink). Arrows indicate invading MCF-7 cells. VO = transfection of Vector Only, PBF = transfection of HA-tagged PBF.

6.3.2 Overexpression of PBF Mutants 1 and 2 does not affect the invasiveness of MCF-7 cells

To determine which functional domains of PBF are important for its ability to enhance MCF-7 cell invasion, cells were transiently transfected with VO, PBF, PBF Mutant 1 or PBF Mutant 2 and invasion assays conducted.

For this experiment, based on the initial validation a single time point (48 hours) and cell density (1×10^5) was chosen. Although wild type PBF retained its ability to induce the invasiveness of MCF-7 cells (187 ± 52 cells compared to VO (82 cells), $P < 0.05$, $N=10$), PBF Mutant 1 and Mutant 2 did not induce cell invasion.



*Figure 6-3 Effect of wild type and mutant PBF overexpression on the invasiveness of MCF-7 cells. 1×10^5 MCF-7 cells were seeded into the upper compartments of BD falcon™ cell culture inserts coated with growth factor reduced BD matrigel™. After 48 hours the cells which were able to migrate through to the underside of the filter were fixed, stained and counted using a light microscope at 20 x magnification. ($N=10$, * $p < 0.05$). Mean values \pm the standard error of the mean are shown.*

6.3.3 The enhanced invasiveness of MCF-7 observed after PBF transfection is not due to an increase in cell proliferation

MCF-7 cells were transfected with VO or PBF. Their proliferation was then measured after 24 and 48 hours using MTT assays. PBF did not significantly increase the proliferation of MCF-7 cells after 24 hours although it did marginally increase the proliferation of MCF7 after 48 hours (1.1 ± 0.1 fold $P < 0.05$, $N=48$).

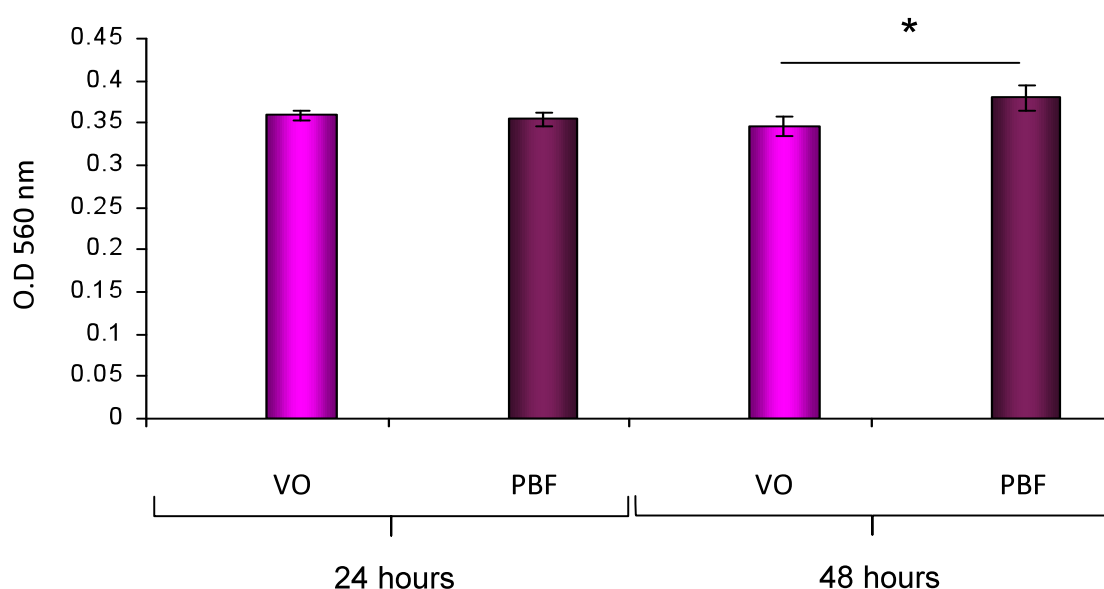


Figure 6-4 Proliferation of MCF-7 cells after transfection with vector only (VO) or PBF. Proliferation is measured as an absorbance as active cells take up the yellow tetrazolium salt MTT and reduce it to form purple crystals, which are then solubilised in DMSO. (* $p < 0.05$, $N=3$ experiments with 16 replicates).

6.3.4 Oestrogen increases the invasiveness of MCF-7 cells

Since the treatment of MCF-7 cells with diethylstilbestrol at a final concentration of 10 nM significantly upregulated PBF protein expression levels (see Chapter 3, Figure

2-3), we hypothesised that oestrogen may increase the invasiveness of MCF-7 cells by upregulating PBF expression.

To test this hypothesis, MCF-7 cells were treated with 10 nM diethylstilbestrol or vehicle and invasion assays conducted. Again a single time point (48 hours) and cell density (1×10^5 cells) were investigated. 10 nM diethylstilbestrol significantly up-regulated the invasiveness of MCF-7 cells (76 ± 13 cells compared to vehicle (56 cells), $P < 0.001$, $N=9$).

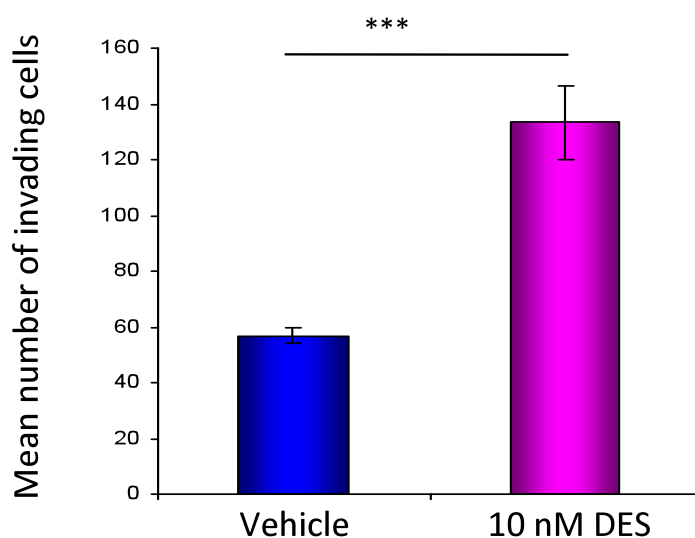


Figure 6-5: Effect of 10 nM diethylstilbestrol (DES) on the invasiveness of MCF-7 cells (***) $P < 0.001$, $N=9$).

6.3.4.1 PBF specific siRNA treatment abrogates the effect of oestrogens on the invasiveness of MCF-7 cells

To determine if the increase in invasion observed after treatment of MCF-7 cells with 10 nM diethylstilbestrol was a direct consequence of its effects on PBF expression levels, MCF-7 cells were simultaneously incubated with 50 nM PBF specific siRNA and treated with 10 nM diethylstilbestrol. For this experiment, cells were seeded at 1×10^5 cells

per insert and invasion assays harvested after 24 hours. The later time point of 48 hours was not used as the PBF specific siRNAs have a transient effect and optimisation experiments (Section 6.2.4) showed that PBF expression levels began to rise again after this time point. (Figure 6-6). In parallel experiments to the invasion assays cells, were seeded into 6 well plates and protein harvested after 24 hours to monitor knockdown. Western blotting confirmed PBF knockdown was successful, reducing native expression by 80 to 90 % (Figure 6-7).).

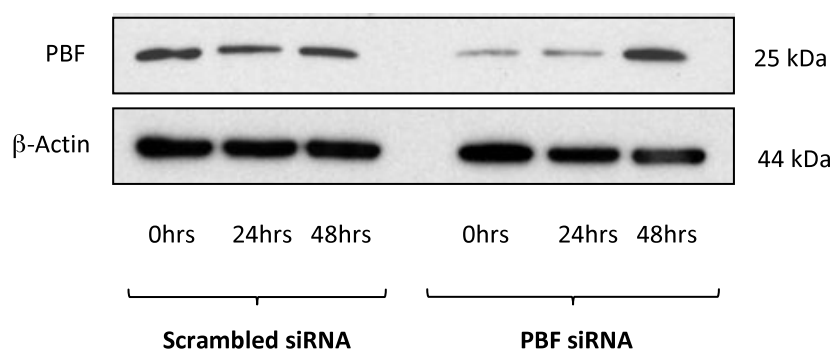


Figure 6-6. Western blot showing successful knockdown of PBF using a PBF specific siRNA compared to the scrambled control. PBF protein levels remained reduced 0 hrs and 24hrs after re-seeding the MCF-7 cells in which PBF protein expression had been knocked down using a PBF specific siRNA, although protein levels rose again 48 hrs post re-seeding.

When MCF-7 cells were incubated with a scrambled control siRNA, oestrogen significantly induced the invasiveness of MCF-7 cells (86 ± 17 cells compared to vehicle (17 cells), $p < 0.01$, $N=4$). However the effect of oestrogens was abolished when MCF-7 cells were incubated with a specific PBF siRNA (a reduction of 8 ± 14 cells compared with vehicle (35 cells), non-significant, $N=4$), suggesting that oestrogen induction of MCF-7 cell invasion may be mediated via PBF (Figure 6-7).

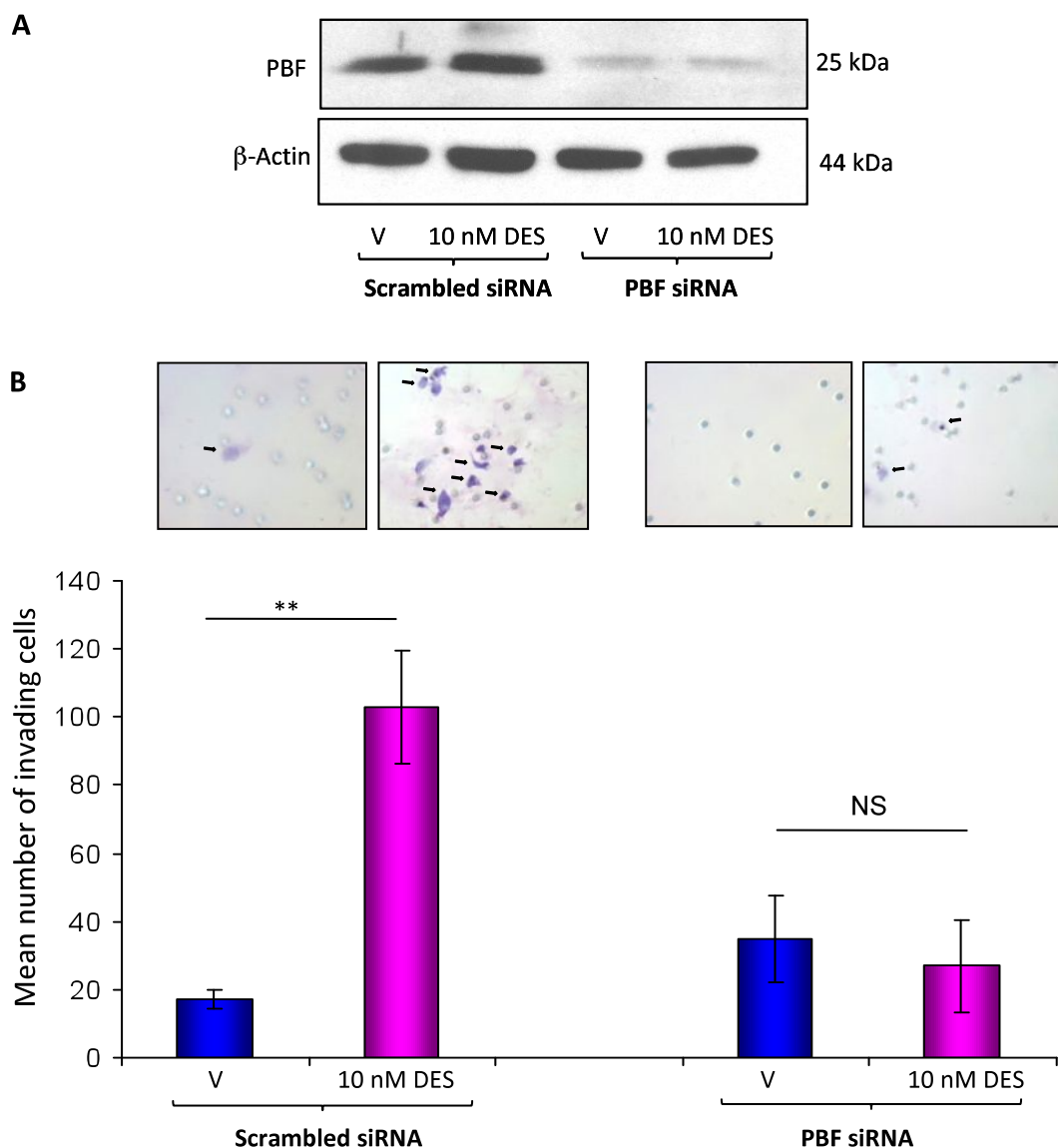


Figure 6-7 A. Western blot showing successful knockdown of PBF using a PBF specific siRNA compared to the scrambled control. B. Effect of simultaneously incubating MCF-7 cells with PBF siRNA and 10 nM diethylstilbestrol (DES) on the invasiveness of MCF-7 cells. (** $P < 0.01$, $N = 4$). NS = non significant.

6.3.5 The enhanced invasiveness of MCF-7 cells observed after oestrogen treatment is not due to an increase in cell proliferation

MCF-7 cells were treated with diethylstilbestrol and 17β -estradiol at final concentrations of 10 nM and 20 nM. Their proliferation was then measured using MTT

assays. Oestrogen treatment of MCF-7 cells did not result in significant changes in cell proliferation (Figure 6-8).

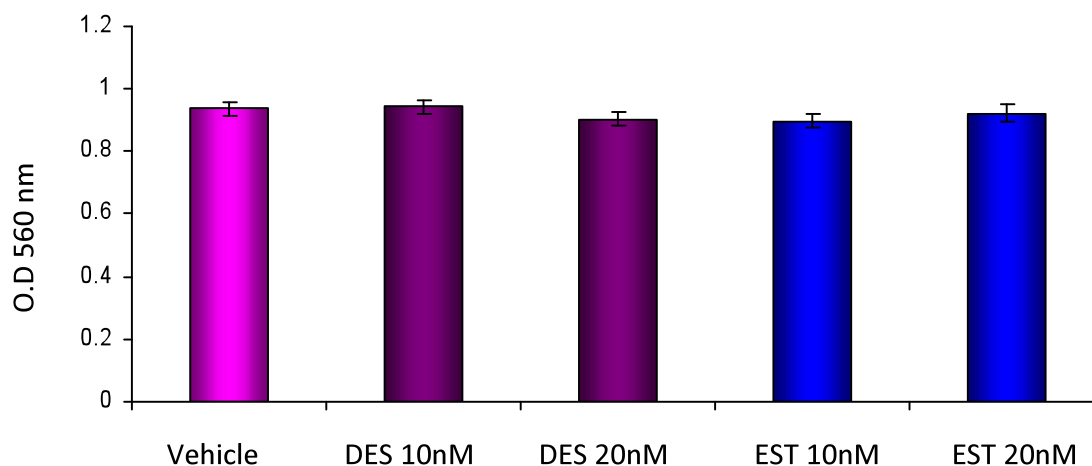


Figure 6-8 Proliferation of MCF-7 cells after treatment with diethylstilbestrol (DES) and 17- β estradiol (EST) at final concentrations of 10 nM and 20 nM (N=16).

6.4 Discussion

In previous chapters, I determined that PBF mRNA and protein is upregulated in response to oestrogen, and that a small region of the PBF promoter is responsive to oestrogen. I also demonstrated that PBF mRNA and protein is overexpressed in breast tumours compared to normals. As the function of PBF is relatively undefined I decided to investigate the consequences of PBF overexpression by assessing its role in invasion. To this end MCF-7 cells were transiently transfected with PBF or treated with 10 nM diethylstilbestrol and invasion assays were conducted.

6.4.1 Elevated PBF expression can induce MCF-7 cell invasion

The data obtained showed that over-expression of PBF, achieved through transient transfection of HA-PBF or treatment with diethylstilbestrol at a final concentration of 10

nM, significantly increased the invasiveness of MCF-7 cells. As the increase in invasion observed after oestrogen treatment could have been due to factors unrelated to its effect on PBF, siRNA technology was employed to ameliorate oestrogen's effect on PBF mRNA and protein levels. In the presence of a scrambled siRNA, treatment of MCF-7 cells with 10 nM diethylstilbestrol still significantly increased the invasiveness of MCF-7 cells, but this was abolished when PBF was simultaneously knocked down using a specific PBF siRNA. Together this data implies that the increased invasiveness of MCF-7 cells observed after oestrogen treatment is specific to its effect on PBF over-expression.

MTT assays were conducted to investigate whether the increase in invasion observed after PBF overexpression was simply due to an increase in cell number. As 10 nM diethylstilbeterol or 10 nM 17 β -estradiol did not induce MCF-7 cell proliferation and because the increase in proliferation observed after HA-PBF transfection was only 1.1 fold compared to at least a 2.5 fold induction of invasion, proliferation per se is not responsible for the increase in invasion observed.

As I have shown that oestrogens induce the invasiveness of MCF-7 breast cancer cells and because this effect is abrogated when PBF is knocked down, PBF could serve as a therapeutic target to prevent oestrogen from stimulating breast cells to invade, and may reduce the progression of early breast cancer (localised) to advanced (metastatic) disease.

6.4.2 The ability of PBF to induce invasion is abolished by the deletion of amino acids 149-180 and 29-93

Having determined that raised PBF expression increased cell invasion, two PBF mutants were employed to determine which domains of PBF are required for it to carry out this function.

PBF mutant 1 has 30 amino acids from the C-terminal deleted, removing both the NLS and the tyrosine based sorting signal. Nuclear localisation signals are short stretches of amino acids that mediate the transport of proteins into the nucleus (Cokol et al. 2000). Typically, deletion of the NLS disrupts nuclear import (Cokol et al. 2000). A monopartite motif is characterised by a cluster of basic residues preceded by a helix-breaking residue (Cokol et al. 2000). However PBF contains a bipartite motif, consisting of two clusters of basic residues separated by 9-12 residues (Chien and Pei 2000).

The tyrosine based sorting signal found at the C-terminus of PBF is an YXX Φ -type signal, where Y is tyrosine, X is any amino acid (these tend to be hydrophilic) and Φ is a bulky hydrophobic residue (Bonifacino and Traub 2003). YXX Φ signals are known to be widely involved in protein sorting. The function of YXX Φ motifs have been implicated in endocytosis, in the targeting of transmembrane proteins to lysosomes and lysosome-related organelles, as well as the sorting of a subset of proteins to the basolateral plasma membrane of polarised epithelial cells (Bonifacino and Traub 2003). The YXX Φ of PBF does not conform to the typical lysosomal or endocytotic signal (Bonifacino and Traub 2003), however, YXX Φ signals can also bind to adaptor proteins (AP) as they are recognised by the μ subunits of these complexes (Bonifacino and Traub 2003). The μ 2 subunit of AP-2 exhibits both high avidity and broad specificity, allowing it to interact with YXX Φ signals even when their placement is suboptimal (Bonifacino and Traub 2003). Recent work in our group has shown that PBF Mutant 1 gets stuck in the plasma

membrane (Smith et al. 2009) indicating that the tyrosine based sorting signal found at the C-terminus of PBF is a endoytic signal which internalises it from the plasma membrane.

PBF mutant 2 has the amino acids between 29 and 93 deleted; this removes the PSI domain, the two putative glycosylation sites at amino acids 45 and 54 and the last 3 amino acids of the signal peptide. PSI domains are usually approximately 50 amino acids in length and contain 8 cytosine residues, pairs of which are linked by dishulphide bonds (Bork et al. 1999). They have been termed PSI domains as they are common to plexins, semaphorins and integrins. However, the function of this domain is still unknown and can be described only vaguely as an extracellular putative binding domain (Bork et al. 1999).

A signal peptide is a short peptide chain that directs the transport of a protein to the endoplasmic reticulum, mitochondrial matrix or the peroxisome. In both eukaryotic and prokaryotic cells, proteins are allowed entry into the secretory pathway only if they are endowed with a signal peptide (von Heijne 1990).

As PBF Mutant 1 and 2 did not induce the invasion of MCF-7 cells, it implies that the NLS, the tyrosine based sorting signal, the PSI domain, the signal peptide and the glycosylation sites are all potentially important for this function. To find out exactly which of these domain are critical for invasion, PBF mutants containing deletions of these domains in isolation are required. Unfortunately the exact mechanism by which PBF can induce invasion and which functional domains are responsible for this process are beyond this body of work. However, I have shown that PBF induces invasion through Matrigel, an action which could be abrogated both by siRNA treatment and specific mutation, suggesting PBF has a specific effect.

Chapter 7

Investigation into the secretion of PBF

7.1 Introduction

In Chapter 6 overexpression of wild type PBF either through oestrogen treatment or transient transfection induced the invasiveness of MCF-7 cells. However 2 mutants which lacked amino acids 149-180 or 29-93 could no longer carry out this function. PBF Mutant 2 lacks the amino acids between residues 29-93 and therefore does not contain a complete signal peptide and will not be glycosylated at the potential glycosylation sites found at amino acids 45 and 54. As mentioned in Section 6.4.2, proteins are allowed entry into the secretory pathway only if they are endowed with a signal peptide (von Heijne 1990). N-glycosylation has also proven to be a key determinant of enzyme secretion (Skropeta 2009). In the majority of cases, elimination of all putative N-glycosylation sites of an enzyme results in significantly reduced protein secretion levels (Skropeta 2009). One reason for this may be because polysaccharides linked at the amide nitrogen of asparagine in the protein confer stability on some secreted glycoproteins, and aid the maintenance of quaternary structure. Prevention of glycosylation may result in intracellular degradation due to improper folding (Skropeta 2009) (Bolt et al. 2007).

A possible reason that PBF Mutant 2 failed to induce invasion is because it lost its capacity for secretion. Secreted proteins such as cytosine protease, cathepsin B and matrix metalloproteinase (MMP)-13 are responsible for the degradation of the extracellular matrix (Sameni et al. 2008), a process which is important for allowing tumour cells to invade local tissue (Kohrmann et al. 2009). However as the PSI domain is also deleted in Mutant 2, we cannot rule out the possibility that this may also induce invasion.

In this Chapter we set out to investigate if PBF is a secreted protein and if the deletion of amino acids 149-180 and 29-93 as in PBF mutants 1 and 2 results in altered secretion.

7.2 Materials and methods

7.2.1 Cell culture and plasmids

MCF-7 cells were maintained as described in section 2.2.1.

PBF-HA and PBF-HA Mutants 1 and 2 have previously been described in section 6.2.1.

7.2.2 Detection of PBF secretion by Western blotting

7.2.2.1 Transient transfections

Cells were seeded into 6 well plates at a density of 2×10^5 cells per well. 24 hours later, transient transfections were performed using 6 μ l Fugene 6 (Roche) with 2 μ g DNA per well, following the manufacturer's instructions. 24 hours post transfection medium was changed to 1 ml of RPMI 1640 medium (Gibco) supplemented with 2 % fetal bovine serum (FBS), L-Glutamine (2 mM), penicillin (10^5 U/l) and streptomycin (100 mg/ml). After a further 24 hours, the cell medium and cell lysate were harvested.

7.2.2.2 Harvesting of cell medium

Cell medium was removed from MCF-7 cells and centrifuged at 13,000 g for 5 minutes, to remove cellular debris. The supernatant was then added to 3 times the volume

of 100 % ethanol and centrifuged at 2,500 rpm for 15 minutes. Finally, the pellet was resuspended in RIPA buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % v/v igeal CA-630, 6 mM sodium deoxycholate, 1 mM EDTA) containing 60 µl/ml protease inhibitor cocktail (containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstainA, E-64, bestain, leupeptin and aprotinin).

7.2.2.3 Harvesting of cell lysate

Harvesting of cell lysate was previously described in section 2.2.6

7.2.2.4 Western blotting

Cell medium (80 µg) was incubated at 37°C for 30 minutes whilst cell lysates (30 µg) were incubated at 95°C for 5 minutes with an equal volume of Laemmli sample buffer (Bio-Rad) containing 43 mg/ml DL-Dithiotheriol (DTT), and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described in section 2.2.7.

The membrane was probed with primary antibody, 1 µg/ml mouse monoclonal anti-HA.11 antibody (Covance Research Products), in 5 % non-fat milk in TBS-T at 4°C for 16 hours. The secondary antibody, 650 ng/ml HRP-conjugated polyclonal rabbit anti-mouse immunoglobulin (Dako), was added in 5 % non-fat milk in TBS-T at room temperature for 1 hour.

7.2.3 Validation of PBF immunoprecipitation

7.2.3.1 Transfection and protein extraction

Cells were seeded into 6 well plates at a density of 1×10^5 cells per well. Twenty four hours later, transient transfections were performed using 6 μ l FUGENE 6 (Roche) with 2 μ g DNA (PCI neo or HA-PBF) per well. 24 hours post-transfection, protein was harvested as described in section 2.2.6.

7.2.3.2 Immunoprecipitation and Western blotting

5 μ l of rabbit polyclonal anti-PBF antibody (Smith et al. 2009) was added to cell lysate fractions and left rotating for 16 hours at 4°C before 5, 10, 20, 40 or 100 μ l of goat anti-rabbit serum (a kind gift from Dr Margaret Eggo, University of Birmingham) were added. Reactions were left for 72 hours at 4°C to allow immunocomplexes to form.

Immunocomplexes were pelleted by centrifugation at 13,000 g for 15 minutes and washed in 100 μ l of PBS containing 1 % Triton X-100. Immunocomplexes were resuspended in Laemmli sample buffer (Bio-Rad) containing 43 mg/ml DL-Dithiotheriol (DTT), before being incubated at 37°C for 30 minutes. Immunocomplexes were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described in section 2.2.7, to confirm that PBF protein had been pulled down.

The membrane was probed with primary antibody, 1 μ g/ml mouse monoclonal anti-HA.11 antibody (Covance Research Products), in 5 % non-fat milk in TBS-T at 4°C for 16 hours and secondary antibody, 650 ng/ml HRP-conjugated polyclonal rabbit anti-mouse immunoglobulin (Dako), in 5 % non-fat milk in TBS-T at room temperature for 1 hour.

7.2.4 Detection of PBF secretion by immunoprecipitation

7.2.4.1 Transient transfection and radiolabelling of newly synthesised PBF

Cells were seeded into 6 well plates at a density of 8×10^4 cells per well. Twenty-four hours later, transient transfections were performed using 6 μ l FUGENE 6 (Roche) with 2 μ g DNA per well. After a further twenty-four hours, cells were maintained with 1 ml of medium containing 2/3 standard medium and 1/3 leucine free equivalent, along with 10 μ Ci L-leucine [3,4,5- 3 H] (MP Biomedicals).

7.2.4.2 Harvesting cell medium and cell lysate

To obtain the cell medium fraction, 24 hours after the radioactive medium had been added it was removed from the cells and centrifuged at 13,000 g for 15 minutes to remove any floating cells. To obtain cell lysate fractions, protein was harvested as in section 2.2.6.

7.2.4.3 Immunoprecipitation

5 μ l of rabbit polyclonal anti-PBF antibody (Smith et al. 2009) or 5 μ l normal rabbit control serum (4 μ g/ μ l. A kind gift from Dr Margaret Eggo, University of Birmingham) were added to the cell medium and lysate fractions and left for 16 hours at 4 $^{\circ}$ C rotating, before 20 μ l of goat anti-rabbit serum was added. Reactions were left for 72 hours at 4 $^{\circ}$ C to allow immunocomplexes to form.

Immunocomplexes were pelleted by centrifugation at 13,000g for 15 minutes. Pellets were washed in 100 μ l of PBS containing 1 % Triton X-100, before being

resuspended in 200 μ l 0.1 M NaOH and 2 ml of ScintiSafe 3 (Fisher Scientific). The disintegrations per minute (dpm) were then measured using a 2500-TR liquid scintillation analyzer (Packard). Allowing the percentage of total PBF secreted into the medium to be calculated.

7.2.5 Statistical analysis

Data were analysed using Sigma Stat (SPSS Science Software UK Ltd). The Kolmogorov-Smirnov test determined whether data followed a normal distribution. If the data set to be tested followed a normal distribution, it was analysed using a two sample Student's t-test. However, if the data set was non-parametric, the Mann Whitney Rank Sum test was used for comparison between two groups of data. To compare more than two groups of data the one way analysis of variance test was employed if the data followed normal distribution. The Kruskal Wallis test was used for non-parametric data.

7.3 Results

7.3.1 PBF is a secreted protein, as detected by Western blot analysis

To determine if PBF is a secreted protein, MCF-7 cells were transfected with HA tagged wild type or Mutant PBF (see Section 6.2.1).

48 hours post-transfection, both cell lysate and medium were harvested and subjected to Western blotting (Figure 7-1). Wild type PBF is a putative glycoprotein (Yaspo et al. 1998), which occasionally runs as a doublet between approximately 25 and 37 kDa. In previous chapters only one PBF band at around 25 kDa was detected as the PBF antibody apposed to an antibody for the HA tag was used. PBF Mutant 1 and Mutant

2 was, as anticipated, smaller, and ran at approximately 25 and 20 kDa respectively. Wild type PBF, Mutants 1 and Mutant 2 were successfully detected in the whole cell lysate. Wild type PBF and Mutant 1 were also detected in the cell medium, suggesting they are secreted proteins. Mutant 2, however, was not apparent in the cell medium. These data support our earlier hypothesis that a complete, functional signal peptide and/or glycosylation of PBF are necessary for its secretion. However we are not able to rule out the possibility that the PSI domain is also important for secretion, as this has also been mutated.

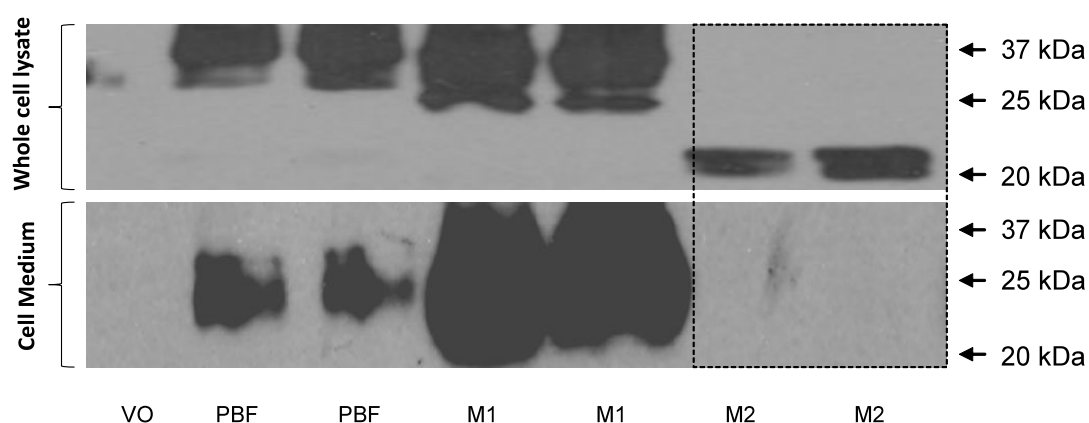


Figure 7-1: Western blot of cell medium and whole cell lysate extracted from MCF-7 cells. Membranes were probed with a mouse monoclonal anti-HA.11 antibody, therefore endogenous PBF was not detected. The Western blot of the whole cell lysate shows that all transfections were successful. M1 = Mutant 1, M2 = Mutant 2, PBF = wild type PBF.

7.3.2 PBF is a secreted protein, as detected by immunoprecipitation

7.3.2.1 Validation of PBF immunoprecipitation

To investigate our initial findings that PBF maybe a secreted protein, a second method to detect secretion was employed. Cell lysate was obtained from MCF-7 cells which had been transfected with wild type HA tagged PBF. PBF was then

immunoprecipitated from the lysate by addition of 5 μ l of rabbit polyclonal anti-PBF antibody and 5, 10, 20, 40 or 100 μ l of goat anti-rabbit serum. To confirm that PBF had been precipitated, immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis, and membranes probed with mouse monoclonal anti-HA.11. Western blots show that PBF was specifically pulled down from the cell lysate (Figure 7-2). For subsequent studies, 20 μ l of goat anti-rabbit serum was chosen as the optimal dose to pull down PBF.

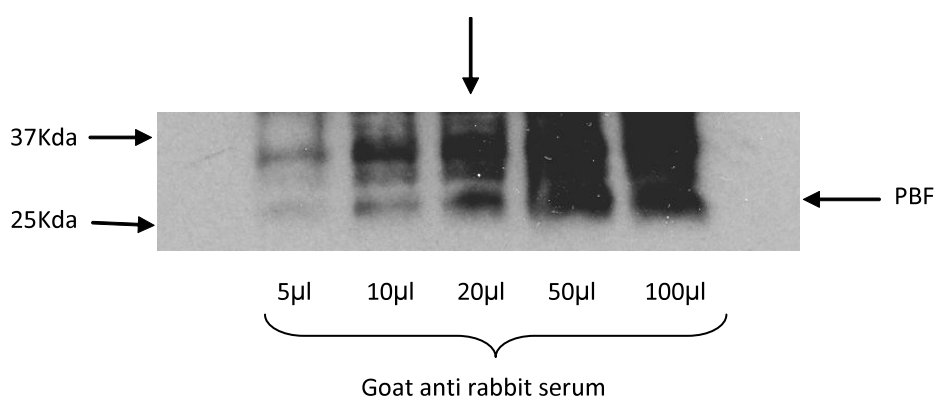
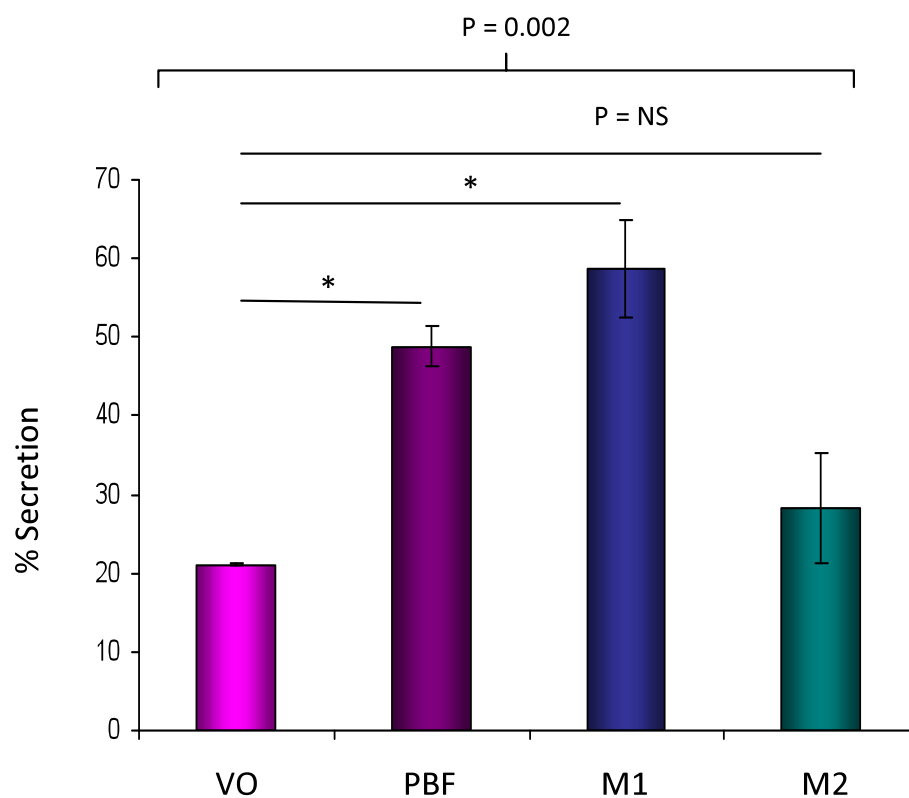


Figure 7-2: Western blot of HA tagged PBF after it had been immunoprecipitated from cell lysates obtained from HA-PBF transfected MCF-7 cells. Different volumes of goat anti-rabbit serum were tested to determine the optimal quantity required to form immunocomplexes with the anti-PBF antibody (\downarrow arrow).

7.3.2.2 Detection of PBF secretion by immunoprecipitation

To investigate whether PBF secretion could be detected by immunoprecipitation MCF-7 cells were transfected with wild type PBF, Mutant 1 or Mutant 2, and newly synthesised proteins were radiolabelled with L-leucine [3 H]. PBF was then precipitated from both cell lysate and cell medium using a rabbit polyclonal anti-PBF antibody and the dpm of each fraction was measured. This allowed the percentage of total PBF secreted into the medium to be calculated.

The results confirmed our initial Western blot findings that PBF is a secreted protein. Overexpression of wild type PBF was associated with an increase in secretion (29 ± 2.6 percent secretion compared to VO, $N=3$, $p<0.05$). Mutant 1 was also secreted (39 ± 6.2 percent secretion compared to VO, $N=3$, $p<0.05$). By contrast Mutant 2 was not significantly secreted (9 ± 7.0 percent compared to VO, $p=$ non significant) (Figure 7-3). As an anti-PBF antibody was employed in VO treatments, these data show that approximately 20% of endogenous PBF is secreted (Figure 7-3).



*Figure 7-3: The percentage of total PBF secreted into the medium extracted from MCF-7 cells. Newly synthesised PBF was radiolabelled with L-leucine [3,4,5-³H] and pulled down from the cell medium and whole cell lysate using a rabbit polyclonal anti-PBF antibody. Disintegrations per minute (dpm) were measured on a scintillation counter allowing the percentage of total PBF secreted into the medium to be calculated (N=3, * $P < 0.05$). Statistics were calculated using one-way Anova which gave an overall significance of $P = 0.002$.*

7.4 Discussion

All proteins that enter the secretory pathway contain an endoplasmic reticulum signal sequence at the N-terminus. It is the interaction of the signal peptide with signal recognition particle on the ribosome which assures that secretory proteins are synthesised on the rough endoplasmic reticulum. Secretory proteins are translocated into the lumen of the endoplasmic reticulum as synthesis proceeds (Halban and Irminger 1994). Newly made proteins in the endoplasmic reticulum lumen or membrane are incorporated into transport vesicles, which either fuse with the cis-Golgi or with each other to form the cis-Golgi reticulum (Harvey Lodish et al. 1999). Proteins destined to be secreted move by cisternal migration to the trans-Golgi reticulum. From the trans-Golgi network a secretory protein is sorted into one of two types of vesicles. Proteins which are secreted constitutively are sorted in the trans-Golgi network into transport vesicles that immediately move and fuse with the plasma membrane, releasing their contents by exocytosis (Harvey Lodish et al. 1999). If a protein is not continuously secreted it is sorted in the trans-golgi network into secretory vesicles that are stored inside the cell awaiting a stimulus for exocytosis (Harvey Lodish et al. 1999). Secreted proteins undergo various modifications as they mature in the secretory pathway and the majority of secreted proteins are glycosylated (Halban and Irminger 1994) (Figure 7-4). PBF contains

an N-terminal signal peptide between amino acids 1-32 and two putative glycosylation sites at amino acids 45 and 54, suggesting that it could be a secreted protein.



Figure 7-4. The secretory pathway of protein synthesis and sorting. Taken from (Harvey Lodish et al. 1999).

In Chapter 6, overexpression of wild type PBF increased the invasiveness of MCF-7 cells, an effect which was abrogated when PBF was mutated so that it lacked either the C-terminal 30 amino acids (Mutant 1) or those found between residues 29-93 (Mutant 2). As Mutant 2 lacked a full signal peptide and did not contain the 2 potential glycosylation sites at amino acids 45 and 54, it was hypothesised that this may prevent the putative secretion of PBF, thereby preventing PBF from being able to induce invasion. To test this hypothesis we firstly needed to address the question whether PBF was a secreted protein.

Two independent experiments were conducted which showed that wild type PBF when overexpressed by transient transfection was secreted into the medium collected from MCF-7 cells. In addition, the second set of experiments showed that approximately 20 % of endogenous PBF is secreted. To determine if PBF is constitutively secreted or if its secretion is regulated, time course experiments would need to be conducted. If PBF was detected in the medium at all time points this would suggest that it is constitutively secreted. Interestingly, in this Chapter PBF ran as a doublet between approximately 25 and 37 kDA, whereas in previous chapters PBF generally ran as a single band. As PBF contains two potential glycosylation sites at amino acids 45 and 54, the higher molecular weight band may represent a glycosylated form of PBF. To determine what each of the PBF bands represent, work employing glycosylation inhibitors is currently being undertaken by our group.

In the same set of investigations we also concluded that PBF Mutant 2 was not secreted. This backs up the hypothesis that a complete functional signal peptide and/or glycosylation at amino acids 45 and 54 are critical for PBF secretion. However as the PSI domain of PBF is also deleted in Mutant 2, we cannot rule out the possibility that this could influence secretion. To answer this question, additional PBF mutants with

specifically abrogated glycosylation sites and/or the signal peptide removed would need to be created.

We have also shown that PBF Mutant 1 is secreted, suggesting that the C-terminal 30 amino acids containing the tyrosine based sorting signal and the NLS do not carry out functions of relevance to the secretion of PBF.

As neither Mutant 1 nor 2 were capable of significantly promoting the invasive capacity of MCF-7 cells, and because Mutant 1 is secreted whilst Mutant 2 is not, secretion alone cannot be responsible for PBF being able to induce invasion. Against this, it is possible that following secretion Mutant 1 is non-functional. This is entirely feasible as for a protein to be active it must retain its correct localisation as well as function. Unfortunately the exact mechanisms by which PBF induces invasion are beyond the scope of this thesis. To investigate such processes further, additional and more specific PBF mutants would need to be created and further invasion and secretion assays carried out in the presence and absence of oestrogen and PBF siRNA.

Chapter 8

Final conclusions and future studies

The work described within this thesis began by investigating the effects of oestrogen and progesterone on the expression of the three functionally related genes PBF, PTTG and separase. It was founded on the hypothesis that oestrogen and progesterone given to postmenopausal women in the form of HRT increases their risk of developing breast cancer partly by upregulating PBF, PTTG and separase expression, which in turn increase mitosis and cause the accumulation of genetic mistakes. In addition, as distant metastasis is the primary cause of death in breast cancer patients, and because an important step in the metastatic process is the invasion of tumour cells into surrounding host tissue (Mangala et al. 2007), it was hypothesised that by upregulating these genes, oestrogen and progesterone might increase the invasiveness of breast cells.

8.1 The PBF promoter is regulated by oestrogen

Previous studies have demonstrated that PTTG and separase are regulated by oestrogen (Heaney et al. 2002; Pati et al. 2004). PBF is a relatively uncharacterised protein which has been implicated in endocrine neoplasms (Stratford et al. 2005), and after studying its promoter I found it to harbour putative consensus EREs and PREs. One of the initial aims of this study therefore was to determine if PTTG, PBF and separase were upregulated in ER α and progesterone receptor positive breast cancer cells following treatment with oestrogen and progesterone. Oestrogen and progesterone treatment were investigated alone and in combination as breast cancer risk is larger for current users of combined oestrogen/ progesterone preparations than for oestrogen only preparations (Cuzick 2008). Despite the fact that PTTG mRNA expression in pituitary GH₃ cells has been shown to be upregulated by oestrogen (Heaney et al. 2002) PTTG mRNA was not regulated by oestrogen or progesterone in MCF-7 cells. In keeping with a previous report

(Pati et al. 2004), PTTG protein expression levels were regulated by oestrogen alone or in combination with progesterone. As the same oestrogen was employed (diethylstilbestrol), the differences observed are most likely due to the different cell types used. The upregulation of PTTG protein expression without significant mRNA changes could conceivably be due to post-translational effects, leading to stabilisation of the protein. However, such mechanisms were not the focus of the current investigation. As PTTG degradation is regulated by ubiquitination, further investigations could focus on treating MCF-7 cells with ubiquitination inhibitors before exposure to hormones; if the hypothesis that oestrogen represses the ubiquitination of PTTG is correct, this would show the same levels of PTTG expression in all treatments.

One previous study indicated that separase mRNA is upregulated in MCF-7 cells in response to the combined treatment of oestrogen and progesterone (Pati et al. 2004). I found that separase mRNA and protein were not regulated by oestrogen or progesterone in the same cell type. As the precise oestrogens and progestones used were not reported the difference in our findings may be because different hormonal compounds were employed. However, our experiments were carried out multiple times with thorough controls, and we were unable to replicate the findings of Pati et al (2004).

In contrast to PTTG and separase, PBF mRNA and protein expression levels were upregulated in response to oestrogen and progesterone. The data suggested that the PBF promoter may be directly regulated by oestrogen and progesterone, although the most significant effects on PBF mRNA and protein expression levels were observed after treatment of MCF-7 cells with oestrogen alone. One caveat to these experiments is that although I investigated diethylstilbestrol, I did not examine the effects of 17β -estradiol in combination with progesterone on PBF mRNA and protein expression. The reason for this

is that the effects of 17β -estradiol were examined retrospectively, after it was realised that it would be important to investigate the actions of a naturally occurring oestrogen in addition to the synthetic diethylstilbestrol. Subsequently, given the larger magnitude of effect, we concentrated predominantly on the actions of oestrogen rather than progesterone.

Parallel work employed luciferase assays to determine if the PBF promoter is regulated by oestrogen. Whilst cloning 1 kb of the PBF promoter, an 18 bp repeat region (gccccctccGGTCAcgcctc), which varied in length between individuals in the population and contained a putative consensus ERE-half site (GGTCA), was identified. This prompted us to also investigate if this region in isolation was oestrogen responsive. To this end, once 1kb of the PBF promoter upstream of the transcriptional start site was cloned into the pGL3 basic vector (pGL3basic_PBFpromoter), the region between -1181 and -510 relative to the translational start site was deleted, (pGL3basic_PBFshort_promoter), thus leaving behind only the EREs between bases -510 to -211, which contains the 18 bp hypervariable region. Dual luciferase assays using the pGL3basic_PBFpromoter construct indicated that the first 1 kb of the PBF promoter upstream of the transcriptional start site was not responsive to oestrogen or progesterone treatment. However, when the pGL3basic_PBFshort_promoter construct was employed, the 18 bp repeat region which containing the ERE-half sites (GGTCA) was activated by oestrogen treatment. The effects of progesterone were not investigated with this construct as by deleting the region between -1181 and -510 relative to the translational start site we removed the consensus PRE. When the pGL3basic_PBFpromoter construct was used, very high constitutive readings were recorded on the Luminometer even in the absence of hormones. As the pGL3 basic vector lacks eukaryotic promoter and enhancer sequences, the expression of luciferase activity in cells transfected with this plasmid depends on the

insertion and proper orientation of a functional promoter upstream of the luciferase gene, suggesting that the first 1 kb of the PBF promoter upstream of the transcriptional start site is very active. This is not surprising given the high number of regulatory sequences (ERE, PRE, SP1 and AP1 binding sites) found within this region of the PBF promoter (Figure 2-1), and given that PBF appears to be an abundant protein (Smith et al. 2009). The presence of these multiple sites may have been masking any potential oestrogen effect on the 18 bp repeat region, giving an explanation into the differences observed between the two constructs. Although I have shown that the region of the PBF promoter between -510 and -211 is responsive to oestrogen promoter assays are artificial systems and in these studies I have isolated a tiny region of a large promoter. Further studies such as chromatin immunoprecipitation (CHIP) and electrophoretic mobility shift assays (EMSAs) should be employed to examine whether oestrogen binds these EREs.

As the activity of the short promoter region was induced 1.3 to 1.8-fold, and PBF mRNA was induced 1.5 to 3-fold at identical timepoints and at identical doses of oestrogen, these data indicate that the short promoter region -510 to -211 confers most, but not all, of PBFs responsiveness to DES and EST. These promoter assays have therefore uncovered some of the functional EREs in the PBF promoter, but others which lie further upstream in the PBF promoter are likely to be undiscovered. This is because EREs which are located far away from the promoter are capable of interacting with the promoter by a looping mechanism (Sathya et al. 1997). Indeed when the association of the ER with the complete nonrepetitive sequence of human chromosomes 21 and 22 was mapped by combining chromatin immunoprecipitation with tiled microarrays, whilst a few of the ER binding sites were found directly adjacent to ER target genes, most were found at significant distances including several >100 kb away (Carroll et al. 2005). To address the possibility that there are EREs slightly further upstream of the transcriptional start site

I attempted to clone 2, 3 and 4 kb of the PBF promoter into the pGL3 basic vector. However technical difficulties in the PCR amplification of these large fragments prevented me from being able to create the necessary constructs and this line of investigation could not be continued within this time frame.

8.2 The PBF promoter contains an 18 bp repeated region, containing a consensus ERE half site which differs in length between individuals in the population

As I demonstrated that the region of the PBF promoter which lies between -211 and -510 relative to the translational start site is activated by oestrogen, and because the presence of multiple EREs results in synergistic activation of genes (Sathya et al. 1997), this could pre-dispose some people to an increased sensitivity to oestrogen therapy and may relate to tumour behaviour and prognosis. To determine the allele frequencies within the population, the PBF promoter was amplified by PCR and then sequenced. After sequencing 122 DNA samples, the overall allele frequencies were 0.8 % (1 ERE), 77.9 % (3 EREs), 2.5 % (4 EREs), 16.8 % (5 EREs) and 2 % (6 EREs). To determine if the ERE-half sites found within this 18 bp region are capable of working synergistically, and to investigate whether an increased/decreased number of repeats results in the PBF promoter being more or less sensitive to oestrogen, the pGL3basic_PBFshort_promoter construct could be mutated in future experiments so that it contains variable numbers of repeats, and then luciferase assays conducted before and after oestrogen treatment. I attempted to undertake these experiments, but because of the highly repetitive nature of this region of the promoter, mutagenesis experiments consistently failed to work.

During this study, DNA from 3 normal breast tissues and 27 tumour samples were analysed. Normal samples were homozygous for 3 repeats whilst the majority of the tumour samples were heterozygous for 3 and 5 repeats. Additionally, when the repeat frequencies between colorectal normal and tumour samples were compared, there was a statistically significant increase in the number of 5 alleles in the tumours compared to the normals. Together these data suggest that PBF in tumour cells may be regulated by a

higher number of tandemly repeated ERE-half sites, and may be more susceptible to the overexpression of PBF after exposure to oestrogens. To address this hypothesis we would need to correlate the number of ERE-half sites in the PBF promoter to PBF mRNA expression levels within the same tissues, a body of work which will be discussed in section 8.3. It would also be necessary when resources become available to increase the sample size of our normal cohort.

8.3 PBF is overexpressed in breast tumours compared to normals

So far my work has revealed that a small region of the PBF promoter which contains variable number of ERE-half sites is responsive to oestrogen, and that PBF mRNA and protein expression levels are induced in MCF-7 cells following oestrogen treatment. To put this into context with our initial hypothesis that oestrogen/progesterone treatment given to postmenopausal women causes an increased risk of breast cancer because it stimulates the expression of PBF, it was necessary to investigate whether or not PBF is overexpressed in breast tumours compared to normals.

To determine if PBF mRNA and protein was upregulated in breast tumours compared to normals, quantitative real time PCR and immunohistochemistry experiments were carried out on formalin fixed and paraffin embedded tissues sections mounted on glass slides. These studies showed that PBF mRNA and protein was low or absent in normal breast specimens, whereas it was strongly expressed in breast tumour specimens. Although this thesis shows that both PBF mRNA and protein expression levels are upregulated in breast tumour samples compared to normals, N numbers for the normals are low. Ideally we would have liked additional normal breast specimens, however these resources were not available to me.

DNA was also extracted from some of the normal and tumourous breast specimens slides so that the number of ERE-half sites in the PBF promoter could be identified and related back to PBF mRNA and protein expression. Despite a relatively small number of formalin fixed paraffin embedded samples yielding both mRNA and promoter sequencing data, when the 18 bp repeat length data from Chapter 4 and the mRNA expression data from Chapter 5 were compared, 14 % of heterozygous (3/5) and 75 % of homozygotes (3/3) samples had undetectable levels of PBF expression, implying that a greater number of EREs results in higher levels of PBF expression. Low N numbers are attributed to the difficulty in successfully extracting RNA and DNA from the same small fixed section of tissue. Although numbers were too low to draw any real conclusions these observations suggest that larger scale association studies would be a useful future target to determine if this is a predisposing site for instability in the initiation or progression of ER α positive breast cancer. This is because a greater number of EREs in a breast tumour would potentially mean a greater response to circulating oestrogen and hence higher expression of a known transforming gene. When the repeat length data from Chapter 4 and the PBF protein expression data were compared, no significant association was identified. Sequencing a polymorphic region of DNA from FFPE TMAs is technically difficult, which necessarily meant a further reduction in our sample, gene expression measured through immunohistochemistry is semi-quantitative and both of these factors may have hampered our statistical power.

As we had detailed demographic patient information encompassing tumour grade, vascular invasion, lymph node stage, Nottingham Prognostic Index and ER status, this information was correlated to PBF protein staining intensity and percentage expression. A positive association was identified with PBF expression and ER status ($P < 0.001$), whereby 59% of ER positive patients and 18% of ER negative patients had PBF

expression levels above 50%, supporting our data that PBF is upregulated by oestrogen. The intensity of PBF staining and the percentage of PBF expression did not correlate with tumour grade, vascular invasion, lymph node stage or the Nottingham Prognostic Index. Similarly, the intensity of PBF staining did not correlate with ER status. Previously work from our group has demonstrated an association between PBF expression and thyroid tumour recurrence (Stratford et al. 2005), therefore the lack of associations between clinical outcome and PBF expression was surprising. If we had a matched normal: tumour cohort we could determine the fold induction of PBF expression in tumours compared to normals. This would remove the natural variation in PBF expression between individuals in the population and would allow a much more detailed interrogation of the association between promoter polymorphism and clinical outcome.

8.4 PBF is a secreted protein and its overexpression increases the invasiveness of MCF-7 cells

Distant metastasis rather than primary tumour is the main cause of death in patients with breast cancer (Mangala et al. 2007). An important step in the metastatic process is the invasion of tumour cells into surrounding host tissue (Mangala et al. 2007). Our initial hypothesis suggested that by upregulating PBF oestrogen may induce the invasiveness of breast cells leading to the progression from early (localised) to advanced (metastatic) disease. To determine if our hypothesis was correct, BD matrigel based assays were conducted. These data show that overexpression of PBF achieved by transient transfections and oestrogen treatment induced the invasiveness of MCF-7 cells. As the actions of oestrogen did not necessarily have to be working via the upregulation of PBF, we used two siRNA constructs simultaneously with oestrogen treatment to knock down PBF protein expression. Given that the increased invasive capacity of MCF-7 cells

observed after oestrogen treatment was abolished when MCF-7 cells were simultaneously incubated with PBF specific siRNAs compared to the scrambled control, these experiments demonstrate that oestrogen induction of MCF-7 cellular invasion was mediated through PBF. PBF could therefore serve as a therapeutical target to prevent oestrogen from stimulating breast cells to invade, reducing the progression of early breast cancer (localised) to advanced (metastatic) disease.

To determine if the increase in invasion observed after the overexpression of PBF was simply due to an increase in cellular proliferation, MTT assays were conducted. These data show that PBF overexpression marginally (10%) increased the proliferation of MCF-7 cells. As PBF overexpression by transient transfection stimulated invasion by at least 2.5 fold, proliferative changes could not solely account for the increase in invasion observed. Similarly, oestrogen treatment of MCF-7 cells did not induce their proliferation, so could not be responsible for the increase in invasion observed after oestrogen treatment.

To try and elucidate the mechanism by which PBF was causing an increase in invasion, two PBF mutants were created. Mutant 1 consisted of amino acids 1-149 only and hence lacked the NLS and the tyrosine based sorting signal. Mutant 2 had a deletion of amino acids 29-93 and therefore lacked a complete signal peptide, the two putative glycosylation sites at amino acids 45 and 54, and a PSI domain. Both of these mutants lost the ability to induce invasion, suggesting that the NLS, the tyrosine based sorting signal, the signal peptide, the glycosylation sites and the PSI domain are important regions either alone or in combination that are required for PBF to induce invasion. To elucidate the exact regions of PBF which are important in inducing invasion more precise mutations of PBF would need to be created. Overall these experiments have demonstrated that PBF

induces invasion through Matrigel, an action that could be abrogated both by siRNA treatment and specific mutation, indicating that PBF has a specific effect.

Secreted proteins such as cytosine protease, cathepsin B and matrix metalloproteinase (MMP)-13 are responsible for the degradation of the extracellular matrix (ECM) (Sameni et al. 2008). Degradation of the ECM is a process which is necessary for tumour cells to invade the surrounding tissue, intravasate (enter) and extravasate (leave) blood vessels, and build new metastatic formations (Kohrmann et al. 2009). Proteins which are secreted from the cell have to enter the secretory pathway, and in order to do this they must be endowed with a signal peptide (von Heijne 1990). Glycosylation is also an important pre-requisite for secreted proteins, as in the majority of cases elimination of all putative N-glycosylation sites of an enzyme results in significantly reduced protein secretion levels (Skropeta 2009). As the signal peptide in Mutant 2 has been truncated, and because it lacks the two putative N-glycosylation sites at position 45 and 54, it may no longer be secreted. This could provide a mechanism by which Mutant 2 loses its ability to induce invasion. To address this theory we had to initially determine whether PBF was a secreted protein.

Two independent experiments were conducted which showed that wild type PBF when overexpressed by transient transfection was secreted into the medium collected from MCF-7 cells. In addition, the second set of experiments which used an antibody which could detect endogenous PBF secretion showed that approximately 20 % of endogenous PBF is secreted over a 24 hour time point. When the secretion of Mutants 2 and 3 was also investigated, it was found that whilst Mutant 2 was not secreted Mutant 1 was. This suggests that secretion does not in fact play a role in the mechanism which allows PBF to induce invasion, as we would expect Mutant 1 to be able to induce invasion

and this does not happen. It is feasible however that by removing the N-terminal 30 amino acids in Mutant 1 we have rendered the protein inactive once it has been secreted.

In summary, we have shown that PBF is a secreted protein which can induce the invasiveness of MCF-7 cells. However the exact mechanisms by which PBF is capable of inducing invasion are beyond this thesis. To elucidate such mechanism, more precise mutations of PBF would need to be created and further invasion assays carried out in the presence and absence of oestrogen and PBF siRNA.

8.5 Mechanisms of oestrogen induced transformation of breast tissue

The work described in this thesis has demonstrated that oestrogen can modulate the activity of the PBF promoter, with oestrogen consequently inducing both its mRNA and protein expression levels. The region of the PBF promoter which showed oestrogen responsiveness contains an 18 bp repeated sequence housing a putative consensus ERE half-site which varies in length between individuals in the population. I have shown that individuals may be homozygous or heterozygous for between 1 and 6 repeats, with the most common repeat length being 3. More than three quarters of breast tumours are oestrogen responsive (Benson et al. 2009) and I have shown that PBF protein and mRNA expression levels are increased in breast tumours compared to normals. Further, higher PBF mRNA expression levels correlate with an increasing number of ERE half-sites in the PBF promoter. The precise mechanism by which PBF may induce tumourigenesis is yet to be determined. However I have established that PBF is a secreted protein and that its overexpression increases the invasiveness of MCF-7 breast cancer cells. Oestrogen induction of MCF-7 cell invasion was abrogated when PBF was knocked down through siRNA implying that its actions are mediated through the overexpression of PBF. As the

invasion of tumour cells into surrounding host tissue is an important step in the metastatic process, targeting PBF overexpression in women exposed to hormones may serve as therapeutic strategy to overcome metastatic disease which at present is still incurable (Cufer 2007). Additionally, as I have shown that PBF is overexpressed in breast tumours compared to normals, and that PBF is capable of inducing invasion of breast cells, it may be worthwhile screening women for PBF expression and targeting it therapeutically in those patients with raised levels. This may prevent the progression from early (localised) disease to its metastatic counterpart. As PBF is a secreted protein this screening process could be made easy by detecting levels in the blood. However additional experiments would need to be conducted to investigate if PBF reaches the blood stream. This could be investigated by developing a sandwich-enzyme linked immunosorbent assay (ELISA).

Throughout this thesis all experiments have been conducted in MCF-7 breast cancer cells. To further this work I would like to investigate the regulation of PBF by oestrogen as well as its secretion and induction of invasion in additional breast cell lines and primary cells. I have tried to carry out some of this work in MCF-12A cells a non-tumourigenic cell line established from tissue taken at a reduction mammoplasty, and MDA-MB-231 human Caucasian breast adenocarcinoma cells isolated from pleural effusions of a breast cancer patient. As both MCF-12A and MDA-MB-231 cells are ER negative I had to co-transfect PBF with the ER α cDNA a process which was problematic as transfection efficiency was relatively low in both cases. However there are other ER positive breast cancer cell lines available such as T47Ds which could be employed in future studies. Although desirable, primary culture of breast cells may prove difficult because of the complexities involved in obtaining breast tissue. However, the work presented in this thesis suggests that PBF is a gene which merits further study in the context of breast cancer.

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Publications relating to this thesis

Vicki E. Smith, Martin L. Read, Andrew J. turnell, **Rachel J. Watkins**, John C. Watkinson, Gred D. Lewy, Jim C.W. Fong, Sally R. James, Margeret C. Eggo, Kristein Boelaert, Jayne A. Franklyn and Christopher J. McCabe. (2009) A novel mechanism of sodium iodide symporter repression in differentiated thyroid cancer. *Journal of Cell Science* **122** (pt18) 3393-402

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