Mechanisms of TNFα action in post-menopausal breast cancer

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ERRATA

P10 figure legend last line: "active" for "detected"

P13 para 2, line 12: "our" for "out"

P14 para 3, line 9: swap "expression" and "aromatase" and read "More detailed analysis of the precise role for these promoter elements in regulating aromatase expression…"

P19 para 3, line 3: insert "a" and "b" in to citations and read "Tian et al. 2005a; Tian et al. 2005b"

P20 para 2, line 10: delete "alpha"

P23 para 2, line 8: insert "a" and "b" in to citations and read "Utsumi et al. 2005a; Utsumi et al. 2005b"

P26 para 3, last line: inset "2011" in to citation and read "Zaldivar et al. 2011"

P102 para 2, line 1: "The source of the excess TNFα" for "The source to the excess TNFα"

P105 para 2, line 6: ""cells" for "cell"

P105 para 3, line 4: "an" for "a"

P108 para 1, line 5: "estrogen" for estrogens"

P119: Remove additional 0 in Supplementary Figure 1A

P180 header: "Appendix 6" for "Appendix 7"

ADDENDUM

P 13 line 4: insert "Prostaglandin E2" and read "Prostaglandin E2 (PGE2)

P15 line 10: citations should read "Zhao et al. 1995a; Zhao et al. 1995b"

P20 para 2, line 14: delete "Prostaglandin E2" and replace with PGE2

P20: Add at the end of section 1.7.2

1.7.3 EGR transcription factors

The Early Growth Response (Egr) genes comprise of four family members: *Egr1* (Sukhatme et al. 1987), *Egr2* (Joseph et al. 1988), *Egr3* (Patwardhan et al. 1991) and *Egr4* (Crosby et al. 1991). These early growth response genes encode for a family of transcription factors with highly conserved DNA-binding domains, comprised of three zinc-finger motifs (O'Donovan et al. 1999). Each family member recognises the same CG-rich DNA consensus sequence, although Egr4 has been shown to have a reduced affinity for this site (Swirnoff and Milbrandt 1995). In binding to response elements within their target gene promoters they are able to modulate transcription levels, although the precise mechanisms by which this occurs is poorly understood.

The best characterised of the Egr transcription factors is Egr1, the first member of the family to be cloned in 1987 (Sukhatme et al. 1987). Egr1 is known to regulate the transcription of a wide variety of genes, including *peroxisome proliferator-activated receptor gamma 1* (Fu et al. 2002), *insulin-like growth factor 2* (Bae et al. 1999), *transforming growth factor β-1* (Liu et al. 1996), *tumour necrosis factor α* (Kramer et al. 1994) and *Egr1* itself (Cao et al. 1993). The ability of Egr1 to activate transcription is governed by its phosphorylation state, as only via its phosphorylation by casein kinase II may it translocate into the nucleus to mediate gene expression (Jain et al. 1996). The application of phosphatase inhibitors to fibroblast cells induces sustained gene transcription and hyperphosphorylation of Egr1, highlighting the importance of phosphorylation in its regulatory processes (Cao et al. 1992). At the mRNA level, Egr1 transcription is regulated via response elements in its promoter, which contains putative binding sites for Sp1, NFkβ and cAMP as well as a number of serum response elements (Thiel and Cibelli 2002). The serum responsive elements have been shown to promote Egr1 expression, whereas the cAMP site mediates its down regulation in synovial fibroblasts of rheumatoid arthritis patients (Aicher et al. 1999a). Deletion of the NFkB responsive element in 3T3-L1 mouse adipose fibroblasts resulted in increased Egr1 expression, suggesting that Egr1 is a downstream target of NFkB, and that its binding confers negative regulation (Aicher et al. 1999b).

Egr1 has been found to be essential in differentiation of macrophages (Nguyen et al. 1993) and T-lymphocyte proliferation (Perez-Castillo et al. 1993) *in vitro*, reflecting its essential role in the immune system. Additionally, Egr1 plays a role in

neural biology, specifically in regulating processes related to neuronal plasticity (Brinton et al. 1998). The generation of an Egr1 knockout mouse model (Lee et al. 1995) contradicted earlier findings in that growth and differentiation could proceed normally in these animals, who were phenotypically normal. Their immune system and brain architecture were not affected by the absence of Egr1, despite a high expression of Egr1 in these areas during the development of normal wild-type mice. The authors hypothesised that the high degree of conservation between members of the Egr family as well as their co-ordinate regulation could mean Egr1 can be functionally compensated for by its family members. In fact, functional compensation within the Egr family has been demonstrated in leydig cells, whereby Egr4 is able to compensate for Egr1 in the steroidogenesis pathway in Egr1 null mice (Tourtellotte et al. 2000). Conversely, opposing effects between Egr family members has also been shown. Whilst Egr1 has an anti-adipogenic action during adipocyte differentiation (Boyle et al. 2009), Egr2 is known to promote this process (Chen et al. 2005). The functional role of Egr family members and the relationship between them requires further elucidation.

P28 para 3, line 12: delete "been" and read "It has recently been shown that..."

P42: Insert at start of section 4.1.4 "Cell lines were obtained from the American Type Culture Collection"

P106 para 1, line 6: Delete "(Figure 5)"

P123 para 1, line 12: "suggests" for "demonstrates"

References: add the following references in the appropriate spaces

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General Declaration

Monash University: Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes [2] original papers published in peer reviewed journals and [1] unpublished publications. The core theme of the thesis is exploring the roles and regulation of TNFα in post-menopausal breast cancer. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within Prince Henry's Institute and the Department of Biochemistry and Molecular Biology under the supervision of Dr. Colin Clyne and Dr. Kevin Knower.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 3, Chapter 4 and Chapter 5, my contribution to the work involved the following:

Thesis chapter	**Publication title**	**Publication status***	**Nature and extent of candidate's contribution**
3	Involvement of the Early Growth Response genes in TNFα induced aromatase expression in breast adipose	Published online first: Breast Cancer Research and Treatment, 22nd Jan 2013	80% Completed all experimental data, wrote manuscript
4	NFκB and MAPK signalling pathways mediate TNFα-induced Early Growth Response gene transcription leading to aromatase expression	Accepted in Biochemical and Biophysical Research communications, 10th Feb 2013	80% Completed all experimental data, wrote manuscript
5	Estradiol increases production of TNFα from ER+ breast tumour epithelial cells	Submitted to Tumor Biology, Feb 2013	80% Completed all experimental data, wrote manuscript

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Literature Review

Chapter 3: Transcriptional regulation of PI.4 by TNFα

Chapter 4: TNFα signalling in BAFs

Chapter 5: Regulation of TNFα in the ER+ breast tumour microenvironment

Chapter 6: General discussion, future directions and conclusion

LIST OF TABLES

Chapter 1: Literature Review

Appendices

ABSTRACT

Approximately one in nine women in Australia are diagnosed with breast cancer throughout their lifetime. Major risk factors, including increased age and adiposity, coincide with prolonged exposure to circulating oestrogens in the body. As a consequence, two thirds of post-menopausal women with breast cancer will have an oestrogen receptor positive (ER+) tumour, responsive to oestrogen for growth and proliferative advantage. Aromatase is the key enzyme responsible for conversion of androgens to oestrogens, and to date, inhibition of oestrogen production via aromatase inhibitors are the frontline treatment in targetting post-menopausal breast cancers. Despite this, inhibition of aromatase throughout the body results in adverse side effects.

Understanding the transcriptional control of the *CYP19A1* gene that encodes aromatase is therefore a critical avenue to the development of breast specific targeted therapeutics. *CYP19A1* has a number of upstream promoters that drive transcription in a tissue-specific manner to restrict expression to oestrogen-producing tissues. Serum levels of the inflammatory factor Tumour Necrosis Factor-α (TNFα) also increases with age and adiposity and is closely correlated to breast cancer risk. TNFα is one major regulator of the breast adipose-specific promoter I.4 (PI.4). However, the signalling mechanisms that drive TNFα production in breast cancer, and the pathways by which it stimulates aromatase expression, are not well understood. The overall aim of this thesis was to determine how TNFα acts upon breast adipose fibroblasts (BAFs) to stimulate oestrogen production, and how in turn TNFα is regulated in tumour epithelial cells.

Using primary human BAFs as a model, this thesis demonstrates that TNFα stimulates expression of the Early Growth Response (EGR) transcription factors via activation of the NFκB and MAPK signalling pathways. These transcription factors then stimulate activity of PI.4, via a defined short distal promoter region. However, this does not appear to be the result of a direct interaction between EGR factors and promoter I.4; rather, intermediate factors induced by the EGRs appear to bind to the aromatase promoter to mediate transcription. Increased PI.4 activity then leads to increased aromatase activity and therefore oestrogen production from BAFs surrounding an ER+ breast tumour. Studies on a panel of breast cancer cell lines show that this oestrogen may then stimulate TNFα expression and secretion from ER+ breast cancer cells. Thus, a positive feedback loop is established that maintains TNFα

production and ultimately drives oestrogen signalling to promote further ER+ breast cancer growth.

Excess local production of oestrogen within a breast tumour microenvironment is the major driver or ER+ tumour development, and identifying the factors and mechanisms which contribute to oestrogen biosynthesis is critical to restricting this process. The data presented in this thesis establishes TNFα as a critical, tumour-derived signalling factor which drives oestrogen biosynthesis in BAFs surrounding the tumour. These findings enhance our understanding of how currently used drug-based treatments inhibit breast cancer growth, and potentially open up avenues for novel ER+ breast cancer therapeutics based on the action of TNFα.

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I would like to say a huge thank you to my supervisors Dr. Colin Clyne and Dr Kevin Knower for all their guidance over the years, from the start of honours and for five difficult but very rewarding years. Thank you for pushing me towards my potential and encouraging me to keep going when everything in the lab was invariably failing. Also, thanks for allowing me to jet off on so many overseas trips throughout the course of my candidature, I promise I did some work in between all the sight-seeing!

Thank you to all the members of the Cancer Drug Discovery laboratory for both helping me out with the little things, giving me plenty of inspiration and for the many gossip sessions that kept lab work interesting. Also thank you to the Metabolism and Cancer lab members for lending a hand over the years. To the fellow students, we've had a lot of fun times and great social functions throughout the time. All this has contributed greatly for keeping my head screwed on straight. To all the students I've shared an office with, thanks for the little chats that broke up the work and put everything in to a little perspective.

I would have gone crazy were it not for keeping up my many hobbies: trumpet playing, netball and basketball. So a big thanks for my friends in the Phil (especially the conductors for putting so much hard work in to making every season fun and successful), my netball teams (the mighty Screaming Fireflies – four times runners up and eventual champions in 2012 – and the Musicians not Netballers) and my basketball team (I just started playing but I'm loving it). The fun times and challenges these bring a smile to my face all the time and hope it can continue for years to come. GO FLIES!!!!!! A shout out to all the stats crew at the Australian Open, watching tennis is the best summer job ever.

Thank you to Ivan for no reason at all.

Thanks to my family for all the support over the years, and for putting up with the thesis-itis. And finally a big thanks to my most awesomest Mark. We started out just as I was starting my PhD, and you have been one step ahead to guide me all the way as we tackled the highs and lows of a postgraduate research degree together. I loved being able to celebrate every little achievement with you, and taking you out to a fancy dinner with poster prize money. Being with you makes me forget about all the stresses of work, and you always make me laugh (even though its mostly lame jokes and constant teasing). I can't wait to see where we go next, but I know we'll dominate at it!

Publications and abstracts arising from this thesis

Peer-reviewed journal articles

To SQ, Knower KC and Clyne CD (2013). "NFκB and MAPK signalling pathways mediate TNFα-induced Early Growth Response gene transcription leading to aromatase expression". *Biochemical and Biophysical Research Communications*, manuscript in press (accepted 10th Feb 2013)

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To SQ, Simpson ER, Knower KC and Clyne CD (2010) "The role of TNFα in oestrogen biosynthesis and breast cancer" 53rd Australian Endocrine Society Meeting, Sydney, Australia. Invited presentation

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To SQ, Takagi K, Miki Y, Suzuki K, Abe E, Yang Y, Sasano H, Simpson ER, Knower KC and Clyne CD (2011), "Characterisation of EP2 prostanoid receptor methylation in post-menopausal breast cancer" Southern Health Research Week, Monash Medical Centre. Poster presentation

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ABBREVIATIONS

5aza	5-aza-2'-deoxycytidine
AIs	Aromatase Inhibitors
AP1	Activating Protein 1
ArKO	Aromatase Knockout
ATP	Adenosine triphosphate
BAFs	Breast adipose fibroblasts
β-Gal	β-Galactosidase
bp	base pairs
BSA	bovine serum albumin
CAFs	Cancer associate fibroblasts
C/EBPα	ccaat enhancer brinding protein alpha
ChIP	Chromatin Immunoprecipitation
°C	degrees Celsius
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E_2	estradiol
EDTA	ethylene diamine tetraacetic acid
EGR	Early Growth Response
ELISA	Enzyme linked immunological surface assay
EMSA	Electromobility shift assay
ER	Oestrogen Receptor
ER+	Oestrogen Receptor Positive
ER-	Oestrogen Receptor Negative
FCS	Fetal Calf Serum
g	grams
GAS	interferon γ activation site
GRE	glucocorticoid responsive element
GREB1	growth regulation by estrogen in breast cancer 1
HIF-1α	Hypoxia Inducible Factor 1 alpha
IGF2	insulin-like growth factor 2
IL-6	interleukin 6
IL-11	interleukin 11
LB	Lauria Broth
LUC	muciferase
M	molar
mg	milligram
ml	millilitre
mM	millimolar
min	minutes
mRNA	messenger ribonucleic acid
ng	nanogram
nM	nanomolar
PII	promoter II
PI.3	promoter I.3

PI.4	promoter I.4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
PPARγ	peroxisome proliferator-activated receptor gamma
qRT-PCR	quantitative real time polymerase chain reaction
RLU	relative light units
ROR	retinoic acid receptor related orphan receptor
RNA	ribonucleic acid
sec	seconds
SERM	Selective Estrogen Receptor Modulator
SGBS	Simpson-Golabi-Behmel Syndrome
siRNA	short interfering ribonucleic acid
SP1	Specificity Protein 1
TBE	tris buffered saline
TNFα	Tumour Necrosis Factor-alpha
TNFR1	Tumour Necrosis Factor Receptor type 1
TNFR2	Tumour Necrosis Factor Receptor type 2
TSA	Trichostatin A
µg	micrograms
µl	micro litre
µM	micro molars
UV	ultraviolet
V	volts
VEGF	Vascular endothelial growth factor

CHAPTER 1

Literature review

1.1 Introduction: Breast cancer

Breast cancer is the most commonly diagnosed form of cancer in Australian women, with approximately one in nine women affected with the disease throughout their lifetime (www.bcna.org). Nationally around 11,700 new cases are reported annually with approximately 2,600 deaths, making breast cancer the leading cause of death amongst women in Australia (www.nbcf.org.au). Whilst instances of breast cancer appear to be on the rise, mortality rates have shown steady decline throughout the past decade. This can in part be attributed to better management of the disease by medical professionals and greater awareness within the community leading to earlier diagnosis and greater survival outcomes. However, research scientists have also developed a more advanced understanding of the genetic and molecular basis of breast cancer. This research effort has identified diagnostic markers for early detection as well as novel approaches for therapy, a combination of which provides a better prognosis for cancer sufferers.

Whilst a great number of risk factors have been identified as being associated with the onset of breast cancer, including ethnicity, radiation exposure and alcohol consumption (Moulder and Hortobagyi 2008), the most significant correlation with breast cancer comes with increasing age and obesity (Metcalf 1974). In fact, approximately 87% of newly diagnosed cases are in women who are post-menopausal (www.nbcf.org.au). Many studies have sought to understand the factors which cause deregulation and breast carcinogenesis, with the hope of dramatically lowering mortality rates. Medical research has identified molecular pathways which are differentially regulated in cancerous tissues. One of the most intensely studied is the oestrogen biosynthesis pathway and its many levels of regulation, as irregularities are often observed in patients with breast cancer. The oestrogen-producing enzyme aromatase is of particular interest, as blocking its action is seen as a key therapeutic strategy.

This literature review discusses current knowledge of aromatase, and how the cytokine TNFα plays an important role in its regulation in the breast. The mechanisms through which aromatase is stimulated in normal breast adipose and breast cancer, the roles TNFα has to play in breast cancer pathology and its relevance as a novel drug target will be discussed. This literature review is made up primarily of a review article accepted in the Journal of Interferon and Cytokine Research, as well as a review article published in the Journal of Steroid Biochemistry and Molecular Biology. However, current opinions and recent findings have

been incorporated as appropriate. Refer to Appendix 1 and Appendix 2 for the original review articles.

1.2 The importance of oestrogen biosynthesis in breast cancer

Oestrone, oestriol and oestradiol form the biologically active group of hormones collectively known as oestrogens (Chen et al. 2008). Enzymatically catalysed in a series of rate-limiting steps from cholesterol in specific tissues, oestrogens may circulate as an endocrine hormone or act locally on target cells (Bulun et al. 2005). In humans, oestrogen acts as the primary female sex hormone, regulating processes related to the growth, differentiation and physiology of the reproductive system whilst also exerting its effects in bone, liver, brain and cardiovascular systems (Pearce and Jordan 2004). Oestrogen mediates gene transcription by binding to the nuclear Oestrogen Receptors ERα (Jensen 1962) and ERβ (Kuiper et al. 1996).

Oestrogen action is also behind pathological states seen in breast, ovarian (Pujol et al. 1998), colon (Campbell-Thompson et al. 2001) and prostate cancers (Chen et al. 2008). The link between oestrogens and breast cancer was first postulated as early as 1934 (Geschickter et al. 1934), when an early study demonstrated the ability of prolonged exposure to oestrogens to cause lesions on the breast. Work in this area has progressed rapidly since then, and our understanding of the role oestrogens play in breast cancer is now much more advanced.

Oestrogen excess has been shown to be a major contributing factor to the risk of developing breast cancer (Miller 2006), with up to 70% of postmenopausal tumours expressing ERα and therefore classed as ER+. Oestrogens are therefore strongly associated with continuing development and progression of breast carcinomas (Manning et al. 1990). Tumours may source oestrogen from the circulating hormone (Huang et al. 1997), but the primary method for oestrogen-responsive growth in postmenopausal women is the paracrine effects of oestrogen locally produced by undifferentiated breast adipose fibroblasts (BAFs) surrounding malignant cells (Agarwal et al. 1996; Chetrite et al. 2000). In fact, oestrogen concentrations within the local breast tumour environment are up to ten times higher than circulating levels (van Landeghem et al. 1985). Adjuvant anti-oestrogen therapies are highly important in the treatment of such cancers (Johnston 2001).

1.2.1 Selective Oestrogen Receptor Modulators

Treatment options for ER+ breast tumours have progressed beyond traditional surgery and chemotherapy techniques, and now many patients are treated with drugs post operatively to combat tumour growth. Selective Oestrogen Receptor Modulators, or SERMs, are commonly used with proven success in restricting tumour growth.

SERMs comprise a group of ERα ligands which have a distinct pharmacological profile different to that of endogenous oestrogens. Tamoxifen is the original and most widely used amongst the non-steroidal anti-estrogens, although raloxifene is also commonly used (Fuchs-Young et al. 1995). These exert their actions by competitively binding to the ERα thereby preventing endogenous oestrogens from binding and signalling (Terenius 1970). The elucidation of the ERα structure has led to an understanding of how SERMs bind to ERα and differentially regulate gene expression in specific tissues (Osborne et al. 2000). They are proven to significantly improve breast cancer survival, as well as reduce the incidence of breast cancer amongst those at high risk of developing the disease (Group 1998; Fisher et al. 2005). Also used clinically is the steroidal anti-oestrogen fulvestrant (ICI 182,780) which has no agonist activity and a one hundred-fold higher affinity to the ERα than other SERMs (Howell 2000).

The clinical use of SERMs is unfortunately accompanied by wide-ranging side effects, stemming from the fact that although they are largely antagonistic in breast, they appear to act as an agonist in other tissues such as uterus and bone (Lyman and Jordan 1985; Miki et al. 2009). The use of Tamoxifen is associated with cardiovascular symptoms, with hot flushes and vaginal dryness being the most commonly reported side effects (Love et al. 1991). Consistent with its oestrogenic effects on bone and liver, Tamoxifen results in a maintenance of bone mineral density (Zidan et al. 2004) and reduction in total serum cholesterol levels (Rutqvist and Mattsson 1993). Worryingly, there is a significantly increased risk of developing endometrial cancer, due to thickening of the endometrial wall and hyperplasia (Fisher et al. 1994).

1.3 Aromatase and its role in oestrogen biosynthesis

P450arom, or aromatase, is a member of the large P450 superfamily of catalytic enzymes. Its function is to catalyse the final key step in the conversion of androgens to biologically active

oestrogens. It localises to the endoplasmic reticulum of oestrogen-producing cells, such as the ovarian granulosa (Means et al. 1991), placental synctrophoblast (Toda et al. 1992), bone (Nawata et al. 1995), brain (Wagner and Morrell 1997), skin fibroblasts (Berkovitz et al. 1989) and adipose tissue (Bulun and Simpson 1994), where it appears to be the marker of an undifferentiated pre-adipocyte phenotype (Cleland et al. 1983).

The aromatase enzyme complex is comprised of two polypeptides, the ubiquitously expressed flavoprotein NADPH-cytochrome P450 reductase and the cytochrome P450arom (Durham et al. 1985), and it is the expression of this second polypeptide that governs the capacity of the cell to produce oestrogens (Bulun et al. 2005). The aromatase enzyme complex binds C_{19} steroid substrates and catalyses the aromatisation reaction which leads to the formation of phenolic A ring structures typical of biological oestrogens (Thompson and Siiteri 1974; Akhtar et al. 1982).

Much of what is known about the actions of aromatase comes from the study of mice deficient in the enzyme. These ArKO mice contain targeted disruptions of the *Cyp19* gene encoding for aromatase, causing an almost complete abolishment of oestrogen production in homozygous animals (Honda et al. 1998). Loss of oestrogen was associated with a number of related phenotypes, including underdeveloped external genitalia and uteri, and ovulation failure leading to infertility in females (Fisher et al. 1998). Males showed immature sexual behaviour and disrupted spermatogenesis, a combination of which resulted in reduced fertility (Robertson et al. 2001). ArKO mice of both sexes displayed increased adiposity, demonstrating the importance of oestrogen in maintaining lipid homeostasis (Jones et al. 2000). Aromatase deficiency has also been clinically reported in humans with rare mutations in the gene encoding aromatase. Inability of the placenta to convert androgens to oestrogens in aromatase deficient female foetuses results in excess androgens in utero, and thus ambiguous genitalia at birth (Shozu et al. 1991). At puberty, there is a lack of breast development, acne and a slowing of bone age, however most cases of aromatase deficiency are detected at this stage and treated with hormone replacements (Ito et al. 1993; Conte et al. 1994; Morishima et al. 1995). A recent case of an adult with aromatase deficiency not treated with oestrogen therapy revealed severe bone and joint problems, increase adiposity and minimal development of secondary sexual characteristics (Lin et al. 2007). Similarly to the ArKO mice, males with an aromatase deficiency suffer from obesity and associated metabolic

disorders. Osteoporosis, impaired fertility and loss of libido are also reported (Carani et al. 1997; Deladoey et al. 1999; Maffei et al. 2004).

Genetic alterations resulting in aromatase excess have also been reported in the medical literature, and in males results in severe prepubertal gynocomastia (Berkovitz et al. 1985). Females with aromatase overexpression underwent premature onset of puberty, had an enlarged uterus and irregularities of the menstrual cycle (Stratakis et al. 1998; Martin et al. 2003). Both sexes displayed a premature fusion of growth plates resulting in overall short stature. These phenotypes were due to high serum oestrogen levels and accompanying low androgen levels, and effectively treated with aromatase inhibitors (Shozu et al. 2003). Taken together, clinical observations from patients deficient for or overexpressing aromatase highlight the many non-reproductive functions for oestrogen and the importance of the aromatase enzyme in biologic processes.

1.3.1 Aromatase inhibitors in breast cancer therapy

Aromatase is crucial to the development of oestrogen-dependant breast tumours, and its upregulation is considered a signature of advancing disease (Sasano and Ozaki 1997). The increased gene expression and enzyme activity is coupled with heightened oestrogen production, enabling ER+ tumours to further proliferate. Blocking the action of oestrogen in ER+ tumours is seen as an effective method for slowing hormone-responsive growth, and the ER antagonist Tamoxifen has been revolutionary in this regard. Due to the many side effects associated with Tamoxifen use and the resistance that can develop, novel drug therapies have been increasingly available. As such, aromatase inhibitors (AIs) such as Arimidex, Femara and Aromasin are emerging at the forefront of endocrine manipulation in the treatment of breast cancer (Hong et al. 2007). These drug-based therapies can be used on Tamoxifen resistant patients (Noberasco et al. 1995) and often compliment traditional forms of cancer treatment. They result in highly efficient global inhibition of aromatase in the body, inhibiting enzyme action by greater than 97% (Geisler et al. 2002). Large clinical trials have demonstrated their superior effectiveness over Tamoxifen in the treatment of postmenopausal ER+ breast cancer, with AI treatment resulting in a reduced tumour size when compared to Tamoxifen treatment (Smith 2003).

Unfortunately, due to the fact that aromatase inhibitors block aromatase action and therefore oestrogen production throughout the entire body, a number of side effects have been

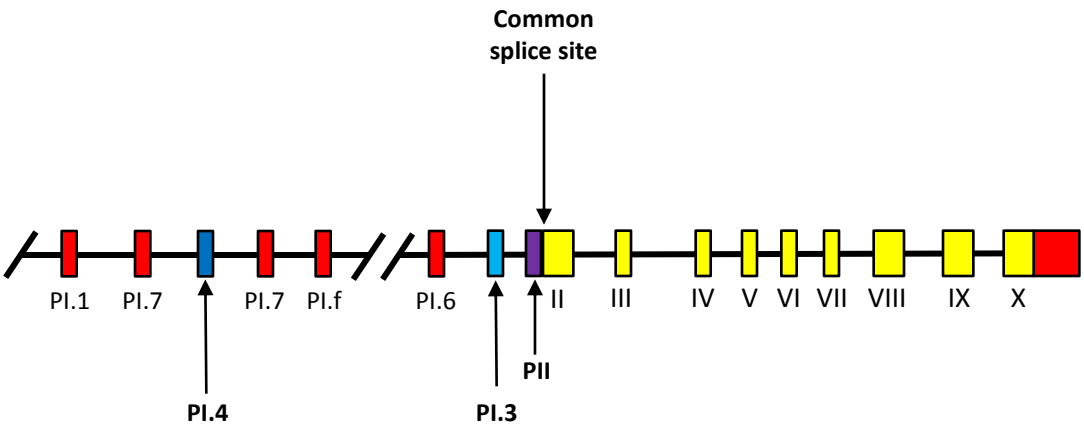

Figure 1.1: The tissue-specific promoters of *CYP19A1*.

The first exon of *CYP19A1* is comprised of eight distinct tissue-specific promoters, which are all spliced onto a common site slightly upstream of the ATG start site. The coding region remains identical for each transcript, however the 5' untranslated region varies according to the tissue of origin. Key promoters discussed in this literature review are indicated by the arrows.

Promoter	Tissue specific expression
PI.1	Placenta
PI.2	Placenta
PI.4	Adipose, bone, skin, breast cancer
PI.7	Endothelia
PI.f	Brain
PI.6	Bone
PI.3	Adipose, breast cancer
PII	Ovary, adipose, breast cancer

Table 1.1: Tissue specific expression of the *CYP19A1* promoters.

The promoters that constitute exon I of the CYP19A1 gene in humans are listed in the left hand column. The tissues in which the corresponding promoters are detected are listed in the right hand column.

associated with their use. These include joint pain and stiffness (Crew et al. 2007), accelerated bone loss resulting in osteoporosis and increased risk of fractures (McCloskey et al. 2007; Mouridsen et al. 2009), hypercholesterolemia (Coates et al. 2007), heightened incidence of adverse cardiovascular events (Buzdar et al. 2006) and hot flushes (Goss et al. 2005). Research is currently directed towards the development of breast-specific aromatase inhibitors, in order to achieve blockade of oestrogen production only in the breast and limit undesirable side effects (Safi et al. 2005).

1.4 Tissue specific regulation of the aromatase gene *CYP19A1*

Aromatase is encoded by the *CYP19A1* gene, located on chromosome 15 band q21 of the human genome (Chen et al. 1988). Whilst it was first cloned and characterised some years ago (Means et al. 1989; Harada et al. 1990; Toda et al. 1990), modern advances such as the human genome project have allowed for greater research potential. The full length of *CYP19A1* is 123kb (Sebastian and Bulun 2001), of which the coding region accounts for a mere 30kb (Shozu et al. 2003). The upstream 93kb contains eight tissue-specific promoters that control the highly tissue-specific expression of *CYP19A1*, corresponding to sites of oestrogen production (Figure 1.1 and Table 1.1). Differential use of these promoters in different tissues gives rise to specificity, and this may be detected through exon-specific RT-PCR (Agarwal et al. 1995). Promoters are transcribed and spliced on to a common junction immediately upstream of the ATG translational start site, resulting in the same aromatase protein being produced despite transcripts with differing 5' untranslated regions (Mahendroo et al. 1993).

In the tumour-free breast, upwards of 50% of aromatase transcripts are derived from the distal promoter I.4 (PI.4) lying 73kb upstream of the common splice site. The remainder of transcripts are derived from the combined contribution of promoter I.3 (PI.3) and promoter II (PII) (Bulun et al. 1993; Agarwal et al. 1996). However, in the presence of a tumour, there is a significant upregulation of PI.3 and PII *CYP19A1* transcripts accounting for up to 80% of total transcripts. This promoter switching results in an overall 3-4 fold increase in aromatase transcripts in the tumour-bearing breast (Bulun et al. 1993).

1.4.1 A role for epigenetics?

In addition to transcriptional regulation of PI.3/PII expression via the action of tumour derived signalling factors, it is becoming increasingly clear that epigenetic changes within the

breast tumour microenvironment, namely DNA methylation and histone modifications, also play an important role in aromatase regulation.

Dynamics of epigenetic regulation

Epigenetics describes a broad range of DNA and histone modifications that may modulate gene expression without altering the underlying coding sequence. This encompasses DNA methylation as well as histone modifications including acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP ribosylation. In combination, these processes work to regulate gene expression, mediate X-chromosome inactivation and facilitate genomic imprinting.

DNA is organised within the cell nucleus as a packaged structure known as chromatin. This dynamic configuration consists of DNA strands wrapped around histone proteins, and is consistently changing according to the transcriptional and replication needs of the cell. Epigenetics is the means by which chromatin structure is configured to prevent or allow access of transcriptional machinery to target genes, thus controlling gene expression levels. When a genomic region is in a tightly packed chromatin structure, transcription is repressed. Conversely, loosely packed chromatin allows to transcription of local genes to initiate (Li et al. 2007). DNA methylation and histone modifications work to determine the open or closed state of chromatin, and therefore exert heavy influence on gene expression.

DNA methylation is a phenomenon that occurs in both prokaryotes and eukaryotes (Hutchinson 1989; Noyer-Weidner and Trautner 1993). In mammals, DNA methylation occurs only on cytosine residues that are immediately 5' of a guanine, described as a CpG site (Razin and Riggs 1980). This dinucleotide is under-represented in the mammalian genome, however they appear to occur in clusters referred to as CpG islands. CpG islands are defined as nucleotide stretches greater than 200bp in length with a CG percentage of at least 60% (Robertson 2002), and occur in the promoter regions of approximately 50% of genes (You and Jones 2012). However, methylation of CpG sites outside of CpG islands and also within coding exons can effect gene expression. Hypermethylation of promoter CpG regions leads to a recruitment of histone modifying enzymes to the chromatin, resulting in acetylation of local histones and compaction of the chromatin structure to prevent access of transcriptional machinery (Nan et al. 1998). Thus, an inverse correlation exists between promoter methylation status and transcription levels, whereby hypermethylayion results in low gene

expression and hypomethylayion results in high gene expression. Methylation of even a single CpG site within a gene regulatory region may also effect gene expression by interfering with the binding of transcription factors to their response elements (Demura and Bulun 2008).

Cytosine methylation is the responsibility of three distinct DNA methyltransferase enzymes: DNMT1, which maintains existing methylation patterns (Song et al. 2012), and DNMT3a and DNMT3b, which establish de novo methylation of CpG sites during developmental stages (Okano et al. 1999) but also to a lesser degree participate in the maintenance of methylation patterns during DNA replication (Jones and Liang 2009). All three catalyse the transfer of methyl groups from S-adenosyl-L-methionine to the 5' position of cytosines as part of a CpG dinucleotide (You and Jones 2012). DNA methylation often works in synergy with histone modifications to regulate gene expression. Hypermethylation of CpG sites leads to a recruitment of methyl-binding proteins, which in turn recruit histone deactylases (HDACs) to the region and repress gene transcription by closing chromatin structure (Boyes and Bird 1992; Nan et al. 1998). Eighteen different HDAC genes have been identified to date subdivided in to two major subgroups (Reichert et al. 2012), however all function to deacetylate not only lysine residues in histone tails but non-histone proteins including transcription factors (Choudhary et al. 2009). The action of HDACs is counterbalanced by histone acetyltransferases which are able to introduce acetylation to histones, loosening their structure in order to become more accessible to transcriptional machinery (Furdas et al. 2012).

Epigenetic regulation of aromatase expression

It has previously been demonstrated that *CYP19A1* transcription can be epigenetically regulated. Global inhibition of DNA methylation with 5-Aza-2'-deoxycytidine (5aza) results in elevated *CYP19A1* mRNA in human endometrial stromal cells, human hepatoma cells and chicken fibroblasts (Leshin 1985; Dannenberg and Edenberg 2006; Izawa et al. 2007). Furthermore, DNA methylation can be correlated with promoter-specific *CYP19A1* expression in sheep and cows promoters 1.1 and 1.5/2 (Furbass et al. 2001; Vanselow et al. 2005; Furbass et al. 2008; Vanselow et al. 2008) and human skin adipose fibroblasts (Demura and Bulun 2008). There is also evidence that methylation of CpG sites within the PI.3/PII region which overlap with the one of the cAMP response elements (CRE) interferes with CREB1 binding.

Our group has published results in BAFs indicating that *CYP19A1* expression via tissue specific promoters is not dependent on the methylation status of CpG sites within the promoter. An examination of eleven CpG sites within PI.3/PII showed that treatment with PGE2 did not alter the methylation status of the promoter despite an increase in transcriptional activity. Treatment of BAFs with 5aza did result in an increase in total *CYP19A1* transcripts, however this was not directly due to demethylation of the promoter. This suggested that epigenetic regulation of aromatase expression lay upstream of *CYP19A1* itself (Knower et al. 2010) (Appendix 3).

To further this hypothesis, we investigated whether epigenetic regulation of the PGE2 receptor EP2 was one such upstream factor. DNA methylation status of the EP2 promoter has previously been shown to have prognostic value in cases of non-small cell lung cancer, where increased methylation is a positive marker, and neuroblastomas, where more advanced tumours exhibit a methylated EP2 promoter. An inverse correlation between EP2 promoter methylation and mRNA expression was demonstrated in the breast cancer cell lines MCF7 (ER+), MDA-MB-231 (ER-) and MCF10A (normal epithelial), and transcript levels were increased in cells treated with 5aza as well as the histone deactylase inhibitor Trichostatin A (TSA). Treatment of BAFs with 5aza also increased EP2 mRNA levels. However, an evaluation of matching normal and cancer-associated stroma of nine clinical samples representing different tumour subtypes revealed no inverse correlations between EP2 promoter methylation and mRNA expression. Out findings suggest that epigenetic regulation of aromatase expression lies still further upstream of the PGE2 pathway than its receptor EP2 (To et al. 2012) (Appendix 4). Further components of the upstream aromatase regulatory network may also be under epigenetic regulation, and these are yet to be identified.

1.4.2 Regulation of PromoterI.3/Promoter II

In pre-menopausal women, oestrogen biosynthesis, and consequently aromatase activity, is primarily localised to the ovary. Here, *CYP19A1* expression is mediated through the proximal promoter II (PII) (Means et al. 1991) in response to FSH signalling (Jenkins et al. 1993). Low levels of PII transcript are detected in normal breast adipose, however in cases of ER+ breast cancer there is a switch to the preferential use of PII driven by the actions of tumour derived factors. Due to the close proximity of PI.3 to PII – the TATA box of each promoter is

separated by 215bp – they are thought to share common cis-regulatory elements that control their expression (Zhou et al. 1997).

Within the PI.3/PII region, key regulatory elements that have been identified include two CREB responsive elements (CREs) and two SF-1 binding sites. PI.3/PII are stimulated by the actions of tumour-derived factors, in particular Prostaglandin E2 (PGE2) (Zhao et al. 1996). The binding of PGE2 to its G-protein coupled receptors EP1, EP2, EP3 and EP4, which have all been implicated in breast cancer pathology (Richards and Brueggemeier 2003; Subbaramaiah et al. 2008; Thorat et al. 2008), leads to an increase in cellular cyclic AMP levels and activation of the PKA and PKC pathways (Regan 2003). Cyclic AMP responsive element binding protein 1 (CREB1) is phosphorylated and activated by PKA, translocating in to the nucleus and binding to both CREs within PI.3/PII (Sofi et al. 2003). Whilst SF-1 is expressed and associated with PI.3/PII activation in the ovarian granulosa cells (Lynch et al. 1993), BAFs express the SF-1 homologue Liver Receptor Homologue-1 (LRH-1) which is able to bind to the same consensus sequence. LRH-1 is overexpressed in approximately 50% of breast cancers, and its expression is strongly correlated with aromatase expression in the adipose adjacent to a tumour (Clyne et al. 2002). PKA and PKC dependent pathways regulate expression of LRH-1, which then binds to the SF-1 elements within PI.3/PII (Clyne et al. 2002). Additionally, PKA and PKC also regulate LRH-1 activity via the regulation of co-activators such as PGC1α, which interacts with LRH-1 to enhance its transcriptional activity in BAFs (Safi et al. 2005). PGE2-induced PI.3/PII activity is also inhibited by the actions of melatonin, a naturally secreted pineal hormone, suggesting a protective effect against excess oestrogen production (Knower et al. 2012) (Appendix 5).

ATF-2 and c-Jun are additional transcription factors which interact with PI.3/PII in response to PKA/PKC pathways and p38 and JNK1/MAPK signalling pathways, although the precise molecular mechanisms and the nature of the promoter interaction are yet unclear (Chen et al. 2007). Additional consensus DNA binding elements have been identified, and these include a TGF-β response element, a glucocorticoid response element (GRE), an AP-1 site and an additional third CRE. Analysis of these novel sites revealed that deletion of the TGF-β and GRE sites resulted in an increase of PI.3/PII activity, whilst deletion of the AP-1 and additional CRE reduced promoter activity (Zhou et al. 1997). More detailed analysis of the precise role for these promoter elements in regulating expression aromatase in the breast has yet to be carried out.

1.4.3 Regulation of Promoter I.4

Once a woman reaches menopause, the ovaries cease hormonal production and responsibility for oestrogen biosynthesis switches primarily to the adipose tissue. This occurs largely in breast adipose, with a low level of aromatase expression maintained in this tissue via the use of distal promoter I.4 (PI.4) (Zhao et al. 1995). The PI.4 region has a number of consensus response elements proximal to its transcription start site and also within the transcribed region. This includes an interferon γ activation site (GAS) (Zhao et al. 1995), glucocorticoid responsive element (GRE), Sp1 binding site (Zhao et al. 1995), retinoic acid receptor related orphan receptor site (ROR) (Odawara et al. 2009) and activating protein-1 binding site (AP-1) (Zhao et al. 1996). PI.4 activity can be stimulated *in vitro* by cytokines such as TNFα, oncostatin M (OSM), Interleukin-6 (IL-6) or Interleukin-11 (IL-11) (Zhao et al. 1995; Zhao et al. 1995; Zhao et al. 1996) in conjunction with the synthetic glucocorticoid Dexamethasone (DEX) (Simpson et al. 1981). IL-6, IL-11 and OSM were previously shown in human adipose to mediate PI.4 transcription via the Jak/STAT pathway and use of the upstream GAS element (Zhao et al. 1995).

The mechanisms by which TNFα is able to activate PI.4 are not yet clear, although a previous study has suggested that the upstream AP-1 element may be involved (Zhao et al. 1996). Whilst it has been shown many times in cell culture, attempts to obtain *in vivo* evidence of a cytokine-glucocorticoid interaction have not been successful. Studies on women given Dexamethasone during therapy found that aromatase activity, and consequently oestrogen conversion, were not enhanced (Reed et al. 1986). Similar studies in monkeys gave the same result (Longcope 1987). *In vitro*, TNFα has been shown to induce *CYP19A1* PI.4 activity in pachytene spermatocytes (Bourguiba et al. 2003) and EM1 endometrial cells (Salama et al. 2009) without the presence of DEX, however again this has not been shown *in vivo*. The reasons for the inconsistency between *in vitro* and *in vivo* are yet to be clarified. However, although pachytene spermatocytes are able to express aromatase in the presence of TNFα alone, the addition of DEX does enhance this effect (Carreau et al. 2004).

Activities of PI.4 as well as proximal promoters II and I.3 are all significantly increased in the tumour and surrounding pre-adipocytes in women with oestrogen-responsive breast cancer (Irahara et al. 2006), explaining the increase in local oestrogen production. Cytokines, and in particular TNFα, therefore play a critical role *in vivo* in both a disease-free adipose and breast-

tumour environments relating to aromatase activity and oestrogen regulation. TNFα is found co-expressed with aromatase in disease-free adipose, confirming its part in regulating expression (Purohit et al. 2002). Additionally, *TNFα* and *CYP19A1* mRNA are found co-localised within a breast tumour microenvironment but not within a normal breast (Irahara et al. 2006). TNFα may also help to prevent the differentiation of tumour-associated fibroblasts into mature adipocytes, thereby ensuring the tumour has a constant source of oestrogen from cells which are able to express aromatase (Meng et al. 2001), as aromatase expression is lost following differentiation (Clyne et al. 2002). The origins and properties of this critical cytokine will now be examined.

1.5 The TNF cytokine superfamily

A factor which could induce necrosis of tumours in mice was first isolated from serum (Carswell et al. 1975; Haranaka and Satomi 1981). The animal itself was producing this tumour necrosis factor from macrophages and lymphocytes, capable of inducing death in a number of cell types not restricted to cancer cells (Granger et al. 1969). This discovery was heralded as a potential new therapy for cancer, whereby the growth of a tumour could be retarded using this molecule. Consequently, many groups quickly turned to finding, cloning and characterising the human tumour necrosis factor (TNF). The human genomic clone was first isolated by from a genomic library and was called TNFα (Shirai et al. 1985). The cDNA sequence and recombinant protein sequence was found to be approximately 80% homologous to the earlier published mouse sequence (Pennica et al. 1984; Marmenout et al. 1985). Although systemic toxicity proved an insurmountable barrier to the use of TNFα as an anti-tumour agent, the study of this molecule and its related family members has remained important to the understanding of apoptosis, immune defences and inflammation (Locksley et al. 2001).

Since TNFα was isolated, a large number of related cytokines forming the TNF superfamily have also been identified, and these include LT-α (Nedwin et al. 1985), Fas ligand (Suda et al. 1993), TRAIL (Wiley et al. 1995), RANKL (Anderson et al. 1997) and LT-β (Browning et al. 1993). Most of these are produced by cells of the immune system, where they are able to regulate a broad range of processes related to cell proliferation, survival, differentiation and apoptosis. Their rapid mode of action makes them ideal for action in pathogen-reactive cells (Locksley et al. 2001). Additionally, TNF cytokines have been implicated in a number of

pathological states and as such inhibitors of TNF action have been trialled in the treatment of rheumatoid arthritis (Maini and Taylor 2000) and inflammatory bowel disease (Bruin et al. 1995). Potential for TNF based therapies in osteoporosis (Fujita et al. 1990), autoimmune disorders (Satoh et al. 1989) and atherosclerosis (Fukuo et al. 1997) have also been hypothesised.

Amongst the TNF superfamily, TNFα has a unique property in that it displays proinflammatory effects in addition to its proliferation and apoptotic abilities (Tracey et al. 1986). The wide ranging effect of TNFα on a number of different cell types has made it an intently studied target for not only disease therapies but also for understanding basic cellular processes and their deregulation.

1.6 Biological properties of TNFα

TNFα is produced primarily by macrophages and monocytes of the immune system (Kornbluth and Edgington 1986), but production can also occur in lymphoid cells, endothelial cells, neuronal tissue, adipose fibroblasts and tumour cells (Wajant et al. 2003). In its pro form, TNFα is a transmembrane protein (mTNFα) and clustered as a homotrimer (Kriegler et al. 1988; Tang et al. 1996). This pro form itself is biologically active, allowing for cell-to-cell contact transfer of signalling messages (MacEwan 2002). Most biological responses to TNFα, however, have been attributed to the soluble homotrimer that is cleaved from the transmembrane form (Li et al. 2002). TNF alpha converting enzyme (TACE) is responsible for the proteolytic cleavage of membrane-bound TNFα into its soluble form (sTNFα) (Black et al. 1997), allowing signalling beyond adjacent cells.

The TNF superfamily of ligands also has an associated superfamily of receptors, of which 27 have been characterised (MacEwan 2002). They have in common between 2 and 4 cysteine rich repeats within the extracellular domain, as well as a death domain in their intracellular domain (Darnay and Aggarwal 1999). TNFα specifically signals via tumour necrosis factor receptor type 1 (TNFR1) and tumour necrosis factor receptor type 2 (TNFR2). Both are able to bind soluble and membrane forms of TNFα, however TNFR1 has a higher affinity for sTNFα (Grell et al. 1998) whilst TNFR2 can only be fully activated by mTNFα (Grell et al. 1995). These receptors may have their extracellular domains cleaved to form soluble receptor fragments which could act to compete for and attenuate TNFα signalling (Wallach et al. 1991). Differences lie in their tissue distribution, as TNFR1 has been found constitutively

expressed in most tissues and is thought to be the primary mediator of TNFα signalling. TNFR2 has thus far been isolated to cells of the immune system, where its role is more pronounced in the lymphoid system (Wajant et al. 2003). As a consequence, much more is understood about signal transduction by TNFR1, although it is hypothesised that TNFR2 may function in a similar way due to conserved domains within the intracellular regions it shares with TNFR1 (Baker and Reddy 1998).

Mouse models with homozygous deletions of TNFα or either of its receptors display phenotypes associated with immune system development and response. TNFα and TNFR1 null mice display retarded development of lymphoidal tissue and immature B cells (Korner et al. 1997; Ettinger et al. 1998), whilst mice lacking TNFR2 show both impaired response and survival amongst the T cell population (Kim and Teh 2001; Kim and Teh 2004). Immune response against pathogens in TNFα and TNFR compromised mice varies according to which experimental pathogen animals are exposed to. For example, greater sensitivity is shown to *Mycobacterium tuberculosis* (Flynn et al. 1995) and *Lysteria monocytogenes* (Rothe et al. 1994) exposure, but enhanced immune response to rabies virus and cerebral malaria is seen, resulting in better survival rates compared to wildtype animals (Lucas et al. 1997; Camelo et al. 2000). Adding another level of complexity, TNFα and TNFR1 null mice die from exposure to *Taxoplasma gondii*, however TNFR2 null animals survive (Deckert-Schluter et al. 1998). Additional findings have indicated that TNFα and its receptors may also contribute to downregulation of immune components upon completion of a successful host response (Kollias et al. 1999). Taken together, these findings indicate that the role of TNFα and its individual receptors in infectious disease is dependent on the type of pathogen involved and the stage of infection.

1.7 TNFα signalling

Binding of the TNFα ligand to its cell surface receptors triggers an intracellular signalling cascade that ultimately leads to biological response. TNFα may induce apoptosis directly by recruitment and activation of caspase-8 via a death-domain in the intracellular regions of its receptor (Yeh et al. 1998; Zhang et al. 1998). Signalling pathways also activated by TNFα include the NFκB, JNK (Natoli et al. 1997) and MAPK (Vietor et al. 1993) pathways.

1.7.1 NFκB signalling

The most well recognised signalling pathway induced in response to TNFα is that of nuclear factor kappa B, or NFκB. NFκB is a transcription factor which binds to responsive elements of target genes to mediate their expression. Importantly, activation of NFκB by TNFα promotes cell survival (Beg and Baltimore 1996; Van Antwerp et al. 1996) and its inhibition *in vitro* promoted TNFα-mediated apoptosis (Wang et al. 1996).

In its inactive state, TNFR is prevented from auto-activation by a silencer protein bound to its intracellular death domain known as SODD (Jiang et al. 1999). Upon binding of the TNFα ligand, the receptor trimerizes (Banner et al. 1993) and SODD is displaced by a death-domain adaptor protein, TRADD (Hsu et al. 1995). This serves as a platform for the assembly of the TNFR signalling complex, which includes the TNF receptor-associated factor 2 (TRAF2) and receptor-interacting kinase (RIP). TRAF2 in turn recruits an IKK complex to the TNFR signalling complex (Devin et al. 2000). An IKK complex is crucial in NFκB activation, as it contains a heterodimer of IKKα and IKKβ kinases. Knockout of either of the IKK components in mice leads to severely impaired TNFα-induced NFκB signalling and associated abnormalities in development, with embryonic lethality resulting in IKKβ null animals (Li et al. 1999; Tanaka et al. 1999; Li et al. 2000). Upon activating phosphorylation by RIP, the IKK complex phosphorylates the inhibitory component of NFκB, IκBα (Zandi et al. 1997). In an unstimulated cell, IκBα interacts with NFκB in the cytoplasm, binding to and masking its nuclear localisation signal (Beg et al. 1992; Henkel et al. 1992). Phosphorylation by the IKK complex marks IκBα for ubiquitination and subsequent degradation by the proteosome, allowing NFκB to translocate into the nucleus and mediate gene transcription (Roff et al. 1996).

In the nucleus, NFκB transcribes a host of genes in response to TNFα signalling including those encoding cytokines, cell surface receptors, growth factors, other transcription factors, and genes associated with metabolism (Tian et al. 2005; Tian et al. 2005). The response of genes to TNFα via the NFκB pathway seems to be dependent on the length of exposure to the TNFα ligand, with a different set of genes seemingly induced following short or sustained treatment (Tian et al. 2005). Recently, a mode for rapid induction of NFκB target genes in response to TNFα has been described (Ainbinder et al. 2002). Using the A20 gene as an example of one which is rapidly induced by TNFα, it was shown that NFκB interacts with a

pre-formed transcription initiation complex which is constitutively present due to the activities of the Sp1 transcription factor. Regardless of the mechanism, NFκB is clearly a critical pathway in TNFα signal transduction.

1.7.2 MAPK signalling

Binding of TNFα to its receptors also activates the c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (MAPK) and their downstream targets. Activation of TNFR1 leads to binding of TRAF2 to the intracellular domain of the receptor, and it is this adaptor protein that is thought to initiate phosphorylation and activation of JNK and p38 MAPK kinases (Reinhard et al. 1997). This is known to be transient activation, although it can be a prolonged response under apoptotic conditions (Ichijo et al. 1997; Carpentier et al. 1998). The JNK kinases are activated through a cascade involving activation of MEKK1 and MMK7 kinases which are downstream of TNFR1 activation (Lange-Carter et al. 1993; Tournier et al. 2001). Crosstalk with the NFκB pathway may also occur, with MEKK1 also able to activate the IκBα alpha complex which eventuates in NFκB translocation in to the nucleus (Lee et al. 1997). JNK is known to phosphorylate and activate AP-1 transcription complexes (Treisman 1996), which have previously been hypothesised to bind to and activate *CYP19A1* via the AP-1 site within the PI.4 region (Zhao et al. 1996). Little is known about signalling events leading to aromatase induction, however Prostaglandin E2 activation of *CYP19A1* expression via PI.3/PII may also involve p38MAPK and JNK signalling mechanisms in BAFs (Chen et al. 2007).

1.8 TNFα and breast cancer

As a potent signalling molecule, TNFα has been found within the tumour micro-environment of several cancer types, including pancreatic (Karayiannakis et al. 2001), renal (Yoshida et al. 2002), prostate (Pfitzenmaier et al. 2003), kidney, lung, bladder, oesophageal, melanoma and leukaemia (Mantovani et al. 2000). In a breast tumour environment, increased concentrations of TNFα have been detected in the breast cyst fluid and breast tumour cytosol, both of which are able to stimulate oestrogen production (Macdiarmid et al. 1994). Furthermore, the presence of TNFα has been strongly correlated to a metastatic, invasive breast tumour phenotype (Leek et al. 1998; Yin et al. 2009). Serum concentrations of TNFα are also higher in patients with more advanced breast cancers (Sheen-Chen et al. 1997), and can be used as a predictive marker for response to chemotherapy treatments (Berberoglu et al. 2004). This

observation is consistent with clinical findings and animal studies suggesting that endogenously secreted TNFα in the presence of a tumour exerts a proliferative response rather than tumour regression (Mochizuki et al. 2004; Zarovni et al. 2004), contrary to the initial isolation of TNFα as a potential anti-cancer agent.

TNFα appears to be particularly critical to the development and progression of the oestrogen-responsive breast tumour type. As previously mentioned, the activity of aromatase is regulated by TNFα in adipose tissue (Purohit et al. 1996). Other key enzymes in the oestrogen-synthesis pathway such as esterone sulfatase and oestradiol- 17β-hydroxysteroid dehydrogenase Type 1 have also been shown to be responsive to TNFα for increased activity (Duncan et al. 1994; Newman et al. 2000). TNFα can also modulate expression of genes in ER+ breast cancer cell lines that lead to promotion of cell growth and proliferation, metastasis, loss of cell cycle control, and degradation of the extracellular matrix (Jeoung et al. 1995; Yin et al. 2009; Li et al. 2012). Additionally, oestrogen has been found to increase the expression of TNFR1 but not TNFR2, in human BAFs, thereby potentiating the response of these cells to TNFα (Deb et al. 2004).

1.9 TNFα and oestrogen biosynthesis

TNFα is one of several cytokines known to play an important role in the synthesis of active oestrogens through modulation of the activity or transcriptional regulation of a number of key enzymes in the steroidogenic pathway.

1.9.1 Oestrogen biosynthesis

The process of biological oestrogen production begins in the mitochondrion, where cholesterol is enzymatically converted to active oestrogens through a number of rate limiting steps. The activity of three main enzymes is responsible for the production of biologically active oestrogens: oestrone sulfatase, oestradiol-17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1) and aromatase. Aromatase converts androstenedione to oestrone via an aromatisation reaction, whilst estrone sulfatase forms oestrone from the oestrone sulfate precursor. 17β-HSD type 1 then catalyses the reduction of oestrone into oestrodiol, the biologically active form of oestrogen (Figure 1.2).

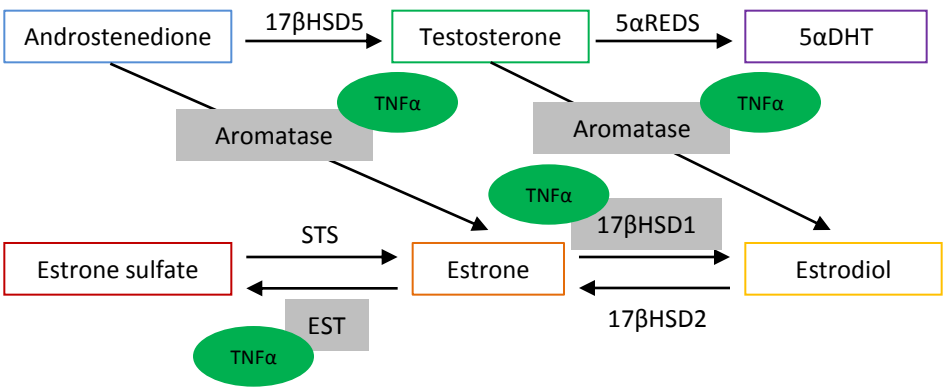

Figure 1.2: Intratumoural oestrogen biosynthesis pathway.

Active oestradiol is synthesised from androgen precursors through a series of enzymatic steps. Three of these enzymes - aromatase, estrone sufatase and 17βHSD1 - are known to be regulated either at the transcriptional of activity level by TNFα.

1.9.2 Aromatase

As discussed previously in section 1.4.3, TNFα is thought to play a critical role in transcriptional regulation of the aromatase encoding gene *CYP19A1* via its distal adipose-specific promoter PI.4. The precise signalling pathways and transcription factors by which this occurs are as yet unknown, however strong evidence has been presented for a correlation between levels of *TNFα* mRNA and *CYP19A1* transcripts in distinct normal and tumour breast compartments (Irahara et al. 2006). TNFα is also shown to have positive stimulatory effects on aromatase activity in osteoblasts (Shozu and Simpson 1998) and adipocytes (Zhao et al. 1997). In contrast, TNFα inhibits the induction of aromatase by FSH in rat ovarian granulosa cells (Ghersevich et al. 2001) and cultured porcine sertoli cells (Mauduit et al. 1993). Additionally, whilst TNFα has no effect on basal levels of aromatase in skin fibroblasts, it is able to inhibit induction by stimulating factors (Emoto et al. 1991). TNFα also appears to have stage-dependent effects on aromatase activity during sperm development, with pachytene spermatocytes showing increased *CYP19A1* mRNA levels in response to TNFα stimulation, whereas there is a decrease in aromatase transcripts in round spermatids treated with TNFα (Bourguiba et al. 2003). Taken together, these findings indicate that TNFα may have tissue and promoter-specific effects on aromatase gene transcription and therefore oestrogen biosynthesis.

1.9.3 Estrone sulfatase (EST)

Following aromatisation of androstenedione, most of the resulting oestrone is converted to oestrone sulfate, which is the primary circulating form of oestrogens as it has a much longer half life than other forms (Ruder et al. 1972). Oestrone sulfate is thought to form a reserve of biologically inactive oestrogens ready to be converted into the active hormone firstly through its conversion back to oestrone via the action of estrone sulfatase (Purohit et al. 2002). Expression of estrone sulfatase mRNA is reported to be robust in most breast tumours when compared with normal breast, and this is associated with a larger tumour volume and poorer prognostic outcomes (Utsumi et al. 1999; Utsumi et al. 1999; Utsumi et al. 2000; Suzuki et al. 2003; Honma et al. 2006). In addition to increased expression, activity of estrone sulfatase has also been shown to be heightened in breast cancer tissue compared with healthy breast (Santner et al. 1993; Evans et al. 1994).

Little is known about the role that cytokines, and in particular TNFα, may play in the regulation of estrone sulfatase expression and activity in the breast tumour microenvironment. TNFα, along with IL-6, has been shown to increase the activity but not transcript levels of estrone sulfatase in primary human fibroblasts derived from both normal and malignant breast tissue (Purohit et al. 1996). This suggests that these cytokines are involved in the post-translational modifications of the active enzyme rather than its transcriptional regulation (Purohit et al. 2002). Recently however, it was demonstrated in the ER+ breast cancer cell line MCF7, the ER- breast cancer cell line MDA-MB-231 as well as two prostate cancer cell lines that treatment with TNFα significantly increased mRNA expression of estrone sufatase via the PI-3 kinase/Akt pathway (Suh et al. 2011). Therefore, targetting the TNFα-mediated increases in estrone sulfatase transcription and activity could potentially reduce the bioavailability of oestrogens to an ER+ breast tumour.

1.9.4 17β-HSD type 1

Oestrone must be reduced to oestrodiol in order to achieve its full biological activity, and this is mediated through the action of 17β-HSD type 1 (Dumont et al. 1992). Expression of 17β-HSD type 1 is strongly correlated with ER+ as well as progesterone receptor positive breast cancers, indicating local synthesis of oestrodiol (Ariga et al. 2000). Overall metabolism of oestrogens favours inactivation of oestrodiol over conversion of estrone to oestrodiol, however within tumour tissue there is preferential reduction to the biologically active oestrodiol (Beranek et al. 1984). This suggests that factors within the tumour are preferentially driving the formation of oestrodiol, and TNFα may be a contributing factor. An early investigation into the role of cytokines in breast cancer revealed that TNFα stimulates the conversion of estrone to oestrodiol in MCF7 breast cancer cells (Duncan et al. 1994). TNFα also increases the activity of 17β-HSD type 1 in the ER+ cell line T47D and ER- cell line MDA-MB-231 (Duncan and Reed 1995), indicating that it acts in a variety of tumour microenvironments. Outside of the breast, TNFα has also been found to stimulate activity of 17β-HSD type 1 in endometrial glandular epithelial cells (Salama et al. 2009). Taken together, these finding indicate that TNFα plays a critical role *in vivo* to increase the bioavailability of active oestrogens.

1.10 Anti-adipogenic actions of TNFα

As well as promoting oestrogen biosynthesis in cancer-associated fibroblasts, TNFα also plays a major role in maintaining these fibroblasts in an undifferentiated state thus ensuring a constant source of oestrogen producing cells for the tumour.

The desmoplastic reaction is critical in maintaining oestrogen supply to the ER+ breast tumour (Deb et al. 2004). This dense layer of undifferentiated fibroblasts immediately adjacent to the malignant epithelial cells gives such tumours their characteristic hard consistency (Bianco et al. 1995), and many factors secreted by the tumour are directed towards maintaining this layer of undifferentiated cells. This is important in breast tumour pathology as only undifferentiated fibroblasts maintain the capacity to express aromatase and produce active oestrogens, a feature lost once differentiation into mature adipocytes occurs (Clyne et al. 2002). Accumulation of pre-adipocytes does not appear to be the natural reaction to growth stimuli, since mouse 3T3-L1 pre-adipocytes treated with a combination of known growth factors initially proliferate, but eventually differentiate into mature adipocytes under the same conditions (Schmidt et al. 1990; Boney et al. 1998). ER+ breast tumours must therefore both cause an accumulation of pre-adipocytes and at the same time prevent them from differentiating into mature adipocytes. TNFα appears to be a major driver of this process.

The two critical transcription factor families that commit the pre-adipocyte towards differentiation in to a mature fat cell and maintain its phenotype are CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor-gamma proteins (PPARs) (Cao et al. 1991; Brun et al. 1996). These proteins work to transactivate adipocyte-specific gene expression, and inhibition of their expression or activity can lead to inhibition of pre-adipocyte differentiation as stimulated by known differentiation factors, or de-differentiation of committed adipocytes (Tamori et al. 2002). Two key family members are C/EBPα and PPARγ, which together act synergistically to drive mature adipocyte differentiation (Meng et al. 2001). TNFα can repress the expression of both C/EBPα and PPARγ in pre-adipocytes, directly inhibiting their capacity to differentiate (Zhang et al. 1996). This has been demonstrated in 3T3-L1 cells, mouse adipose tissue and primary human mammary adipocytes (Hu et al. 1995; Guerrero et al. 2009). The source of this TNFα appears to be from the tumour epithelial cells themselves, as conditioned media collected from MCF7

and T47D breast cancer cell lines show an enrichment of the cytokine. Treatment of 3T3-L1 preadipocytes with this conditioned media leads to a reduction in C/EBPα and PPARγ expression (Guerrero et al. 2009). Melatonin, a naturally secreted hormone from the pineal gland, has been shown to interfere with this process, down regulating the expression of TNFα in epithelial cells whilst inhibiting aromatase expression in the surrounding fibroblasts (Alvarez-Garcia et al. 2012; Knower et al. 2012). Further elucidation of other factors capable of restricting TNFα secretion from epithelial cells and therefore its role in the desmoplastic reaction will need to be uncovered in order to improve options for breast cancer therapy.

1.11 Sources of TNFα within the breast tumour microenvironment

Heightened concentrations of TNFα are found within the breast tumour microenvironment, with several different cell types thought to contribute to cytokine production. Tumour-associated macrophages and lymphocytes, which make up to 50% of the total tumour volume (Kelly et al. 1988), are thought to be the primary contributors. TNFα is associated largely with the immune response, and in the healthy body is mainly produced by cells of the immune system (Kornbluth and Edgington 1986). Tumour epithelial cells secrete chemo-attractants such as IL-8 and MCP-1, which leads to an invasion of macrophages and lymphocytes into the breast tumour matrix (Leonard and Yoshimura 1990; Baggiolini et al. 1994) These immune cells in turn respond to soluble factors secreted from breast cancer cells by upregulating their production of TNFα (Eichbaum et al. 2011). Conditioned media from cultured monocytes and lymphocytes of breast cancer patients are able to stimulate aromatase activity in human breast adipose fibroblasts, and this can be attributed to the high levels of TNFα also detected in the media (Singh et al. 1997).

Evidence has suggested that tumour cells are themselves able to produce TNFα in order to achieve proliferative advantage, although this has not been confirmed for breast cancer epithelial cells. However, several of the main factors known to stimulate TNFα production are present within a breast tumour microenvironment, suggesting that TNFα secretion by tumour epithelial cells is a contributing factor to disease pathology. TNFα is able to positively auto-regulate its own expression in murine 3T3-L1 preadipocytes (Neels et al. 2006), and given the abundance of the cytokine surrounding tumour epithelial cells it is possible that it upregulates its own expression. Oestrogen increases the expression and secretion of TNFα in cells in uterine and lactotrope cells (Zaldivar et al. ; De et al. 1992), and whilst this has not been

confirmed in breast cancer cells, raises the possibility that a positive feedback loop between TNFα and oestrogen exists within a breast tumour microenvironment. Supporting this hypothesis is the finding that oestrogen increases the expression of the TNFα receptor TNFR1 in human breast adipose fibroblasts, thereby potentiating the response of these cells to TNFα (Deb et al. 2004).

Epigenetic regulation, specifically DNA methylation, is another poorly defined area of TNFα in the context of ER+ breast cancer. It has been established in neuronal cells, hematopoietic stem cells and macrophages that TNFα expression is under epigenetic regulation (Sullivan et al. 2007; Pieper et al. 2008). Indeed, a screen for genes differentially expressed following DNA demethylation with 5aza showed that TNFα and many of its target genes showed increased expression (Kim et al. 2012). Epigenetic mechanisms are known to factor in the regulation of oestrogen biosynthesis as well as many other key breast cancer processes (Knower et al. 2010), and the elucidation of its role in TNFα regulation is critical to our overall understanding of the importance of DNA and histone methylation and acetylation.

1.12 Clinical associations between TNFα and breast cancer risk

1.12.1 Advanced age and TNFα

Advanced age is a major risk factor in the development of breast cancer. Approximately 70% of post-menopausal cases are diagnosed as ER+ tumours, suggesting that processes within the post-menopausal endocrine system are altered as such that peripheral oestrogens, particularly those in the breast, are being upregulated. Increased levels of plasma TNFα in older individuals may help to provide an explanation.

As well as being a critical pro-inflammatory immune cytokine, TNFα is also implicated in a number of disease pathologies. These include rheumatoid arthritis (Maini and Taylor 2000), inflammatory bowel disease (Bruin et al. 1995), osteoporosis (Fujita et al. 1990) and atherosclerosis (Fukuo et al. 1997). Most of these conditions affect older individuals, suggesting that increasing concentrations of TNFα in those with advanced age contribute to common diseases associated with aging. Breast cancer could be one other such disease.

Animal models were initially investigated to establish a link between aging and increasing TNFα levels. Aged mice and rats show a significantly increased secretion of cytokines from the T-helper cells of their immune systems, and this is likely to account for the increased

peripheral oestrogen synthesis also observed (Chorinchath et al. 1996; Morin et al. 1997). A number of studies have since examined this association in a large human cohort and uncovered similar associations between increasing age and higher levels of measured serum TNFα (Paolisso et al. 1998).

1.12.2 Obesity and TNFα

In addition to advanced age, rates of breast cancer occurrence are significantly higher in obese women, with increased weight strongly associated with a higher risk of developing not only breast but many other forms of cancer (Basen-Engquist and Chang 2011). Obese breast cancer patients also show higher mortality rates, greater metastasis to distal sites, larger tumour mass and overall poorer prognosis when compared to non-obese breast cancer patients (Maruthur et al. 2009; Hauner et al. 2011). Again, strong associations between TNFα and obesity may help to provide an explanation.

In addition to being produced by cells of the immune system, TNFα is also produced in adipose tissue, including mature adipocytes, stromal-vascular cells and preadipocytes (Hube et al. 1999; Weisberg et al. 2003; Fain et al. 2004). Initially shown in animal models (Hotamisligil et al. 1993), TNFα levels are also markedly increased in the adipose tissue of obese individuals. This has been shown clinically as obese patients record a higher serum concentration of TNFα than age-matched healthy weight individuals. This effect was decreased upon surgery-mediated weight loss (Hotamisligil et al. 1995). TNFα may indeed be one of the driving forces behind obesity and insulin resistance, as mice lacking a functional TNFα protein or receptor are protected from diet-induced obesity and insulin resistance (Schreyer et al. 1998; Nieto-Vazquez et al. 2008). The mechanism resulting in increased TNFα production in states of obesity is however undefined, and this knowledge may help explain why obese individuals are at higher risk of breast cancer. It has been recently been shown that TNFα positively regulates its own transcription and secretion in adipose tissues, perhaps explaining how high levels of the cytokine are maintained in obesity (Neels et al. 2006).

Significantly, adipose tissue is also the major site of oestrogen conversion in post-menopausal women, highlighting a link between increased TNFα and oestrogen production. The increased risk of developing breast-cancer in obese women may therefore not only be associated with the increased oestrogen production from the higher volume of fat cells, but also with the

increased production of TNFα which may further drive oestrogen production. Studies in mice have shown that obesity is associated with increased aromatase activity and TNFα expression in the mammary gland (Subbaramaiah et al. 2011; Chen et al. 2012), supporting this hypothesis.

1.13 The potential for TNFα-targeted breast cancer therapies

Given its important role in many facets of breast cancer development, progression and maintenance, TNFα represents an attractive yet challenging therapeutic target. As demonstrated, TNFα plays vital roles in maintaining and upregulating local oestrogen biosynthesis as well as preventing the differentiation of oestrogen-producing pre-adipocytes into mature adipocytes. Reducing the capacity of the breast tumour to do either of those things would severely restrict its growth and proliferative potential.

Numerous clinical trials have already investigated the effectiveness of anti-TNFα therapies for the treatment of a number of associated diseases such as septic shock, rheumatoid arthritis, Chrones disease and even multiple sclerosis (Shimamoto et al. 1988; Elliott et al. 1993; van Dullemen et al. 1995; Hohlfeld 1996) (see Table 1.2). Currently used in the clinic are a number of approved monoclonal antibodies as well as a soluble TNFα receptor for the treatment primarily of rheumatoid arthritis patients (Thalayasingam and Isaacs 2011). Concerns were initially raised about their potential to increase a patient's risk of developing certain forms of cancer, however it is now clear that cancer incidence and prognosis were no worse in patients treated with anti-TNFα therapies compared to those who had not received the treatment (Askling et al. 2005; Raaschou et al. 2011). Due to its importance in the immune response, the most significant risk appears to be in susceptibility to infectious diseases. Indeed, rheumatoid arthritis patients treated with anti-TNFα therapies appear to be at a greater risk of developing skin infections, soft-tissue infections and septic arthritis (Dixon et al. 2006; Galloway et al. 2011). The risk appears to be higher in the first six months of treatment, and is enhanced with advanced age and concurrent use of glucocorticoid treatments (Askling et al. 2007; Strangfeld et al. 2011). Tuberculosis, listeria, salmonella and legionella infections also appear at a higher rate in anti-TNFα treated patients (Dixon et al. 2006; Dixon et al. 2010).

TNFα-targetting monoclonal antibodies have so far been demonstrated to retard mouse mammary tumour growth *in vivo*, as well as inhibiting proliferation of skin cancer cells (Scott

	Inflaximab	Adalimumab	Golimumab	Etanercept	Certolizumab
Type[1]	Monoclonal antibody	Monoclonal antibody	Monoclonal antibody	P75TNFR/ Fc fusion protein	PEGylated humanised Fab fragment
Recognising ligands[1]	TNF	TNF	TNF	TNF and LTα3	TNF
Molecular Weight[1]	150kDa	150kDa	150kDa	150kDa	95kDa
Half life (days) [1]	8-10	10-14	12 ± 3	3	14
Uses	Chrones Disease[2], Rhumatoid arthritis[3], psoriatic arthritis[4], psoriasis[5]	Chrones Disease[6], Rhumatoid arthritis[7], psoriasis[8],	Rheumatoid arthritis[9], psoriatic arthritis[10]	Rheumatoid arthritis[11], psoriatic arthritis[4], plaque psoriasis[8] and ankylosing spondylitis[12]	Chrones Disease[13], Rhumatoid arthritis[14]

Table 1.2: Summary of currently available anti-TNFα therapies

References as indicated within the table in superscript: [1](Thalayasingam and Isaacs 2011), [2](van Dullemen, van Deventer et al. 1995), [3](Harriman, Harper et al. 1999), [4](Woolacott, Bravo Vergel et al. 2006), [5](Kirby, Marsland et al. 2001), [6](Bultman, de Haar et al. 2012), [7](den Broeder, van de Putte et al. 2002), [8](Chastek, Fox et al. 2012), [9](Zhou, Jang et al. 2007), [10](Yang, Epstein et al. 2011), [11](Wakabayashi, Sudo et al. 2011), [12](Dougados, Braun et al. 2012), [13](Schreiber 2011), [14](Weinblatt, Fleischmann et al. 2012).

et al. 2003). Furthermore, TNFα null mice show lower rates of induced tumour formation than the wildtype, with TNFα neutralising antibodies again able to slow rates of tumour growth (Warren et al. 2009). This demonstrates a potential for the use to anti-TNFα therapies in the clinic. TNFα is a ubiquitous cytokine, and its critical role in the pro-inflammatory immune response means that it is present in many tissues as well as circulating plasma. To target its actions in breast cancer would therefore require a therapy to limit undesirable side-effects. Indeed, a case of primary breast tumour has been reported in a patient undergoing long-term anti-TNFα treatment for rheumatoid arthritis, suggesting that tumour development not regression may in fact result from anti-TNFα therapies (Pattanaik et al. 2011). Further work to elucidate the precise molecular pathways by which TNFα acts and how it is produced to excess within a breast tumour microenvironment are required so that further downstream components may be targeted. For example, agents stabilizing microtubules in breast fibroblasts inhibit TNFα-induced aromatase activity (Purohit et al. 1999). Identifying other similar pathways would hopefully result in a more specific blockade of TNFα action in breast cancer without compromising its critical immune function.

1.14. Summary and future perspectives

The research presented in this review highlights the many complexities of the role TNFα has to play within the breast tumour microenvironment. Not only is it implicated in the transcription and activation of key oestrogen-producing enzymes aromatase, estrone sulfatase and 17β-HSD type 1, but it plays a key part in maintaining supporting stroma adjacent to the tumour epithelial cells in an undifferentiated state so that they may continue to produce oestrogen. It is found in abundance within the breast tumour microenvironment, with the tumour itself thought to contribute to its production (Figure 1.3). Our understanding of the molecular basis for the actions of TNFα in breast cancer is more developed, but many questions are yet to be answered. For example, it has been well established that TNFα induces expression of the aromatase gene *CYP19A1* and that this is via its adipose specific promoter. What has not been identified is by which signal transduction pathway this is occurring, and what specific transcription factors and cis-acting elements are being activated to initiate this response. Defining the exact mechanisms by which TNFα stimulates aromatase transcription is important if we are to target more specifically in the breast this key oestrogenic enzyme. Our understanding of how TNFα contributes to the upregulation of the other oestrogen-forming enzymes is not much more advanced, for whilst their increased activity has been

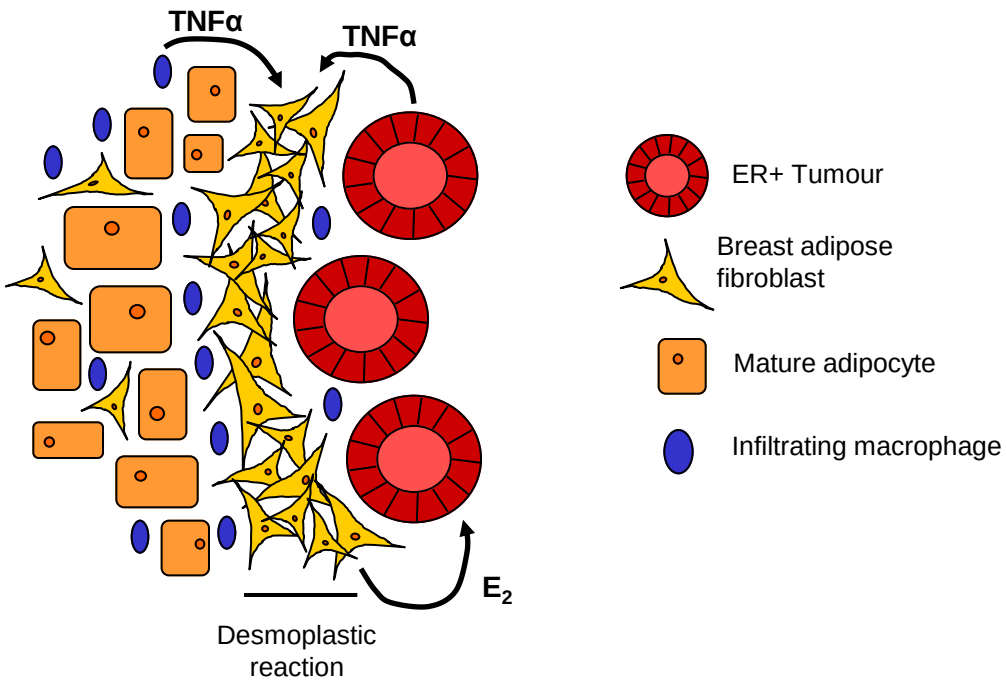

Figure 1.3: Summary of TNFα origins and action in the ER+ breast tumour microenvironment.

TNFα is thought to be derived from both macrophages that have infiltrated the tumour matrix, and from the ER+ breast tumour epithelial cells themselves. TNFα acts to maintain undifferentiated fibroblasts within the desmoplastic reaction, and to promote estrogen biosynthesis within these same cells.

correlated with the presence of TNFα, whether this key cytokine is acting at the transcriptional or translation level and how this is occurring has not been uncovered. Oestrogen formation is a precisely regulated process involving many genes and enzymes, and consideration of all components of the pathway must be given in order to effectively limit hormone production.

Targetting TNFα formation and action has also been proposed as a potential novel method for treating ER+ breast cancer. This would not only contribute to lowering the production of oestrogen in the stromal cells, but effect the desmoplastic reaction so critical to the tumour's pathology. As discussed, TNFα sequestering monoclonal antibodies are in clinical use for the treatment of several inflammatory-related diseases. Although mouse models have shown a positive response to TNFα antibodies with respect to slowed breast tumour growth, such a treatment has to date not been trialled in humans specifically for the treatment of breast cancer. The effects an anti-TNFα treatment would have on the immune system need to be considered, as increased rates of infection have been shown when TNFα is targeted as a signalling molecule. In conclusion, TNFα is a complex cytokine implicated in not only the pathology of ER+ breast cancer but also in the risk of developing the disease. It presents an attractive target for therapeutic intervention due to its multi-function role in the tumour microenvironment, however our limited understanding of the molecular basis for its actions hinder its translational development in to clinical use. Further research in to the basic mechanisms of TNFα secretion and action within the breast is required before we are able to consider it as a drug development target.

1.15. Thesis aims and outline

The aim of this Ph.D. thesis is to examine the roles and regulation of TNFα in the breast tumour environment. Though it has long been established that TNFα is a factor capable of inducing aromatase gene transcription via the distal adipose-specific promoter I.4, the transcription factors induced and the response elements within the promoter responsible for the induction are not yet established. Furthermore, understanding the signalling pathways involved will help in the development of inhibitors of processes. This thesis also seeks to understand the source of excess TNFα within the breast tumour microenvironment, and to determine the factors involved in regulating TNFα secretion.

In vitro and *in vivo* studies have described TNFα as being able to upregulate *CYP19A1* transcripts in a number of cell types, including breast adipose fibroblasts. However, the precise transcription factors that are induced by TNFα signalling and how these interact with the adipose-specific promoter I.4 to activate *CYP19A1* transcription are poorly defined. In this study, it is identified that the Early Growth Response transcription factor family are induced in response to TNFα treatment in BAFs, and that overexpression of EGR2 results in an increase in *CYP19A1* total and PI.4 mRNA transcripts. The region of the promoter PI.4 responsible for mediating EGR induced promoter activity was narrowed down to a 41bp region, however the EGRs are not binding directly or indirectly as part of a transcriptional complex to this region. This suggests that the EGRs are acting indirectly by inducing expression of an intermediate factor, which in turn is then acting within the short promoter region via a novel transcription factor binding site. Furthermore, siRNA-mediated knockdown of all four EGR family members results in a reduction of *CYP19A1* transcripts via PI.4. This work is published in Breast Cancer Research and Treatment (2013, vol. 138(1) pp. 193-203), and is described in **Chapter 3**.

TNFα signals via a number of pathways, most notably NFκB and the MAPK pathway. Whether these are active in BAFs, and whether their activity is further stimulated with TNFα treatment is not explored. This study sought to determine what signalling pathways were active in BAFs, and whether inhibitors of these pathways could attenuate induction of TNFα target genes involved in *CYP19A1* transcription. It is demonstrated that NFκB and MAPK signalling pathways are involved in upregulation of the EGR family members previously described as part of TNFα induced *CYP19A1* expression. Inhibition of these pathways inhibits EGR as well as *CYP19A1* expression. This manuscript is accepted for publication in Biochemical and Biophysical Research Communications (accepted Feb 10th 2013), and is described in **Chapter 4**.

Concentrations of TNFα within the ER+ breast tumour microenvironment are significantly higher than in the circulating plasma, and the source of this excess TNFα is thought to primarily come from infiltrating immune cells such as macrophages and monocytes. It has been hypothesised, however not experimentally explored, that the tumour itself may respond to local oestrogen to upregulate the secretion of TNFα as a tumour derived factor. Oestradiol upregulates mRNA expression and protein secretion of TNFα from the ER+ breast cancer cell

lines, and that this is mediated via ERα. This work was submitted to Tumor Biology, and is described in **Chapter 5**.

In conclusion, the results presented in the following chapters will examine the biological effects of TNFα on regulating oestrogen biosynthesis in the breast by looking at the pathways and transcription factors activated to initiate aromatase gene transcription. The origins and regulation of TNFα from the ER+ tumour will also be explored, providing a more complete picture on the function of this cytokine in breast cancer. These findings will detail a critical regulatory network in breast cancer involving TNFα and oestrogen, and uncover novel targets for therapeutic development.

CHAPTER 2

Materials and methods

2.1 MATERIALS

2.1.1 Ethics approval

Human tissues were obtained from the Avenue Plastic Surgery (Surgeon Dr. Allan M Kalus) and from Corymbia House (Surgeons Mr. Tam Dieu and Mr. James Leong) with full patient consent. Human ethics approval was obtained from the Southern Healthcare Network Human Research Ethics Committee under the project title "Aromatase in adipose – relationship to aging and cancer" with the reference number 00109B.

2.1.2 Chemicals and reagents

All chemicals were obtained from departmental stocks and were of analytical grade or the highest purity available

Acetone	Merck
Acrylamide/BIS 39:1	Bio-rad
Agar	Merck
Agarose LE – analytical grade	Promega
Ammonium persulfate	Sigma Aldrich
Ampicillin	Sigma Aldrich
AMV-RT 5x buffer	Promega
B-Mercapoethanol	Sigma Aldrich
BAY-11-7082	Calbiochem
Boric Acid	Sigma Aldrich
Bovine Serum Albumin	Sigma Aldrich
Bromophenol Blue	Sigma Aldrich
Buffer H	Promega
Dexamethasone	Sigma Aldrich
Dimethyl sulphoxide	Sigma Aldrich
Dithiothreitol	Sigma Aldrich
Deoxyribonucleotides	Bioline
DNase/RNase free water	Gibco

EDTA	Sigma Aldrich
Estradiol	Sigma
Ethanol	Merck
Ethidium Bromide	Sigma Aldrich
Ficoll	Sigma Aldrich
Formaldehyde	Merck
Fugene 6 transfection reagent	Roche
Glycerol	Sigma Aldrich
Glycine	Sigma
Human recombinant TNFα	Sigma
ICI 182-780	Tocris Bioscience
Isopropanol	Merck
NaCl	Sigma Aldrich
Nucleofector cell line solution V	Lonza
Oligo dT primers	Promega
Orange G loading buffer	Sigma Aldrich
Phenylmethanesulfonyl fluoride	Sigma Aldrich
Random hexamer primers	Promega
Sodium dodecyl sulphate	Sigma Aldrich
SP600125 - Inhibitor of JNK1,2,3 and c-Jun	Cell Signalling
TEMED	Biorad
TNFα human recombinant 10µg/ml	Sigma
Tris base	Sigma Aldrich
Tryptone	Oxoid
U0126 - Inhibitor of MEK1/2	Cell Signalling
Xylene Cyanole FF	Sigma Aldrich
Yeast Extract	Sigma Aldrich

2.1.3 Cell Culture reagents

All reagents were kept under sterile conditions

75cm^2 culture flask	Nunc
175cm^2 culture flask	Nunc
6-well culture plates	Nunc
12-well culture plates	Nunc
Antimitotic/antibiotic	Gibco
Biotin	Sigma Aldrich
Dubecco's Modified Eagles Medium (DMEM)	Gibco
Dubecco's Phosphate Buffered Saline	Gibco
DMEM/F-12 1:1	Gibco
Fetal Calf Serum	Gibco
HEPES buffer	Sigma Aldrich
Kanamycin	Gibco
L-Glutamine	Gibco
MEM amino acids	Gibco
MEM vitamins	Gibco
Non-essential amino acids	Gibco
Pantothenic acid	Sigma Aldrich
Penicillin/Streptomycin	Gibco
RPMI Medium	Gibco
Sodium Pyruvate	Sigma Aldrich
Tryple Express	Gibco
Weymouths Medium	Gibco

2.1.4 Cell lines

All cell lines were subcultured under suppliers prescribed conditions

COS7 (ATCC CRL-1651)
HS578t (ATCC HTB-126)
MCF7 (ATCC HTB-22)
MCF10A (ATCC CRL-10317)
T47D (ATCC HTB-133)

2.1.5 Kits

All kits were utilised as per manufacturer's instructions

ChIP-IT express kit	Active Motif
Dual-Luciferase kit	Promega
DNA-free	Ambion
GoTaq PCR Master Mix	Promega
Human TNFα-HS Immunoassay	R&D systems
Quick-change site-directed mutagenesis	Agilent Technologies
pGEM-T Easy	Promega
RNeasy mini kit	QIAGEN
TnT quick-coupled transcription/translation	Promega
Triple Prep	GE Healthcare
Wizard gel extraction	Promega
Wizard DNA purification	Promega
Wizard Miniprep	Promega
Wizard Maxiprep	Promega

2.1.6 Enzymes

All DNA restriction and modifying enzymes were obtained from commercial sources and used as per manufacturer's instructions

AMV-RT	Promega
DNAase 1	Ambion
Pfu DNA polymerase	Fermentas
Restriction Endonucleases	Promega
	New England Biolabs
T4 DNA Ligase	Promega
T4 Kinase	Promega

2.1.7 Radiochemicals

^{32}P-γ-ATP	Perkin Elmer

2.1.8 Nucleic acid molecular weight standards

1kb Plus DNA ladder	Invitrogen

2.1.9 Equipment

3550 UV Microplate reader	Biorad
ABI 7900T Sequence Detection System	Applied Biosystems
Film Developer	Agfa
Humidifying incubator	NU Air
isCUBE-X thermal cycler	Integrated Sciences
Laminar flow hood	Clean air
Light cycler PCR machine	Roche
Microcentrifuge	Eppendorf
Nanodrop spectophotometre	Thermo Scientific
S220 focused ultrasonicator	Covaris
Typhoon Variable Mode Imager	GE Healthcare
Ultracentrifuge	Beckman
Vortex	Selby

2.1.10 Antibodies

Anti-FLAG monoclonal	Sigma Aldrich
Goat Egr4	Santa Cruz
Rabbit Egr1	Santa Cruz
Rabbit Egr2	Santa Cruz
Rabbit Egr3	Santa Cruz

2.1.11 Vectors

pcDNA3	Clonetech
pGEM-T	Promega
pGL-3	Promega
pTAL	Clonetech

2.1.12 Buffers and solutions

Composition of buffers used can be located in Appendix

B-galactosidase assay buffer
ChIP lysis buffer
EMSA gel shift buffer
EMSA TBE sample buffer
EMSA running gel
Orange G Loading buffer
TBE buffer

2.1.13 Bacterial strains

JM109 competent cells	Promega
XL-1 blue competent cells	Agilent Technologies

2.1.14 Bacterial Media

Liquid media

Lysogeny Broth	1% (w/v) NaCl
	1% (w/v) tryptone
	0.5% (w/v) yeast extract

Solid media

Lysogeny-agar plates: 1.5% (w/v) bactoagar was added to Lysogeny broth prior to autoclaving. Antibiotics were added from sterile stock solutions following autoclaving and cooling to less than 55°c.

2.2 GENERAL METHODS

2.2.1 Isolation of nucleic acids

Minipreps

The Wizard SV Miniprep system (Promega) was used to isolate plasmid DNA according to manufacturer instructions.

Maxipreps

The Wizard SV Maxiprep system (Promega) was used to isolate plasmid DNA according to manufacturer instructions.

RNA isolation

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) according to manufacture instructions. Genomic DNA contaminants were removed by treatment with DNase1 (Ambion) according to manufacture instructions.

2.2.2 Analysis of nucleic acids

Agarose gel electrophoresis

The appropriate weight of DNA resolution grade agarose was mixed with 1X TBE and boiled in the microwave until dissolved. The solution was allowed to cool for 5 minutes. 1.5µl of ethidium bromide per 100ml of gel solution was incorporated, then the gel was poured in to the electrophoresis gel apparatus and allowed to set. The gel running buffer was 1X TBE. Plasmid DNA or restriction digested DNA was made up to a final volume of 10µl with 6X orange G loading buffer. A voltage of 100 volts was passed through the gel until the DNA had migrated toward the positive cathode as desired. The agarose gel was then visualised by exposure to ultraviolet (UV) light.

Quantification

2µl of DNA/RNA sample was measured at an absorbance of 260nm against a blank water control in a photo spectophotometre (Thermo Scientific).

Sequencing

Automated sequencing was carried out using the ABI Prism 377 automated sequencer according to manufacturer instructions. Specific oligonucleotides for sequencing reactions are outlined in specific chapters.

2.2.3 Cloning of nucleic acids

Restriction enzyme digestion

DNA was digested with appropriate restriction enzymes and respective buffers supplied by the manufacturers. To ensure complete digestion, 2-5 units of enzyme was added per 1µg of DNA and the reaction was incubated between 1hr and overnight.

Isolation of DNA from agarose gels

DNA fragments of interest were visualised under UV light. Fragments were excised with a scalpel and placed in to a 1.5ml eppendorf tube. The DNA way purified from the gel fragment using the Wizard SV Gel Extraction kit (Promega) according to manufacture instructions.

Ligation of restriction fragments to vector DNA

Ligation reactions were performed in a total 10µl reaction containing 1X ligation buffer (Promega), 1 U of T4 DNA Ligase (Promega), 50ng of prepared vector and 3 molar excess of fragment. Ligations were performed overnight at 4°C or 15°C.

2.2.4 Transformation of plasmid DNA into bacteria

Heat shock transformation

50µl of $CaCl_2$ competent cells were removed from -80°c and allowed to thaw on ice. 50ng of plasmid or 2µl of ligation mix was then added to cells and incubated for 20mins on ice. Cells were then heat shocked at 42°c for 45secs and allowed to recover on ice for 2mins. 1ml of LB media was added to cells and the reaction was incubated for 1.5hr at 37°C on a rotating shaker. Cells were then spun down at 500rpm in a microcentrifuge. Transformed cells were plated on to LB agar plates containing the required antibiotic and incubated overnight at 37°C.

2.2.5 Amplification of nucleic acids

Reverse transcription

A minimum of 200ng total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega) primed by either random hexamers or oligo dT primers, depending of the intended PCR product.

Polymerase chain reaction

Block PCR

Following reverse transcription, the cDNA product was used for PCR amplification. Separate reactions were carried out for individual genes. The total 25µl was comprised of 1X GoTaq Green Master Mix (Promega), 0.5µM of each specific primer and 1µl cDNA product made up to total volume using PCR grade water. Reactions were amplified in an isCUBE-X thermal cycler (Integrated Sciences). All amplifications were initiated with a single cycle 95°C denaturation step for 2mins before denaturing, annealing and extension cycling phases. Amplifications were ended with a final 5min extension cycle at 72°C. For semi-quantitative comparisons of gene expression, PCR reactions were restricted to linear phase of amplification by limiting the number of PCR cycles. PCR products were visualised by agarose gel electrophoresis.

LightCycler capillary PCR

Real time quantification of mRNA levels was conducted using the LightCycler system (Roche) with Fast Start Master SYBR Green I. Standard curves were produced by amplification of DNA, serially diluted to within the pg quantity range, from the gene of interest. Standard curves were used as a reference to determine the amount of mRNA amplified from samples. dCt calculation of crossing point values were also used to calculate absolute fold change gene expression values. All values obtained were standardised to amplification levels of the housekeeping genes *18S* and *β-ACTIN*.

Applied Biosystems PCR

Real time quantification of mRNA levels was conducted using the ABI 7900T sequence detection system (Applied Biosystems) with Power SYBR green detection. Standard curves

were produced by amplification of DNA, serially diluted to within the pg quantity range, from the gene of interest. Standard curves were used as a reference to determine the amount of mRNA amplified from samples. dCt calculation of crossing point values were also used to calculate absolute fold change gene expression values. All values obtained were standardised to amplification levels of the housekeeping genes *18S* and *β-ACTIN*. Specific TaqMan Gene expression assays for the ABI 7900T sequence detection system were also used as detailed in the specific chapters and appendices.

Oligonucleotides

Gene specific oligonucleotides used for amplification of transcripts or plasmids are outlined in specific chapters and appendices. In most instances, the literature was used as a reference to obtain established primer sets. When primers were unavailable, oligonucleotides were designed through the web-based program Primer3 (http://frodo.wi.mit.edu/primer3/). Primers for the purpose of qRT-PCR were designed to span introns to allow for the detection of genomic contamination through size differentials. Oligonucleotides used for mutagenesis were designed as described according to manufacture instructions.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quickchange site directed mutagenesis kit (Stratagene) following the manufacturers protocol.

2.2.6 Tissue culture

All tissue procedures were performed in laminar flow tissue culture hoods using sterilised materials. All cell lines were grown as an adherent monolayer, and incubated at 37°C with 5% CO_2 in a humidifying incubator.

Thawing and freezing of cells

Cell lines were removed from liquid nitrogen and quickly thawed in a 37°C water bath with agitation. Thawed cells were then spun down and excess media aspirated to limit the carryover of DMSO. Cells were then resuspended and added to flasks containing pre-warmed growth medium. For freezing, cells were taken from flasks and pelleted by centrifugation. Cells were then resuspended in media containing 10% DMSO, aliquoted into 1ml cryogenic

tubes and placed in a Cyro 1°C freezing container at -80°C. The following day, cells were transferred for storage in liquid nitrogen.

Sub-culturing of cells

Visual inspection was carried out daily and subcultivation took place once cells were at 80-90% confluency. Media was aspirated, cells washed with PBS then incubated with 2ml Tryple Express (Invitrogen) for 2-3 minutes at 37°C to digest anchor proteins responsible for attaching cells to the surface of the flask. Cells were then diluted into flasks containing fresh medium.

Isolation of Breast Adipose Fibroblasts (BAFs)

Human breast adipose fibroblasts were isolated by collagenase digestion of whole adipose tissue obtained with full ethical consent from women undergoing elective reduction mammoplasty as described by Ackerman et al (1981). Briefly, adipose tissue is separated and incubated with collagenase solution in a plastic bottle at 37°C for 1 hour with vigorous shaking. The digested solution is filter sterilised then spun down to collect a cell pellet, which is washed twice with Kreb's Bicarbonate Buffer. Cells are finally resuspended and seeded in primary culture medium.

Fugene Transfection

Cells were counted using a haemocytometer and seeded in 12-well plates at a density of 40000 cells per 1ml of media. Cells were then grown for 24hrs to enable adhesion and growth prior to the addition of DNA. DNA was added using the Fugene 6 Transfection Reagent (Roche). Briefly, 3µl of Fugene reagent per 1µg of DNA was incubated with serum-free media for 5mins at room temperature. This was then added to prepared DNA, with the total reaction incubated for 30mins at room temperature before addition to cells. In most cases, cells were harvested after 48hrs for assaying.

Nucleofection

Transfection of SGBS and BAFs was performed using electroporation by the Amaxa Nucleofector kit, Amaxa Biosystems (Lonza) using cell line solution V and the pre-adipocyte program T-030. One 175cm^2 flask of cells was used per transfection, and re-seeded in to a single well of a 6-well plate. In most cases, cells were harvested after 48hrs for assaying.

2.2.7 Solutions for breast adipose fibroblast preparation

1M NaOH

Add 40 grams NaOH to 1L of MilliQ water and store at 4°C.

7.5% $NaHCO_3$

Add 75 grams of $NaHCO_3$ to 950mL of MilliQ water. Adjust pH to 7.4 using 1M NaOH. Add water to 1L and store at 4°C.

10X enriching solution

Penicillin/Streptomycin	(10,000u/mL)	100mL
Kanamycin	(100X)	100mL
Non-essential amino acids	(100X)	50mL
Sodium Pyruvate	(100mM)	50mL
MEM amino acids	(50X)	50mL
MEM vitamins	(50X)	50mL
HEPES buffer	(1M)	100mL

Filter sterilise and store at 4°C.

Weymouths medium with enriching solution

To one 500mL bottle of Weymouths plus phenol red, add 5mL antibiotic-antimycotic, 15mL foetal calf serum and 50mL 10X enriching solution. Store at 4°C.

2.2.8 Reporter assays

Cell lysis

Following 48hrs transfection (unless otherwise stated), culture media was removed from plates and cells were washed with 1ml PBS per well. 120µl of 1X Reporter Lysis Buffer (Promega) was added to each well and placed on to a shaker at room temperature for 10mins to lyse cells. Complete collection of lysed cells was ensured by pippetting of lysis buffer. Cells were then collected and placed in to a 1.5ml eppendorf tube and spun down at top speed for 2mins at room temperature to collect cell pellet. The supernatant was then used for reporter assays.

β-Galactosidase assay

30µl of cell lysate was added to a 96-well flat-bottomed clear microplate. 30µl if 2X β-galactosidase assay buffer was then added and the reaction incubated at 37°C for at least 10mins or until colour development was observed. Absorbance was measured at 420nm in a microplate reader for determination of β-galactosidase activity.

Luciferase assay

50µl of cell lysate was added to a 96-well luciferase assay plate. 50µl of reconstituted 2X luciferase assay buffer was then added to each well. Luminescence readings at 405nm were instantly measured for determination of luciferase activity.

2.2.9 Protein production

Proteins were produced using the TnT quick-coupled transcription/translation system (Promega). Translation of protein was verified by running a small aliquot on an SDS-PAGE gel and probing with the appropriate antibody.

2.2.10 Electrophoretic Mobility Shift Assays (EMSA)

Probe Preparation

Annealing

2.25µg of forward and reverse oligonucleotide were added together in a 1.5ml eppendorf tube with 5µl of 10X buffer H (Promega) and made up to 50µl with water. Tubes were incubated in a heat block at 85°C for 5mins. The heat block was then switched off and the tubes allowed to cool slowly in the heat block to room temperature. Annealed probes were stored at -20°C.

Probe labelling

Annealed probes were labelled with γP_{32} ATP (Perkin Elmer) using the T4 Kinase enzyme (Promega). In a 1.5ml eppendorf tube 100pmol of annealed oligonucleotides, 5µl of 10X kinase buffer, 4µl of γP_{32} ATP and 1µl of T4 Kinase enzyme were added, with the total volume made up to 50µl with water. The reaction was incubated for 30mins at 37°C. 2µl of

0.5M EDTA was then added to the tube to stop the reaction, and tubes were again incubated for 10mins at 70°C.

Purification of labelled probes

To isolate radio-labelled probes, the samples were eluted through a G-50 column. Briefly, prepared columns with resin were placed in to a 2ml collection tube. The labelled reaction was slowly applied on to the column and then spun down for 2mins at 735x*g*. The flow through was collected and purified probes stored at -20°C.

Binding Reaction

In a 1.5µl eppendorf tube, 5µl *in vitro* translated protein was combined with 1µl labelled probe, 2µl 0.1M DTT, 1µl of 10mg/ml BSA, 1µl 0.5mg/ml salmon sperm and 4µl of 5X gel shift buffer, with the total volume made up to 20µl with water. Binding reactions were incubated at room temperature for 20mins. For supershift assays, binding reactions were initially incubated for 10mins. 1µl of the appropriate antibody was then added and the total reaction incubated for a further 10mins at room temperature.

Electrophoresis

4µl of 6X TBE sample loading buffer was added to each of the binding reactions. The entire sample was then loaded on to a 4% polyacrylamide gel that had been equilibrated for 30mins, and migrated at 180V in a 0.5X TBE buffer. After sufficient migration, the gel was dried and exposed to radiographic film overnight at -80°C prior to development and imaging.

2.2.11 Chromatin Immunoprecipitation (ChIP)

Treatment and cross-linking of cells

BAFs were grown to ~60% confluency and then starved for 24hrs prior to treatment for 24hrs. To cross link chromatin, cells were incubated in 10mls warmed serum-free media containing 1% formaldehyde for 10mins. Reaction was stopped with the addition of 1ml 1.25M glycine and incubated for a further 10mins.

Sonication shearing of chromatin

Following cross linking, cells were washed twice with ice cold PBS and then scraped with a rubber policeman in 3mls PBS containing PMSF. Cells were spun down for 5mins at 3000rpm at 4°C to collect cell pellet, and PBS thoroughly removed. Cells were resuspended in 130µl ChIP lysis buffer containing PMSF and incubated on ice for 5mins. Sonication shearing of chromatin was performed on the Covaris

2.2.12 Enzyme linked immunosorbent assay (ELISA)

Detection of TNFα protein concentrations was carried out using the Human TNF-alpha Quantikine HS ELISA (R&D systems) following manufacture protocols.

2.2.13 Statistical analysis

Data was analysed using tools within the GraphPad Prism 5 statistical software package. Generally, a one-way analysis of varience (ANOVA) or Student's T-test were performed. The data is presented in this thesis as mean ± standard error of means with $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) considered statistically significant.

CHAPTER 3

Transcriptional regulation of PI.4 by TNFα

Preface to Chapter 3

The cytokine TNFα is abundantly present in the ER+ breast tumour microenvironment. It plays an important role in the upregulation of local oestrogen production through its ability to transcriptionally activate the aromatase encoding gene *CYP19A1* in breast BAFs. Whilst it has been experimentally established for some time that TNFα acts via the adipose specific distal *CYP19A1* promoter I.4, the precise transcription factors and cis-elements activated by TNFα stimulation are undetermined. In this Chapter, the aim was to elucidate the transcription factors which are activated by TNFα in BAFs which are in turn responsible for *CYP19A1* transcription via PI.4.

In a manuscript published in Breast Cancer Research and Treatment (2013, vol. 138(1) pp. 193-203, doi: 10.1007/s10549-013-2413-5; Pubmed ID 23338760), it was demonstrated that the Early Growth Response (EGR) transcription factor family are upregulated by TNFα in BAFs, and that these transcription factors increase the activity of PI.4. This response is mediated via a short 41bp regulatory region towards the 3' end of the promoter region (-27/+14). Although required for full activity, there is no direct or indirect binding of the EGRs to this region suggesting the involvement of at least one other intermediate factor. Regardless, the EGR transcription factors form a critical component of TNFα induced PI.4 transcriptional activation, as siRNA mediated knockdown of all four EGRs results in a significant decrease in total *CYP19A1* transcription via PI.4.

This data establishes for the first time the transcriptional network which, in part, results in adipose-specific aromatase expression as stimulated by TNFα. PI.4 is not only primarily expressed in normal breast, but upregulated in the estrogen-producing adipose immediately adjacent to an ER+ tumour. Therefore, understanding how this promoter is transcriptionally activated is a critical gap in knowledge required for targeted breast-specific aromatase inhibition. By determining downstream factors responsible for TNFα induced aromatase expression, more specific targets may be considered.

PART B: Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3: Transcription regulation of PI.4 by TNFα

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Completed all experimental data, wrote the manuscript	80

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Evan Simpson	Edited the manuscript, financial support	
Kevin Knower	Conceived and helped plan the study, edited the manuscript.	
Colin Clyne	Conceived and helped plan the study, edited the manuscript, financial support	

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Prince Henry's Institute, Level 3 Block E Monash Medical Centre**

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	Evan Simpson 	
Signature 2	Kevin Knower	
Signature 3	Colin Clyne	

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EPIDEMIOLOGY

Involvement of early growth response factors in TNFα-induced aromatase expression in breast adipose

Sarah Q. To · Evan R. Simpson · Kevin C. Knower · Colin D. Clyne

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Abstract Expression of the oestrogen producing enzyme, aromatase, is regulated in a tissue-specific manner by its encoding gene *CYP19A1*. In post-menopausal women, the major site for oestrogen production in the breast is the adipose, where *CYP19A1* transcription is driven by the distal promoter I.4 (PI.4). Transcripts via this promoter are also elevated in breast adipose fibroblasts (BAFs) adjacent to a tumour. PI.4 expression is stimulated by a number of cytokines, and TNFα is one such factor. The transcriptional mechanisms induced by TNFα to stimulate PI.4 are poorly characterised. We show that the early growth response (Egr) transcription factors play an important role in the TNFα-induced signalling pathway resulting in elevated PI.4 transcription. TNFα treatment of BAFs increases mRNA levels of all four Egr family members, with *EGR2* being the most highly expressed. Overexpression of *EGR2* causes an increase in endogenous *CYP19A1* expression in preadipocyte Simpson–Golabi–Behmel syndrome cells, driven by increases in PI.4-specific transcripts. PI.4 luciferase reporter activity is increased in a dose-dependent manner by EGR2, EGR3 and EGR4, with EGR2 showing the most potent activation of promoter activity. Deletion analysis indicates that this promoter activity is being indirectly mediated by a short region of the promoter not containing any previously characterised binding sites, and we further show that EGR2 does not bind directly or indirectly to this promoter region. However, siRNA knockdown of the Egrs reduces the total and PI.4-derived *CYP19A1* transcription in BAFs. These studies unveil a novel component of the aromatase gene regulatory network and further enhance the complexity of oestrogen production in the breast.

Keywords Aromatase · Breast cancer · Adipose fibroblasts · TNFα · Early growth response

Kevin C. Knower and Colin D. Clyne have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10549-013-2413-5) contains supplementary material, which is available to authorized users.

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Introduction

Oestrogen biosynthesis occurs via a series of enzymatic steps from a cholesterol precursor. A key enzyme responsible for this process is the cytochrome P450 aromatase, which performs the conversion of androgens into oestrogens [1]. Aromatase expression is present in oestrogen producing tissue such as the brain, skin, placenta, gonads and adipose [2]. In pre-menopausal women, the ovaries are the main site for oestrogen production [3, 4]; however, in post-menopausal women, peripheral tissues such as the breast adipose are the major sites for oestrogen synthesis [3, 5]. Additionally, breast tumour epithelial cells are also known to express aromatase and contribute to local oestrogen production [6, 7].

Regulation of aromatase activity is mainly at the transcriptional level of its encoding gene, *CYP19A1*. Regulation of *CYP19A1* expression is achieved through the use of a number of tissue-specific promoters spanning up to ~93 kb upstream of the translation start site [8]. Each promoter constitutes an untranslated first exon with specific response elements and is spliced on to a common site 38 bp upstream of the ATG start site to produce a protein identical from exons II through X [9]. The use of these alternative promoters is the basis by which aromatase expression can be regulated in different tissues by various factors, including growth factors, hormones and cytokines [10]. A low level of aromatase expression occurs in breast adipose fibroblasts (BAFs) of disease-free women; however, levels in the presence of an oestrogen receptor-positive (ER+) tumour are strongly elevated up to three- to fourfold, driven primarily through the use of proximal promoters I.3 and II (PI.3, PII) which are highly active in the presence of a tumour [11, 12]. This upregulation provides oestrogen to the tumour, thereby stimulating growth and proliferation. Consequently, aromatase inhibitors are a commonly used therapy for the treatment of post-menopausal ER+ breast cancer [13].

The distal PI.4, which lies approximately 73 kb upstream of exon II, principally mediates *CYP19A1* transcription in normal human BAFs [14]. Several positive *cis*-acting elements have been identified within PI.4 and its 5′ flanking region, including an AP-1 site, an interferon γ activation site (GAS), a glucocorticoid response element (GRE), a Sp1 binding site [15] and a retinoic acid receptor-related orphan receptor response element [16]. In cultured BAFs, PI.4 is stimulated by the combined action of glucocorticoids, such as dexamethasone (DEX) [15], and class I cytokines or TNFα [17–19]. DEX stimulates the glucocorticoid receptor to bind to the GRE [15] within PI.4, whilst the class I cytokines stimulate the JAK/STAT pathway, with phosphorylated STAT3 acting via the GAS element [17]. The mechanisms by which TNFα stimulates PI.4 expression are currently poorly defined.

As an important pro-inflammatory cytokine, TNFα is present within the tumour microenvironment of many cancer types, including pancreatic [20], renal [21], prostate [22], kidney, lung, bladder, oesophageal, melanoma and leukaemia [23]. In breast cancer, increased concentrations of TNFα have been detected in the breast cyst fluid and breast tumour cytosol, both of which are able to stimulate oestrogen production [24]. Furthermore, the presence of TNFα has been strongly correlated to a metastatic, invasive breast tumour phenotype [25, 26]. This observation is consistent with clinical findings and animal studies suggesting that endogenously secreted TNFα in the presence of a tumour exerts a proliferative response rather than tumour regression [27, 28], contrary to the initial isolation of TNFα as a potential anti-cancer agent.

PI.4-derived *CYP19A1* transcripts have previously been reported to be abundant in breast cancer specimens [29, 30], and this is likely attributed to the greater abundance of cytokines within the tumour microenvironment. Indeed, *CYP19A1* transcripts are strongly correlated with TNFα levels in breast tumours, an association which in not present in the disease-free breast [30]. Accordingly, in vitro experiments have shown significant increases in TNFα production in ER+ epithelial cell lines, a result enforced by the presence of secreted TNFα in conditioned media [31]. Infiltrating macrophages and lymphocytes which comprise a large proportion of the tumour stromal mass may also contribute a major source of cytokines [32, 33]. As well as contributing to disease pathology, increased serum levels of TNFα have been correlated with hallmark risk factors of developing post-menopausal ER+ breast cancer, namely advanced age and increased weight [34–36]. It has already been demonstrated that blockade of TNFα signalling by application of a neutralising antibody can retard mouse mammary tumour growth [37, 38], indicating a potential for the development of similar therapeutic strategies in humans.

We hypothesise that TNFα induces a distinct signal transduction pathway in the human breast to stimulate *CYP19A1* transcription via PI.4, thereby regulating local oestrogen biosynthesis. In this study, we demonstrate that the early growth response (Egr) transcription factors play a key role in the pathway mediating PI.4 transcription in the breast following induction by TNFα.

Materials and methods

Plasmids

A PI.4 luciferase reporter construct containing 1,018 bp of the 5′ regulatory region upstream of exon I.4 and subsequent deletion constructs have been previously described by our group [39]. The two shortest 5′ deletion constructs, −95/+14 and −27/+14, were created by PCR and restriction enzyme digest, followed by cloning into pGL3-basic luciferase reporter vector (Promega). Expression vectors encoding human *EGR1*, *EGR2*, *EGR3* and *EGR4* with a FLAG tag were prepared by PCR amplification from human BAFs and subcloned into pcDNA3.1 vector (Promega). Egr/Sp1 binding site mutants were prepared using the Quickchange site-directed mutagenesis kit (Stratagene) following the manufacturers protocol. The specific mutations induced were mutant one (GGGCGGGGC → TTTCGGGGC); mutant two (GGGCGGGGC → GGGCGTTT); mutant three (GGGCGGGGC → TTTTTTTTC).

Cell culture

BAFs were isolated from breast reduction mammoplasty by collagenase digestion and cultured as previously described [40] with appropriate human ethics approval. COS7 cell line was cultured in DMEM supplemented with 10 % fetal bovine serum, penicillin G-sodium (100 units/ml) and streptomycin sulphate (100 μg/ml) (Invitrogen) and L-glutamine (Invitrogen). Human pre-adipocyte cell line Simpson–Golabi–Behmel Syndrome (SGBS) has been previously described as morphologically, biochemically and functionally similar to pre-adipocytes isolated from human donors [41]. They express aromatase in a similar fashion to human BAFs [42]. SGBS cells were cultured at subconfluency in DMEM supplemented with 10 % FCS, penicillin/streptomycin, biotin (33 μM) and pantothenic acid (17 μM) (Sigma). To stimulate TNFα-dependent cell signalling pathways, BAFs were grown to ~60 % confluency and then incubated in serum-free media containing 0.1 % BSA (Sigma) for 24 h. Serum-free media containing 5 ng/ml TNF (Sigma) was then added to the cells for the specified period of time.

Transient transfection and reporter assay

COS7 cells were co-transfected with PI.4 luciferase reporter constructs or the promoter-less pGL3-basic vector, expression vectors or the empty pcDNA3.1 vector using Fugene 6 transfection reagent (Roche). β-Galactosidase was transfected as an internal control vector. After a 48-h transfection, firefly luciferase activity was measured using the Luciferase Assay System (Promega) and β-galactosidase activity was measured by β-galactosidase assay (Promega). SGBS cells were transfected using electroporation (Nucleofector kit V, Amaxa Biosystems, Lonza, Walkersville, MD, USA) using cell line solution V and the pre-adipocyte program T-030. Cells were lysed for RNA collection or luciferase assays 48 h after transfection.

siRNA transfection

siRNA pools directed against EGR1 (sc-29303), EGR2 (sc-37827), EGR3 (sc-35268) and EGR4 (sc-37829) as well as control siRNA-A (sc-37007) (Santa Cruz) were transfected into BAFs using electroporation (Nucleofector kit V, Amaxa Biosystems, Lonza, Walkersville, MD, USA) using cell line solution V and the pre-adipocyte program T-030. 24 h after transfection, cells were serum starved in serum-free media containing 0.1 % BSA for 24 h. Cells were then treated with serum-free media containing 5 ng/ml TNFα (Sigma) and 250 nM DEX (Sigma) for 24 h.

Quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Mini kit (QIAGEN). cDNA synthesis was performed on a minimum of 200 ng of total RNA using avian myeloblastosis virus reverse transcriptase (Promega) primed by oligo dT primers. Quantitative RT-PCR (qRT-PCR) of *EGR1*, *EGR2*, *EGR3* and *EGR4* transcripts was performed using SYBR green detection on the ABI 7900T sequence detection system. *18S* or *β-ACTIN* transcripts were also detected as an internal RNA loading control. Detection of total *CYP19A1* transcripts was performed on the Roche LightCycler System (Roche Diagnostics) using Fast Start Master SYBR Green I or the ABI 7900T sequence detection system using the *CYP19A1* TaqMan Gene Expression assay (Hs00903413_m1) (Applied Biosystems). Primer sequences are as follows: *EGR1* (sense, CAGTGGCCTA GTGAGCATGA; anti-sense, CCGCAAGT GGATCTTGG TAT); *EGR2* (sense, TTGACCAGATG AACGGAGTG; anti-sense, GTTGAAGCTGG GGAAGTGAC); *EGR3* (sense, CAATCTGTACCCCGAGGAGA; anti-sense, GGAAGGAG CCGGAGTAAGAG); *EGR4* (sense, CGCGCTCCTCGT CAAGTC; anti-sense, GCTCA AGAA GTCGCCTGCTC); total *CYP19A1* (sense, TTGGA AA TGCTGAACCCGAT; anti-sense, CAGGAATCTGCCG TGGGGAT); *18S* (sense, CGGCTACCACATCCAAGGA; anti-sense, GCTGGAATTACCGCGGCT) and *β-ACTIN* (sense, TGCGTGACATT GCGTGACATTAAGGAGAAG; anti-sense, GCTCGTA GCTCTTCTCCA). Detection of PI.4 transcripts was performed on the ABI 7900T sequence detection system as previously described [43].

Electrophoretic mobility shift assay

In vitro translation of proteins was carried out using the TnT quick-coupled transcription/translation system (Promega). Probes were labelled with ^{32}P-γ-ATP (Perkin Elmer) using T4 Kinase (Promega) and purified using Microspin G50 columns (Amersham Biosciences). Following binding of in vitro-translated proteins with or without an anti-FLAG antibody to probes, reactions were run on a 0.5 % TBE acrylamide gel in 0.5× TBE buffer. The gel was then exposed to radiographic film for detection. The sequences of the probes used are as follows: PI.4 (AAACTGGCTCCTGGCTCCAAGTAGAACGTGACCA ACTGGA); *EGR* positive control (GGATCCAGCGGG GGCGAGCGGGGGCGA); *EGR* negative control (GGAT CCAGCTAGGGCGAGCTAGGGCGA).

Chromatin immunoprecipitation

BAFs were serum starved in serum-free media containing 0.1 % BSA (Sigma) for 24 h. Cells were then treated with serum-free media containing 5 ng/ml TNF (Sigma) and

250 nM DEX (Sigma) for 24 h. Cells were fixed in serum-free media containing 1 % formaldehyde for 10 min. The reaction was stopped with 1.25 M glycine. DNA was sheared using sonication and immunoprecipitated with an EGR2 antibody (Santa Cruz). Chromatin was then extracted using the chromatin immunoprecipitation (ChIP)-IT express chromatin immunoprecipitation kit (Active Motif). Primers used for ChIP PCR are as follows: PI.4 (sense, ATGACCAACCAAGACTAAGAG; anti-sense, CAGTTGGTCACGTTCTACTTGG) and MMP9 (sense, AGGCTGCTACTGTCCCCTTT; anti-sense, CTCCCTGACAGCCTTCTTTG).

Statistical analysis

Student's *T* test was applied to assess statistical significance of result comparisons. Analysis was performed using Graph Pad Prism 5 statistical software.

Results

Expression of Egr transcription factors in response to TNFα

TNFα has previously been shown to induce expression of the Egr transcription factors in endothelial cells, kidneys and osteoblast-like cells [44–46]. The potential role of the Egrs in BAFs has not yet been studied. Therefore, qRT-PCR was performed to assess the effects of TNFα on the expression of Egr transcription factors in human BAFs. mRNA levels of *EGR1*, *EGR2*, *EGR3* and *EGR4* were increased in BAFs in a time-dependent manner in response to TNFα stimulation (Fig. 1a–d), with significant upregulation seen after approximately 20 min. Induction of *EGR2* expression was shown to be the highest of the family members in response to TNFα, with a maximal sevenfold induction observed after 90 min of treatment. This compares with a sixfold *EGR3* induction. The weakest response to TNFα treatment in BAFs was *EGR1* mRNA, where a twofold induction was observed. *EGR4* expression was very weakly detected, and although results indicated a moderate induction response to TNFα stimulation, this was not significant. Basal expression levels indicated that in BAFs, *EGR2* is the most highly expressed, followed by *EGR3* and *EGR1* (data not shown).

Overexpression of Egr2 increases CYP19A1 expression via PI.4 in SGBS cells

The human pre-adipocyte cell line SGBS was used as a model to study the effects of Egr overexpression on endogenous *CYP19A1* expression. This cell line has previously been described to express *CYP19A1* in a similar fashion to isolated primary human BAFs and also respond to external stimuli with respect to regulation of aromatase

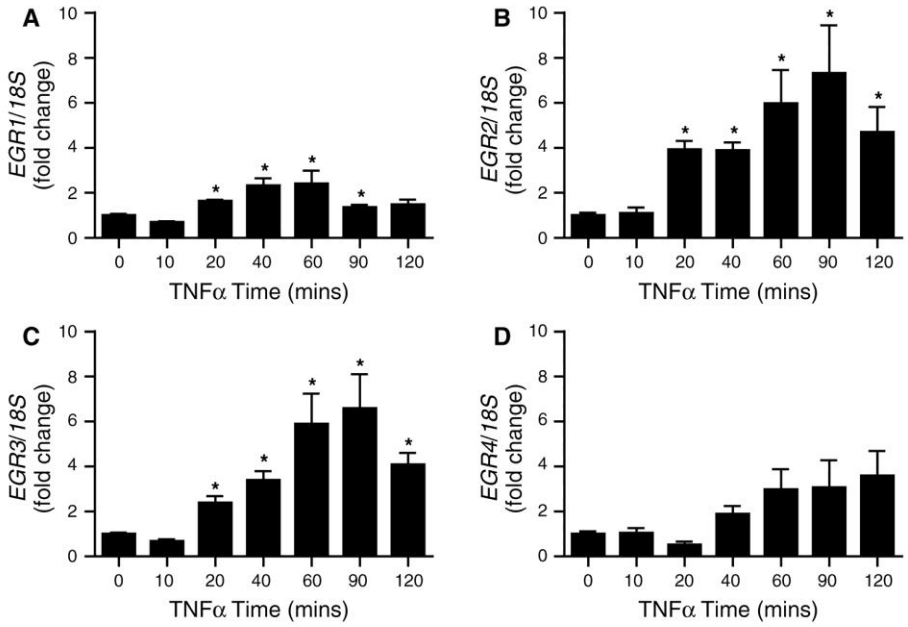


Fig. 1 qRT-PCR on RNA isolated from BAFs stimulated over a time course period with TNFα (5 ng/ml). Data obtained from three independent experiments conducted in triplicate. *Error bars* represent standard error of mean, data were analysed by independent *T* tests of grouped means (*$p < 0.05$)

expression [42]. We chose EGR2 as a representative Egr family member, as previous studies have indicated it may promote adipogenesis [47, 48]. Overexpression of *EGR2* in SGBS cells (Fig. 2a) led to a significant sevenfold increase in endogenous *CYP19A1* expression compared to empty vector control (Fig. 2b). This was driven by an increase in PI.4-specific transcripts (Fig. 2c). PI.3/II transcripts could not be detected in either control or EGR2 overexpressed cells (data not shown).

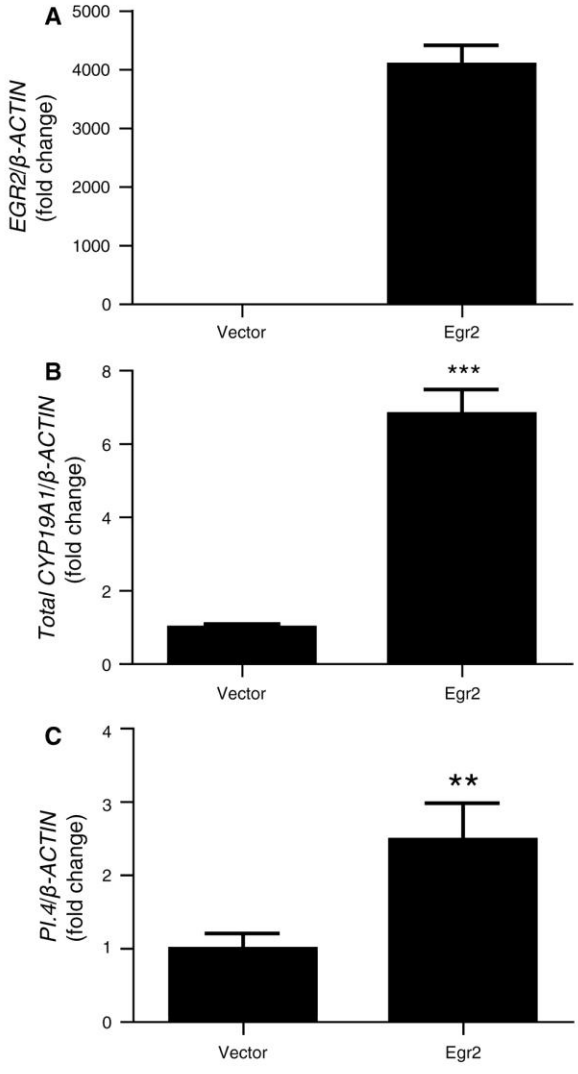


Fig. 2 **a** qRT-PCR showing overexpression of *EGR2* in SGBS cells that have been transfected with 2 μg EGR2 expression plasmid as a fold increase over *EGR2* levels in empty vector transfected cells. Representative qRT-PCR from three independent experiments. **b** qRT-PCR on SGBS cells transfected with an empty vector control or EGR2 expression plasmid showing total *CYP19A1* levels normalised to *β-ACTIN*. **c** Promoter-specific qRT-PCR demonstrating a significant increase in PI.4-specific transcripts in SGBS cells overexpressing *EGR2*. Data obtained from three independent experiments. *Error bars* represent standard error of mean, data were analysed by independent *T* tests of grouped means (**$p < 0.01$, ***$p < 0.001$)

Egrs activate PI.4 reporter activity

The potential for the family of Egr transcription factors to directly interact with PI.4 to mediate *CYP19A1* transcription was assessed via a luciferase reporter assay. *EGR2* was able to increase PI.4 reporter activity in SGBS cells (Supplementary Fig. 1); however, the greater sensitivity of the assay in COS7 cells led us to perform further experiments using this cell line. Co-transfection of a PI.4 luciferase reporter construct with an *EGR1* expression vector into COS7 cells did not result in any detectable luciferase induction (Fig. 3a). However, co-transfection of an *EGR2* expression vector resulted in a dose-dependent increase in luciferase activity up to a maximal 70-fold (Fig. 3b). Similar results were obtained with *EGR3* (Fig. 3c) and *EGR4* (Fig. 3d) co-transfection, with 30- and 8-fold maximal inductions observed, respectively. EGR1 moderately increased luciferase activity of the empty pGL3-basic control vector; however, EGR2, EGR3 and EGR4 showed no such activity (Supplementary Fig. 2).

Deletion of an Egr response element within PI.4 does not lower Egr2-driven PI.4 luciferase activity

Promoter analysis of the PI.4 regulatory region revealed that the previously identified Sp1 binding site may also serve as an overlapping Egr binding site. To confirm if this was the site responsible for mediating the Egr-induced PI.4 activity, a series of 5′ deletion constructs containing progressively shorter lengths of PI.4 upstream of a luciferase reporter were utilised (Fig. 4). The 41 bp sequence of the shortest construct (−27/+14) did not contain the potential overlapping Egr/Sp1 response element that has previously been identified [15]. Co-transfection of these reporter constructs together with the most potent inducer of PI.4 activity, EGR2, resulted in elevated luciferase reporter activity in all constructs, up to and including the shortest sequence lacking any of the previously characterised response elements (Fig. 4). Additionally, mutation of the Egr/Sp1 site did not abolish EGR2-, EGR3- or EGR4-induced PI.4 reporter activity (Supplementary Fig. 3).

EGR2 does not bind to PI.4

In order to determine whether there was direct interaction of EGR2 to PI.4 sequences, we radioactively labelled the 41 bp sequence that constituted the shortest luciferase construct and used it as a probe for electrophoretic mobility shift assay (EMSA). No specific binding of in vitro-translated EGR2 could be detected to the PI.4 probe, as the

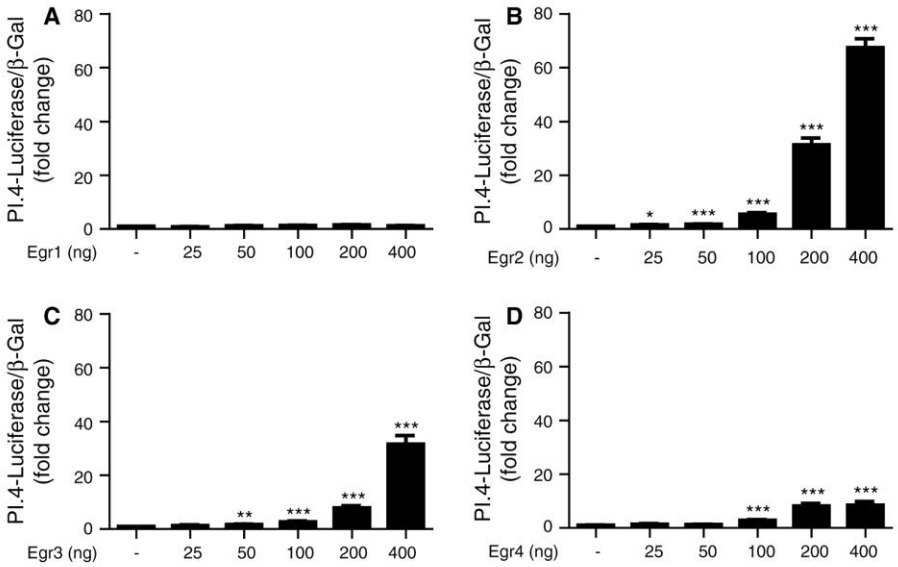


Fig. 3 Luciferase assays showing **a** EGR1-, **b** EGR2-, **c** EGR3- and **d** EGR4-induced PI.4 activity in COS7 cells. Increasing concentrations (ng) of EGR expression plasmid were co-transfected with a PI.4 luciferase reporter. Data shown are from three independent experiments conducted in duplicate. Results are expressed as mean luciferase activity in relative light units (RLU) normalised to β-galactosidase activity, with *error bars* representing standard error of mean. *T* test was used to assess statistical significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

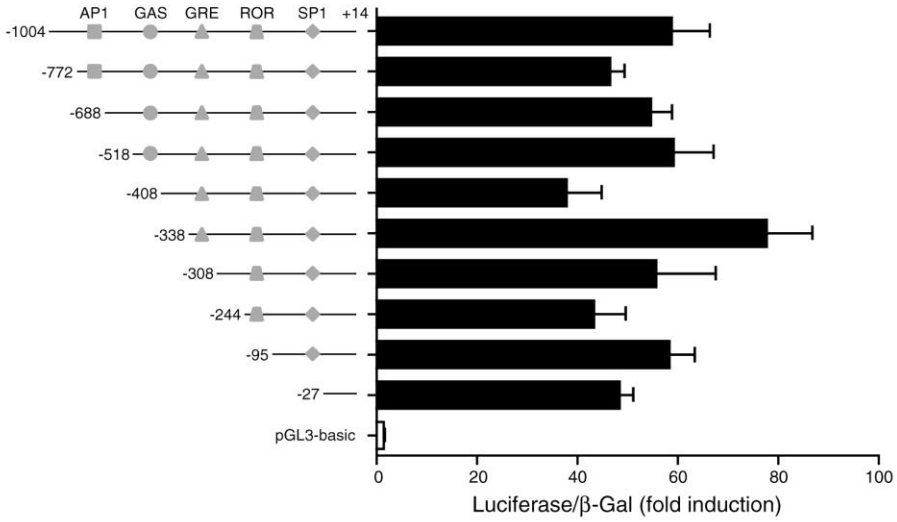
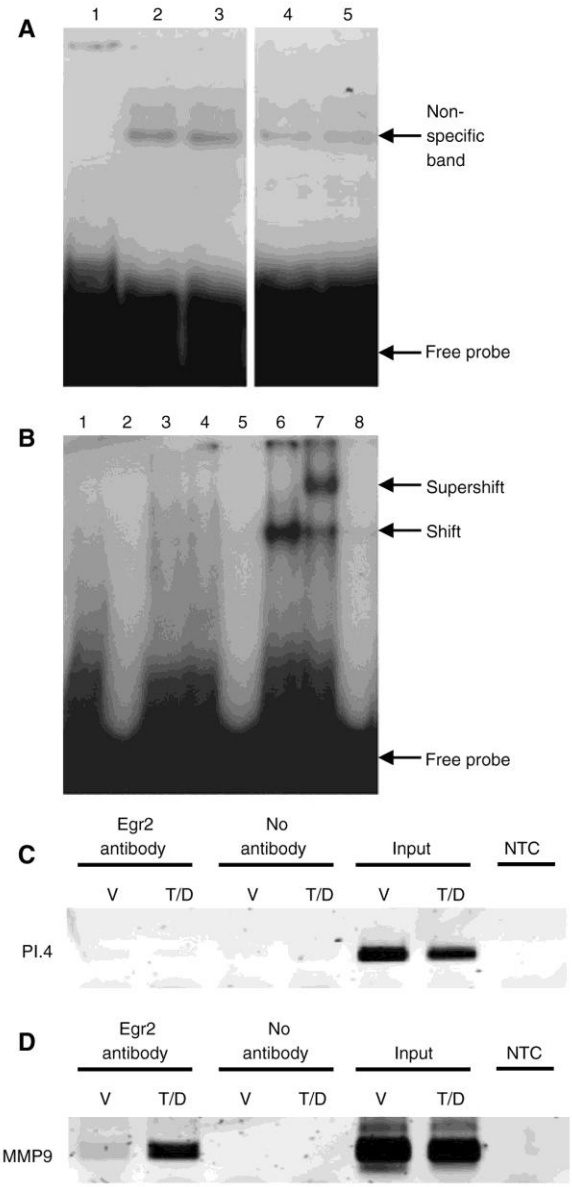


Fig. 4 Luciferase assays showing Egr2 induction of serially 5′-deleted PI.4-luciferase reporter constructs in COS7 cells. Data shown are from three independent transfections conducted in duplicate, with results expressed as mean luciferase activity in RLU normalised to β-galactosidase activity. *Error bars* represent standard error of mean

addition of an anti-FLAG antibody did not cause a supershift in any band observed (Fig. 5a). This is despite the in vitro-translated EGR2 being able to bind specifically to a positive control Egr consensus binding sequence probe (Fig. 5b). We also assessed whether EGR2 was binding indirectly as part of a transcriptional complex through use of ChIP. ChIP PCR spanning the PI.4 region including the putative Sp1 site and the 41-base pair region of interest (+56/+215) was performed on chromatin immunoprecipitated with an EGR2 antibody. No basal of TNFα/DEX-induced binding of Egr2 to PI.4 could be detected (Fig. 5c), although biding of EGR2 to an Egr site on the MMP9 promoter as a positive control was detected (Fig. 5d) as described by Shin et al. [49].

siRNA knockdown of the Egr family reduces basal levels of CYP19A1

Despite showing that the Egr transcription factor family was not directly mediating the induction of PI.4 in response



◀**Fig. 5** **a** EMSA demonstrating that there is no specific binding of any of the Egr family members to the PI.4 region demonstrated to be mediating promoter activity. *Lane 1* contains the ^{32}P-labelled PI.4 probe alone. Incubation of this probe with an in vitro-translated pcDNA3.1 empty vector control results in the formation of a non-specific band shift (*lane 2*), and addition of an anti-FLAG antibody does not alter this complex (*lane 3*). Incubation of in vitro-translated Egr2 results in the same non-specific band (*lane 4*), and the failure of an anti-FLAG antibody to supershift the complex confirms that there is no specific binding (*lane 5*). **b** EMSA showing binding of in vitro-translated Egr proteins to a positive control Egr consensus binding sequence probe. *Lane 1* contains the ^{32}P-labelled Egr consensus binding sequence probe, whilst *lane 2* contains a ^{32}P-labelled mutated Egr consensus binding sequence probe (Egr+). *Lane 3* shows that incubation of in vitro-translated pcDNA3.1 empty vector control results in no band complex formation, nor does the addition of an anti-FLAG antibody (*lane 4*). In vitro-translated pcDNA 3.1 also does not bind to the mutated Egr consensus binding sequence probe (Egr−) (*lane 5*). In vitro-translated EGR2 does cause a band shift complex with the Egr consensus binding sequence probe (*lane 6*), and a supershift created by the addition of an anti-FLAG antibody confirms this binding is specific (*lane 7*). This in vitro-translated EGR2 does not bind to the mutated Egr consensus binding sequence probe (*lane 8*). **c** ChIP showing no occupancy of EGR2 on PI.4 in the presence (*T/D*) or absence (*V*) of TNFα and DEX. Immunoprecipitation was performed with an anti-EGR2 antibody on chromatin isolated from BAFs. No antibody and no template (NTC) were used as negative controls. **d** ChIP showing increased occupancy of EGR2 on the MMP9 promoter following treatment with TNFα and DEX. Immunoprecipitation was performed as described for PI.4 using the same chromatin

to TNFα, we wanted to demonstrate the importance of these factors in aromatase regulation. siRNA constructs directed against each of the four EGR family members were transfected into BAFs in order to determine the effect of EGR knockdown on *CYP19A1* transcript levels. siRNA transfection resulted in an approximately 50 % reduction in *EGR1* transcripts, 80 % reduction in *EGR2* transcripts and 80 % reduction in *EGR3* transcripts. EGR4 transcripts were not detected in control or siRNA-transfected cells (Fig. 6a). Knockdown of all four EGRs resulted in a 50 % reduction in basal total *CYP19A1* transcripts and 30 % reduction in total *CYP19A1* transcripts in BAFs treated with TNFα and DEX (Fig. 6b). Semi-quantitative PCR was performed to assess the relative contribution of PI.4-specific transcription to the overall reduction in the total *CYP19A1* transcripts detected. In TNFα/DEX-stimulated BAFs, PI.4 transcription was strongly reduced in cells transfected with EGR siRNAs. This contributes to the reduction in total *CYP19A1* transcripts also observed, showing that the Egr transcription factors are critical to PI.4 activation (Fig. 6c).

Discussion

In the present study, we have demonstrated that the family of EGR transcription factors, primarily through work using EGR2, activates *CYP19A1* expression via PI.4-specific transcripts. This is a downstream effect of TNFα stimulation in BAFs, which is physiologically relevant given the increasing basal levels of this cytokine in obese and ageing women and increased concentrations in an ER+ breast tumour microenvironment.

TNFα is integral to two interrelated processes within the ER+ breast tumour microenvironment. Firstly, it plays a significant role in the desmoplastic reaction, maintaining a population of dense undifferentiated BAFs adjacent to a tumour. It does this by inhibiting the expression of key pro-adipogenic proteins such as C/EBPα and PPARγ [31, 50]

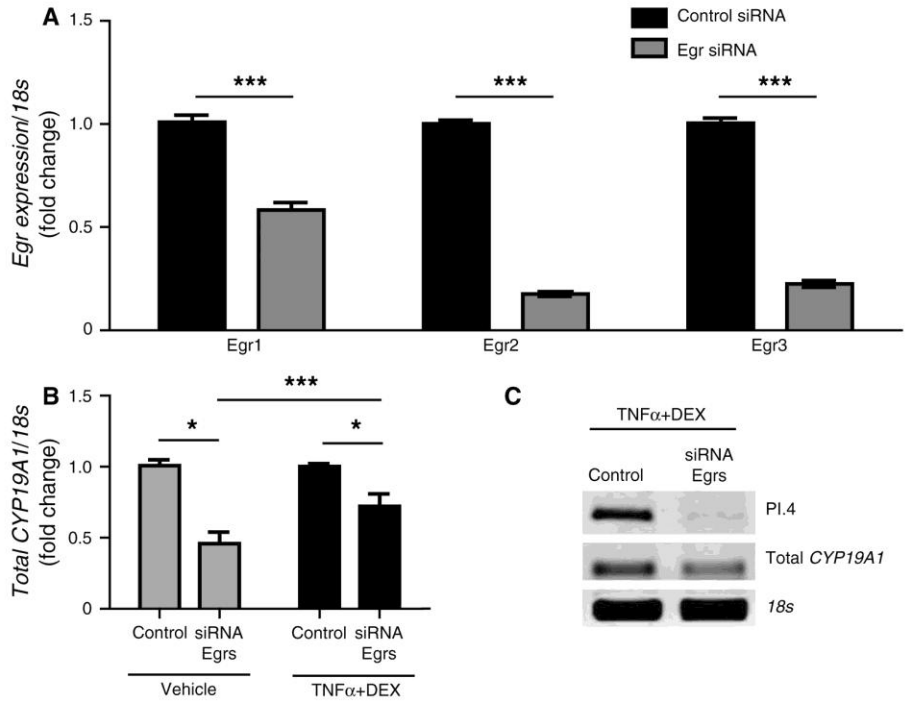


Fig. 6 siRNA knockdown of Egr transcription factors in BAFs. **a** RT-PCR showing transcript levels of the Egr family members in BAFs transfected with a control siRNA or a pool of Egr-targeted siRNAs. **b** RT-PCR showing levels of total *CYP19A1* transcripts in BAFs transfected with a control siRNA or a pool of Egr-targeted siRNAs, treated with either vehicle or TNFα and DEX for 24 h. **c** Semi-quantitative PCR showing PI.4-specific and total *CYP19A1* expression in BAFs transfected with a control siRNA or a pool of EGR-targeted siRNAs and treated with TNFα/DEX. *Error bars* represent standard error of mean, *T* test and a two-way ANOVA were used to assess statistical significance ($*p < 0.05$, $***p < 0.001$)

to prevent maturation. This is critical, as only in their undifferentiated form may these pre-adipocytes express the necessary enzymes, including aromatase, to synthesise oestrogens [51]. Whilst the means by which TNFα participates in the desmoplastic reaction are well defined, this is the first study to elucidate some of the molecular mechanisms responsible for TNFα-mediated oestrogen biosynthesis via regulation of *CYP19A1* transcription.

We show TNFα induced the upregulation of EGR family gene expression in BAFs, leading indirectly to an increase in PI.4-specific *CYP19A1* transcripts. What remains to be uncovered, however, is whether this same effect is occurring in ER+ breast tumour epithelial cells. Aromatase transcripts are also detectable in the epithelial cells as well as the surrounding BAFs, and TNFα is known to contribute to the tumour phenotype of ER+ epithelial cells [7, 26]. TNFα treatment of MCF7 cells, an ER+ breast epithelial cell line, results in time-dependent upregulation of EGR2 and EGR3 in a similar pattern to that observed in BAFs (data not shown). This suggests that EGRs may be contributing to TNFα-induced *CYP19A1* expression in breast epithelial cells also, although whether TNFα is a contributing factor to increased aromatase expression, particularly via PI.4, in epithelial cells remains to be uncovered.

Our results indicate a critical 41 base pair region encompassing the PI.4 transcriptional start site which appears to be responsible for EGR2-mediated induction. This is in contrast to previous studies which have suggested the AP-1 element further upstream is activated in BAFs in response to TNFα [18]. 5′ deletion analysis of the ~1 kb region encompassing all identified upstream elements revealed that promoter activity was retained when the AP-1 element was removed, suggesting that this response element is not involved in transcriptional induction. Additionally, the SP1 site previously found to bind proteins in response to serum [15] was not required for EGR2 induction, despite the response elements for SP1 and EGR2 being similarly CG rich [52]. Mutation of this site to disrupt any potential EGR2 binding did not result in abolishment of luciferase response (Supplementary Fig. 3), indicating that EGR2 effects were not direct. This finding was supported by ChIP showing no basal or TNFα/DEX-enriched binding of EGR2 to the same region. Due to the number of different cytokines and glucocorticoids able to

induce PI.4, it is possible that these elements are indeed involved in PI.4 transcription, but not in response to TNFα.

In our studies, we identified that overexpression of EGR2 in the pre-adipocyte cell line SGBS caused an increase in endogenous PI.4-specific *CYP19A1* transcripts, indicating that Egr-dependent signalling mechanisms played a direct role in *CYP19A1* expression. EGR2 has previously been identified as a pro-adipogenic factor, activating key proteins involved in differentiation of pre-adipocytes into mature adipocytes [47, 48]. As previously discussed, TNFα is a key factor which helps to maintain the dense undifferentiated fibroblastic area adjacent to a tumour. Our studies suggest that TNFα signalling leads to an upregulation of *EGR2*, followed by a resultant increase in *CYP19A1* expression. These findings are seemingly in contrast to previous studies, which showed that an increase in *EGR2* transcript could promote adipogenesis. The differentiated cells would therefore lose their ability to express aromatase and thus synthesise oestrogen. Further work is needed in order to further define the role of *EGR2* in adipogenesis and oestrogen biosynthesis, to assess whether it indeed promotes or represses the desmoplastic reaction and tumour progression.

siRNA-mediated knockdown of all four EGR family members revealed that basal levels of *CYP19A1* transcripts were decreased as well as TNFα/DEX-stimulated levels via PI.4, although not to as great an extent. This suggests that the Egr transcription factors contribute to the TNFα/DEX-mediated response; however, other independent factors are also involved which still maintain levels of *CYP19A1*. A reduction in aromatase transcripts upon siRNA knockdown of EGR transcription factors does, however, present the possibility that the EGRs could be targeted in anti-aromatase therapies. Given the multi-factorial nature of aromatase regulation in breast cancer via a number of distinct promoters and regulatory pathways, a gene silencing approach would need to involve a number of factors. We have identified in this study that the EGR family of transcription factors may form part of such targeted therapies. siRNA-directed gene silencing therapies have already been trialled for the treatment of bladder cancer [53], melanoma [54] and prostate cancer [55], targeting different genes and using a number of delivery techniques. Silencing of EGR transcription factor action may be a possible therapeutic approach in inhibiting aromatase activity in ER+ breast cancer, although the ubiquitous nature of their expression may present challenges in translation to the clinic.

The specific downstream factor which is directly responsible for PI.4 activation in response to TNFα is still to be determined. Temporally, *EGR2* acting as an intermediate factor in the context of PI.4 transcription is consistent with this transcription factor being an early response factor, exerting its effects readily upon activation [56]. Conversely, in vitro cultured cells require around 24 h of stimulation by known factors before *CYP19A1* transcripts may be detected [18]. This lapse in time frame would allow for a continuation of the TNFα-induced signalling pathway downstream of EGR2 activation, allowing it to further transcribe or activate other factors. Few target genes of EGR2 have been identified, and its main role that has been characterised to date is in brain and nervous system development [57, 58]. EGR2 has been identified as a transcriptional activator of ErbB2, associated with poor prognosis in approximately 20 % of human breast cancers [59], indicating it may have a role in tumour progression. More target genes have been identified for EGR1, and these include HIF-1α [60], VEGF, Jun proteins and IGF2 [61]. All of these have previously been indicated as playing a role in the progression of breast cancer [62–65]. Additionally, JunB and JunD are known to activate *CYP19A1* gene transcription via the proximal tumour PII [62]. Given the common DNA binding site shared by the Egr family members [56], it is possible that EGR2 may also regulate the expression of these genes and thus play a pivotal role in breast cancer progression. What remains to be seen is whether any of these factors are able to bind directly to PI.4 in order to activate its transcription and contribute specifically to oestrogen excess in ER+ tumours. Analysis of the 41 base pair sequence pertaining to our PI.4 region of interest reveals a number of potential transcription factor binding sequences, including those for c-Myb, C/EBPβ and HIF-1α. Interestingly, HIF-1α has previously been demonstrated to be a downstream target of both TNFα and the Egr transcription factors [66, 67], lending itself as a potential candidate for further investigation. Identification of factors involved both downstream and upstream of EGR2 activation would allow us a better understanding of the mechanisms driving TNFα-induced oestrogen biosynthesis and pave the way for manipulation of such processes for therapeutic intervention.

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Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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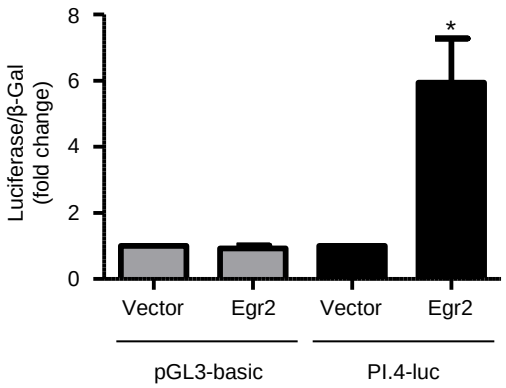
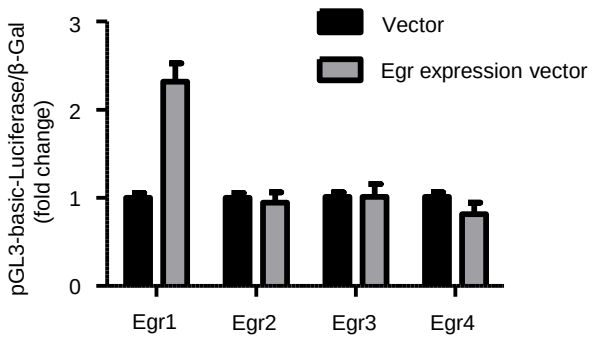
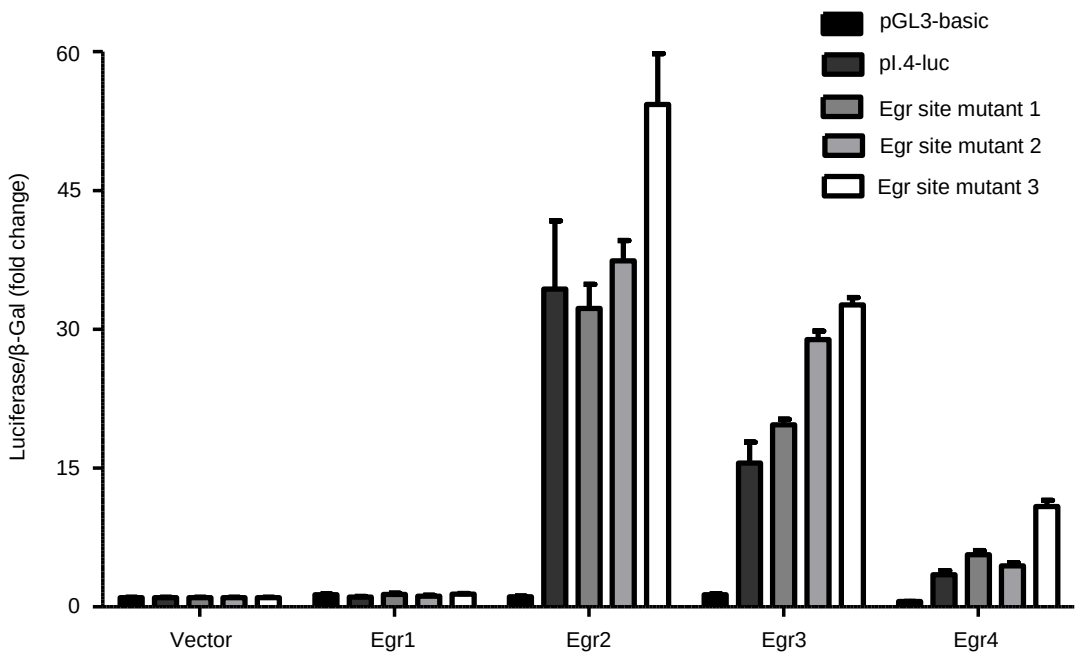


Supplementary Figure 1: Luciferase assay showing EGR2 (1.2µg) induction of a PI.4-luc reporter construct in SGBS cells. Data shown is from three independent transfections, with results expressed as mean luciferase activity in RLU normalised to β-galactosidase activity. Error bars represent standard error of mean.



Supplementary Figure 2: Luciferase assay showing EGR1 (400ng), EGR2 (400ng), EGR3 (400ng) and EGR4 (400ng) induction of the empty pGL3-basic luciferase vector in COS7 cells. Data shown is from three independent transfections, with results expressed as mean luciferase activity in RLU normalised to β-galactosidase activity. Error bars represent standard error of mean.



Supplementary Figure 3: Luciferase assay showing EGR1, EGR2, EGR3 and EGR4 induction of a PI.4-luc reporter construct containing a mutation in the potential Egr consensus binding site in COS7 cells. Data shown is from three independent transfections conducted in duplicate, with results expressed as mean luciferase activity in RLU normalised to β-galactosidase activity. Error bars represent standard error of mean.

CHAPTER 4

TNFα induced NFκB and MAPK signalling activates aromatase

Preface to Chapter 4

In Chapter 3, the research focus was on determining what transcription factors and cis-elements were activated by TNFα in BAFs to initiate transcription of PI.4-specific *CYP19A1* transcripts. EGR2 and EGR3 transcription factors were found to be upregulated in BAFs stimulated by TNFα, and these initiated promoter reporter activity of PI.4. The transcriptional regulation of EGR2 and EGR3, as primarily it has been the representative family member EGR1 which has been examined in the literature. In Chapter 4, the aim was to determine the signal transduction pathways initiated by TNFα that are upstream of EGR2 and EGR3 in BAFs, ultimately responsible for adipose-specific aromatase induction.

In a manuscript accepted to Biochemical and Biophysical Research Communications (10th February 2013, doi: 10.1016/j.bbrc.2013.02.058), it was demonstrated that the NFκB pathway is active in BAFs, and that its activity is further stimulated upon TNFα treatment. When BAFs were treated with the NFκB pathway inhibitor BAY-11-7082, the TNFα and Dexamethasone induced PI.4-specific and total *CYP19A1* induction was dose dependently reduced. This may be a consequence of the initial down regulation of TNFα-induced *EGR2* and *EGR3* transcription, which is inhibited by BAY-11-7082. Similarly, inhibition of the MAPK signalling component MEK 1/2 had the same effect by decreasing transcript levels of *EGR2*, *EGR3* and *CYP19A1* transcripts mediated via PI.4. However, when a chemical inhibitor of Jnk1,2,3 signalling was applied to BAFs, the same effects were not seen, indicating that this component of MAPK signalling was not critical to *EGR* regulation or aromatase induction.

The data in this Chapter highlights the signalling cascades downstream of TNFα stimulation that are required for PI.4-specific aromatase transcription via the important role of the EGR transcription factors. Inhibition of key signalling pathways often abnormally activated in cancers decreases expression of aromatase in BAFs. These findings demonstrate the involvement of two well known signalling pathways in the oestrogen biosynthesis pathway, and could be utilised in the development of novel therapeutics.

PART B: Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 4: NFκB and MAPK signaling mediates TNFα induced PI.4 activity

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Completed all experimental data, wrote the manuscript	80

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Kevin Knower	Conceived and helped plan the study, edited the manuscript.	
Colin Clyne	Conceived and helped plan the study, edited the manuscript, financial support	

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Prince Henry's Institute, Level 3 Block E Monash Medical Centre**

Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.

Signature 1	Kevin Knower	
Signature 2	Colin Clyne	

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NFκB and MAPK signalling pathways mediate TNFα-induced Early Growth Response gene transcription leading to aromatase expression

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ABSTRACT

The Early Growth Response genes *EGR2* and *EGR3* play an important role in mediating TNFα induced aromatase expression via the adipose specific promoter PI.4. The upstream signalling pathway stimulated by TNFα to initiate this is unknown. The aim of this present study was to determine the signalling pathways activated by TNFα which result in *EGR2* and *EGR3* transcription, and ultimately activation of PI.4. The NFκB inhibitor BAY-11-7082 dose-dependently inhibited transcription of *EGR2* and *EGR3* mRNA as well as total and PI.4-specific *CYP19A1* mRNA. The MAPK pathway inhibitor U0126, inhibitor of MEK1/2 had the same effect, however inhibition of c-Jun and JNK1,2,3 with SP600125 did not lead to down-regulation. We provide evidence for the first time that *EGR2* and *EGR3* are regulated by NFκB and MAPK signalling pathways downstream of TNFα stimulation in breast adipose fibroblasts, and that this in turn is upstream of *CYP19A1* transcription via PI.4.

1. Introduction

Approximately 70% of post-menopausal breast cancers are diagnosed as Estrogen Receptor-α positive (ER+), and such tumours are responsive to estrogen for continued growth and proliferative advantage. Adjuvant selective estrogen receptor modulators (SERMs), such as Tamoxifen, are commonly used to block the action of estrogen in breast cancer [1]. ER+ tumours primarily source estrogen from the increased local production in surrounding breast adipose fibroblasts (BAFs) [2].

Aromatase is the key enzyme responsible for the conversion of androgens to estrogens. Its expression is regulated at the transcriptional level by its encoding gene *CYP19A1*. Upstream of the coding region, *CYP19A1* contains a number of tissue-specific promoters which are activated by distinct stimulatory factors in different tissues to convey a specific expression pattern [3,4]. In normal breast adipose and increasingly so in BAFs adjacent to an ER+ tumour, *CYP19A1* is transcribed via its distal adipose-specific promoter I.4 (PI.4), which is located approximately 73 kb upstream of the common splice site [5].

The activity of PI.4 is stimulated through the combined actions of glucocorticoids, such as dexamethasone, and cytokines [6,7], one of which is the pro-inflammatory cytokine tumour necrosis factor α (TNFα) [8]. The specific mechanisms by which TNFα is able to stimulate PI.4 activity remains to be resolved, however we recently demonstrated that the Early Growth Response (EGR) genes form an integral part of the pathway. EGR transcription factors, in particular EGR2 and EGR3, are upregulated upon TNFα stimulation in BAFs, and they in turn increase activity of PI.4 though not through direct binding to the promoter. Additionally, siRNA mediated knockdown of the EGR family members in BAFs significantly decreases expression of *CYP19A1* via PI.4 [9].

Whilst the first characterised family member EGR1 has been extensively studied, little is known about the regulation and biological function of other family members. Structurally similar, each EGR family member contains three zinc-finger DNA binding motifs which recognise the same CG-rich consensus sequence. Transcriptional regulation of EGR2 and EGR3 in the breast is poorly characterised, however EGR3 is thought to be an estrogen responsive gene in the ER+ breast cancer cell line MCF7 [10]. Understanding the molecular mechanisms by which EGRs are activated in the breast is important as it will help develop a clearer picture of how aromatase is upregulated, ultimately leading to the development of novel breast cancer treatments.

TNFα functions via a number of signalling pathways downstream of its main receptor, TNFR1. The best characterised of these are the Nuclear Factor κ-B (NFκB) and Mitogen Activated Protein Kinase (MAPK) signalling pathways. NFκB is hyperactivated in cases of breast cancer, promoting growth through hyper-activation of anti-apoptotic genes [11]. Compared to normal breast, breast cancers contain higher levels of activated Bcl-3, p65, p50 and

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p52, resulting in increased cyclin D1 levels and consequently cell cycle progression [12,13]. Experiments in mouse models of breast cancer have shown that application of the selective NFκB pathway inhibitor BAY-11-7082 can block TNFα-stimulated cell proliferation and cause tumour regression [14]. Components of the MAPK signalling cascade have also been targeted to slow the progression of tumour growth. p38 MAPK is highly overexpressed in both human and mouse metastatic breast cancer cell lines, and correlated with metastatic disease in clinical samples. shRNA mediated knockdown of p38 MAPK in breast cancer cell lines inhibits their proliferation, migration, invasion and colony forming capacity [15]. Inhibition of p38 MAPK signalling with the drug RWJ67657 strongly decreases tumour growth in a mouse xenograft model [16], indicating a clear role for this signalling factor in tumour growth and proliferation.

In this present study, we aim to elucidate the role of both the NFκB and MAPK signalling pathways in mediating TNFα stimulated PI.4-specific *CYP19A1* transcription via the EGR transcription factors. We demonstrate for the first time a direct role for both these signalling pathways in mediating *EGR2* and *EGR3* transactivation leading to PI.4 specific transcription, suggesting that targeted drug therapies directed against these often hyperactivated pathways in cancer may also contribute to inhibiting local estrogen production in the breast.

2. Materials and methods

2.1. Plasmids

NFκB-RE and pTAL constructs were provided by Dr. Simon Chu and have been previously described [17].

2.2. Cell culture

BAFs were isolated from breast reduction mammoplasty by collagenase digestion and cultured as previously described [18]. To stimulate TNFα-dependent pathways, BAFs were grown to ~60% confluency then incubated in serum-free media containing 0.1% BSA (Sigma) for 24 h. Serum-free media containing 5 ng/ml TNFα (Sigma) was then added to cells. For activation of PI.4, 250 nM dexamethasone was added in conjunction with TNFα to serum-free media for 24 h. To inhibit NFκB signalling, BAY-11-7082 (Calbiochem) was added to the culture medium at the specified dose. To inhibit MAPK signalling PD98059, SB203580, SB202190, SP600125 or U0126 (Cell Signalling) was added to the culture medium at the specified dose.

2.3. Transient transfection and reporter assay

BAFs were transiently transfected using electroporation (Nucleofector kit V, Amaxa Biosystems, Lonza, Walkersville, MD, USA) using cell line solution V and pre-adipocyte program T-030. β-galactosidase was transfected as an internal control vector. Cells were incubated for 24 h, then vehicle or TNFα treatment was applied for 24 h. Firefly luciferase activity was measured using the Luciferase Assay System (Promega) and β-galactosidase activity was measured by β-galactosidase assay (Promega).

2.4. Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini kit (QIAGEN). cDNA synthesis was performed on a minimum 200 ng of total RNA using avian myeloblastosis virus reverse transcriptase (Promega) primed by oligo dT or random primers. qRT-PCR of *EGR2* and *EGR3* transcripts was performed using SYBR green detection on the ABI 7900T sequence detection system. *18S* transcripts were detected as an internal RNA loading control. Detection of total *CYP19A1* transcripts was performed on the Roche LightCycler System (Roche Diagnostics) using Fast Start Master SYBR Green I or the ABI 7900T sequence detection system using the *CYP19A1* TaqMan Gene Expression assay (Hs00903413_m1) (Applied Biosystems). PI.4 transcripts were detected on the Roche LightCycler System using Fast Start Master SYBR Green I. Primer sequences are as follows: *EGR2* (sense, TTGACCAGATGAACGGAGTG; anti-sense, GTTGAAGCTGGGGAAGTGAC); *EGR3* (sense, CAATCTGTACCCCGAGGAGA; anti-sense, GGAAGGAGCCGGAGTAAGAG); total *CYP19A1* (sense, TTGGAAATGCTGAACCCGAT; anti-sense, CAGGAATCTGCCGTGGGGAT); PI.4 (sense, GTAGAACGTGACCAACTGG; anti-sense, CACCCGGTTGTAGTAGTTGCAGGCACTGCC); and *18S* (sense, CGGCTACCACATCCAAGGA; anti-sense, GCTGGAATTACCGCGGCT).

3. Results

3.1. The NFkB pathway is constitutively active in BAFs

To determine whether NFκB signalling is active, a luciferase reporter construct under the regulation of a consensus NFκB responsive element motif was transfected into BAFs. Compared to the enhancer-less empty vector control pTAL, the NFκB-RE reporter showed a 6-fold increase in activity under basal conditions. When BAFs were treated with 5 ng/ml TNFα following transfection, this induction was significantly further increased (Fig. 1). This indicates that NFκB signalling is constitutively active in BAFs, and that TNFα further stimulates its activity.

3.2. Inhibition of the NFκB pathway decreases CYP19A1 transcript via PI.4

Having established that NFκB signalling is active in BAFs, an inhibitor of the NFκB pathway was used to determine its role in aromatase expression. BAY-11-7082 is a chemical inhibitor that prevents the activation of the IKK kinase complex. BAFs treated with TNFα/DEX together with BAY-11-7082 at concentrations of 0.5 μM and 1 μM for a period of 24 h showed significantly lower

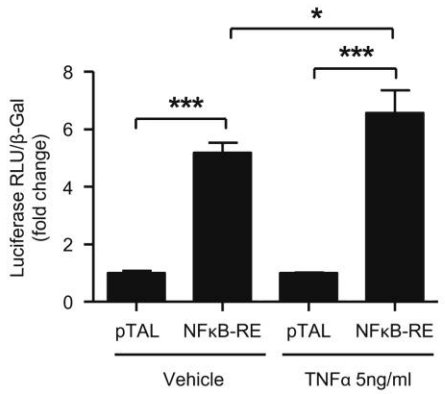

Fig. 1. NFκB is constitutively active in primary human BAFs. BAFs were transfected with either the empty vector reporter pTAL or a reporter construct containing repeating NFκB response elements (NFκB-RE). Following transfection, cells were treated with vehicle control or 5 ng/ml TNFα for 24 h prior to luciferase activity being assessed. Data obtained from three independent experiments. Results are expressed as mean luciferase activity in relative light units (RLU) normalised to β-galactosidase activity, with error bars representing standard error of means. Data was analysed by independent T-test of grouped means ($^{*}p < 0.05$, $^{***}p < 0.001$).

levels of total *CYP19A1* transcripts compared to TNFα/DEX alone (Fig. 2A). This was mirrored by decreases in PI.4 transcripts (Fig. 2B). Higher doses proved toxic to cells after 24 h of treatment.

3.3. Decrease in EGR2 and EGR3 transcript leads to decreased CYP19A1 transcript in response to NFkB inhibition

EGR transcription factors are involved in TNFα-induced *CYP19A1* expression [9]. To determine the role of NKκB in EGR signalling, BAFs were treated for 1 h with BAY-11-7082. *EGR2* expression was dose-dependently inhibited by NFκB inhibition, falling below basal levels at 10 μM BAY-11-7082 (Fig. 2C). Similar effects were seen on *EGR3* mRNA (Fig. 2D), indicating that early loss of EGR transcription factors through NFκB pathway inhibition may result in a decreased capacity to transcribe *CYP19A1*. Alhough higher doses of BAY-11-7082 were toxic in BAFs treated for 24 h, viability was not affected during the shorter 1 h treatment period.

3.4. MAPK signalling is also upstream of EGR2 and EGR3 transcription in response to TNFα

The MAPK signalling cascade is also activated by TNFα binding to its receptor. The effects of two inhibitors targetting distinct branches of the MAPK pathway were examined. SP600125 – which inhibits c-Jun, JNK1,2,3 – showed a dose dependent effect on the reduced expression of *EGR2*, however no significant effect was seen until higher doses (20 μM and 50 μM) of inhibitor were used (Fig. 3A). However when U0126 – inhibitor of MEK1/2 – was added in conjunction with TNFα, the effect on EGR2 expression was immediate from 1 μM dose (Fig. 3B). Conversely, TNFα-induced EGR3 expression showed a more dose-dependent decrease in response to co-treatment with SP600125, with significant reductions observed from 1 μM (Fig. 3C). Again, it appears that U0126 has a more potent effect than SP600125 on EGR3 expression levels, with complete abolishment of TNFα-induced expression at 1 μM. This trend is continued at higher doses, with EGR3 reduced to below basal levels at higher concentrations (Fig. 3D). Other MAPK inhibitors PD98059, SB203580 and SB202190 were also used to test the response of EGR2 and EGR3 transcript to other MAPK inhibitors. All drugs led to a down-regulation of EGR2 and EGR3 transcript in BAFs to differing degrees (Supplementary Fig. 1).

3.5. Inhibition of MAPK signalling decreases expression of CYP19A1 via PI.4

Inhibition of MAPK signalling resulted in down regulation of *EGR2* and *EGR3* transcription in a similar fashion to inhibition of NFκB signalling. Therefore, to determine if MAPK signalling is required for *CYP19A1* expression via PI.4, we treated BAFs with TNFα and Dexamethasone, and co-treated with SP60025 and U0126. Treatment with SP60025 had no effect on total *CYP19A1* transcripts; however inhibition with U0126 significantly down regulated total *CYP19A1* compared to TNFα and DEX treatment alone (Fig. 3E). A similar pattern of promoter I.4-specific transcript levels was observed (Fig. 3F). Inhibition with SP60025 has no effect on PI.4 transcripts, however significant decrease compared to TNFα and DEX alone was observed when cells were co-treated with U0126.

4. Discussion

The signalling pathways downstream of TNFα which lead to transcriptional activation of PI.4 in BAFs are not fully understood. We have shown that both NFκB and MAPK pathways are down-

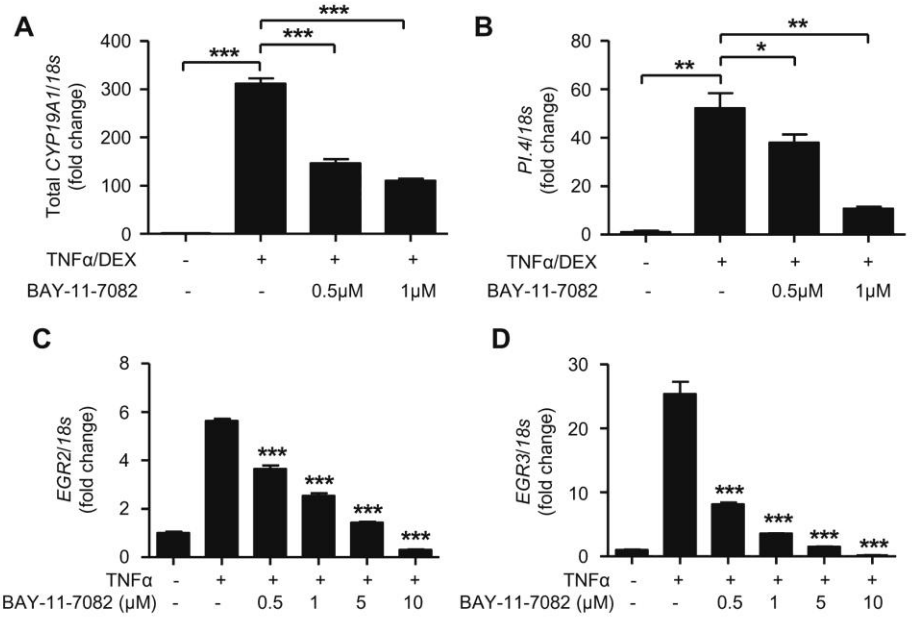

Fig. 2. NFκB inhibition dose dependently decreases *CYP19A1* transcript via PI.4, via early inhibition of EGR2 and EGR3. BAFs were treated for 24 h with TNFα (5 ng/ml) and DEX (250 nM), with or without the NFκB pathway inhibitor BAY-11-7082 (0.5 μM or 1 μM). mRNA expression of (A) *CYP19A1* total and (B) PI.4-specific transcripts were then detected. (C) BAFs were treated for 1 h with TNFα (5 ng/ml) and BAY-11-7082 at varying concentrations. qRT-PCR was then used to detect transcript levels of EGR2 and (D) EGR3. Data obtained from three independent experiments performed in triplicate. Error bars represent standard error of means, data analysed by one-way ANOVA with a Newman–Keuls post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

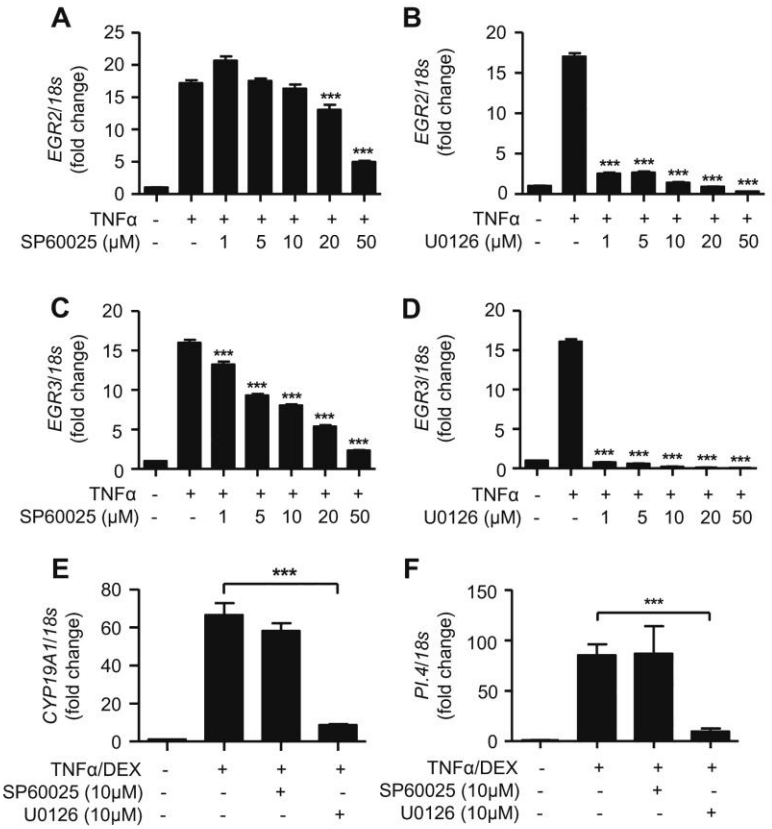

Fig. 3. Inhibition of MEK1/2 decreases EGR2 and EGR3 expression, leading to a decrease in CYP19A1 mRNA via PI.4. BAFs were pre-treated with MAPK pathway inhibitors for 30 min, then treated for 1 h with TNFα (5 ng/ml). qRT-PCR was then used to detect transcript levels of (A and B) EGR2 and (C and D) EGR3. BAFs were treated with TNFα (5 ng/ml) and DEX (250 nM) together with either SP60025 (5 μM) or U0126 (5 μM) for 24 h. Transcript levels of total (E) *CYP19A1* or (F) PI.4 specific transcripts were then detected by qRT-PCR. Data obtained from three independent experiments performed in triplicate. Error bars represent standard error of mean, data analysed by one-way ANOVA with a Newman–Keuls post-test (***$p < 0.001$).

stream of TNFα, leading to transcription of the EGR genes and ultimately *CYP19A1* via the adipose specific promoter PI.4. Inhibition of either NFκB or MAPK signalling leads to decreased expression of *EGR*s and *CYP19A1*.

Little is known about the transcriptional regulation of *EGR2* and *EGR3*, with the majority of information focusing on the initially isolated family member *EGR1*. Given the rapid effects NFκB and MAPK inhibitors on *EGR2* and *EGR3* expression, it is likely there is direct regulation of these two transcription factors by the NFκB and MAPK-induced transcriptional complexes. In T-cells, Vasoactive Intestinal Peptide and pituitary adenylate cyclase-activating polypeptide inhibit nuclear translocation of NFκB and also down regulate expression of *EGR2* and *EGR3*, although no direct link between the two events was established [19]. An analysis of the *EGR2* and *EGR3* regulatory region reveals potential NFκB response elements present in both promoters (data not shown), consistent with a direct interaction. NFκB rapidly induces immediate early response genes following stimulation with TNFα, via a cooperative interaction with Sp1. Transcriptional machinery is constitutively bound to promoters, as mediated by Sp1, and NFκB may then enhance the initiation rate rapidly [20]. Although this was only shown using the representative immediate early gene *A20* in response to TNFα, this is one potential mechanism for rapid transcription of *EGR2* and *EGR3* by NFκB.

EGR transcription showed differential sensitivity to inhibition of different components of the MAPK pathway. Targetting c-Jun and JNK1,2,3 with SP60025 resulted in significant *EGR2* inhibition only at higher 20 μM and 50 μM doses, but a dose-dependent inhibition of *EGR3* expression significant at 1 μM. Conversely, targetting MEK1/2 with U0126 had a much more dramatic effect on EGR transcription. *EGR2* transcripts were reduced to basal levels at 10 μM and below basal levels at higher doses, and *EGR3* transcripts were repressed to basal levels or below immediately from the lowest 1 μM dose used. This suggests a preferential role for MEK1/2 in EGR regulation, versus the c-Jun or JNK component of MAPK signalling. *EGR2* and *EGR3* promoter analysis however does not uncover direct binding sites for any of the inhibited factors. One possible explanation for this is the potential for crosstalk between the NFκB signalling pathway and MAPK signalling components. Several examples of NFκB regulation of MAPK-regulated transcription factors are known, and this may be a mechanism by which inhibition of MAPK leads to a down regulation of *EGR* transcription.

Our results indicate a clear role for NFκB in mediating aromatase induction by TNFα. A number of previous studies have shown a potential role for NFκB signalling in *CYP19A1* transcriptional activation, but our data is provides direct evidence for the first time. A study of primary human endometrial stromal cells found that treatment with the progestin drug dienogest inhibited both NFκB

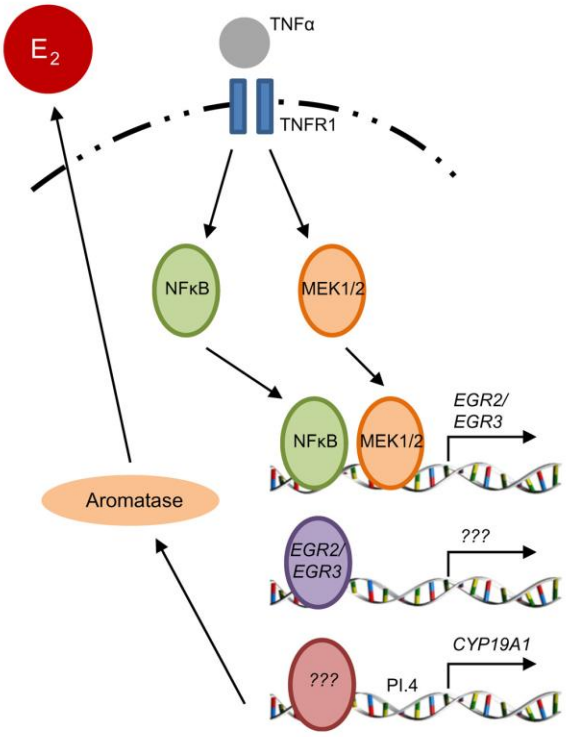

Fig. 4. Model of TNFα induced aromatase expression. In breast adipose fibroblasts, TNFα binds to its receptor TNFR1 to initiate activation of the NFκB and MAPK pathways. NFκB and MEK1/2 lead to transcription of EGR2 and EGR3, which in turn activates *CYP19A1* PI.4 by indirect means of transcribing an intermediate factor.

DNA-binding activity and aromatase expression, suggesting a possible link [21]. In human breast adipose fibroblasts, cell shape-induced aromatase induction can be significantly down regulated by BAY-11-7082. This was independent of the canonical NFκB pathway however, as a specific siRNA directed against the IKKβ component ablated aromatase transcription, but not siRNAs against IKKα or IKK [22]. Increased binding activity of NFκB coupled with increased expression and activity of aromatase is also observed in the inflamed breast adipose tissue of overweight and obese women [23]. Interestingly, NFκB signalling has been implicated in *CYP19A1* transcription via the proximal promoter II, commonly upregulated in breast cancer, with a study in ovarian granulosa tumour cell lines showing direct binding of the p65 subunit to PII, and NFκB inhibitors decreasing PII-driven luciferase response [24]. Our findings suggest that PI.4 may also be downstream of NFκB activation. Despite clear roles of NFκB in breast cancer proliferation and aromatase activation, clinical use of NFkB inhibitors is precluded by adverse effects due to global inhibition of NFκB signalling [25].

MAPK signalling also appears to be downstream of TNFα stimulation, and EGR/PI.4 activation appears to be mediated more specifically by MEK1/2. There are currently conflicting reports within published literature pertaining to role of MAPK in aromatase induction. In human breast adipose fibroblasts, downstream of PGE2 and PKA/PKC activation, p38/MAPK can phosphorylate ATF-2 and c-Jun, which subsequently interact with and upregulate *CYP19A1* PII [26]. Conversely, active MAPK signalling downregulates aromatase transcript and therefore estrogen production in immature sertoli cells [27]. Pharmacologically targetting MEK1/2 presents an attractive option, given its involvement in numerous cancer processes. A number of MEK1/2 inhibitors are currently at the stage of clinical trials, though there is yet to be one developed for full clinical use [28]. Early indicators suggest that they can cause regression of tumours *in vivo* including colorectal, thyroid and myeloma [29–31], although adverse side effects are compounding clinical trials. Our research suggests that with further development of specific MEK1/2 inhibitors, ER+ breast cancer may be amongst carcinomas that may be treated.

In conclusion, we have elucidated the downstream signalling pathways involved in TNFα induced aromatase transcription via EGR2 and EGR3 (Fig. 4). The data presented here represents a significant development in understanding local estrogen production in the breast, and offers new avenues by which therapeutic intervention of ER+ breast cancer may be further developed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.058.

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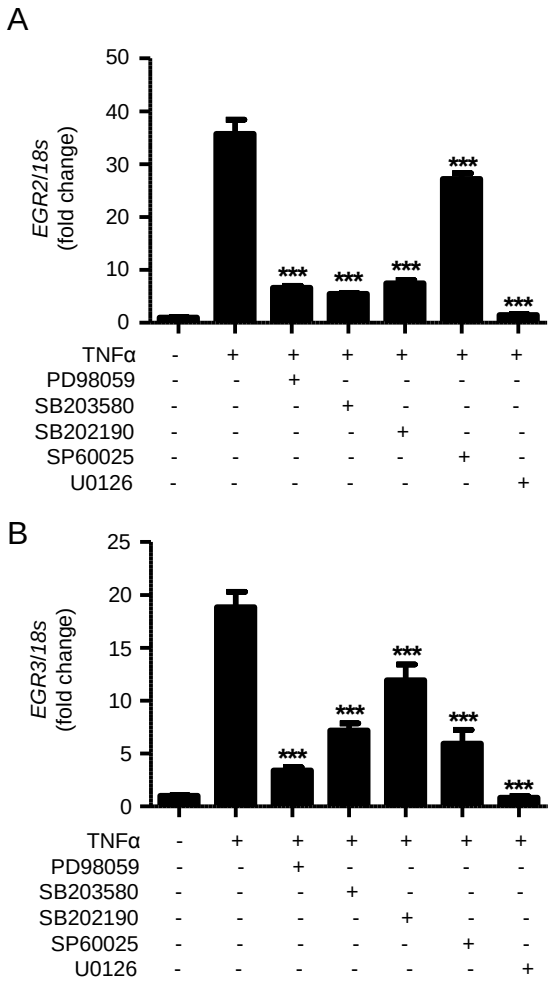

Supplementary Figure 1: Screen of MAPK inhibitors in BAFs. BAFs were pre-treated with inhibitor for 30mins, before TNFα was added for 1hr to assess (A) EGR2 and (B) EGR3 mRNA expression. Concentrations used were as follows: PD98059 (50µM), SB203580 (50µM), SB202190 (20µM), SP60025 (20µM), U0126 (10µM).

CHAPTER 5

Regulation of TNFα by estradiol in ER+ breast tumours

Preface to Chapter 5

In Chapter 3 and Chapter 4, the research focus has been on determining the transcription factors and cis-elements by which TNFα is working to activate aromatase transcription via the adipose-specific promoter PI.4, and the downstream signalling pathway by which this is occurring. From this data, it is clear that TNFα plays a critical role in the local upregulation of oestrogen in the ER+ breast tumour microenvironment through its capacity to stimulate aromatase activity. TNFα is abundantly detected within the breast tumour cytosol, and its presence is correlated with a poor disease prognosis. The source of this increased TNFα is not fully understood. Although the contribution of infiltrating immune cells in the tumour matrix is established, the relative contribution of tumour epithelial cells has not been investigated. The aim of the study was to determine the contribution of ER+ tumour epithelial cells to the secretion of TNFα, and what factors are leading to its upregulation.

In a manuscript submitted to Tumour Biology (February 2013, manuscript ID TUBI-D-13-00097), it was shown for the first time how a positive feedback loop is established between the ER+ tumour and surrounding oestrogen-producing adipose fibroblasts. It was demonstrated that the ER+ breast cancer cell line MCF7 produces a low basal level of TNFα, however transcription and secretion is significantly upregulated by treatment with oestradiol. This process is occurring via interactions with ERα, as ERα antagonist ICI-182780 was able to inhibit this effect. The ER- breast cancer cell line HS578t produce the highest basal levels of TNFα suggesting that TNFα may play a significant role in ER- breast tumour progression, a finding that needs to be further explored.

This data establishes for the first time a positive feedback loop between oestradiol and TNFα in an ERα breast tumour microenvironment. This shows a new method by which anti-oestrogen therapies contribute to retarding tumour growth, and suggests that breaking the reciprocal link between oestradiol and TNFα may be important to the development of novel successful treatment strategies.

PART B: Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 5: Regulation of TNFα by estradiol in tumour epithelial cells

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Conceived and helped plan the study, completed most experimental data, wrote the manuscript	80

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Kyren Lazarus	Helped with data collection, edited the manuscript	
Kevin Knower	Conceived and helped plan the study, edited the manuscript.	
Colin Clyne	Conceived and helped plan the study, edited the manuscript, financial support	

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Prince Henry's Institute, Level 3 Block E Monash Medical Centre**

Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.

Signature 1	Kyren Lazarus	
Signature 2	Kevin Knower	
Signature 3	Colin Clyne	

Estradiol increases production of TNFα from ER+ breast tumour epithelial cells

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Abstract

TNFα plays a critical role in estrogen receptor positive (ER+) breast cancer pathology, both maintaining undifferentiated fibroblasts adjacent to the tumour and also upregulating estrogen-producing factors. High levels of TNFα are detected within the tumour microenvironment, and though infiltrating immune cells are thought to contribute a significant amount of TNFα, the relative role of the ER+ tumour epithelial cells in producing this cytokine as a tumour-derived paracrine signalling factor has not been examined. The aim of this study was to determine the relative contribution of tumour epithelial cells to TNFα production and how this is regulated using a number of breast cancer cell lines. We show that TNFα transcript and secreted protein can be detected in breast cancer cell lines, and that relative expression was lowest in the ER+ cell line MCF7. Treatment of MCF7 cells with estradiol increased TNFα transcript and secretion, an effect which was mitigated in the presence of the ERα antagonist ICI-182780. Our results show that ER+ tumour epithelial cells are a source for

TNFα within a breast tumour microenvironment, enforcing the positive role this inflammatory cytokine has to play in tumour progression.

Keywords: TNFα, estradiol, breast cancer, ERα

Introduction

Tumour Necrosis Factor-α, or TNFα, is a critical pro-inflammatory cytokine primarily produced by the macrophages and monocytes of the immune system [1] and was initially isolated from mouse serum as a potential anti-cancer agent [2, 3]. Further studies however revealed that although it did cause necrosis of some tumours, it actually promoted growth and proliferation of other tumour types, including breast cancer. TNFα has been detected within the breast cyst fluid and breast tumour cytosol of the tumour microenvironment [4], and its presence has been correlated with a metastatic, invasive phenotype and poorer prognosis [5, 6]. Indeed, retardation of TNFα signalling via treatment with a TNFα-antibody results in a slowing of mammary tumour growth in a mouse model [7], and animals null for the TNFα gene show lower rates of induced tumour formation when compared to wildtype [8].

Approximately 70% of breast tumours diagnosed in post-menopausal women are Estrogen Receptor-α positive (ER+), responsive to the critical sex hormone for continued growth and proliferative advantage [9]. Adjuvant hormone-therapy treatment options include Selective Estrogen Receptor Modulators (SERMs), such as Tamoxifen, which has an antagonistic effect in the breast but acts as an ER agonist in other tissues such as uterus and bone [10-12]. Consequently, side effects from Tamoxifen use include an enhanced risk of endometrial and uterine cancer [13]. Activity of the estrogen-converting enzyme aromatase is increased in most ER+ cancers [14], therefore Aromatase Inhibitors (AIs) are also commonly used as breast cancer therapy. AIs inhibit estrogen formation globally, and therefore side effects of estrogen deprivation include cardiac disturbances, bone loss and an increased risk of fractures [15, 16]. Breast-specific inhibition of estrogen action or formation is a high research priority.

Many growth and signalling factors are known to stimulate the increased local production of estrogen in an ER+ breast tumour microenvironment, including TNFα. Treatment of breast cancer cell lines with TNFα modulates gene expression to favour cell growth and proliferation, metastasis, loss of cell cycle control and degradation of the extra-cellular matrix [17, 18]. Importantly, TNFα directly supports the ER+ tumour growth by maintaining the dense layer of undifferentiated fibroblasts in the

desmoplastic reaction which are able to express key estrogen-producing enzymes by working as an anti-adipogenic factor [19]. TNFα also increases the activities of key estrogen-producing enzymes aromatase [20], estrone sulfatase [21] and 17β-HSD type 1 [22], leading to a net increase in the bioavailability of active estrogens to the tumour.

The source to the excess TNFα often found in the tumour is not well understood. Given that under normal conditions TNFα is produced by immune cells, tumour-associated macrophages and lymphocytes which comprise up to 50% of the total tumour volume are key contributors [23, 24]. It has been hypothesised that the breast tumour itself also produces TNFα as a factor that contributes to paracrine signalling within the tumour microenvironment [25]. The source of TNFα and the mechanisms regulating its expression within a tumour microenvironment is a critical gap in knowledge that needs to be filled in order to develop potential therapeutic agents targetting TNFα-formation in the breast, limiting the proliferative potential of ER+ breast tumours.

Given the abundance and importance of estrogens within the ER+ breast tumour microenvironment, one hypothesis proposed has been that this may be one of the factors driving TNFα overexpression in the malignant epithelial cells [25]. Estradiol has previously been shown to increase TNFα expression in the uterus and lactotrope cells [26, 27], demonstrating that it forms part of the TNFα regulatory pathway in some tissues. Expression of the TNFα receptor, TNFR1, is also upregulated in the presence of estradiol in lactotropes [27] and human breast adipose fibroblasts [25]. Autoregulation of its own expression is a proposed mechanism by which TNFα remains consistently expressed in adipose [28], therefore upregulation of TNFR1 by estradiol would allow for increased activation of TNFα signalling. Regulation of TNFα by estradiol has yet to be examined in the context of breast cancer cells, and is important to refining our understanding of the molecular basis behind breast cancer proliferation. In this present study, we present evidence that TNFα is upregulated by estradiol in ER+ breast cancer cell lines.

Materials and Methods

Cell culture

Breast cancer cell lines HS578t (ATCC no. HTB-126), MCF7 (ATCC no. HTB-22), T47D (ATCC no. HTB-133) and MCF10A (ATCC no. CRL-10318) were subcultured under prescribed conditions according to suppliers procedures. For treatment with estradiol (E_2), cells were grown to ~50% confluence in media containing serum. Cells were then incubated in phenol red-free media containing 5% charcoal stripped serum for 72 hours. E_2 (10nM) (Sigma) and/or ICI-182780 (100nM) (Santa Cruz Biosciences) was then added for the described amount of time. An equal amount of vehicle alone, being the agent in which the compound was dissolved, was used as the control.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen). First strand cDNA synthesis was conducted using a minimum 500ng total RNA, and carried out using avian myeloblastosis virus reverse transcriptase (Promega) primed with random primers. Quantitative real-time PCR (qRT-PCR) was carried out using TaqMan gene expression assays specific for *TNFα* (Applied Biosystems Assay # Hs01113624_g1) on the ABI 7900HT Sequence Detection System. *TNFR1* transcripts were also detected on the ABI 7900HT Sequence Detection System using SYBR Green detection methodologies. *GREB1* transcripts were detected on the Light Cycler sequence detection system using SYBR green detection methodologies. *18S* transcripts were also detected as an internal control. Primers used are as follows: *TNFR1* (sense, TCAGTCCCGTGCCCAGTTCCACCTT; anti-sense, CTGAAGGGGGTTGGGGATGGGGTC); GREB1 (sense, GTGGTAGCCGAGTGGACAAT; anti-sense, AAACCCGTCTGTGGTACAGC) and *18S* (sense, CGGCTACCACATCCAAGGA; anti-sense, GCTGGAATTACCGCGGCT).

ELISA assay

Conditioned media from breast cancer cell lines was collected and concentrated using the Vivaspin 20 centrifugal concentrators (Sartorius). Concentration of TNFα in the conditioned culture media was

determined using the Quantakine HS ELISA for Human TNFα (R&D Systems) following the manufacturers protocol.

Statistical analysis

Student's T-test or one-way ANOVA was applied to assess statistical significance of result comparisons. Analysis was performed using Graph Pad Prism 5 statistical software.

Results

Relative expression of TNFα in breast cancer cell lines

To investigate whether breast cancer cells have the capacity to produce TNFα, we performed qRT-PCR to assess whether *TNFα* mRNA could be detected and the relative levels of transcript in different cell lines. All cell lines expressed *TNFα* mRNA under basal conditions (Figure 1A). MCF7 (ER+) and MCF10A (normal epithelial) cells showed relatively low levels of *TNFα* mRNA, whereas T47D (ER+) cells expressed higher levels. The ER- cell line HS578t demonstrated the highest levels of relative *TNFα* mRNA expression. Consistent with mRNA expression, secreted protein as measured by ELISA revealed relatively low levels of TNFα in MCF7 conditioned media. The highest levels of secreted TNFα were detected in HS578t conditioned media (Figure 1B). All cell lines also expressed the TNFR1 receptor, with MCF10A cells showing the highest relative levels of expression (Figure 1C).

E_2 increases expression of TNFα mRNA in MCF7 cells

To determine whether estradiol (E_2) treatment caused an increase in TNFα transcript in breast cancer cells, MCF7, T47D, HS578t and MCF10A were treated with 10nM E_2 for various times up to 24hrs. As expected, the two ER- cell lines showed no responsiveness to E_2 treatment with respect to TNFα mRNA levels. The ER+ cell line MCF7 showed significantly upregulated transcript levels of TNFα following 6hrs and 16hrs of E_2 treatment. This effect was not present following 24hrs of treatment, suggesting that upregulation of TNFα by E_2 is an early response effect. Interestingly, E_2 appeared to

down regulate TNFα expression in the normal epithelial cell line MCF10A (Figure 2A). Expression of GREB1, known to be strongly induced by E_2 via ERα, was measured as a positive control for active estradiol (Supplementary Figure 1). To determine whether increases in *TNFα* transcription were translated in to higher levels of the secreted protein, concentrations of TNFα in MCF7 conditioned media were measured using ELISA. The conditioned media from MCF7 cells treated with E_2 contained a significant two-fold higher concentrations of TNFα (2pg/ml) compared to the conditioned media of vehicle treated MCF7 cells (1pg/ml) (Figure 2B). To confirm that this was an exclusive effect to ER+ cells, we demonstrated that there is no change in secreted TNFα levels in HS578t cells treated with or without E_2 (Figure 2C). There is no effect of E_2 treatment on the expression of TNFR1 in tumour epithelial cells (Figure 2D).

ERα mediates E_2 induced increases in TNFα production

To confirm that observed increases in TNFα mRNA expression were due specifically to the effects of E_2 activating the ERα receptor, MCF7 cells were co-treated with the ERα antagonist ICI-182780 (ICI). This resulted in a significant decrease in TNFα transcript 16hrs post-treatment (Figure 3A). ICI also inhibited E_2-mediated upregulation of TNFα secretion at 24hrs, though not to basal levels (Figure 3B). The effectiveness of the ICI treatment was confirmed by quantifying levels of *GREB1* transcript, which were significantly down regulated in cell treated with E_2 and ICI compared to those treated with E_2 alone (Figure 3C).

Discussion

Tumour epithelial cells secrete many growth factors and cytokines which signal in an autocrine and paracrine manner to maintain their proliferative advantage. In this present study, we demonstrate for the first time that expression and secretion of the tumour-derived cytokine TNFα is upregulated by estradiol in a Estrogen Receptor-α dependent mechanism.

Our findings establish an important positive feedback relationship between the ER+ tumour and the surrounding estrogen-producing adipose fibroblasts. TNFα serves to maintain these surrounding

fibroblasts in an undifferentiated state, thus conserving their estrogen-producing capacity [29]. TNFα is also critical in regulating the expression and activity of key enzymes involved in the estrogen biosynthesis pathway, therefore increases in TNFα signalling to surrounding fibroblasts results in a net increase in estrogen levels within the breast tumour microenvironment [20, 22, 30]. In turn, we have shown that estrogen may then upregulate transcript and secreted levels of TNFα in ER+ breast tumour cells (Figure 5). This positive feedback cycle needs to be disrupted in order to effectively target and block estrogen formation in the breast.

Whilst the data presented indicates an increase in TNFα transcript levels in response to estradiol, this effect was only observed in MCF7 ER+ breast cancer cells and not in the other ER+ cell line studied, T47D. mRNA expression of *GREB1* following estradiol treatment at all measured time points indicate that there is activation of *GREB1* transcription in T47D, however the fold increase of transcripts is not as high as in MCF7 (Supplementary Figure 1). One possible explanation is a differential responsiveness of the two cell lines to estradiol, despite both being ER+. Whilst MCF7s show a dose-dependent increase in cell number in response to estradiol treatment, no such effect occurs with T47D cells [31]. Alternatively, differences in their mRNA and protein expression profile may result in differential regulation of TNFα and GREB1. A recent proteomic analysis of the two cell lines found that 164 proteins were either upregulated or down regulated in MCF7 compared to T47D. Whilst TNFα and GREB1 were not amongst the proteins identified in either cell line, it is possible that components of their regulatory network are differentially regulated between the two cell lines. Importantly, transcript levels of ERα were substantially higher in MCF7s, suggesting greater responsiveness to estradiol stimulation [32]. ER+ breast cancer cell lines share a large number of transcriptome similarities with ER+ clinical tumour samples [33], and our data indicating differences in the regulation of TNFα expression between MCF7 and T47D cells could reflect subtle differences in the vast range of breast cancer pathologies which may be diagnosed.

Interestingly, our data shows that levels of TNFα in the ER+ cell line MCF7 are relatively low compared to the ER- cell line HS578t. This is evident at both the mRNA and secreted protein levels

where significantly higher amounts were detected in the HS578t cells. However, we did show that estradiol had no effect on TNFα secretion levels in the ER- cells, confirming the specificity of the ER in mediating the increases observed in the MCF7 cells. Little is known about what roles TNFα signalling may play in ER- breast tumours, however given the abundance of the protein detected it may contribute to maintaining the proliferative potential of the tumour. Studies have suggested that NFkB signalling, for which TNFα is a key activator, is highly active in ER- tumours and is a key positive regulator of proliferation in these cells [34]. TNFα has also been shown to increase CD44 expression and therefore cell migration in the ER- cell line MDA-MB-231 [35]. The TNFα-neutralising antibody Infliximab also suppresses bone metastasis of MDA-MB-231 cells in an in vivo model, again suggesting an important role for TNFα in cell migration [36]. Our findings that TNFα appears to be abundantly expressed in ER- breast cancer cells warrants further investigation in to its role in ER- breast cancer progression.

TNFR1 is upregulated in response to estradiol in human breast adipose fibroblasts [25], however we show that the same response does not occur in the tumour epithelial cells. In the mouse pre-adipocyte cell line 3T3-L1, TNFα positively autoregulates its own expression through paracrine signalling [28]. Given that there is no increased expression of TNFR1 observed concurrently with upregulated TNFα, this is unlikely to be an active mechanism in ER+ tumour cells. TNFα has been demonstrated to cause a down-regulation of ERα expression in MCF7 cells [37], suggesting that ER+ tumour epithelial cells may limit their responsiveness to the TNFα they are producing by expressing lower levels of receptor. Consistent with this, our data suggests that the highest relative expression of TNFR1 is in the normal epithelial cell line MCF10A, whereas the two ER+ cell lines studied both expressed the lowest relative levels of TNFR1.

To further expand on these studies, the underlying pathways resulting in estradiol-induced TNFα expression would need to be elucidated. Analysis of the upstream promoter region of TNFα reveals two potential EREs which may be directly activated to drive increased TNFα transcription and consequently secretion (data not shown). Further work to determine if ERα can bind to these sites to

initiate transcription, or whether there are intermediate factors are involved, is required. Our findings also suggest a novel way in which SERM therapy could combat ER+ breast tumours. By blockade of the estrogen receptor, TNFα production in tumour cells in response to estradiol is diminished. This reduces the capacity of tumours to maintain the surrounding dense layer of undifferentiated fibroblasts, and also lowers its estrogens-producing potential. TNFα has many roles in breast cancer pathology, and breaking the positive cycle of its production and action within an ER+ breast tumour microenvironment is critical for successful clinical outcomes.

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Disclosure

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Figure legends

Figure 1 Relative expression levels of TNFα and TNFR1 in breast cancer cell lines. (A) qRT-PCR on RNA isolated from breast cancer cell lines MCF7, T47D, HS578t and MCF10A showing relative TNFα mRNA expression levels. (B) ELISA showing secretion of TNFα protein in the conditioned media of cell lines. Cell lines were cultured for three days prior to removal of media and concentration for ELISA. (C) qRT-PCR on breast cancer cell line RNA showing relative mRNA expression levels of TNFR1. Data obtained from three independent experiments conducted in triplicate. Error bars represent standard error of mean

Figure 2 Estradiol increases TNFα production in MCF7 cells. (A) qRT-PCR showing expression levels of *TNFα* mRNA in breast cancer cell lines treated with E_2 (10nM) for 0, 6, 16 or 24hrs. (B) TNFα secretion in MCF7 conditioned media following 24hr treatment with vehicle or E_2 (10nM) as measured by ELISA. (C) TNFα secretion in HS578t conditioned media following 24hr treatment with vehicle or E_2 (10nM) as measured by ELISA. (D) qRT-PCR showing expression levels of *TNFR1* mRNA in breast cancer cell lines treated with E_2 (10nM) for 0, 6, 16 or 24hrs. Data obtained from three independent experiments conducted in triplicate. Error bars represent standard error of mean, data was analysed by independent T-tests of grouped means and one-way ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

Figure 3 ERα mediates TNFα upregulation by E_2 in MCF7 cells. (A) qRT-PCR showing mRNA expression levels of TNFα in MCF7 cells following 16hrs of treatment with E_2 (10nM) alone or in conjunction with the ERα antagonist ICI-182780 (100nM). (B) TNFα secretion in MCF7 conditioned media following 24hr treatment with vehicle, E_2 (10nM) or E_2 and ICI-182780 (100nM) as measured by ELISA. Data obtained from three independent experiments conducted in duplicate. Error bars represent standard error of mean, data was analysed by independent T-tests of grouped means (*$p<0.05$, **$p<0.01$)

Figure 4 Schematic representation of the relationship between estrogen and TNFα in the ER+ breast tumour microenvironment. TNFα is produced by the ER+ breast tumour epithelial cell as stimulated by estrogen. TNFα then signals to the surrounding undifferentiated adipose fibroblasts, leading to an increase in activity of estrogen-forming enzymes aromatase, estrone sulfatase and 17β-HSD1, resulting in increased estrogen production. This estrogen then signals back to the tumour epithelial cells, causing sustained secretion of TNFα. At the same time, estrogen increases expression of TNFR1 in the undifferentiated fibroblasts, increasing their receptivity to TNFα signalling

Supplementary Figure 1 GREB1 expression in E2 treated MCF7 cells. qRT-PCR showing mRNA expression levels of *GREB1* in (A) MCF7 cells and (B) T47D cells treated with E2 (10nM) for 0, 6, 16

or 24hrs. Error bars represent standard error of mean, data was analysed by one-way ANOVA (**p<0.01, ***p<0.001)

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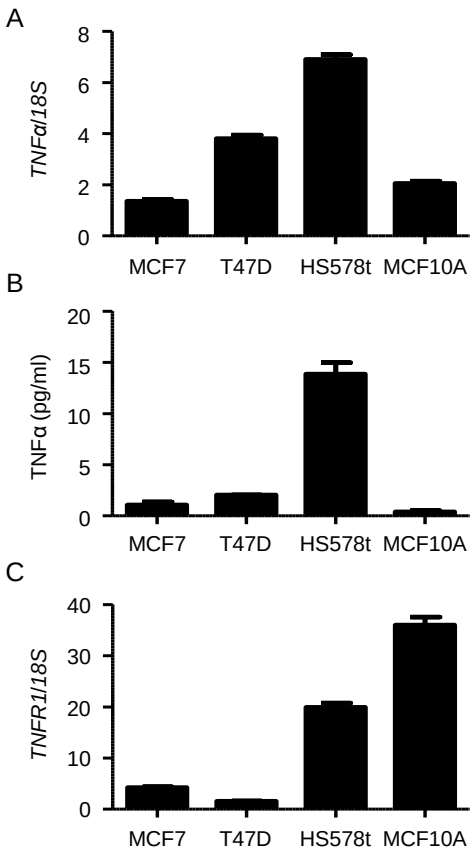


Figure 1: Relative expression levels of TNFα and TNFR1 in breast cancer cell lines. (A) qRT-PCR on RNA isolated from breast cancer cell lines MCF7, T47D, HS578t and MCF10A showing relative TNFα mRNA expression levels. (B) ELISA showing secretion of TNFα protein in the conditioned media of cell lines. Cell lines were cultured for three days prior to removal of media and concentration for ELISA. (C) qRT-PCR on breast cancer cell line RNA showing relative mRNA expression levels of TNFR1. Data obtained from three independent experiments conducted in triplicate. Error bars represent standard error of mean.

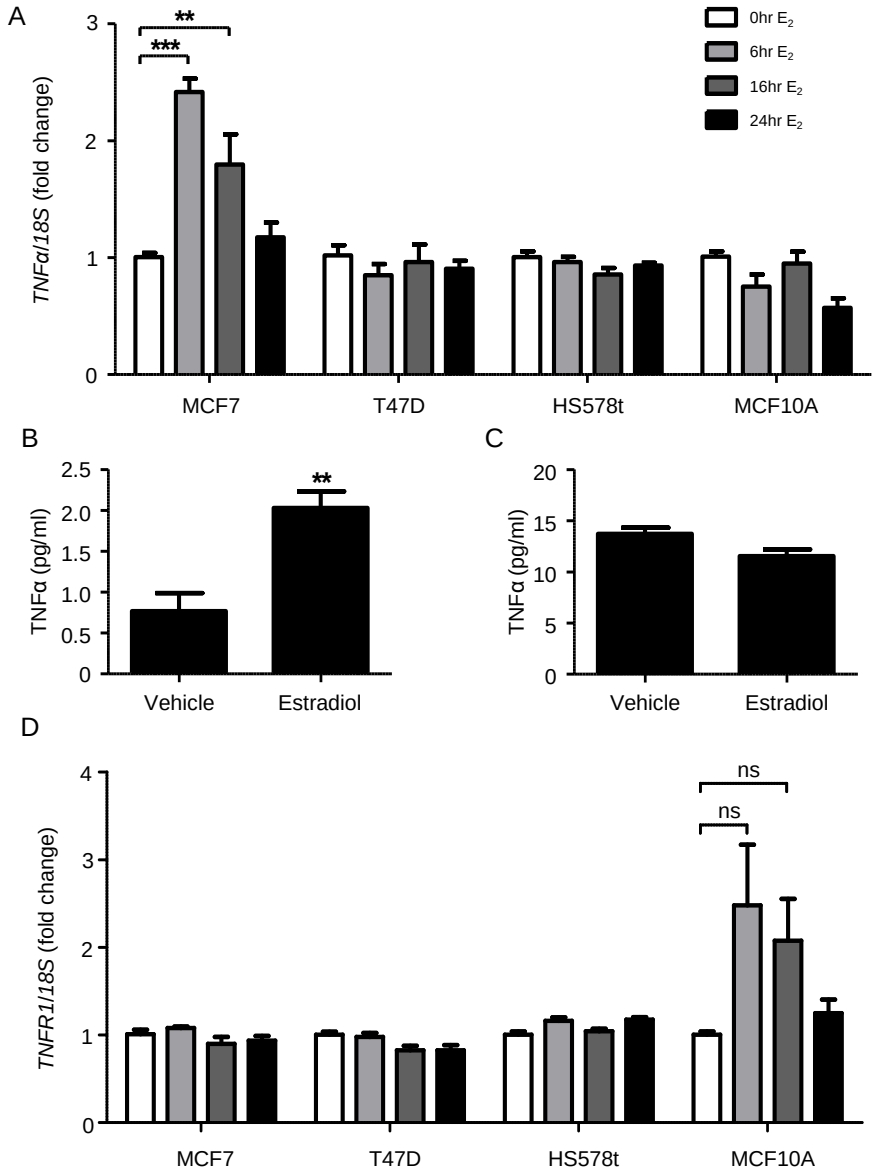


Figure 2: Estradiol increases TNFα production in MCF7 cells. (A) qRT-PCR showing expression levels of *TNFα* mRNA in breast cancer cell lines treated with E_2 (10nM) for 0, 6, 16 or 24hrs. (B) TNFα secretion in MCF7 conditioned media following 24hr treatment with vehicle or E_2 (10nM) as measured by ELISA. (C) TNFα secretion in HS578t conditioned media following 24hr treatment with vehicle or E_2 (10nM) as measured by ELISA. (D) qRT-PCR showing expression levels of *TNFR1* mRNA in breast cancer cell lines treated with E_2 (10nM) for 0, 6, 16 or 24hrs. Data obtained from three independent experiments conducted in triplicate. Error bars represent standard error of mean, data was analysed by independent T-tests of grouped means and one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ns = not significant).

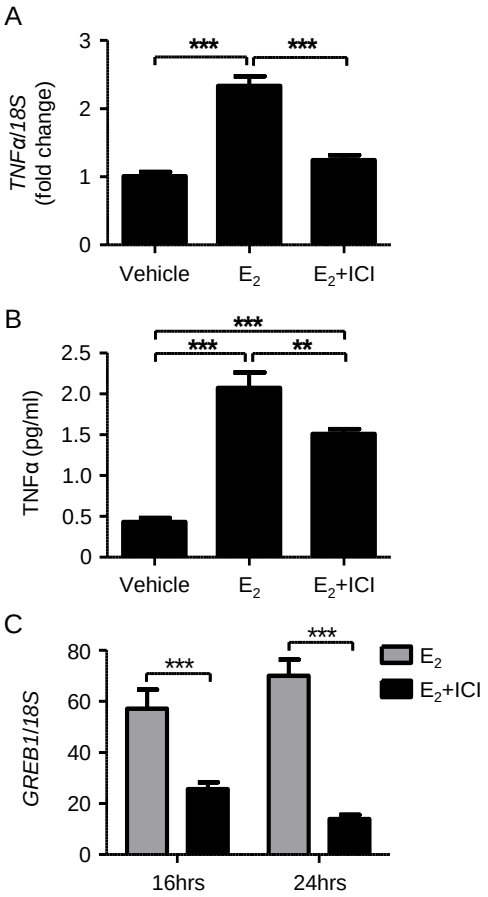


Figure 3: ERα mediates TNFα upregulation by E_2 in MCF7 cells. (A) qRT-PCR showing mRNA expression levels of TNFα in MCF7 cells following 16hrs of treatment with E_2 (10nM) alone or in conjunction with the ERα antagonist ICI-182780 (100nM). (B) TNFα secretion in MCF7 conditioned media following 24hr treatment with vehicle, E_2 (10nM) or E_2 and ICI-182780 (100nM) as measured by ELISA. Data obtained from three independent experiments conducted in duplicate. Error bars represent standard error of mean, data was analysed by independent T-tests of grouped means (*$p<0.05$, **$p<0.01$).

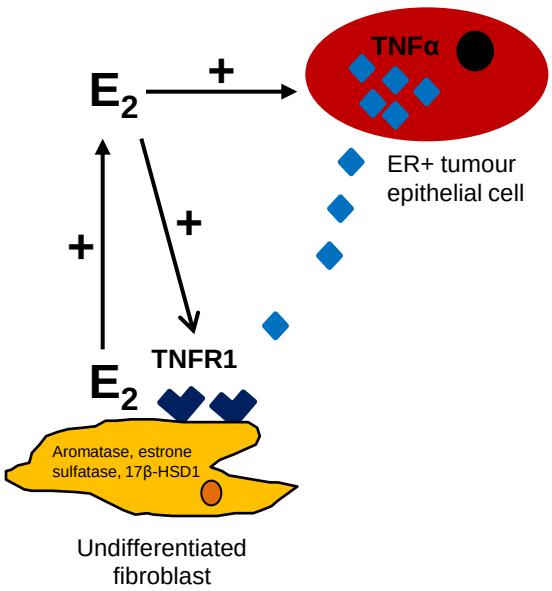
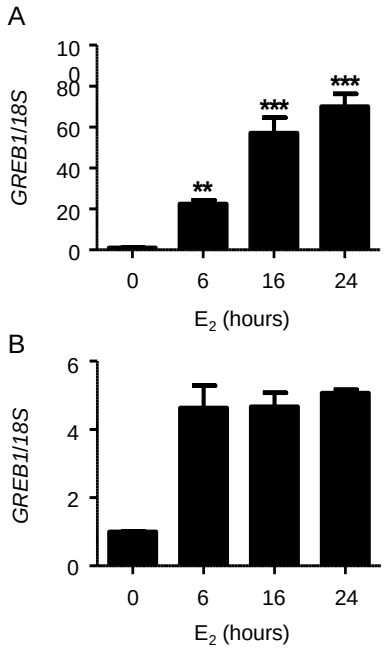


Figure 4: Schematic representation of the relationship between estrogen and TNFα in the ER+ breast tumour microenvironment. TNFα is produced by the ER+ breast tumour epithelial cell as stimulated by estrogen. TNFα then signals to the surrounding undifferentiated adipose fibroblasts, leading to an increase in activity of estrogen-forming enzymes aromatase, estrone sulfatase and 17β-HSD1, resulting in increased estrogen production. This estrogen then signals back to the tumour epithelial cells, causing sustained secretion of TNFα. At the same time, estrogen increases expression of TNFR1 in the undifferentiated fibroblasts, increasing their receptivity to TNFα signalling.



Supplementary Figure 1 GREB1 expression in E2 treated MCF7 cells. qRT-PCR showing mRNA expression levels of *GREB1* in (A) MCF7 cells and (B) T47D cells treated with E2 (10nM) for 0, 6, 16 or 24hrs. Error bars represent standard error of mean, data was analysed by one-way ANOVA (**p<0.01, ***p<0.001).

CHAPTER 6

General discussion, future directions and conclusion

6.1 Overview

The critical oestrogen-producing enzyme aromatase is commonly overexpressed in ER+ breast carcinomas. This is due to increased transcriptional activation of its encoding gene *CYP19A1*, driven by stimulating influences from autocrine and paracrine factors. One such factor is the cytokine TNFα, a pro-inflammatory cytokine that activates *CYP19A1* transcription via the distal adipose promoter PI.4. This promoter is active in the normal breast adipose fibroblasts, and increased expression is observed in the fibroblasts immediately adjacent to an ER+ breast tumour (Irahara et al. 2006). This thesis has demonstrated that TNFα stimulates both the NFκB and MAPK signalling pathways in BAFs, which in turn initiates transcription of the Early Growth Response family of transcription factors particularly *EGR2* and *EGR3*. The EGRs in turn activate transcription of PI.4 by indirect mechanisms, upregulating an unknown PI.4 transcription factor (PI.4 TF). This work also demonstrates a positive feedback loop between oestrogen produced by the BAFs and TNFα produced by the tumour epithelial cells. Additionally, it was shown that expression and secretion of TNFα occurs in ER+ tumour cells, and that this is further stimulated by estradiol treatment. The findings of this thesis can be summarized in Figure 6.1.

Promoter I.4 is expressed in bone, skin fibroblasts and breast adipose fibroblasts. It was initially established as the basal promoter expressed at low levels in the normal breast adipose of post-menopausal women to maintain a base level of oestrogen biosynthesis (Zhao et al. 1995). Its role in promoting cancer has more recently come to light, with studies revealing that its relative expression levels in an ER+ tumour are increased (Irahara et al. 2006). Together with the upregulation of transcripts derived from PI.3 and PII, this drives a dramatic upregulation of aromatase and therefore oestrogen production. The upregulation of PI.4 are attributed in part to increased TNFα within the tumour microenvironment from the infiltrating immune cells and from the tumour itself.

6.2 TNFα and breast cancer

This data has enforced the important role TNFα has to play in ER+ breast tumour development, and furthered the understanding of why age and obesity are critical indicators of breast cancer risk. ER+ tumours make up the bulk of cases diagnosed in post-menopausal women and the increased presence of oestrogen-stimulating factors in high-risk women is in part responsible. TNFα is detected in higher concentrations in the serum of overweight and

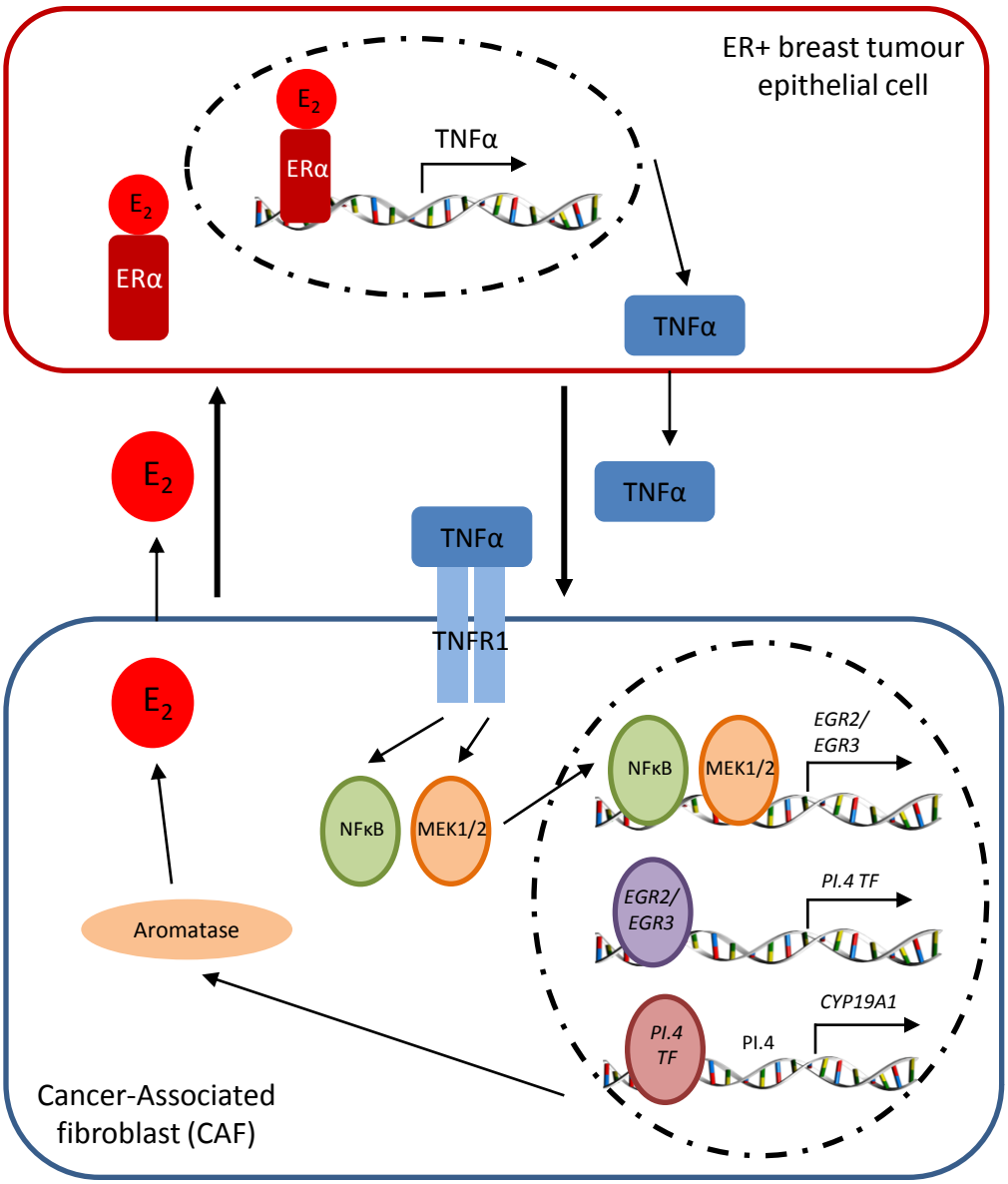


Figure 6.1 Schematic summary of thesis findings. From the data presented in this thesis, a new understanding of the relationship between TNFα and oestrogen within the ER+ breast tumour microenvironment has been established. The signalling pathways induced by TNFα in CAFs stimulates PI.4 via the indirect effects of Early Growth Response transcription factors. Aromatase activity and therefore oestrogen production is increased in CAFs, oestrogen which then feeds to the tumour to further upregulate TNFα secretion.

older women, meaning it may be a key indicator to breast cancer development. Given this, an interesting study may be to correlate serum TNFα with breast cancer onset in a large cohort of older women to determine if heightened presence of TNFα may be of prognostic value in the early detection of breast cancer. Heightened endogenous TNFα levels can lead to increased oestrogen production in the post-menopausal breast where expression of aromatase is mediated by PI.4, increasing the probability of tumour formation. TNFα not only stimulates oestrogen production, but may also stimulate the pro-cancer effects of the NFκB and MAPK pathways to promote tumourogenesis. These pathways are often highly active in breast cancer (Meng et al. 2011; Shostak and Chariot 2011). Once tumour formation has initiated, local TNFα is then produced by the ER+ tumour itself and continues its roles in tumour growth and proliferation through maintaining oestrogen biosynthesis as well as contributing to tumour invasive properties (Yin et al. 2009).

6.3 Implications for breast cancer therapy

The identification of EGR transcription factors as part of the TNFα induced cascade leading to aromatase activation is the first step in elucidating the exact mechanisms by which TNFα contributes to increasing local oestrogen biosynthesis in the breast. Whilst it has been known for some time that TNFα is a key factor in stimulating *CYP19A1* expression via PI.4, the precise transcription factors and cis-elements by which this is occurring have remained elusive. By establishing that TNFα activates the NFκB and MAPK pathways in BAFs, which in turn transcriptionally activate the EGRs, these studies have identified the initial steps in the process. However, ChIP and EMSA show that EGR2 does not bind directly or as part of a transcriptional complex to PI.4, therefore not itself responsible to activating *CYP19A1* transcription. This is consistent with the fact that the EGRs are immediate-acting transcription factors, and are unlikely to still be activating transcription in the 24hr time frame when *CYP19A1* transcripts are detected. An intermediate factor, or factors, is therefore likely being upregulated by the EGRs, which then in turn directly binds to and transcribes PI.4. Identifying these intermediate factors which are directly responsible for TNFα induced PI.4 expression is the next key step in the research area. Using a promoter deletion analysis, these studies have narrowed down the PI.4 region responsible to a 41 base pair region that mediates the EGR effects. The next step in the research process would be to identify the precise transcription factors that are binding to this region, and how they are regulated by the EGRs. Answering

these questions will lead to a detailed evaluation of the most effective part of the TNFα pathway to target in order to attenuate its pro oestrogen-producing capacity.

Determining the precise TNFα-induced transcription factor or factors utilised in BAFs and in breast cancer is a critical step forward, however to be able to target these pathways in novel breast cancer therapeutics further understanding is required. The major drawback of current adjuvant aromatase inhibitor treatment regimes is that they result in the global blockade of aromatase expression, leading to adverse side effects such as bone loss, cardiac disturbances and hypercholesterolemia. An ideal therapy for patients with an ER+ over expressing aromatase would be one where aromatase is inhibited only locally in the breast. Therefore, identifying transcription factors exclusively utilised in breast-specific aromatase expression that are overexpressed in cancer is an important aim of the research field. TNFα induced pathways and transcription factors which activate PI.4 may represent candidates for further investigation.

Whilst PI.4 is expressed in normal post-menopausal breast and overexpressed in breast cancer, it is also detected in bone (Shozu and Simpson 1998) and skin fibroblast cells (Harada 1992; Harada et al. 1993). Whilst the stimulatory factors specifically responsible for PI.4 induction in skin fibroblasts are unknown, TNFα appears to in fact decrease aromatase transcription in osteoblasts (Enjuanes et al. 2003). Blockade of TNFα with a neutralising antibody in mouse models has demonstrated that targetting TNFα itself is sufficient to cause breast tumour regression. Given that TNFα affects aromatase expression in bone, and the fact that it carries critical immune system function, direct attenuation of TNFα would likely also result in bone side effects and a compromise of vital immune system function. Never the less, an investigation in to the effectiveness of current anti-TNFα therapies used in the treatment of inflammatory diseases such as arthritis and psoriasis could be warranted. Their application in breast cancer therapy has not yet been trialled, and *in vitro* and *in vivo* studies to test their effect on breast cancer cell proliferation may be an interesting first step. Given the hypothesis that the primary role of TNFα is to inhibit fibroblast differentiation and stimulate oestrogen production, *in vitro* experiments would best be served through observation of co-culture experiments. In this instance, it may be observed whether addition of anti-TNFα drugs to the culture media promotes differentiation of fibroblasts and lowers oestrogen concentrations. *In vivo* experiments could confirm whether mouse mammary tumours are regressed when treated with these drugs, furthermore whether this is due to lowered local oestrogen concentrations.

Mouse studies would also allow for an evaluation of potential side effects arising from anti-TNFα therapies.

A more specific approach would be to further develop our understanding of the TNFα induced pathways, and use such knowledge to develop breast-specific inhibitors. TNFα has established roles in aromatase regulation in spermatocytes, endometrial cells (Bourguiba et al. 2003; Salama et al. 2009) as well as bone and breast adipose, only the latter two of which is relevant to the post-menopausal women for whom such treatments would be targeted at. Further examination of the TNFα-induced pathways leading to PI.4 activation in bone is required to understand the molecular mechanisms involved in that system. Should TNFα induce a specific set of factors in bone that bind to and transcribe PI.4 in a way that is different to breast, then a possibility for breast-specific inhibition arises. The data presented here shows that EGRs form an integral part of the pathway, and that knocking down their expression significantly lowers aromatase expression. siRNA directed therapies aimed at lowering their expression in the breast may be a viable option, however it first must be determined whether the same signalling mechanisms involving the EGRs are utilised in bone derived aromatase transcription. A more specific downstream target of TNFα and the EGRs would be ideal. The identification of direct PI.4 binding factors would allow for more detailed analysis of factors which may be exclusively induced by TNFα in the breast that may represent novel therapeutic targets.

This work has also highlighted how existing therapies may or could be contributing to ER+ breast cancer treatment. By identifying TNFα as a downstream target of ERα signalling, a new mechanism by which SERM therapies such as Tamoxifen function to restrict ER+ tumour growth has been uncovered. Antagonising ERα action in breast tumours may result in a down regulation of TNFα secretion in to the tumour microenvironment, and whilst TNFα would still be present from the contribution of infiltrating immune cells, a restriction on tumour-derived TNFα would contribute to less oestrogen-producing capacity from surrounding fibroblasts. The establishment of NFκB and MAPK as part of TNFα induced aromatase expression also suggests that targetting these pathways may also contribute to restricting ER+ tumour growth. Whilst there are no NFκB inhibitors in clinical trials, MAPK inhibitors have been tested in the treatment of melanoma (Falchook et al. 2012) and thyroid cancer (Hong et al. 2011). Such trials may also be beneficial to assess the efficacy of MAPK inhibitors in the treatment of ER+ breast cancer.

6.4 Epigenetic regulation of aromatase expression

Whilst no data relating to the epigenetic regulation of gene expression in breast cancer was presented in the results chapters of this thesis, this area of research was a recurring theme in publications produced during candidature. Future directions may involve integrating results from this thesis with other studies performed to attain a greater understanding of epigenetic regulation of oestrogen biosynthesis in breast cancer.

Studies in to the epigenetic regulation of *CYP19A1* expression in BAFs revealed that PI.4 was the primary mediator of 5aza induced upregulation. This however was not due to direct changes to the methylation status of the limited number of CpG sites found within the PI.4 region, suggesting upstream factors were responsible (Knower et al. 2010). The work presented here may suggest further insight in to these mechanisms. Further experiments to determine whether the EGRs or their downstream targets are epigenetically regulated may help us elucidate the epigenetic mechanisms that regulate *CYP19A1* expression. Analysis of the promoter regions of *EGR2* and *EGR3* reveal substantial CpG islands in both (data not shown), suggesting that demethylation within their promoter region, and therefore increased expression, could be behind the increased PI.4 seen when BAFs are treated with 5aza. This is seemingly in contrast with previous data indicating that methylation within exon 1 of *EGR2* actually conveyed an enhancer-like activity leading to increased expression in cancer cell lines, although breast cancer cells were not amongst those examined (Unoki and Nakamura 2003). Aberrant methylation of *EGR3* is also found in T-cell leukaemia cells, its expression increased by 5aza treatment (Yasunaga et al. 2004). Significantly, a screen of MCF7 cells treated with melatonin revealed EGR3 to be oncogenic and down regulated via epigenetic mechanisms to in part mediate the anti-cancer effects of melatonin (Lee et al. 2012). Given that the DNA binding site for all the EGRs is CG rich, another possibility is that downstream activation of its target genes is either disrupted or enhanced through methylation-induced alterations to the capacity of EGRs to bind to its consensus sequence. Further studies in to how DNA methylation and other epigenetic processes are influencing PI.4 expression though the EGRs are certainly warranted.

Epigenetic mechanisms may be involved further upstream still, at the level of TNFα expression itself. Although it does not have a classical CpG island in its promoter, a number of CpG sites are found and their methylation status can be inversely correlated with *TNFα*

mRNA expression levels in monocytes, macrophages and brain cells (Sullivan et al. 2007; Pieper et al. 2008). Recently, genome-wide profiling of differentially expressed genes following demethylation in MCF7 cells revealed strong involvement of TNFα mediated cancer pathways (Kim et al. 2012). *TNFα* itself showed inverse correlation between promoter methylation levels and mRNA expression in both MCF7 and MCF10A cells, although only relative methylation levels as measured by methylation specific PCR were taken, not specific methylation of individual CpG sites. To further explore the epigenetic mechanisms regulating *TNFα* in breast tumour epithelial cells, it would be interesting to treat a variety of cell lines with 5aza and also the histone deactylase inhibitor TSA to observe the relative contribution of DNA methylation and histone acetylation. Having already established a role for estradiol in regulating TNFα in tumour epithelial cells, it would then be interesting to investigate whether this is linked to epigenetics. Oestradiol can modify the expression of key epigenetic modulating enzymes including the DNA methyltransferases and histone deacetylases (Krusche et al. 2005; Cui et al. 2009). This is demonstrated in the MCF7 cell line, which upregulates mRNA and protein expression of HDAC6 in response to estradiol treatment (Saji et al. 2005). Microarray analysis reveals many overlapping targets of estradiol and DNA methylation in MCF7 cells, although TNFα was not amongst those genes identified (Putnik et al. 2012). Sequencing of individual CpG sites within the TNFα promoter would reveal whether its methylation status is inversely correlated with expression levels. Further sequencing after estradiol treatment would reveal whether there is a relationship between estradiol and epigenetics with regards to TNFα expression in breast tumour epithelial cells.

6.5 Conclusion

The work presented here has broad implications for the development of novel, breast-specific aromatase inhibitors for the treatment of ER+ breast cancers, and reveals new ways in which previously utilised treatment strategies may be aiding in tumour regression. Further expansion to knowledge on the pathways and molecular mechanisms resulting in both TNFα expression in the breast and its capacity to stimulate estrogen production will enhance treatment design potential.

APPENDICES

Appendix 1: Origins and actions of TNFα in post-menopausal breast cancer

Review article in press in *The Journal of Interferon and Cytokine Research*

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This review article is based on the literature review in Chapter 1, and focuses on the broad topic of my thesis research: The role of TNFα in post-menopausal breast cancer. My contribution to the manuscript was 90%, with Kevin Knower and Colin Clyne providing reviewing and editing assistance.

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REVIEWS

Origins and Actions of Tumor Necrosis Factor α in Postmenopausal Breast Cancer

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Tumor necrosis factor α (TNFα) has many roles in both physiological and pathological states. Initially thought to cause necrosis of tumors, research has shown that in many tumor types, including breast cancer, TNFα contributes to growth and proliferation. The presence of TNFα—derived from the tumor and infiltrating immune cells—within a breast tumor microenvironment has been correlated with a more aggressive phenotype, and the postmenopausal ER+ subtype of breast cancers appears to strongly respond to its many pro-growth signaling functions. We discuss how TNFα regulates estrogen biosynthesis within the breast, affecting the activity of the key estrogen-synthesizing enzymes aromatase, estrone sulfatase, and 17β-HSD type 1. Additionally, we describe the anti-adipogenic actions of TNFα that are critical in preventing adjacent estrogen-producing adipose fibroblasts from differentiating, ensuring that the tumor maintains a constant source of estrogen-producing cells. We examine how the increased risk of developing breast cancer in older and obese individuals may be linked to the levels of TNFα in the body. Finally, we evaluate the feasibility of targeting TNFα and its associated pathways as a novel approach to breast cancer therapeutics.

Introduction

Estrone, estriol, and estrodiol form the biologically active group of hormones collectively known as estrogens (Chen and others 2008). Enzymatically catalyzed in a series of rate-limiting steps from cholesterol in specific tissues, estrogens may circulate as an endocrine hormone or act locally on target cells (Bulun and others 2005). In humans, estrogen acts as the primary female sex hormone, regulating processes related to the growth, differentiation, and physiology of the reproductive system while also exerting its effects in bone, brain, liver, and the cardiovascular system (Pearce and Jordan 2004). Estrogen action also contributes to the pathological states seen in breast, ovarian (Pujol and others 1998), colon (Campbell-Thompson and others 2001), and prostate cancers (Chen and others 2008). The link between estrogens and breast cancer was first postulated as early as 1934 when an early study demonstrated the ability of prolonged exposure to estrogens to cause legions on the breast (Geschickter and others 1934). Work in this area has progressed rapidly since then, and our understanding of the role that estrogens play in breast cancer is now much more advanced. Accompanying this has been the development of a range of therapeutic drugs to target the action of estrogen in breast cancer.

Local estrogen production is a major contributing factor to the risk of developing breast cancer (Miller 2006), with up to 70% of postmenopausal tumors expressing the estrogen receptor and therefore classed as ER+ tumors. Estrogens are strongly associated with continuing development and progression of disease pathology (Manning and others 1990). Many factors have been identified within the breast tumor microenvironment that are able to stimulate increased local estrogen production. One that has risen to prominence recently is the proinflammatory factor tumor necrosis factor-α (TNFα). This cytokine helps to link the role of the invading immune system in tumor development and to explain the altered breast cancer risk that comes with advanced age and obesity (Reed and Purohit 1997). In this review, we explore the molecular basis for TNFα regulation of estrogen in breast cancer and explore how it contributes to disease risk. We also assess the suitability of TNFα as a therapeutic target for ER+ breast cancer treatments.

TNFα: A Pro-Inflammatory, Pro-Cancer Cytokine

A factor that could induce necrosis of tumors in mice was first isolated from serum (Carswell and others 1975; Haranaka and Satomi 1981). The mouse itself was producing TNF from macrophages and lymphocytes, capable of inducing death in a number of cell types not restricted to cancer cells (Granger and others 1969). This discovery was heralded as a potential new therapy for cancer, whereby the

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growth of a tumor could be retarded using this molecule. Consequently, the human genomic clone was quickly isolated and the cDNA and protein sequence found to be 80% homologous to the mouse TNFα (Marmenout and others 1985; Pennica and others 1984; Shirai and others 1985). Although systemic toxicity proved to be an insurmountable barrier to the use of TNFα as an anti-tumor agent, the study of this molecule and its related TNF superfamily members has remained important to the understanding of apoptosis, immune defenses, inflammation, and cancer processes (Locksley and others 2001).

In humans, TNFα is produced primarily by the macrophages and monocytes of the immune system (Kornbluth and Edgington 1986), but production has also been detected in lymphoid cells, endothelial cells, neuronal tissue, adipose fibroblasts, and tumor cells (Wajant and others 2003). TNFα signals via 2 receptors: type 1 (TNFR1) and type 2 (TNFR2). Both bind TNFα with equal high affinity (Grell and others 1995; Grell and others 1998); however, differences lie in their tissue distribution. TNFR1 is constitutively expressed in most tissues and is thought to be the primary mediator of TNFα signaling in the body. TNFR2 expression has thus far been isolated to the cells of the immune system, where its role is more pronounced in the lymphoid system (Wajant and others 2003). As a consequence, much more is understood about signal transduction via TNFR1.

TNFα has the unique property among its related family members in that it displays proinflammatory effects in addition to its proliferation and apoptotic abilities (Tracey and others 1986). The wide ranging effects of TNFα on a number of different cell types have made it an intently studied target not only for disease therapies but also for understanding basic cellular processes and their regulation.

TNFα and Breast Cancer

As a potent signaling molecule, TNFα has been found within the microenvironment of several cancer types, including pancreatic (Karayiannakis and others 2001), renal (Yoshida and others 2002), prostate (Pfitzenmaier and others 2003), kidney, lung, bladder, oesophageal, melanoma, and leukemia (Mantovani and others 2000). In a breast tumor environment, increased concentrations of TNFα have been detected in the breast cyst fluid and breast tumor cytosol, both of which are known to stimulate estrogen production (Macdiarmid and others 1994). Furthermore, the presence of TNFα has been strongly correlated to a metastatic, invasive breast tumor phenotype (Miles and others 1994; Leek and others 1998). Serum concentrations of TNFα are also higher in patients with more advanced breast cancers (Sheen-Chen and others 1997), and can be used as a predictive marker for response to chemotherapy treatments (Berberoglu and others 2004). This observation is consistent with clinical findings and animal studies suggesting that endogenously secreted TNFα in the presence of a tumor exerts a proliferative response rather than tumor regression (Mochizuki and others 2004; Zarovni and others 2004), contrary to the initial isolation of TNFα as a potential anti-cancer agent.

TNFα appears to be particularly critical to the development and progression of the estrogen-responsive breast tumor type. The activity of key estrogen-biosynthesis enzymes have been shown to be increased by TNFα in adipose tissue, namely, aromatase (Purohit and others 2002), estrone sulfatase (Newman and others 2000), and 17β-hydroxysteroid dehydrogenase type 1 (Duncan and others 1994). TNFα can also modulate expression of genes in ER+ breast cancer cell lines that lead to the promotion of cell growth and proliferation, metastasis, loss of cell cycle control, and degradation of the extracellular matrix (Jeoung and others 1995; Yin and others 2009; Li and others 2012).

Sources of TNFα Within the Breast Tumor Microenvironment

Heightened concentrations of TNFα are found within the breast tumor microenvironment, with several different cell types thought to contribute to cytokine production. Tumor-associated macrophages and lymphocytes, which constitute up to 50% of the total tumor volume (Kelly and others 1988), are thought to be the primary contributors. TNFα is associated with immune response, and in the healthy body is mainly produced by cells of the immune system (Kornbluth and Edgington 1986). Tumor epithelial cells secrete chemoattractants such as Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), which leads to an invasion of immune cells into the breast tumor matrix (Leonard and Yoshimura 1990; Baggiolini and others 1994). These immune cells in turn respond to soluble factors secreted from breast cancer cells by upregulating their production of TNFα (Eichbaum and others 2011). Conditioned media from cultured monocytes and lymphocytes of breast cancer patients are able to stimulate aromatase activity in human breast adipose fibroblasts, and this can be attributed to the high levels of TNFα also detected in the media (Singh and others 1997).

Evidence has suggested that tumor cells are themselves able to produce TNFα in order to achieve proliferative advantage, although this has not been confirmed of breast cancer epithelial cells. However, several of the main factors known to stimulate TNFα production are present within a breast tumor microenvironment, suggesting that TNFα secretion by tumor epithelial cells is a contributing factor to disease pathology. TNFα is able to positively auto-regulate its own expression in murine 3T3-L1 preadipocytes (Neels and others 2006), and given the abundance of the cytokine surrounding tumor epithelial cells it is possible that it upregulates its own expression. Estrogen increases the expression and secretion of TNFα in cells in uterine and lactotrope cells (De and others 1992; Zaldivar and others 2011), and while this has not been confirmed in breast cancer cells, raises the possibility that a positive feedback loop between TNFα and estrogen exists within a breast tumor microenvironment. Supporting this hypothesis is the finding that estrogen increases the expression of the TNFα receptor TNFR1 in human breast adipose fibroblasts, thereby potentiating the response of these cells to TNFα (Deb and others 2004).

Epigenetic regulation, specifically DNA methylation, is another poorly defined area of TNFα in the context of ER+ breast cancer. It has been established in neuronal cells, hematopoietic stem cells, and macrophages that TNFα expression is under epigenetic regulation (Sullivan and others 2007; Pieper and others 2008). Indeed, a screen for genes differentially expressed following DNA demethylation with 5-aza-2′-deoxycytidine showed that TNFα and many of its target genes showed increased expression (Kim and others 2012). Epigenetic mechanisms are known to factor in the

regulation of estrogen biosynthesis as well as many other key breast cancer processes (Knower and others 2010), and the elucidation of its role in TNFα regulation is critical to our overall understanding of the importance of DNA methylation and histone modifications.

TNFα Regulation of Estrogen Biosynthesis

Estrogen biosynthesis

The process of biological estrogen production begins in the mitochondrion, where cholesterol is enzymatically converted to active estrogens through a number of rate limiting steps. The activity of 3 main enzymes is responsible for the production of biologically active estrogens: estrone sulfatase, estradiol-17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1), and aromatase. Aromatase converts androstenedione to estrone via an aromatisation reaction, while estrone sulfatase forms estrone from the estrone sulfate precursor. 17β-HSD type 1 then catalyzes the reduction of estrone into estrodiol, the biologically active form of estrogen (see Fig. 1). TNFα

Aromatase

Aromatase is the key enzyme responsible for the conversion of androgens to estrogens. Its highly tissue-specific expression is regulated at the transcriptional level of its encoding gene *CYP19A1* via tissue-specific promoters (Agarwal and others 1995). In premenopausal women, aromatase expression primarily localizes to the ovary. Here, *CYP19A1* expression is mediated via the proximal cAMP-responsive promoter II in response to FSH signaling (Means and others 1991; Jenkins and others 1993). Once a woman reaches menopause, the ovaries cease estrogen production, and the source of oestrogen biosynthesis switches primarily to the adipose tissue. This occurs largely in breast adipose, with a low level of aromatase expression maintained in this tissue via the use of distal promoter I.4 (PI.4) (Zhao and others 1995a). PI.4 activity can be stimulated *in vitro* by cytokines such as TNFα, oncostatin M (OSM), IL-6, or IL-11 (Zhao and others 1995b, 1996) in conjunction with the synthetic glucocorticoid dexamethasone (DEX) (Simpson and others 1981).

OSM, IL-6, and IL-11 have previously been demonstrated to act via the Jak/STAT pathway and the upstream GAS element within PI.4 in breast adipose fibroblasts (Zhao and others 1995b). The mechanisms by which TNFα is able to activate PI.4 are not yet clear, although a previous study has suggested that an upstream AP-1 element may be involved (Zhao and others 1996). While it has been shown many times *in vitro*, attempts to obtain *in vivo* evidence of a cytokine-glucocorticoid interaction have not been successful. Studies on women given DEX during therapy found that aromatase activity and, consequently, estrogen conversion were not enhanced (Reed and others 1986). Similar studies in monkeys gave the same result (Longcope 1987). *In vitro*, TNFα has been shown to induce *CYP19A1* PI.4 activity in pachytene spermatocytes (Bourguiba and others 2003) and EM1 endometrial cells (Salama and others 2009) without the presence of DEX; however, again this has not been shown *in vivo*. The evidence points to a critical role for TNFα in aromatase regulation, and elucidating its mode of action will be important in the treatment of estrogen-driven breast cancers and other diseases.

Estrone sulfatase

Following aromatisation of androstenedione, most of the resulting estrone is converted to estrone sulfate, which is the primary circulating form of estrogens as it has a much longer half-life than other forms (Ruder and others 1972). Estrone sulfate is thought to form a reserve of biologically inactive estrogens ready to be converted into the active hormone firstly through its conversion back to estrone via the action of estrone sulfatase (Purohit and others 2002). Expression of estrone sulfatase mRNA is reported to be robust in most breast tumors when compared with normal breast, and this is associated with a larger tumor volume and poorer prognostic outcomes (Utsumi and others 1999a, b, 2000; Suzuki and others 2003; Honma and others 2006). In addition to increased expression, activity of estrone sulfatase is heightened in breast cancer tissue compared with healthy breast (Santner and others 1993; Evans and others 1994).

Little is known about the role that cytokines, and in particular TNFα, may play in the regulation of estrone sulfatase expression and activity in the breast tumor microenvironment. TNFα, along with IL-6, increases the activity but not transcript levels of estrone sulfatase in primary human fibroblasts derived from both normal and malignant breast tissue (Purohit and others 1996). This suggests that these cytokines are involved in the post-translational modifications of the active enzyme rather than its transcriptional regulation (Purohit and others 2002). Recently, however, it was demonstrated in the ER+ breast cancer cell line MCF7, the ER- breast cancer cell line MDA-MB-231, as well as 2 prostate cancer cell lines that treatment with TNFα significantly increased mRNA expression of estrone sulfatase via the PI-3 kinase/Akt pathway (Suh and others 2011). Therefore,

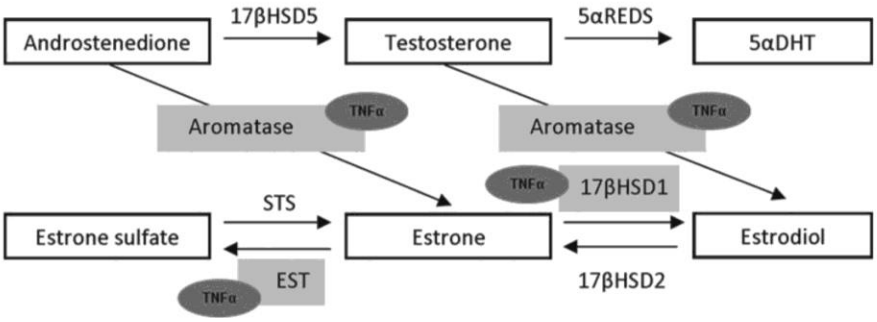

FIG. 1. Tumor necrosis factor-α (TNFα) and its effect on the intratumoral estrogen biosynthesis pathway. Schematic representation of the enzymatic conversion from androgens to active estrogens, and the stages at which TNFα is thought to play a key regulatory role.

targeting the TNFα-mediated increases in estrone sulfatase transcription and activity could potentially reduce the bioavailability of estrogens to an ER+ breast tumor.

17β-HSD type 1

Estrone must be reduced to estrodiol in order to achieve its full biological activity, and this is mediated through the action of 17β-HSD type 1 (Dumont and others 1992). Expression of 17β-HSD type 1 is strongly correlated with ER+ as well as progesterone receptor-positive breast cancers, indicating local synthesis of estrodiol (Ariga and others 2000). Overall metabolism of estrogens favors inactivation of estrodiol over conversion of estrone to estradiol; however, within tumor tissue there is preferential reduction to the biologically active estrodiol (Beranek and others 1984). This suggests that factors within the tumor are preferentially driving the formation of estrodiol, and TNFα may be a contributing factor. An early investigation into the role of cytokines in breast cancer revealed that TNFα stimulates the conversion of estrone to estrodiol in MCF7 breast cancer cells (Duncan and others 1994). TNFα also increases the activity of 17β-HSD type 1 in the ER+ cell line T47D and ER- cell line MDA-MB-231 (Duncan and Reed 1995), indicating that it acts in a variety of tumor microenvironments. Outside of the breast, TNFα has also been found to stimulate activity of 17β-HSD type 1 in endometrial glandular epithelial cells (Salama and others 2009). Taken together, these finding indicate that TNFα plays a critical role *in vivo* to increase the bioavailability of active estrogens.

Anti-Adipogenic Actions of TNFα in the ER+ Breast Tumor Microenvironment

As well as promoting estrogen biosynthesis in cancer-associated fibroblasts, TNFα also plays a major role in maintaining these fibroblasts in an undifferentiated state, thus ensuring a constant source of estrogen-producing cells for the tumor.

The desmoplastic reaction is critical in maintaining estrogen supply to the ER+ breast tumor (Deb and others 2004). This dense layer of undifferentiated fibroblasts immediately adjacent to the malignant epithelial cells gives such tumors their characteristic hard consistency (Bianco and others 1995), and many factors secreted by the tumor are directed toward maintaining this layer of undifferentiated cells. This is important in breast tumor pathology as only undifferentiated fibroblasts maintain the capacity to express aromatase and produce active estrogens, a feature lost once differentiation into mature adipocytes occurs (Clyne and others 2002). Accumulation of preadipocytes does not appear to be the natural reaction to growth stimuli, since mouse 3T3-L1 preadipocytes treated with a combination of known growth factors initially proliferate, but eventually differentiate into mature adipocytes under the same conditions (Schmidt and others 1990; Boney and others 1998). ER+ breast tumors must therefore both cause an accumulation of preadipocytes and at the same time prevent them from differentiating into mature adipocytes. TNFα appears to be a major driver of this process (Chae and Kwak 2003).

The 2 critical transcription factor families that commit the preadipocyte toward differentiation in to a mature fat cell and maintain its phenotype are CCAAT/enhancer binding protein (C/EBP) and preoxisome proliferator-activated receptor-gamma proteins (PPARs) (Cao and others 1991; Brun and others 1996). These proteins work to transactivate adipocyte-specific gene expression, and inhibition of their expression or activity can lead to inhibition of preadipocyte differentiation as stimulated by known differentiation factors, or dedifferentiation of committed adipocytes (Tamori and others 2002). Two key family members are C/EBPα and PPARγ, which together act synergistically to drive mature adipocyte differentiation (Meng and others 2001). TNFα can repress the expression of both C/EBPα and PPARγ in preadipocytes, directly inhibiting their capacity to differentiate (Zhang and others 1996). This has been demonstrated in 3T3-L1 cells, mouse adipose tissue, and primary human mammary adipocytes (Hu and others 1995; Chae and Kwak 2003; Guerrero and others 2009). The source of this TNFα appears to be from the tumor epithelial cells themselves, as conditioned media collected from MCF7 and T47D breast cancer cell lines show an enrichment of the cytokine. Treatment of 3T3-L1 preadipocytes with this conditioned media leads to a reduction in C/EBPα and PPARγ expression (Guerrero and others 2009). Melatonin, a naturally secreted hormone from the pineal gland, has been shown to interfere with this process, down regulating the expression of TNFα in epithelial cells while inhibiting aromatase expression in the surrounding fibroblasts (Alvarez-Garcia and others 2012; Knower and others 2012). Further elucidation of other factors capable of restricting TNFα secretion from epithelial cells and therefore its role in the desmoplastic reaction will need to be uncovered in order to improve options for breast cancer therapy.

Clinical Associations Between TNFα and Breast Cancer Risk

Advanced age and TNFα

Advanced age is a major risk factor in the development of breast cancer. Approximately 70% of postmenopausal cases are diagnosed as ER+ tumors, suggesting that processes within the postmenopausal endocrine system are altered as such that peripheral estrogens, particularly those in the breast, are being upregulated. Increased levels of plasma TNFα in older individuals may help to provide an explanation.

As well as being a critical pro-inflammatory immune cytokine, TNFα is also implicated in a number of disease pathologies. These include rheumatoid arthritis (Maini and Taylor 2000), inflammatory bowel disease (Bruin and others 1995), osteoporosis (Fujita and others 1990), and atherosclerosis (Fukuo and others 1997). Most of these conditions affect older individuals, suggesting that increasing concentrations of TNFα in those with advanced age contribute to common diseases associated with aging. Breast cancer could be one other such disease. Animal models were initially investigated to establish a link between aging and increasing TNFα levels. Aged mice and rats show a significantly increased secretion of cytokines from the T-helper cells of their immune systems, and this is likely to account for the increased peripheral estrogen synthesis also observed (Chorinchath and others 1996; Morin and others 1997). A number of studies have since examined this association in a large human cohort and uncovered similar associations between increasing age

and higher levels of measured serum TNFα (Paolisso and others 1998).

Obesity and TNFα

In addition to advanced age, rates of breast cancer occurrence are significantly higher in obese women, with increased weight strongly associated with a higher risk of developing not only breast but many other forms of cancer (Basen-Engquist and Chang 2011). Obese breast cancer patients also show higher mortality rates, greater metastasis to distal sites, larger tumor mass, and overall poorer prognosis when compared to nonobese breast cancer patients (Maruthur and others 2009; Hauner and others 2011). Again, strong associations between TNFα and obesity may help to provide an explanation.

In addition to being produced by cells of the immune system, TNFα is also produced in adipose tissue, including mature adipocytes, stromal-vascular cells, and preadipocytes (Hube and others 1999; Weisberg and others 2003; Fain and others 2004). Initially shown in animal models (Hotamisligil and others 1993), TNFα levels are also markedly increased in the adipose tissue of obese individuals (Hotamisligil and others 1995). This has been shown clinically as obese patients record a higher serum concentration of TNFα than age-matched healthy weight individuals. This effect was decreased upon surgery-mediated weight loss (Hotamisligil and others 1995). TNFα may indeed be one of the driving forces behind obesity and insulin resistance, as mice lacking a functional TNFα protein or receptor are protected from diet-induced obesity and insulin resistance (Schreyer and others 1998; Nieto-Vazquez and others 2008). The mechanism resulting in increased TNFα production in states of obesity is, however, undefined, and this knowledge may help explain why obese individuals are at higher risk of breast cancer. It has been recently been shown that TNFα positively regulates its own transcription and secretion in adipose tissues, perhaps explaining how high levels of the cytokine are maintained in obesity (Neels and others 2006).

Significantly, adipose tissue is also the major site of estrogen conversion in postmenopausal women, highlighting a link between increased TNFα and estrogen production. The increased risk of developing breast-cancer in obese women may therefore not only be associated with the increased estrogen production from the higher volume of fat cells, but also with the increased production of TNFα which may further drive estrogen production. Studies in mice have shown that obesity is associated with increased aromatase activity and TNFα expression in the mammary gland (Subbaramaiah and others 2011), supporting this hypothesis.

The Potential for TNFα-Targeted Breast Cancer Therapies

Given its important role in many facets of breast cancer development, progression, and maintenance, TNFα represents an attractive yet challenging therapeutic target. As demonstrated, TNFα plays vital roles in maintaining and upregulating local estrogen biosynthesis as well as preventing the differentiation of estrogen-producing preadipocytes into mature adipocytes. Reducing the capacity of the breast tumor to do either of those things would severely restrict its growth and proliferative potential.

Numerous clinical trials have already investigated the effectiveness of anti-TNFα therapies for the treatment of a number of associated diseases such as septic shock, rheumatoid arthritis, Chrones disease, and even multiple sclerosis (Shimamoto and others 1988; Elliott and others 1993; van Dullemen and others 1995; Hohlfeld 1996) (see Table 1). Currently used in the clinic are a number of approved monoclonal antibodies as well as a soluble TNFα receptor for the treatment primarily of rheumatoid arthritis patients (Thalayasingam and Isaacs 2011). Concerns were initially raised about their potential to increase a patient's risk of developing certain forms of cancer; however, it is now clear that cancer incidence and prognosis were no worse in patients treated with anti-TNFα therapies compared to those who had not received the treatment (Askling and others 2005; Raaschou and others 2011). Due to its importance in

Table 1. Current Anti-Tumor Necrosis Factor-A Drug Therapies and Their Applications

	Inflaximab	*Adalimumab*	*Golimumab*	*Etanercept*	*Certolizumab*
Type[1]	Monoclonal antibody	Monoclonal antibody	Monoclonal antibody	P75TNFR/Fc fusion protein	PEGylated humanized Fab fragment
Recognizing ligands[1]	TNF	TNF	TNF	TNF and LTα3	TNF
Molecular weight[1]	150 kDa	150 kDa	150 kDa	150 kDa	95 kDa
Half-life (days)[1]	8–10	10–14	12 ± 3	3	14
Uses	Chrones disease,[2] rheumatoid arthritis,[3] psoriatic arthritis,[4] psoriasis[5]	Chrones disease,[6] rheumatoid arthritis,[7] psoriasis[8]	Rheumatoid arthritis,[9] psoriatic arthritis[10]	Rheumatoid arthritis,[11] psoriatic arthritis,[4] plaque psoriasis[8] and ankylosing spondylitis[12]	Chrones disease,[13] rheumatoid arthritis[14]

Summary of currently available anti-TNFα therapies. References as indicated within the table in superscript: [1]Thalayasingam and Isaacs (2011), [2]van Dullemen and others (1995), [3]Harriman and others (1999), [4]Woolacott and others (2006), [5]Kirby and others (2001), [6]Bultman and others (2012), [7]den Broeder and others (2002), [8]Chastek and others (2013), [9]Zhou and others (2007), [10]Yang and others (2011), [11]Wakabayashi and others (2012), [12]Dougados and others (2012), [13]Schreiber (2011), [14]Weinblatt and others (2012).
TNF, tumor necrosis factor.

the immune response, the most significant risk appears to be in susceptibility to infectious diseases. Indeed, rheumatoid arthritis patients treated with anti-TNFα therapies appear to be at a greater risk of developing skin infections, soft-tissue infections, and septic arthritis (Dixon and others 2006; Galloway and others 2011). The risk appears to be higher in the first 6 months of treatment, and is enhanced with advanced age and concurrent use of glucocorticoid treatments (Askling and others 2007; Strangfeld and others 2011). Tuberculosis, listeria, salmonella, and legionella infections also appear at a higher rate in anti-TNFα-treated patients (Dixon and others 2006, 2010).

TNFα-targeting monoclonal antibodies have so far been demonstrated to retard mouse mammary tumor growth *in vivo*, as well as inhibiting proliferation of skin cancer cells (Scott and others 2003). Furthermore, TNFα null mice show lower rates of induced tumor formation than the wild type, with TNFα-neutralizing antibodies again able to slow rates of tumor growth (Warren and others 2009). This demonstrates a potential for the use to anti-TNFα therapies in the clinic. TNFα is a ubiquitous cytokine, and its critical role in the pro-inflammatory immune response means that it is present in many tissues as well as circulating plasma. To target its actions in breast cancer would therefore require a therapy to limit undesirable side effects. Indeed, a case of primary breast tumor has been reported in a patient undergoing long-term anti-TNFα treatment for rheumatoid arthritis, suggesting that tumor development not regression may in fact result from anti-TNFα therapies (Pattanaik and others 2011). Further work to elucidate the precise molecular pathways by which TNFα acts and how it is produced to excess within a breast tumor microenvironment is required so that further downstream components may be targeted. For example, agents stabilizing microtubules in breast fibroblasts inhibit TNFα-induced aromatase activity (Purohit and others 1999). Identifying other similar pathways would result in a more specific blockade of TNFα action in breast cancer without compromising its critical immune function.

Summary and Future Perspectives

The research presented in this review highlights the many complexities of the role TNFα has to play within the breast tumor microenvironment. Not only is it implicated in the transcription and activation of key estrogen-producing enzymes aromatase, estrone sulfatase, and 17β-HSD type 1, but it plays a key part in maintaining supporting stroma adjacent to the tumor epithelial cells in an undifferentiated state so that they may continue to produce estrogen. It is found in abundance within the breast tumor microenvironment, with the tumor itself thought to contribute to its production (Fig. 2).

Our understanding of the molecular basis for the actions of TNFα in breast cancer is more developed, but many questions are yet to be answered. For example, it has been well established that TNFα induces expression of the aromatase gene *CYP19A1* and that this is via its adipose-specific promoter. What has not been identified is by which signal transduction pathway this is occurring, and what specific transcription factors and *cis*-acting elements are being activated to initiate this response. Defining the exact mechanisms by which TNFα stimulates aromatase transcription is important if we are to target more specifically in the breast this key estrogenic enzyme. Our understanding of how TNFα contributes to the upregulation of the other estrogen-forming enzymes is not much more advanced, for while their increased activity has been correlated with the presence of TNFα, whether this key cytokine is acting at the transcriptional or translation level and how this is occurring has not been uncovered. Estrogen formation is a precisely regulated process involving many genes and enzymes, and consideration of all components of the pathway must be given in order to effectively limit hormone production.

Targeting TNFα formation and action has also been proposed as a potential novel method for treating ER+ breast cancer (Reed and Purohit 1997). This would not only contribute to lowering the production of estrogen in the stromal cells, but effect the desmoplastic reaction so critical to the

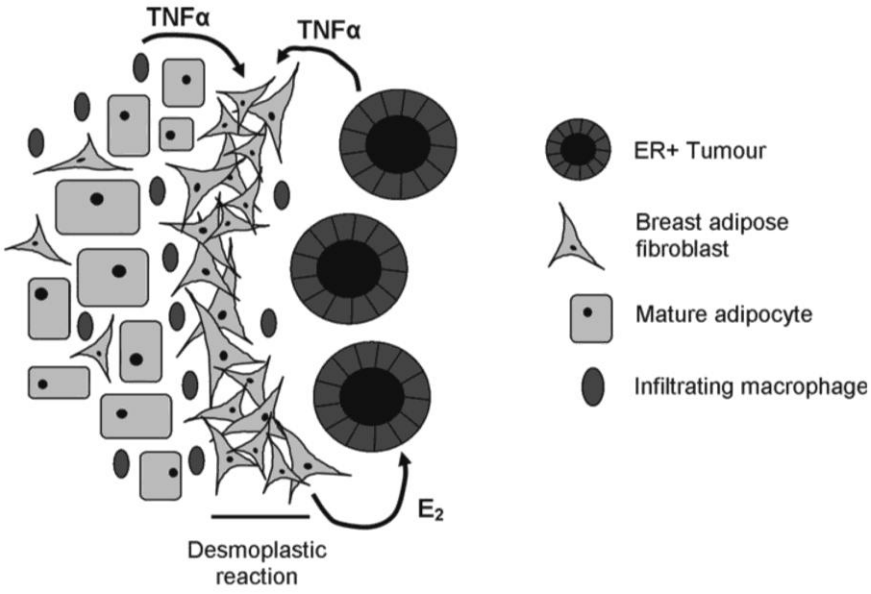

FIG. 2. Model of TNFα formation and action within the breast tumor microenvironment.

tumor's pathology. As discussed, TNFα sequestering monoclonal antibodies are in clinical use for the treatment of several inflammatory-related diseases. Although mouse models have shown a positive response to TNFα antibodies with respect to slowed breast tumor growth, such a treatment has to date not been trailed in humans specifically for the treatment of breast cancer. The effects an anti-TNFα treatment would have on the immune system need to be considered, as increased rates of infection have been shown when TNFα is targeted as a signaling molecule.

In conclusion, TNFα is a complex cytokine implicated in not only the pathology of ER+ breast cancer but also in the risk of developing the disease. It presents an attractive target for therapeutic intervention due to its multi-function role in the tumor microenvironment; however, our limited understanding of the molecular basis for its actions hinders its translational development in to clinical use. Further research in to the basic mechanisms of TNFα secretion and action within the breast is required before we are able to consider it as a drug development target.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Appendix 2: Intracrine oestrogen production and action in breast cancer: an epigenetic focus

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Review article written in collaboration with Kevin Knower and Colin Clyne. My contribution was 25%, having written the section entitled "Overview of the dynamics of epigenetic regulation" and co-writing the section entitled "Oestrogen regulation of epigenetic modifying enzymes in the breast" and also reviewing the overall manuscript. This was an invited review article for a special issue of the Journal of Steroid Biochemistry and Molecular Biology on steroids and cancer.

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Review

Intracrine oestrogen production and action in breast cancer: An epigenetic focus

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ABSTRACT

Epigenome changes have been widely demonstrated to contribute to the initiation and progression of a vast array of cancers including breast cancer. The reversible process of many epigenetic modifications is thus an attractive feature for the development of novel therapeutic measures. In oestrogen receptor α (hereinafter referred to as ER) positive tumours, endocrine therapies have proven beneficial in patient care, particularly in postmenopausal women where two-thirds of tumours are oestrogen dependent. However, resistance to such therapies is a common feature amongst individuals. In the current review, we discuss the influence that epigenetics has on oestrogen dependent breast cancers, in particular (i) the production of intracrine oestrogen in postmenopausal women, (ii) the action of oestrogen on epigenetic processes, and (iii) the links between epigenetics and endocrine resistance and the current advancements in epigenetic therapy that target this process.

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Contents

1. Introduction

The majority of postmenopausal breast cancers are dependent on oestrogens, particularly the biologically potent 17β-estradiol (E2), for sustained growth and proliferation [1]. In premenopausal women, oestrogens arise from the ovary and behave in a classic endocrine manner through circulation, acting on target tissues that express specific oestrogen receptors [2]. Following menopause, oestrogen ceases to be produced by the ovaries, and circulating levels are reduced. Despite this, adrenal androgen substrates remain in abundance allowing for the conversion to oestrogens. In postmenopausal women this conversion occurs in peripheral tissues such as muscle, bone, skin and the brain [3–6]. In the breast, adipose tissue becomes the major source of local oestrogen production through the expression of metabolising enzymes and the peripheral conversion of androgens [4]. The transformation of this non-classical hormone producing site into an intracrine source of intra-tumoural oestrogen is a key feature of ER-positive breast carcinoma cells.

Our understanding of oestrogen biosynthesis and its actions in the breast tumour microenvironment has led to frontline endocrine therapies such as selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) that are used in the neo- and adjuvant setting [7]. In particular, AIs have become the gold standard for the treatment of postmenopausal women with oestrogen-dependent tumours [8]. One of the major challenges facing endocrine therapy is the development of resistance to prolonged oestrogen inhibition. Determining the cause or how this

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resistance can be alleviated in combination with other therapeutics is therefore an active focus of research. Changes in the epigenome are an underlying facet associated with all cancers studied to date, in particular breast cancer [9]. In this review, we describe current advances in our understanding of the epigenome changes associated with breast cancer that affect oestrogen biosynthesis, that are influenced by oestrogen and those that contribute resistance to endocrine therapy.

2. Overview of the dynamics of epigenetic regulation

Epigenetics describes a broad range of DNA and histone modifications that may modulate gene expression without altering the underlying coding sequence. This encompasses DNA methylation as well as histone modifications including acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP ribosylation. In combination, these processes work to regulate gene expression, mediate X-chromosome inactivation and facilitate genomic imprinting.

DNA is organised within the cell nucleus as a packaged structure known as chromatin. This dynamic configuration consists of DNA strands wrapped around histone proteins, and is consistently changing according to the transcriptional and replication needs of the cell. Epigenetics is the means by which chromatin structure is configured to prevent or allow access of transcriptional machinery to target genes, thus controlling gene expression levels. When a genomic region is in a tightly packed chromatin structure, transcription is repressed. Conversely, loosely packed chromatin allows transcription of local genes to initiate [10]. DNA methylation and histone modifications work to determine the open or closed state of chromatin, and therefore exert a significant influence on gene expression.

DNA methylation is a phenomenon that occurs in both prokaryotes and eukaryotes [11,12]. In mammals, DNA methylation occurs only on cytosine residues that are immediately 5′ of a guanine, described as a CpG site [13]. This dinucleotide is under-represented in the mammalian genome, however they appear to occur in clusters referred to as CpG islands. CpG islands are defined as nucleotide stretches greater than 200 bp in length with a CG percentage of at least 60% [14], and occur in the promoter regions of approximately 50% of genes [15]. However, methylation of CpG sites outside of CpG islands and also within coding exons can effect gene expression. Hypermethylation of promoter CpG regions leads to a recruitment of histone modifying enzymes to the chromatin, resulting in acetylation of local histones and compaction of the chromatin structure to prevent access for transcriptional machinery [16]. Thus, an inverse correlation exists between promoter methylation levels and transcription levels, whereby hypermethylayion results in low gene expression and hypomethylayion results in high gene expression. Methylation of even a single CpG site within a gene regulatory region may also effect gene expression by interfering with the binding of transcription factors to their response elements [17].

Cytosine methylation is mediated by three distinct DNA methyltransferase enzymes: DNMT1, which maintains existing methylation patterns [18], and DNMT3a and DNMT3b, which establish *de novo* methylation of CpG sites during developmental stages [19] but also to a lesser degree participate in the maintenance of methylation patterns during DNA replication [20]. All three catalyse the transfer of methyl groups from S-adenosyl-L-methionine to the 5′ position of cytosines as part of a CpG dinucleotide [15]. A number of mutations of the DNMTs have been reported, and these are associated with colorectal cancer [21], acute myeloid leukaemia [22] and acute monocytic leukaemia [23]. Additionally, overexpression of the DNMTs often occurs in cancer and contributes to the hypermethylation often observed in tumour tissue [24]. More recently, mechanisms of DNA demethylation have come to light, with research suggesting both active and passive means of removing methyl groups. Passive demethylation can occur when there is an error in DNA methylation maintenance during replication, resulting in a loss of methylation on the newly synthesised daughter strand [25]. Active demethylation conversely, relies upon a range of enzymes that can hydroxylate, further oxidise or deaminate methylated cytosines independent of DNA replication. The current model of DNA demethylation involves a two-step process: hydroxylation of methylated cytosines by ten-eleven-translocation (TET) proteins [26], followed by deamination by activation-induced cytidine deaminase (AID) enzymes [27]. Alternatively, it has been proposed that following hydroxylation, cytosines may be further carboxylated and then subsequently entered in to the base excision repair pathway [28]. Another possible explanation for demethylation is that hydroxymethylated cytosines are not recognised by maintenance DNMTs, therefore TET protein-mediated hydroxylation of methylated cytosines would result in a loss of methylation during subsequent replication cycles [29]. Three TET family members have been reported to date, including TET1, TET2 and TET3 [25]. Each appears to have a distinct function within a variety of cellular contexts [30]. Mutations in TET2 have been reported in T-cell lymphoma [31] and acute myeloid leukaemia [32], whilst other TET family members are less well characterised.

DNA methylation often works in synergy with histone modifications to regulate gene expression. Hypermethylation of CpG sites leads to a recruitment of methyl-binding proteins, which in turn recruit histone deactylases (HDACs) to the region and repress gene transcription by closing chromatin structure [16,33]. Eighteen different HDAC genes have been identified to date subdivided in to two major subgroups [34], however all HDACs function to deacetylate not only lysine residues in histone tails but non-histone proteins including transcription factors [35]. The action of HDACs is counterbalanced by histone acetyltransferases which are able to introduce acetylation to histones, loosening their structure in order to become more accessible to transcriptional machinery [36]. Histone methyltransferases and demethylases are also critical to conveying information about chromatin accessibility by controlling specific methylation signatures on histone lysine or argenine residues [37,38]. Other histone modifying enzymes which sumoylate [39], phosphorylate [40] and ubiquinate histones [41], although the dynamics of their regulatory ability are less well characterised.

More recently, the roles that non-coding RNAs (ncRNAs) have to play in epigenetic regulation of gene expression have come to light. These are broadly divided into two classes of long and short ncRNAs, and regulate expression both at the pre- and post-transcriptional level. Long ncRNAs are at least 200 nucleotides long [42] and are derived from diverse origins including gene regulatory regions [43], intergenic regions [44] and even mitochondria [45]. Long ncRNAs primarily act locally on the genomic region from which they are derived, attracting transcription factors or epigenetic modifiers. They may also act as precursors to shorter ncRNAs [46]. Conversely, short ncRNAs exert their effects through post-transcriptional regulation of gene expression. Consisting of microRNAs (miRNAs) [47], endogenous short interfering RNAs (endogenous siRNAs) [48] and PIWI interacting RNAs (piRNAs) [49], short-interfering RNAs differ in their origin, processing and structure, but all work post-transcriptionally to repress expression of not only target genes but also other ncRNAs [42].

3. Epigenetic regulation of oestrogen biosynthesis pathways

Before describing the epigenetic alterations mediating oestrogen production, a brief overview of the key players involved in oestrogen biosynthesis is required. The intracrine production of intra-tumoural oestrogen in breast cancer tissue is mediated

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G Model
SBMB-3933; No. of Pages 8
ARTICLE IN PRESS
K.C. Knower et al. / Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

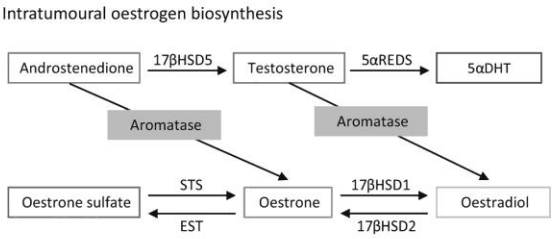

Fig. 1. Biosynthesis cascade of intratumoural oestrogen production in postmenopausal women. 17β-HSD: 17β-hydoxysteroid dehydrogenase isozymes; 5αREDS: 5α reductase; 5αDHT: 5α dihydrotestosterone; EST: oestrogen sulfotransferase; STS: steroid sulfatase.

through a cascade of enzymatic processes (Fig. 1). The cytochrome P450 aromatase enzyme, encoded by the *CYP19A1* gene, provides the key step of oestrogen production through aromatisation of circulating androgens [50], namely androstenedione which remains abundant in postmenopausal women [51]. The enzymatic activity of 17β-hydroxysteroid dehydrogenase (*17β-HSD*) isozymes, steroid sulfatase (*STS*) and oestrogen sulfotransferase (*EST*), provide an integral role in further production of bioactive E2. Interconversion of E2 and estrone (E1) is performed by 17β-HSD isozymes, specifically 17β-HSD type 1 reduces E1 to E2, whereas 17β-HSD type 2 oxidises E2 to E1 [52]. Circulating E1 is in a sulfated form or E1 sulfate (E1-S) and is hydrolyzed by STS to E1 [53], while EST sulfonates oestrogens to biologically inactive oestrogen sulfates [54].

The importance of aromatisation as the rate-limiting step in the production of oestrogen has identified this enzyme as a key therapeutic target and has resulted in the successful development of AIs. However, due to the wide spread expression of aromatase in peripheral tissues in postmenopausal women [55], adverse side effects arise in those women under AI therapy [56], namely decreases in bone mineral density [57,58]. Therefore, we and others have endeavoured to understand the complex regulation of the *CYP19A1* gene in the breast as a means of more targeted inhibition [59–67]. *CYP19A1* expression throughout the body is controlled by a number of tissue-specific promoter elements upstream of untranslated first exons that encode for identical proteins in respective tissues (reviewed in [68]). In postmenopausal breast cancer, *CYP19A1* transcripts derived from the tissue-specific promoters PI.4, PI.3 and PII are predominantly elevated by epithelial paracrine signals and located in adjacent adipose stromal cells [69–79], with transcripts also elevated amongst ER-positive carcinoma cells albeit not to the same extent [80,81].

Epigenetic regulation of aromatase has been demonstrated in both human and non-human tissues (summarised in Table 1). The earliest description demonstrated that inhibition of DNA methylation and histone deacetylation with the cytosine analogue 5-azacytidine or sodium butyrate respectively, resulted in increased aromatase activity in chicken fibroblasts [82]. Similar inhibition of epigenetic silencing processes have also been shown to increase aromatase expression and activity in human endometrial stromal cells [83,84], human hepatoma cells [85], sexual differentiation of the mouse brain [86] and sensitisation of ER-negative breast tumours to AIs [87]. The significance of this latter study will be discussed in later sections. In contrast, the histone deacetylation inhibitor panobinostat was found to repress PI.3/II mediated aromatase expression in breast cell lines and further augmented the effects of the AI letrozole [88].

DNA methylation and histone modifications have also been shown to play a prominent role in the promoter- and tissue-specific transcription of *CYP19A1* in various human [17,83] and non-human tissues [89–100]. As described earlier and outlined in Table 1, regulation of the *CYP19A1* gene is under the control of a number of tissue-specific promoters. Despite the fact that dense CpG islands are lacking proximal to these promoters, low-density CpG regions have still been demonstrated to mediate transcription. For example, CpG methylation status within the cAMP response element (CRE) of PII modulates *CYP19A1* expression in human skin adipose fibroblasts [17]; and that methylation status of PII and chromatin remodelling through histone modifications of PI.1, also coincide with the changes in expression of *CYP19* gene during folliculogenesis and luteinization in the buffalo ovary [96]. Together with DNA methylation and histone modifications, more recent studies have identified new roles that miRNAs, in particular let-7f, may have in targeting and silencing *CYP19A1* [101].

To ascertain the importance of these epigenetic links to aromatase regulation in the breast, using breast adipose fibroblasts (BAFs) we were able to demonstrate that *CYP19A1* was under epigenetic inhibition by DNA methylation, a process mediated *via* the tissue-specific promoter PI.4 [102]. Inhibition of DNA methylation in a number of breast epithelial cells also resulted in elevated *CYP19A1* transcripts, albeit *via* the proximal promoter PII. In both instances PI.4 or PII CpG promoter methylation status did not inversely correlate to mRNA expression [102]. The identification of a non-promoter CpG island region mediating the upregulation of *CYP19A1* in endometrial stromal cells therefore warrants further investigation in the breast, particularly clinically relevant samples [83]. In spite of this, *CYP19A1* is under epigenetic inhibition and deregulation may result in an epigenetic disorder increasing breast cancer risk, a process postulated to also occur in endometriosis [83,84].

Detailed evidence of whether genes encoding EST, STS and 17β-HSD isozymes are under epigenetic regulation in the breast is limited. DNA hypermethylation of the 17β-HSD type 1 promoter is however associated with decreased expression in colorectal cancer [103], a process alleviated when HDACs are inhibited [104]. Given the promising data for the use of 17β-HSD type 1 inhibitors in oestrogen-dependent breast cancers [105–107], a detailed characterisation of the epigenetic control of this gene in the breast clinical setting is warranted. With the aid of breast cancer cell lines, however, Fu and colleagues have demonstrated the contribution that HDACs may form in regulating these enzymes [108]. Treatment with the HDAC inhibitor trichostatin A (TSA) induced *EST* and *CYP19A1* mRNA but suppressed *STS*, *17β-HSD1*, and *17β-HSD2* mRNA in the normal epithelial MCF10A cell lines. In the ER-positive MCF-7 cell line, TSA treatment suppressed *STS* but induced *CYP19A1* [108]. Again, further evaluation of these correlations, particularly in breast tumour associated stromal cells will provide a clearer understanding.

4. Oestrogen regulation of epigenetic modifying enzymes in the breast

The epigenomic actions mediated by E2 through its nuclear receptor ER and associated co-activators and co-repressors involves multiple enzymatic alterations to DNA and protein function that act in the direct regulation of target genes in the breast, a process that lies outside of the scope of this review [109–111]. Furthermore, a recent review on the action of E2 in the regulation of miRNAs has been performed [112], with newer studies confirming a strong association between expression of miRNAs and the clinical outcome of hormone-responsive breast cancers [113]. However, as discussed above, DNMTs and HDACs are critical enzymes involved in the epigenetic regulation of gene expression. Emerging evidence has implicated E2 in modulating the expression of these enzymes, providing a reciprocal link between epigenetics and oestrogen biosynthesis.

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G Model
SBMB-3933; No. of Pages 8
ARTICLE IN PRESS

Table 1
Epigenetic means of aromatase regulation across different species and tissues. 5-aza: 5-azacytidine; 5-aza-dC: 5-aza-2′-deoxycytidine; TSA: trichostatin A; ENT: entinostat; SAHA: suberoylanilide hydroxamic acid.

Epigenetic modifying compounds affecting aromatase expression

Compound	Species	Cell type/tissue	Promoter	Reference
5-Aza, sodium butyrate	Chicken	Fibroblasts	n.d.	[82]
5-aza-dC	Human	Endometrial stromal cells	PII, PI.3, PI.6, non-promoter CpG island	[83,84]
5-aza-dC, TSA	Human	HepG2	n.d.	[85]
TSA	Rat	Brain	PII	[86]
ENT, SAHA	Human	Breast cells: MDA-MB-231, SKBr3, Hs578t	n.d.	[87]
Panobinostat	Human	H295R, MCF7	PII, PI.3	[88]
5-aza-dC	Human	Breast adipose fibroblasts, MCF7, MDA-MB-231, MCF10A	PI.4, PII	[101]

Promoter- and tissue-specific epigenetic regulation of aromatase

Promoter	Species	Cell type/tissue	Regulatory mechanism	Reference
P2	Bovine	Granulosa	DNA methylation	[89,95,99]
PII	Rat	Granulosa	Histone acetylation and methylation	[90]
Gonad	Chicken	Embryonic gonads	DNA methylation, histone methylation	[92]
Gonad	Fish	Gonads	DNA methylation	[93]
PII, PI.1	Buffalo	Ovary	DNA methylation, histone acetylation	[94,96]
PI.1, PI.5/2	Bovine, ovine	Placenta	DNA methylation, histone modifications	[97,98,100]
PI.3/II	Human	Skin fibroblasts	DNA methylation	[17]

miRNAs regulating aromatase expression

miRNA	Species	Tissue/cell type	Reference
let-7f	Human	Breast	[101]

Evidence exists for the regulation of DNMT enzymes by E2, although this is yet to be established in breast cancer cells. In endometrial cancer cells expression of DNMT3b is upregulated [114,115], however, conversely in the normal endometrium all three DNMTs appear downregulated by the actions of E2 [116]. High expression of histone deactylase protein family members HDAC1, HDAC3 correlates with ER-positive positive tumours [117,118], and treatment of the ER-positive breast cancer cell line MCF-7 with E2 increases mRNA and protein expression of HDAC6 [119]. *In vivo* studies have also revealed the importance of epigenome changes for E2 induced mammary carcinogenesis [120]. Through the use of the August Copenhagen Irish (ACI) rat model, Kovalchuk et al. demonstrated that prolonged exposure to E2 resulted in mammary carcinogenesis, a process associated with rapid and sustained loss of global DNA methylation, LINE-1 hypomethylation, loss of histone H3 lysine 9 and histone H4 lysine 20 trimethylation and altered microRNA expression [120].

While E2 is clearly capable of regulating epigenetic modifying enzymes on its own, this process may also be mediated by additional hormones or signalling factors that promote alterations to the epigenome that stimulate breast cancer progression. To identify regulatory cross-talk between oestrogen signalling and DNA methylome pathways, combinatorial treatment of breast cancer cell lines with E2 and inhibitors of DNA methylation has provided a greater insight. Gene expression profiling identified a number of overlapping targets of E2 and DNA methylation that are involved in intracellular signalling cascades, cell proliferation and apoptosis in MCF-7 cells [121]. However of interest, E2 on its own was not capable of demethylating CpG promoter levels of selected genes. In a similar study, inhibition of DNA methylation was found to abrogate E2-induced growth and reduced the expression of DNA repair genes in MCF-7 cells [122]. Both studies reveal the importance that E2, together with DNA methylation alterations, have in breast cancer pathways.

5. Alleviating epigenetic resistance to endocrine therapy

Two-thirds of postmenopausal breast cancers are ER-positive at the time of diagnosis. Unfortunately, half of these women will not respond to first-line treatment with tamoxifen [123], whereas patients who do initially respond develop acquired resistance [124]. It is widely described that epigenetic silencing, either by DNA methylation or histone modifications, of the ER is a prominent feature associated with *de novo* repression in ER-negative cell lines [125–127]. Although not consistent, similar findings are observed in ER-negative tumours where the ER promoter is found to be hypermethylated [128–132]. Acquired resistance to tamoxifen in cell line models and patient tumours have also been attributed to epigenetic silencing [133–136].

The reversible nature of epigenetic modifications through inhibitors of DNA methylation or HDACs has made the resensitisation of ER-negative cells to endocrine therapy a possibility. Indeed, the use of such agents on ER-negative cell lines results in the expression of ER [137–139] and responsiveness to tamoxifen [140]. The use of these agents alone or in combination with endocrine and chemotherapies for the treatment of breast cancer are mainly in Phase I and II trials [141], however results to date using tamoxifen and the HDAC inhibitor vorinostat have been promising [142]. Given the global epigenome changes that do occur in response to these agents, care must be exercised until the long-term benefits of combinatorial treatments are clarified.

While postmenopausal breast cancers acquire resistance to SERMs such as tamoxifen, they also develop resistance to AIs, complicating the treatment of oestrogen-dependent tumours further. As seen with the epigenetic therapeutic potential in ER-negative tumours, HDAC inhibitors are also proving to be beneficial in combinational therapy with AIs or for the treatment of those tumours resistant to AIs. For example, LBH589 (panobinostat), a highly potent HDAC inhibitor that is efficacious at low nanomolar concentrations in preclinical studies [143], was found to abrogate the growth of AI resistant cells both *in vitro* and *in vivo* through the inhibition of NF-kB1 mRNA and protein expression [144]. Furthermore, in combination with the AI exemestane, the HDAC inhibitor entinostat that has been shown pre-clinically to inhibit mechanisms of AI resistance through the down-regulation of activated growth factor signalling pathways, significantly improved the progression-free survival of postmenopausal women with ER-positive advanced breast cancer [145]. The same HDAC inhibitor entinostat sensitises

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G Model
SBMB-3933; No. of Pages 8
ARTICLE IN PRESS
K.C. Knower et al. / Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

ER-negative tumours to the AI letrozole by up-regulating the expression of ER and aromatase [87].

6. Concluding remarks

The actions of oestrogens in postmenopausal breast cancer will remain an intense area of continued research. While the landscape of the epigenome is forever evolving, the cross-talk amongst these two critical processes in the progression of breast cancer will continue to grow. How we apply this knowledge to the development of biomarkers and the advancement of current endocrine therapies is an exciting area of research with much potential [146].

Conflict of interest

The authors declare no conflicts of interest.

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SBMB-3933; No. of Pages 8
ARTICLE IN PRESS
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K.C. Knower et al. / Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

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Appendix 3: Epigenetic mechanisms regulating CYP19 transcription in human breast adipose fibroblasts

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Epigenetic mechanisms regulating CYP19 transcription in human breast adipose fibroblasts

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ABSTRACT

Cytochrome aromatase p450, encoded by the gene *CYP19*, catalyzes the synthesis of estrogens from androgens. In post-menopausal women, adipose becomes the major site for estrogen production, where basal *CYP19* transcription is driven by distal promoter I.4. In breast adipose fibroblasts (BAFs), *CYP19* expression is elevated in the presence of tumour-derived factors through use of promoters I.3 and II. We show for the first time that DNA methylation contributes to *CYP19* regulation in BAFs and breast cell lines. Promoter I.4 and I.3/II-derived mRNA were not dependent on the CpG methylation status within respective promoters. However, inhibition of DNA methylation with 5-aza-2′-deoxycytidine resulted in a significant ~40-fold induction in *CYP19* mRNA expression in BAFs and breast cell lines. These studies uncover a new layer of complexity in the regulation of aromatase where *CYP19* appears to be inhibited by DNA methylation and evokes the possibility that disruption to this epigenetic regulation may give rise to an increase in aromatase levels in the breast.

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1. Introduction

The key enzyme of estrogen biosynthesis, aromatase cytochrome P450, is encoded by the *CYP19A1* gene located on chromosome 15 (commonly referred to as *CYP19*), and is responsible for the conversion of C_{19} steroids to estrogens. In humans, aromatase is expressed in a tissue-specific manner in organs such as the gonad, brain, skin, placenta and adipose (Simpson, 2004). The regulation of *CYP19* in these tissues is complex, with its mRNA expression regulated by a number of tissue-specific promoters each upstream of an untranslated first exon. This upstream regulatory region spans ~93 kb with the various untranslated first exons being spliced to a common splice acceptor site 38 bp upstream of the ATG translational start site to produce protein through exons II–X, identical in all tissue expressed (Bulun et al., 2004). The alternate promoter use underlies the basis for the differential regulation of aromatase expression in a tissue-specific manner by various factors such as hormones, growth factors and cytokines (Bulun and Simpson, 1994a, 1994b). Local estrogen production in the breast of post-menopausal women occurs primarily in the surrounding adipose tissue (Simpson et al., 1997). In disease-free breast adipose fibroblasts (BAFs), aromatase expression is low, however expression is elevated 3–4-fold in the presence of a tumour, contributing to the progression and growth of estrogen receptor-positive tumours (Bulun et al., 1993; Harada, 1997; Thorsen et al., 1982). As a consequence, inhibitors of the aromatase enzyme are currently the most effective and commonly used non-cytotoxic therapeutic in post-menopausal women (Carlini et al., 2007).

Regulation of *CYP19* in BAFs is mediated by three distinct promoters. In the absence of a tumour, the distal promoter I.4 (PI.4) located ~73 kb upstream of exon II is the major driver of transcription and is under the regulation of class I cytokines or tumour necrosis factor-α (TNFα), together with glucocorticoids (Harada et al., 1993; Mahendroo et al., 1993; Zhao et al., 1995a, 1995b). In the presence of a tumour, elevated levels of aromatase expression and subsequent increased estrogen production coincides with a switch from PI.4 transcription to the predominant use of the proximal promoters I.3 (PI.3) and II (PII) located 215 bp from each other (Agarwal et al., 1996; Irahara et al., 2006; Zhou et al., 1997). The secretion of prostaglandin E_2 (PGE_2) by breast tumour epithelial cells is associated with the increased PI.3/II transcriptional activity in surrounding BAFs (Brueggemeier et al., 1999; Schrey and Patel, 1995; Zhao et al., 1996a, 1996b). PGE_2, through both cAMP/PKA- and PKC-dependent signalling, has been shown to induce PI.3/II transcription through the activation of a number of transcription factors including liver receptor homologue 1 (LRH-1), activating transcription factor 2 (ATF-2), CCAAT/enhancer binding protein beta and delta (C/EBPβ and C/EBPδ), cAMP responsive element binding protein (CREB) and jun oncogene (c-Jun) (Clyne et al., 2002; Deb et al., 2006; Kijima et al., 2008; Sofi et al., 2003).

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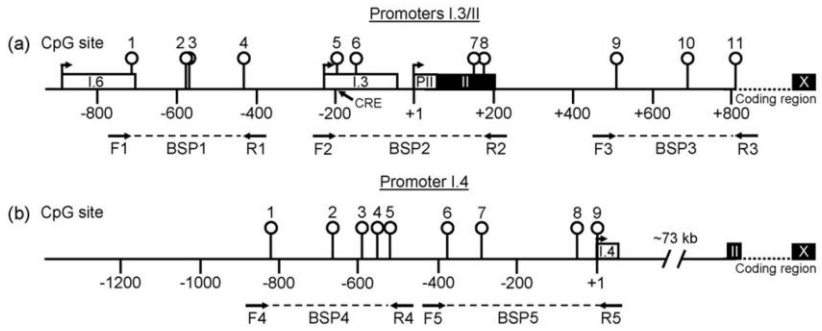

Fig. 1. Schematic representation of CpG dinucleotides within promoter regions (a) I.3/II and (b) I.4 of *CYP19*. CpG dinucleotides are numbered and represented as open circles with their positions shown relative to the transcriptional start site (+1). CpG-5 of PI.3/II lies within a CRE site. Primers used for sodium bisulfite sequencing encompassing CpG dinucleotides are shown below promoters. Three amplicons for promoters I.3/II (BSP1, BSP2 and BSP3) and two amplicons for promoter I.4 (BSP4 and BSP5) were designed. Untranslated exons (open rectangle) I.6, I.3, PII and I.4 and coding exons II and X (closed rectangle).

Since aromatase is a key target in the treatment of breast cancer, understanding the promoter-specific regulation of *CYP19* in BAFs is of fundamental importance. The vast majority of previous studies investigating *CYP19* regulation in BAFs have focussed on its hormonal regulation. While this is clearly important, certain lines of evidence suggest that *CYP19* regulation is also under epigenetic control. DNA methylation of CpG dinucleotides is an important level of transcriptional regulation with its role in the local organisation of chromatin structure and thus long term regulation of gene expression becoming an ever growing focal point in the study of cancer progression. Differential DNA methylation of CpG-islands in promoter regions can account for the tissue-specific expression of genes, such as hypermethylation of tumour suppressors or hypomethylation of oncogenes (Jones and Baylin, 2007). DNA methylation of CpG-poor promoter regions has also been shown as a mechanism of mediating tissue-specific gene transcription through the inhibition of transcription factor binding (Fujii et al., 2006; Jones and Chen, 2006).

Epigenetic regulation of aromatase expression has been demonstrated in a number of instances in both human and non-human tissues. Global inhibition of DNA methylation with the agent 5-aza-2′-deoxycytidine (5-aza-dC) results in elevated *CYP19* mRNA in human endometrial stromal cells (Izawa et al., 2008), human hepatoma cells (Dannenberg and Edenberg, 2006), and chicken fibroblasts (Leshin, 1985). Correlation between DNA methylation and promoter-specific *CYP19* transcription has also been demonstrated in sheep and cattle (promoter regions 1.1 and 1.5/2) (Furbass et al., 2001, 2008; Vanselow et al., 2005; Vanselow et al., 2008) and in skin adipose fibroblasts (Demura and Bulun, 2008). In the latter study, CpG methylation within a cAMP response element (CRE) in the PII region was found to modulate cAMP-stimulated aromatase activity through potential inhibition of CREB transcription factor binding. Therefore, along with the hormonal regulation of aromatase expression, evidence for an additional layer of epigenetic regulation exists; however, this relationship has yet to be explored in the context of aromatase regulation in breast cancer.

In the present study, we therefore sought to examine if epigenetic mechanisms are involved in the regulation of *CYP19* in BAFs by (i) elucidating whether an inverse correlation existed between the DNA methylation status of CpG dinucleotides and promoter-specific transcription employing PI.4 and PI.3/II; and (ii) determining whether *CYP19* was under tonic inhibition by DNA methylation by relieving this mechanism with 5-aza-dC treatment. Data presented in this study reveals that transcription mediated by PI.4 and PI.3/II promoter regions is independent on the methylation status of CpG sites within respective promoters. Inhibition of DNA methylation with 5-aza-dC caused a robust increase in *CYP19* mRNA expression in both BAFs and a number of breast cell lines. In BAFs, this event was mediated through PI.4, potentially via upstream trans-activators or a non-promoter CpG regulatory region. This work reveals an additional layer of aromatase regulation potentially involved in the progression of breast cancer.

2. Results

2.1. Identification of CpG sites within CYP19 promoters I.3/II and I.4

The PI.3/II and PI.4 regions of *CYP19* were screened for CpG dinucleotides. The PI.3/II region was found to have no CpG islands and contained a total 11 CpG sites spanning a ~1.6 kb region proximal to the transcriptional start site of PII, deeming this a CpG-poor region (Fig. 1a). Three primer pairs were designed to produce the amplicons BSP1, BSP2 and BSP3 for sodium bisulfite sequencing of the 11 CpG dinucleotides. Similar to the PI.3/II region, the PI.4 region was also of low CpG complexity and contained 9 CpG dinucleotides located ~800 bp upstream of the transcriptional start site (Fig. 1b). Two primer pairs producing the amplicons BSP4 and BSP5 were designed for sequencing.

2.2. CpG methylation profile of CYP19 promoters I.3/II following stimulation in human breast adipose fibroblasts by PGE_2

To establish whether the methylation status of CpG sites within *CYP19* PI.3/II inversely correlated with transcriptional activity, PI.3/II activity was induced in BAFs with PGE_2 over a 24 h period. qRT-PCR using untranslated exon-specific primers demonstrated a significant ~3-fold induction in both PI.3 and PII driven *CYP19* mRNA in response to PGE_2 compared to vehicle treatment (Fig. 2). PI.3/II transcriptional activity was unresponsive following treatment with the PI.4 stimulants dexamethasone (DEX) and TNF-α (see below). Genomic DNA isolated from BAFs following respective treatments was used for sodium bisulfite sequencing PCR (BSP) and subsequent CpG methylation mapping. Individual CpG site methylation and overall amplicon methylation average was determined following BSP (Fig. 3b and c respectively). In vehicle treated BAFs, methylation levels of 69.4% (BSP1), 77% (BSP2) and 64.3% (BSP3) were observed within respective amplicons. A comparison of amplicon methylation between PGE_2 treated BAFs and vehicle revealed no significant changes in methylation levels with data suggesting moderate elevated levels of methylation in each amplicon – 75% (BSP1), 91% (BSP2) and 72.9% (BSP3). No significant differences in methylation were observed within these amplicons in the DEX/TNFα treated group.

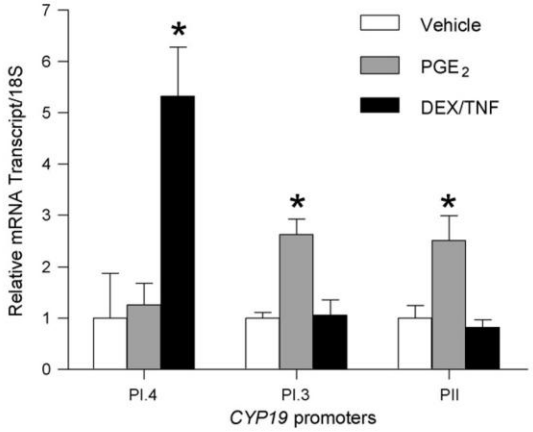

Fig. 2. qRT-PCR on RNA isolated from BAFs stimulated with either PGE_2 or DEX/TNFα for 24 h showing relative PI.4, PI.3 and PII mRNA levels. Data obtained from two independent experiments conducted in triplicate. Error bars represent standard error of means. Data was analyzed by independent *t*-tests of group means ($^*p < 0.05$).

The methylation status of each CpG dinucleotide was compared between vehicle and hormone treated cells. Overall, the 11 CpG dinucleotides demonstrated a stochastic heterogenous level of methylation across the three amplicons, results typical of CpG-poor promoter regions (Fig. 3a) (Eckhardt et al., 2006). In each of the treated groups, CpG-1, -3, -4, -7, -8 and -10 showed comparable hypermethylation; CpG-2 and -11 hypomethylation; and CpG-5, -6 and -9 with large variability. Analysis of mRNA from the breast cell lines MCF-7 (tumour epithelial, ER-positive), MDA-MB-231 (metastatic, ER-negative) and MCF10A (normal epithelial) revealed differing levels of basal PII transcripts (Fig. 4a). Despite the elevated PII mRNA in MCF-7 cells, the PI.3/II region was heavily hypermethylated – 71.9% (BSP1), 82.5% (BSP2) and 76.2% (BSP3) (Fig. 4b). Furthermore, analysis of individual CpG dinucleotide methylation within the cell lines again revealed a high level of heterogeneity among CpG sites, including CpG-5 (Fig. 4c), reflecting the findings in BAFs and again suggesting that PI.3/II transcription is not influenced by CpG dinucleotide methylation.

2.3. CpG methylation is reduced at promoter I.4 following stimulation in BAFs suggesting an inverse correlation with expression

The distal promoter I.4 directs *CYP19* expression in normal BAFs and is regulated by the combined action of glucocorticoids and members of the class I cytokine family or TNFα (Zhao et al., 1995b). PI.4-derived expression was stimulated in BAFs with DEX and TNF-α (Zhao et al., 1996a, 1996b) to determine if an inverse correlation exists between PI.4-specific mRNA expression and CpG methylation. qRT-PCR on RNA isolated from BAFs following DEX/TNFα treatment revealed a ~5-fold stimulation in PI.4 *CYP19* transcription compared to vehicle treated cells, whereas PGE_2 had no affect (Fig. 2). The methylation status of the 9 CpG sites identified within the proximal region of PI.4 (Fig. 1b) was assessed by BSP and compared between treated groups. Following induction with DEX/TNFα, a decreased level of methylation across amplicons BSP4 (Δ −12.3%) and BSP5 (Δ −19.3%) was observed, with BSP5 being statistically different ($p = 0.0047$, Fig. 5b). To identify specific CpG dinucleotides contributing to the hypomethylation observed, the 9 CpG sites were compared between groups. Similar to the PI.3/II region, CpG dinucleotides in PI.4 also exhibited a high level of heterogenous methylation in BAFs – hypermethylation of CpG-1, -2, -4, -6, -7 and -9; hypomethylation of sites CpG-3 and -5; and variable methylation of site CpG-8 (Fig. 5a and c). In BAFs treated with DEX/TNFα, statistically significant differences in CpG-6, -7 and -9 (hypomethylated) and CpG-8 (hypermethylated) were found when compared to vehicle suggesting that these hypomethylated sites may contribute to PI.4 transcription. However, inspection of these sites in PGE_2 treated BAFs revealed that CpG-7 and -8 also showed the same statistically different changes compared to vehicle, implying that these CpG sites may not play a role in PI.4-mediated transcription.

2.4. Inhibition of DNA methylation with 5-aza-dC results in increased CYP19 expression in BAFs

Previous reports have demonstrated that inhibition of DNA methylation with 5-aza-dC results in elevated levels of *CYP19* expression in a number of different cell types (Dannenberg and Edenberg, 2006; Izawa et al., 2008; Leshin, 1985). These findings suggest that methylation is a major factor suppressing *CYP19* expression within regulatory regions of the gene itself and/or

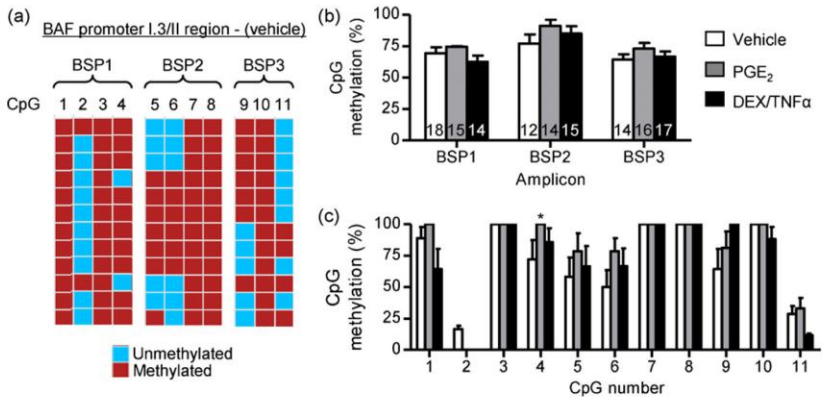

Fig. 3. PI.3/II CpG methylation in BAFs following stimulation. (a) Example of the CpG methylation profile of 11 CpGs in amplicons BSP1, BSP2 and BSP3 spanning PI.3/II from vehicle treated BAFs. Squares within each clone represent a CpG site (numbered) with its status shown as either unmethylated or methylated. (b) Percentage methylation of each amplicon following treatment. Numbers within bars represent the number of clones analyzed per group. Error bars represent standard error of means. DNA was obtained from BAF treatments (Fig. 2) repeated twice in duplicate. (c) Percentage methylation of individual CpG sites following treatment. Error bars represent standard deviation. Means of each treated group were compared to vehicle with one-way ANOVA testing ($^*p < 0.05$).

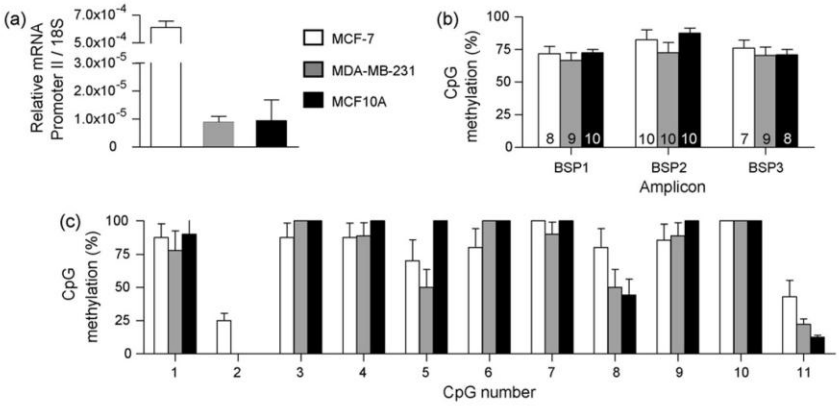

Fig. 4. CpG methylation status of PI.3/II in cell lines with differing PII mRNA expression. (a) qRT-PCR on the relative mRNA expression levels of PII driven *CYP19* mRNA in the breast cell lines MCF-7 (ER-positive), MDA-MB-231 (ER-negative), MCF10A (normal epithelial). Despite the elevated levels of PII mRNA seen in MCF-7 cells, all cell lines are relatively hypermethylated in (b) amplicons (numbers within bars represent the number of clones analyzed per group, error bars represent standard error of means) and (c) individual CpG sites (error bars represent standard deviation).

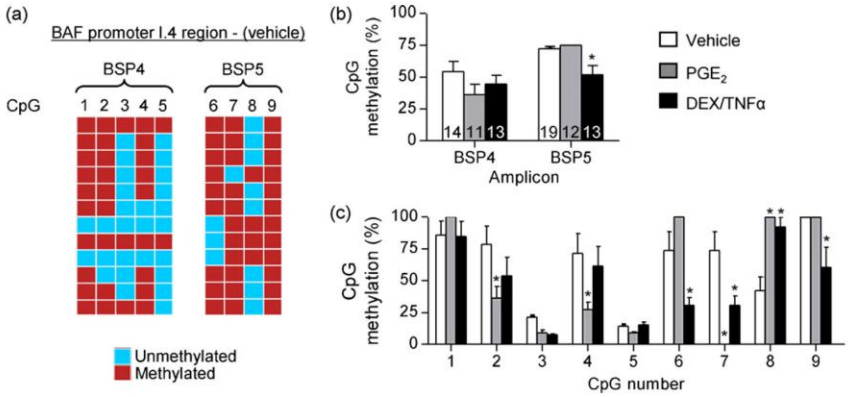

Fig. 5. CpG methylation of PI.4 in BAFs following stimulation with PGE_2 and DEX/TNFα. (a) Example of the CpG methylation profile of 9 CpGs in amplicons BSP4 and BSP5 spanning PI.4 from vehicle treated BAFs. Squares within each clone represent a CpG site (numbered) with its status shown as either unmethylated or methylated. (b) Percentage methylation of each amplicon following treatment revealed hypomethylation in BSP5 following DEX/TNFα treatment compared to vehicle. Numbers within bars represent the number of clones analyzed per group. DNA was obtained from BAF treatments (Fig. 2) repeated twice in duplicate. Error bars represent standard error of means. (c) Percentage methylation of individual CpG sites following treatment shows high variability between CpG site methylation among treatment groups. Error bars represent standard deviation.

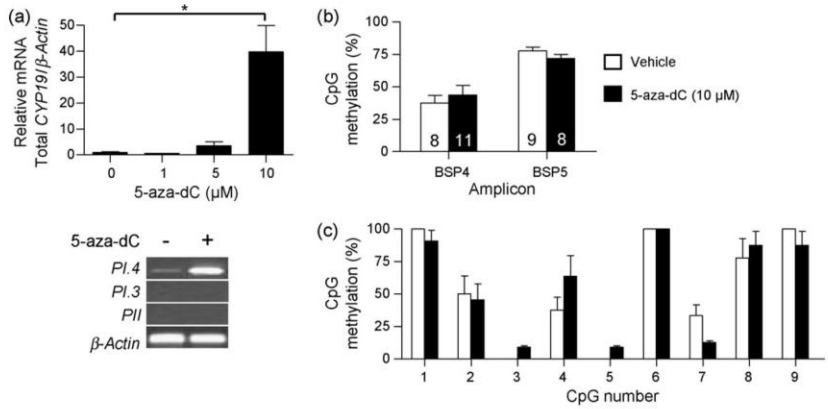

Fig. 6. (a) Inhibition of DNA methylation with 5-aza-dC for 5 days (upper panel) resulted in a dose-dependant activation of total *CYP19* mRNA up to a significant ~40-fold (10 μM, $n = 3$, $^*p = 0.0044$). Data was analyzed by independent *t*-tests of group means. Error bars represent standard error of means. This activity was mediated via PI.4 (lower panel) as assessed by semi-quantitative RT-PCR (10 μM 5-aza-dC). (b) Methylation mapping of the PI.4 region following treatment with 5-aza-dC showed that percentage methylation compared to vehicle was unchanged in amplicons BSP4 and BSP5. Error bars represent standard error of means. Numbers within bars represent the number of clones analyzed per group. (c) Individual CpG sites. Error bars represent standard deviation. Means of each treated group were compared to vehicle with one-way ANOVA testing.

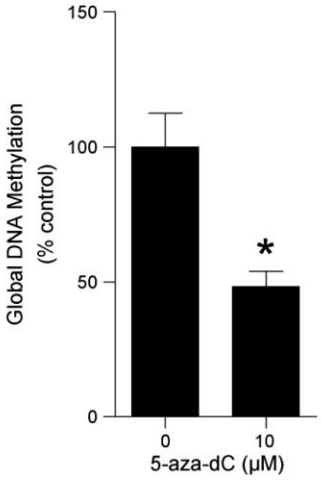

Fig. 7. Global DNA methylation levels are reduced in BAFs following 5-aza-dC treatment. Data obtained using genomic DNA (used in Fig. 6) from two independent experiments done in duplicate. Data was analyzed by independent *t*-tests of group means comparing 10 μM treatments to control. $^{*}p = 0.0097$. Error bars represent standard error of means.

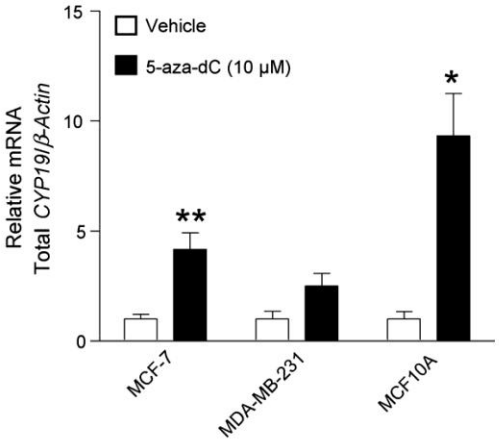

Fig. 8. qRT-PCR of total *CYP19* mRNA in breast cell lines following 5-aza-dC stimulation (10 μM) for 5 days shows elevated levels of transcripts compared to vehicle treated. Data obtained from two independent experiments done in duplicate. Error bars represent standard error of means. Data was analyzed by independent *t*-tests of group means ($^{*}p < 0.05$, $^{**}p < 0.01$).

its trans-acting factors. To determine whether demethylation affected *CYP19* expression in BAFs, cells were treated with 5-aza-dC over 5 days culture. qRT-PCR revealed a considerable significant dose-dependant stimulation of total *CYP19* mRNA up to ~40-fold greater than vehicle treatment (10 μM, Fig. 6a, upper panel). Semi-quantitative exon-specific RT-PCR revealed that increases in total *CYP19* levels in response to 5-aza-dC (10 μM) were mediated through PI.4, whereas PI.3/II-derived transcripts showed little to no expression (Fig. 6a, lower panel). DNA isolated from 5-aza-dC treated BAFs (10 μM) was used to measure the levels of methylation in PI.4 following 5-aza-dC treatment. Surprisingly, no difference in the levels of methylation in amplicons BSP4 and BSP5 was found after 5-aza-dC treatment, despite the elevated levels of PI.4-specific mRNA (Fig. 6b and c). To confirm genomic demethylation had occurred following 5-aza-dC treatment, global DNA methylation levels were quantitated by ELISA. Genomic DNA methylation levels was significantly reduced 50% in BAFs treated with 5-aza-dC (10 μM) compared to control (Fig. 7). The finding that DNA methylation inhibits *CYP19* transcription was not limited to BAFs, as treatment of the breast cell lines with 5-aza-dC also resulted in increases in *CYP19* expression (Fig. 8), albeit through additional promoters, including PII (data not shown).

3. Discussion

The present study presents an analysis on the methylation status of two different spatially separated promoter regions, PI.4 and PI.3/II, of the human *CYP19* locus in the context of BAFs and breast cell lines. The analyzed sequences offer minimal CpG sites spanning large genomic regions and both promoter regions can therefore be characterised as CpG-poor promoters (Brena et al., 2006). Sodium bisulfite sequencing of both promoter regions revealed a heterogenous methylation haplotype among CpG sites, with amplicons by and large hypermethylated, indicative of the low levels of PI.4- and PI.3/II-derived transcripts in BAFs and cell lines. No inverse correlation between CpG methylation and PI.3/II expression following stimulation with the tumour-derived paracrine signalling factor PGE_2 was observed, suggesting that the methylation status of the 11 CpG sites is not critical for cAMP-induced stimulation in BAFs. Given the finding that the methylation status of CpG-5 in the PI.3/II region located within the CRE transcription factor binding site, essential for PII activity (Sofi et al., 2003), could mediate cAMP activity in skin fibroblasts (Demura and Bulun, 2008), our findings suggest that the tissue-specific expression dictated by this particular CpG site may only be limited to such cell types non-responsive to cAMP stimulation. Further studies using DNA and RNA isolated from breast cancer-associated fibroblast cells (CAFs) need to be carried out to get a greater understanding of this mechanism *in vivo*.

Statistically significant decreased methylation was found in amplicon BSP5 of the PI.4 region following stimulation with DEX/TNFα. Despite this, an inverse correlation between PI.4 transcription and methylation was difficult to interpret due to the differing levels of methylation of neighbouring CpG sites. Of those hypomethylated, CpG-6 positioned −174 bp relative to the PI.4 transcriptional start site is located between the critical GAS (−282/−272) and GRE (−133/−119) transcription factor binding sites (Zhao et al., 1995a, 1995b). Future studies are needed to determine whether the methylation status of CpG-6 is capable of mediating PI.4 transcription through the inhibition of trans-acting factors. Further compounding this is the observation that, at least in sheep placentomes, the inverse correlation between methylation and the CpG-poor *Cyp19* promoter 1.5/2 expression is not linear (Furbass et al., 2008). Rather a methylation threshold of 25% determines promoter expression indicating that hypomethylation seems to be necessary, but not sufficient for high gene expression. Therefore, similar to region PI.3/II, PI.4 transcriptional activity may be more reliant on trans-acting factors rather than CpG methylation status.

Inhibition of DNA methylation in BAFs with the agent 5-aza-dC resulted in a robust significant increase in *CYP19* transcription. This data correlates with previous findings in endometrial stromal cells (Izawa et al., 2008) and suggests that DNA methylation is one of the main epigenetic components inhibiting *CYP19* expression levels in BAFs. CpG hypomethylation at PI.4 was not evident following 5-aza-dC treatment of BAFs, despite the observed increase in total *CYP19* transcription being mediated via increased PI.4 stimulation and a demonstration of global demethylation. This result reflects that of cells treated with DEX/TNFα, in that the expression of PI.4 transcripts is independent of the methylation status of CpG sites in the PI.4 promoter. Recent work has demonstrated that the actions of 5-aza-dC may be gene specific, selective towards pro-

moters containing CpG-islands and capable of reactivating genes without promoter hypomethylation (Flotho et al., 2009). Such a situation provides possible mechanisms of *CYP19* activation following 5-aza-dC treatment observed in this study. Specifically, cis- or trans-activating factors of *CYP19* with hypermethylated CpG-islands may be reactivated, alternatively, non-promoter CpGs not identified in this study may be demethylated. Indeed, epigenetic regulation exists for a number of known PI.4 regulators including TNFα, c-jun and c-fos (Chandrasekhar and Raman, 1997; Sullivan et al., 2007; Tao et al., 2000).

Further to our findings in BAFs, the breast cancer cell lines MCF-7, MDA-MB-231 and MCF10A were also found to have increased CYP19 mRNA following 5-aza-dC treatment suggesting that epigenetic mechanisms regulating *CYP19* may not be limited to BAFs but may also contribute to regulation in epithelial cell types. Interestingly, in these cell types additional *CYP19* promoter transcriptional activity was also increased, including PII (data not shown), highlighting the differences of methylomes within different cell types of the breast. Understanding the context of these epigenetic differences in clinical pathological instances and how this may contribute to elevated aromatase expression in breast cancer warrants further investigation. Likewise, investigation into the epigenetic mechanisms mediating the age- and region-dependent elevation in aromatase expression within adipose fibroblasts amongst healthy individuals is also of merit (Bulun and Simpson, 1994b; Cleland et al., 1985; Rink et al., 1996).

Understanding how the methylome changes within a tumour has been a topic of intense basic and clinical investigation. Our knowledge of the events associated with gene-specific DNA hypermethylation in cancer progression is greater than that of those leading to DNA hypomethylation. In breast cancer, the majority of genes identified to be epigenetically regulated by DNA methylation involved in carcinogenesis are those shown to be hypermethylated, with only a few in comparison shown to be hypomethylated (Hinshelwood and Clark, 2008). Our findings in this study lend to the possibility that an epigenetic disorder in which active methylation-dependent suppression mechanisms are disrupted may lead to the hypomethylation of *CYP19* trans-acting factors contributing to breast carcinogenesis, a similar proposed mechanism to that of *CYP19* regulation in endometriosis (Izawa et al., 2008). Indeed, global hypomethylation is well characterised and predominant in neoplasia relative to normal cells (Santini et al., 2001), with hypomethylation the most evident in breast cancers with reductions in up to 50% of 5meC content (Esteller and Herman, 2002). The global hypomethylation found in the breast tumour genome generally occurs in the intragenic and intergenic regions and in repeat sequence elements (Wilson et al., 2007), compared to hypomethylation sites enriched in the 5′-end of widely expressed genes of normal tissues (Shann et al., 2008). The current advancements in genome-wide high throughput DNA methylation mapping will prove beneficial in elucidating trans-acting factors of *CYP19* under epigenetic control in breast cancer (Zilberman and Henikoff, 2007).

The epithelial–stromal microenvironment in breast cancer results in dramatic gene expression changes occurring in every cell type compared to normal tissue (Allinen et al., 2004). Despite these transcriptome changes, genetic alterations are restricted to tumour breast cancer epithelial cells and are not found in the surrounding stroma (Allinen et al., 2004; Qiu et al., 2008). This relationship, together with subsequent findings, demonstrates that epigenetic regulation of genes in the breast stromal compartment underlies the number of changes in gene transcription observed in the presence of a tumour (Fiegl et al., 2006; Hu et al., 2005). The additional layer of epigenetic regulation of *CYP19* identified in this study may therefore encompass the potential epigenetic changes in the surrounding BAFs, and together with hormonal paracrine signalling from cancerous epithelial cells may in turn contribute to the increase in aromatase expression. Therefore, the genetically stable stromal environment may benefit future chemotherapeutic approaches due to lower complexity and poorer chance of developing drug resistance, furthermore, combined with the advancing field of epigenetic therapeutics, a new era of targeting the epigenetic regulation of aromatase in breast cancer may arise.

4. Materials and methods

4.1. Cell culture

Human breast adipose fibroblasts were isolated by collagenase digestion and cultured as previously described (Ackerman et al., 1981). Cell lines MCF-7 (ATCC No. HTB-22), MCF10A (ATCC No. CRL-10317), MDA-MB-231 (ATCC No. HTB-26) were subcultured according to suppliers procedures. To stimulate promoter-specific *CYP19* transcription, BAFs grown to ~60% confluence were initially starved in serum-free media containing 0.1% BSA for 24 h. Serum-free media containing either PGE_2 (1 μM) or DEX (250 nM) and TNFα (10 ng/ml) was added to cells for 24 h to stimulate PI.3/II or PI.4 transcription respectively. To inhibit DNA methylation with 5-aza-dC, cells were grown to ~50% confluence in media containing serum. Growth media was then replaced with media containing 5-aza-dC (Sigma) (1, 5 or 10 μM) and changed daily for 5 days. In all treatments, equal amounts of vehicle alone (the agent in which the compound was dissolved) was used as a control.

4.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini kit (QIAGEN). First strand cDNA synthesis from a minimum of 200 ng of total RNA was performed using avian myeloblastosis virus reverse transcriptase (Promega) primed by random hexamers. Quantitative RT-PCR (qRT-PCR) of total *CYP19* mRNA was performed with SYBR Green detection methodologies with Roche Lightcycler Systems using the primer pair termed RT-7 and RT-8 previously described (Agarwal et al., 1996). Where applicable, PI.4 and PI.3 and PII-driven transcripts were quantitated on the ABI 7900HT Sequence Detection System using the amplification conditions of the primers and dual labelled fluorescent probes previously described (Imir et al., 2007). qRT-PCR amplification of the internal control genes *18S* and *β-actin* was conducted

Table 1
Primers used to amplify *CYP19* promoter regions PI.4 and PI.3/II for sodium bisulfite sequencing.

Amplicon	Primer sequence (5′–3′)	Extension temperature (°C)	$MgCl_2$ (mM)	Product size (bp)
Promoter I.3/II				
BSP1	F1 – TTTTGTATAGGATGTTAGTTGTTTTT	70	4	383
	R1 – CCTTTTTAACCAAAATAACTTTTT			
BSP2	F2 – AATTTGGTTTTTAATTGGGAATGTA	66	3	397
	R2 – TAACAACAAACATAACTTCAAACAC			
BSP3	F3 – ATTTTTTTATTTTGTGTTGTGTTTT	68	5	334
	R3 – ACTACCAAACTCCCATAAAAATC			
Promoter I.4				
BSP4	F4 – ATTTTTAGAAAATTTTGTTATAAATTTAAA	67	4	445
	R4 – AAAACACCCTAAAATATAACCTACAA			
BSP5	F5 – TTGGATATATTATATTATTGGAAAGATGTA	65	4	454
	R5 – ATACCAAAAACCTCCTATCAAACTC			

Extension temperatures and $MgCl_2$ vary for each amplicon using the following cycling conditions – (95 °C for 5 min) × 1 cycle; (95 °C for 20 s, 60 °C for 30 s (decreasing 1 °C/cycle), extension for 1 min 30 s) × 5 cycles; (95 °C for 20 s, 55 °C for 30 s, extension for 1 min 30 s) × 35 cycles; (extension for 5 s) × 1 cycle.

with SYBR Green detection methodologies. Primer sequences for *18S* – (Fwd) 5′-CGG CTA CCA CAT CCA AGG A-3′ (Rev) 5′-GCT GGA ATT ACC GCG GCT-3′; and *β-Actin* (Fwd) 5′-TGC GTG ACA TTG CGT GAC ATT AAG GAG AAG-3′ (Rev) 5′-GCT CGT AGC TCT TCT CCA-3′.

4.3. Sodium bisulfite sequencing

Genomic DNA was isolated from cells using the Wizard Genomic DNA Purification Kit (Promega). DNA was deaminated with sodium bisulfite using the EpiTect Bisulfite Kit (QIAGEN). PCR primers to amplify converted DNA were designed with the online tool MethPrimer (Table 1) (Li and Dahiya, 2002). Amplification of each amplicon was performed using MangoTaq (Bioline) following cycle conditions outlined in Table 1. Amplicon PCR products were cloned using the pGEM-T Easy Vector System (Promega), and positive clones were then sent for sequencing. Cloned sequences were put through quality control procedures with BiQ Analyzer (Bock et al., 2005) and methylation data was attained with the Bisulfite sequencing Data Presentation and Compilation (BDPC) web server tool (Rohde et al., 2008).

4.4. Global DNA methylation quantitation

Genomic DNA (50 ng) isolated from BAFs treated with 0 or 10 μM 5-aza-dC was used to quantitate global DNA methylation levels. An Enzyme-Linked Immunosorbent Assay (ELISA) based method was performed using the Imprint® Methylated DNA Quantification Kit (Sigma) as per instructions.

4.5. Statistical analysis

BSP amplicon and CpG site methylation levels were obtained through the data obtained with the BDPC web server tool. One-way ANOVA analysis was performed to compare the methylation status between collected clone sequences. Independent Student's *t*-tests, where applied, were performed for the comparison of qRT-PCR data. Statistical analysis was done with Microsoft Excel spreadsheet. $^{*}p < 0.05$ unless otherwise stated.

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Appendix 4: Epigenetic mechanisms regulate the prostaglandin E receptor 2 in breast cancer

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Original research article written in collaboration with Kevin Knower, Evan Simpson, Colin Clyne and colleagues in Sendai and Tokyo, Japan. This article is the result of my honours year of study, some of the data of which was completed during my Ph.D. candidature. My contribution to the study was approximately 80%, and constitutes collection of all the experimental data (the patient clinical samples were isolated by colleagues in Japan) as well as preparing the manuscript for publication.

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Epigenetic mechanisms regulate the prostaglandin E receptor 2 in breast cancer

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ABSTRACT

The increase in local oestrogen production seen in oestrogen receptor positive (ER+) breast cancers is driven by increased activity of the aromatase enzyme. *CYP19A1*, the encoding gene for aromatase, is often overexpressed in the oestrogen-producing cells of the breast adipose fibroblasts (BAFs) surrounding an ER+ tumour, and the molecular processes underlying this upregulation is important in the development of breast-specific aromatase inhibitors for breast cancer therapy. Prostaglandin E2 (PGE2), a factor secreted by tumours, is known to stimulate *CYP19A1* expression in human BAFs. The hormonal regulation of this process has been examined; however, what is less well understood is the emerging role of epigenetic mechanisms and how they modulate PGE2 signalling. This present study characterises the epigenetic processes underlying expression of the prostanoid receptor EP2 in the context of ER+ breast cancer. Sodium bisulphite sequencing of CpG methylation within the promoter region of *EP2* revealed that an inverse correlation existed between methylation levels and relative *EP2* expression in breast cancer cell lines MDA-MB-231, MCF7 and MCF10A but not in HS578t and T47D. Inhibition of DNA methylation with 5-aza-2′-deoxycytidine (5aza) and histone deacetylation with Trichostatin A (TSA) resulted in upregulation of *EP2* mRNA in all cell lines with varying influences of each epigenetic process observed. Expression of *EP2* was detected in human BAFs despite a natively methylated promoter, and this expression was further increased upon 5aza treatment. An examination of 3 triple negative, 3 ductal carcinoma in situ and 3 invasive ductal carcinoma samples revealed that there was no change in *EP2* promoter methylation status between normal and cancer associated stroma, despite observed differences in relative mRNA levels. Although EP2 methylation status is inversely correlated to expression levels in established breast cancer cell lines, we could not identify that such a correlation existed in tumour-associated stroma cells.

1. Introduction

The aromatase enzyme is critical to oestrogen biosynthesis, being responsible for the conversion of androgen precursors to estrogens. Aromatase is encoded by the *CYP19A1* gene, located on chromosome 15 of the human genome. Expression of this gene reflects the sites of oestrogen biosynthesis within the body, primarily being localised to organs such as gonads, placenta, brain, skin and adipose [1]. Whilst the ovaries are primarily responsible for oestrogen production in pre-menopausal women, peripheral sites, and in particular the breast adipose, take over following menopause [2]. Regulation of the aromatase enzyme changes throughout a woman's lifetime, and this is mainly via transcriptional regulation of *CYP19A1*.

A characteristic feature of *CYP19A1* is its use of a number of tissue-specific promoters through which transcriptional regulation is achieved [3]. These promoters span up to ~93 kb upstream of the translation start site, and form untranslated first exons which are spliced on to a common site 38 bp upstream of the ATG start site leading to an identical protein being produced [4]. The unique set of response elements found within each promoter is the basis by which each responds to specific stimuli to convey tissue-specific expression of aromatase. In the disease-free breast of a post menopausal woman, a low level of aromatase expression is maintained in the breast adipose fibroblasts (BAFs) [5]. A strong 3–4 fold increase in aromatase expression is observed in the presence of an oestrogen receptor positive (ER+) breast tumour, leading to increases in local oestrogen production in order to sustain tumour growth [6,7]. As a consequence, aromatase inhibitors are currently widely used as a key

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therapeutic approach in the treatment of post-menopausal ER+ breast cancers [8].

Prostaglandin E2, or PGE2, is a major factor known to stimulate aromatase activity. It activates cAMP/PKA- and PKC-dependant signalling pathways [9], resulting in transcription of the proximal promoter II via binding of the cAMP responsive element binding protein (CREB) [10–12]. This is a key promoter involved in *CYP19A1* regulation in the breast, as although it is minimally expressed in normal BAFs, upregulation is observed in breast cancer associated fibroblasts (CAFs) where it becomes the primary mechanism by which transcription occurs [13]. The source of PGE2 in an ER+ tumour microenvironment is primarily the tumour itself, which secretes this paracrine signalling molecule in order to maintain oestrogen production in surrounding CAFs. PGE2 signals via four G-protein coupled prostanoid receptors which increase intracellular cAMP levels: EP1, EP2, EP3 and EP4. EP2 and EP4 are shown to be particularly key to PGE2 signalling in an ER+ breast tumour context [14].

Regulation of aromatase in human breast adipose fibroblasts has, to date, largely been studied in the context of hormone, cytokine and growth factor responses [7]. Recent evidence, however, has suggested that there may be an additional layer of epigenetic regulation. DNA methylation is one essential component of epigenetics, exerting direct control over gene expression through the organisation of local chromatin structure for the allowance or blockage of access by transcriptional machinery [15]. Importantly, epigenetic processes such as DNA methylation are reversible with drug treatments, making these compounds a possible therapeutic agent [16]. It has previously been demonstrated that *CYP19A1* transcription can be epigenetically regulated. Global inhibition of DNA methylation with 5-Aza-2′-deoxycytidine results in elevated *CYP19A1* mRNA in human endometrial stromal cells, human hepatoma cells and chicken fibroblasts [17–19]. Furthermore, DNA methylation can be correlated with promoter-specific *CYP19A1* expression in sheep and cows promoters 1.1 and 1.5/2 [20–23] and human skin adipose fibroblasts [24]. Recently, our laboratory described epigenetic regulation of *CYP19A1* expression in BAFs as well as breast cancer cell lines, presenting evidence for the first time that epigenetics may be involved in progression of an ER+ breast tumour [25].

Upstream components of the PGE2 signalling pathway may be key to understanding the role of epigenetics in estrogens production. The EP2 receptor has been shown to be epigenetically regulated and a number of studies have looked at the significance of this in human disease. A clinically based study associated hypermethylation of *EP2* with positive prognostic outcomes in non-small cell lung cancer [26], whilst conversely another study showed that hypermethylation of the EP2 promoter was associated with increased cell proliferation and an inhibition of apoptosis in advanced neuroblastomas [27]. Our laboratory has previously identified that signalling factors upstream of *CYP19A1* transcription may be under epigenetic regulation, indirectly increasing levels of aromatase in BAFs [25]. In this study, we provide a detailed analysis of the DNA methylation status of *EP2* in breast cancer cell lines, BAFs and tumour-associated stroma cells. We show that although *EP2* methylation status is inversely correlated to mRNA levels in cell lines, no such relationship exists in primary human BAFs or clinical cancer-associated stromal cells.

2. Materials and methods

2.1. Patient information

Subcutaneous adipose tissue was obtained from cancer-free women during breast reduction mammoplasty, approved by the Southern Health Human Ethics Research Committee at Prince Henry's Institute (#B00109). Specimens of triple negative carcinomas, DCIS and IDC of the breast were obtained from Japanese female patients at St. Luke's International Hospital (Tokyo). Relevant clinical data were retrieved from the review of the patients' files. The histologic grade of each specimen was independently evaluated. The ethics committees at St Luke's International Hospital approved the research protocols (2010-509), with informed consent being obtained from these patients before surgery at the institution.

2.2. Cell culture

Primary human breast adipose fibroblasts were isolated by collagenase digestion of subcutaneous adipose tissue and cultured according to methods previously described [28]. Breast cancer cell lines MDA-MB-231 (ATCC no. HTB-26), Hs578T (ATCC no. HTB-126), MCF7 (ATCC no. HTB-22), T47D (ATCC no. HTB-133) and MCF10A (ATCC no. CRL-10318) were subcultured under prescribed conditions according to suppliers procedures. To inhibit DNA methylation, cells were grown to ~50% confluence in media containing serum. Growth media was then replaced with media containing 5-aza-2′-deoxycytidine (Sigma) at 10 μM, and changed daily for 5 days. Trichostatin A (Sigma) at 500 nM was added to the growth medium for the final 24 h of treatment for inhibition of histone deactylation. An equal amount of vehicle alone, being the agent in which the compound was dissolved, was used as the control.

2.3. Laser capture microdissection

Laser capture microdissection was performed according to a previous publication [29]. Briefly, frozen sections of 9 breast carcinoma cases along with their matching normal biopsies were examined under light microscope and stromal cells transferred via laser. DNA and RNA were then recovered with the use of the Illustra TriplePrep kit (GE Healthcare).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen). First strand cDNA synthesis was conducted using a minimum 200 ng total RNA, and carried out using avian myeloblastosis virus reverse transcriptase (Promega) primed with random primers. RNA isolated from laser capture microdissected tissue was amplified using the complete TransPlex whole transcriptome amplification kit (Sigma). Quantitative real time PCR (qRT-PCR) was carried out using TaqMan Gene expression assays (Applied Biosystems) or SYBR green detection methodologies on the ABI 7900HT Sequence Detection System or the System (Roche Diagnostics) using Fast Start Master SYBR Green I to quantitate *EP2* mRNA expression. *18S* or *β-actin* transcripts were also detected as an internal RNA loading control. Primer sequences used are as follows: *EP2* (sense, AGACGGACCACCTCATTCTC; anti-sense, GCCTAAGGATGGCAAAGACC); *18S* (sense, CGGCTACCACATCCAAGGA; anti-sense, GCTGGAATTACCGCGGCT); *β-Actin* (sense, TGCGTGACATTGCGTGACATTAAGGAGAAG; anti-sense, GCTCGTAGCTCTTCTCCA).

2.5. Sodium bisulphite sequencing

Genomic DNA was isolated from cells using the Wizard Genomic DNA purification kit (Promega). Deamination of DNA by sodium bisulphite conversion was carried out using the EpiTect Bisulphite kit (Qiagen) according to manufacturer's instructions. Primers to amplify the CpG island within the *EP2* promoter were obtained according to those previously published [27]. Amplification of the amplicon was performed using GoTaq (Promega) following touchdown PCR conditions. Amplified PCR products were cloned

using the pGEM-T Easy Vector System (Promega), with positive clones sent for sequencing. Sequences were input to the Bisulphite Sequencing DNA Methylation Analysis (BISMA) web server tool for analysis and generation of statistics [30].

2.6. Methylation-sensitive restriction enzyme PCR (msRE-PCR)

250 ng of genomic DNA was digested with either *Hpa*II or *Msp*I for methylation-sensitive enzymatic cleavage overnight at 37 °C. PCR was then performed to amplify both the target *EP2* sequence and a control *IGFII* genomic region. Primer sequences used are as follows: *EP2 msRE-PCR* (sense, ATCGGGCACCCTACTTCTA; anti-sense, GTGAAAGGCAAGGAGCAGAC); IGFII msRE-PCR (sense, CTTGGACTTTGAGTCAAATTGGCC; anti-sense, GAGGGTCGTGCCAATTACATTTCA).

2.7. Statistical analysis

Student's *t*-test or one-way ANOVA followed by Newman–Keuls Multiple Comparison Test was applied to assess statistical significance of result comparisons. Analysis was performed using Graph Pad Prism 5 statistical software.

3. Results

3.1. EP2 methylation status is inversely correlated with mRNA expression in breast cancer cell lines

To gain an initial understanding of the epigenetic mechanisms regulating EP2 receptor expression in the context of breast cancer, an analysis of cell lines was carried out. Using key representative cell lines MCF7 and T47D (tumour epithelial, ER+), MDA-MB-231 and Hs578T (metastatic, ER−), and MCF10A (normal epithelial), we found that basal *EP2* mRNA expression levels varied between the different cell types. Both the ER+ (MCF7) and the ER− (MDA-MB-231) breast cancer cell lines displayed relatively low levels of *EP2* expression. Conversely, the normal epithelial cell line MCF10A showed comparatively higher expression levels (Fig. 2A). In order to establish whether methylation of CpG dinucleotides within the *EP2* promoter were responsible for the discrepancies in mRNA expression observed, sodium bisulphite sequencing and subsequent CpG methylation mapping was performed. Sugino et al. [27] showed in a study of clinical neuroblastoma samples that *EP2* methylation was inversely correlated to mRNA expression in a region encompassing the second half of exon 1 (Fig. 1). We therefore concentrated our bisulphite sequencing on this promoter region. The data revealed an inverse correlation existed between CpG methylation and mRNA expression. MCF-7 cells showed a low basal level of *EP2* mRNA expression and had a hypermethylated promoter (77% CpG methylation). Hypermethylation was also observed in the MDA-MB-231 cells (97%) and this also correlated with a low level of *EP2* mRNA. In contrast, MCF10A cells were hypomethylated at the *EP2* CpG island, reflecting the higher mRNA expression observed (Fig. 2B and C). In contrast to

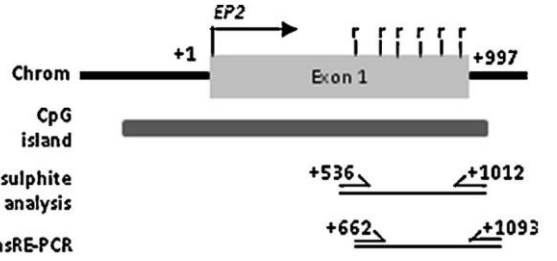

Fig. 1. Schematic representation of the EP2 CpG island region analysed for this study. The span of the CpG island is indicated by the grey strip. Regions for sodium bisulphite sequencing and msRE-PCR as used in this study are indicated. Recognition sites for the restriction enzymes *Hpa*II and *Msp*I are indicated along the chromosome (r). Adapted from Sugino et al. [27].

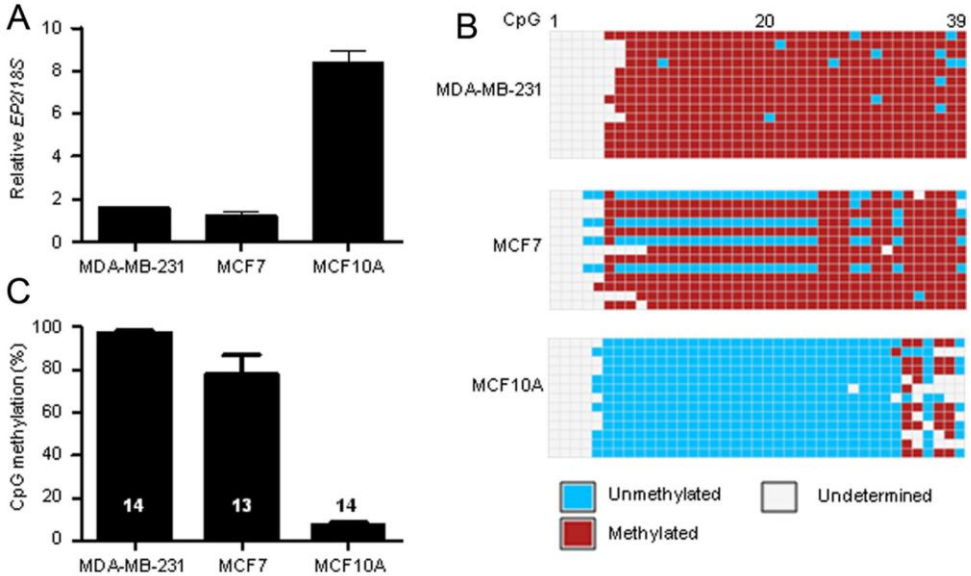

Fig. 2. Methylation and expression of EP2 in breast cancer cell lines. (A) qRT-PCR on RNA isolated from breast cancer cell lines MDA-MB-231, MCF7 and MCF10A showing relative *EP2* mRNA levels. Data obtained from three independent experiments conducted in triplicate. (B) CpG methylation profile of the 39 CpG sites within the analysed *EP2* promoter region. Each row represents an individual clone. Squares within each clone represent an individual CpG site with its status shown as either unmethylated or methylated. Where insufficient sequencing data was obtained to determine the methylation status of a particular CpG site, it is shown as undetermined. (C) Percentage methylation of each cell line across the whole analysed *EP2* promoter region. Numbers represent the number of clones analysed per group. Error bars represent standard error of means.

the inverse correlation observed in these three cell lines, analysis of additional ER+ (T47D) and ER− (HS578t) breast cancer cell lines failed to identify such a correlation (Supplementary Fig. 1), suggesting alternate means of EP2 regulation within these cells.

3.2. Inhibition of DNA methylation and histone deacetylation restores EP2 mRNA expression in MDA-MB-231, MCF7 and MCF10A cells

It has previously been reported that global inhibition of DNA methylation with the DNA methyl-transferase inhibitor 5-aza-2′-deoxycytidine (5aza) results in restoration of EP2 receptor expression in a number of different cell types [26,27,31]. This suggests that DNA methylation is a major mechanism by which mRNA expression of *EP2* is regulated and further correlates to our initial observations. Additionally, it has been reported in non-small cell lung cancer samples that histone deacetylation is also able to regulate EP2 expression, demonstrating an additional layer of chromatin epigenetic regulation [32]. To determine whether this was true in breast cancer cells, representative cell lines previously described were treated with 5aza (10 μM), the histone deactylase inhibitor Trichostatin A (TSA) (500 nM) or a combination of both (Fig. 3). Semi-quantitative confirmation of EP2 promoter demethylation was performed by methylation-sensitive restriction enzyme analysis of the region determined to be inversely correlated to expression in breast cancer cell lines following 5aza treatment (Supplementary Fig. 2). In MDA-MB-231 cells, inhibition of DNA methylation with 5aza significantly restores *EP2* mRNA expression up to 20-fold over vehicle. Inhibition of histone deacetylation with TSA does not alone have any effect on *EP2* expression, however appears to synergistically enhance the effect of 5aza when used in combination, with a 30-fold increase in *EP2* expression over vehicle. In the ER+ cell line MCF7, neither 5aza nor TSA treatment alone could increase *EP2* transcript levels. Interestingly, when both treatments were applied, a significant 6-fold increase in *EP2* mRNA was observed. Similar results were observed in MCF10A cells, where a combination treatment resulted in a significant 3-fold increase in *EP2* mRNA levels. These results support that in addition to DNA methylation, histone acetylation status is a critical determining factor in the regulation of *EP2* mRNA expression in breast cancer. Interestingly, while we did not observe inverse correlations between DNA methylation and mRNA expression in the cell lines T47D and HS578t, *EP2* expression was still altered by the epigenetic modifying agents, including significantly large increases when 5aza and TSA were used in combination in T47D cells (Supplementary Fig. 3).

3.3. EP2 mRNA expression is increased in BAFs following 5aza treatment

As the main proposed site of action for PGE2 within the tumour microenvironment is on the neighbouring stromal cells, the potential for epigenetic regulation of the EP2 receptor was investigated in BAFs. Methylation-sensitive restriction enzyme analysis of the region determined to be inversely correlated to expression in breast cancer cell lines (Fig. 1) showed that under basal conditions, the *EP2* promoter was hypermethylated. Upon treatment with 5aza, this region was subsequently demethylated (Fig. 4A). Endogenous expression of *EP2* in BAFs has previously been demonstrated [14], thus we wished to determine whether further expression could be induced with 5aza treatment. Freshly isolated BAFs were treated with 5aza (10 μM) over a 5 day culture. qRT-PCR revealed that DNA methylation inhibition with 5aza resulted in an increase in *EP2* expression, up to 15-fold over control (Fig. 4B).

3.4. Expression of EP2 is not correlated with methylation status in normal and cancer-associated stroma

In order to establish whether our findings in breast cancer cell lines and *in vitro* cultured BAFs could be applied *in vivo*, breast stromal cells from matching normal and cancerous breast were isolated by laser capture microdissection (LCM). Patients represented a diversity of breast cancer subtypes, namely ER+ ductal carcinoma in situ, ER+ invasive ductal carcinoma and triple negative tumours (Table 1). qRT-PCR was performed to investigate the levels of *EP2* mRNA in normal stroma compared with cancer-associated stroma. Two of the three tumours that were negative for ER immunoreactivity showed lower *EP2* expression in cancer-associated stroma compared to normal. Conversely, four of six ER+ patients showed higher levels of *EP2* mRNA in cancer-associated stroma. Three of these were of patients diagnosed with a DCIS breast cancer subtype (Fig. 5A).

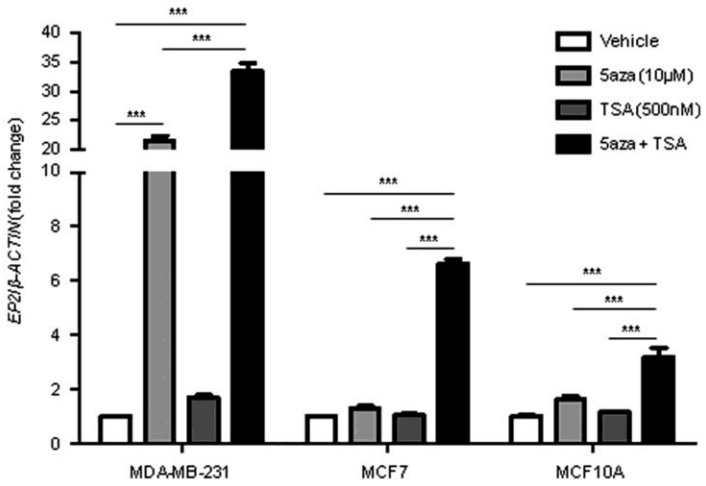

Fig. 3. qRT-PCR on RNA isolated from cell lines treated with 5aza and TSA. Data obtained from three independent experiments conducted in triplicate. Data were analysed by independent *t*-tests of grouped means. ***$p < 0.001$.

Table 1
Clinicopathological information of the nine cases of breast cancer in which matching normal and cancer-associated stromal cells were microdissected. Histologial grade was determined according to Robbins et al. [42], ER and PR immunoreactivity was evaluated according to Allred et al. [43] and HER2 immunoreactivity was evaluated based on the CAP-ASCO guideline [44].

Sample ID	Age	Sex	Histopathological diagnosis	Nottingham's histological grade	ER	PgR	HER2
8T	51	F	Invasive ductal carcinoma, scirrhous carcinoma	Grade II	Negative	Negative	Negative
9T	64	F	Ductal carcinoma in situ	Grade I	Negative	Negative	Negative
24T	36	F	Invasive ductal carcinoma, solid-tubular carcinoma	Grade III	Negative	Negative	Negative
19T	69	F	Ductal carcinoma in situ	Grade II	Positive	Positive	Negative
53T	50	F	Ductal carcinoma in situ	Grade II	Positive	Positive	Negative
111T	66	F	Ductal carcinoma in situ	Grade I	Positive	Positive	Negative
20T	61	F	Invasive ductal carcinoma, papillotubular carcinoma	Grade I	Positive	Positive	Negative
38T	62	F	Invasive ductal carcinoma, solid-tubular carcinoma	Grade III	Positive	Negative	Equivocal
50T	75	F	Invasive ductal carcinoma, solid-tubular carcinoma	Grade I	Positive	Positive	Negative

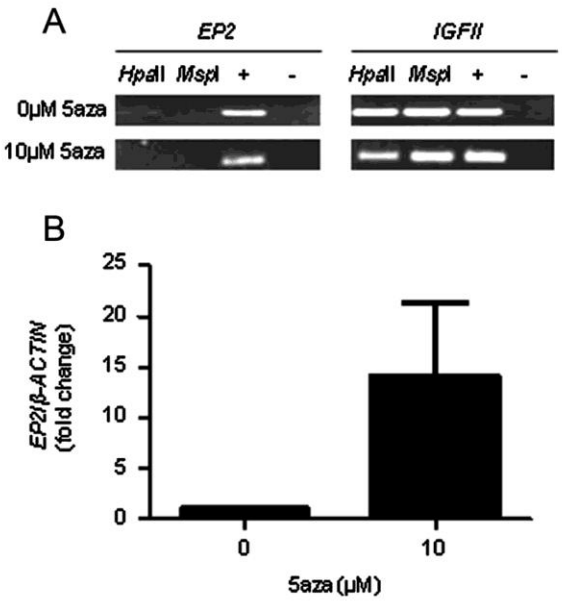

Fig. 4. Demethylation of BAFs using 5aza treatment. (A) msRE-PCR to detect EP2 promoter methylation in BAFs treated with or without 5aza. Genomic DNA was digested with the methylation sensitive enzyme HpaII or the methylation insensitive enzyme MspI. A positive control (+) of undigested DNA and no template control (−) are also shown. A region of the IGFII promoter known to be hypermethylated was amplified as a positive control. (B) qRT-PCR on RNA isolated from BAFs treated for 5 days with 5aza to inhibit DNA methylation. Data obtained from three independent experiments conducted in triplicate.

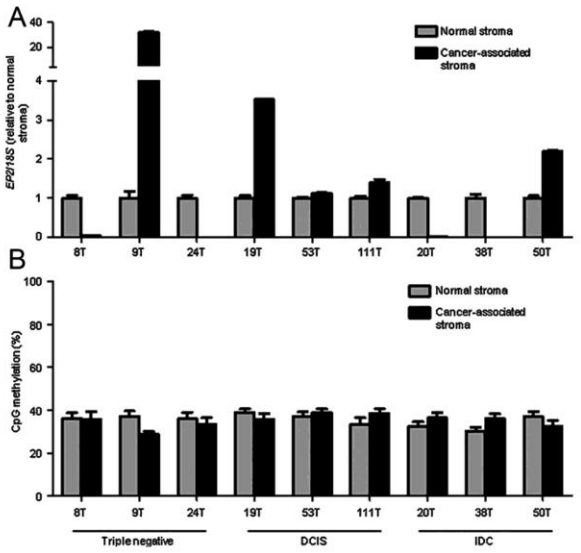

Fig. 5. Methylation and expression of EP2 in clinical breast-cancer associated stroma. (A) qRT-PCR on RNA isolated from matching normal associated stroma and cancer-associated stroma of breast cancer patients as a relative percentage to normal. Experiment was conducted in duplicate, error bars represent standard error of means. (B) Percentage methylation across the analysed *EP2* promoter region from the matching normal associated stroma and cancer-associated stroma of breast cancer patients. Error bars represent standard error of means.

Sodium bisulphite sequencing on each of the matching samples revealed no clear differences in the methylation pattern or the overall average methylation percentage across the *EP2* CpG island between normal and cancer-associated stroma in any of the patients (Fig. 5B). Consistent hypermethylation was observed in CpG numbers 26–28, 31, 32 and 34–38 in all cases, whereas the methylation pattern of CpG numbers 1–6 displayed inconsistent results. Clones were either consistently hypermethylated or hypomethylated, and the extent to which hypermethylation was occurring varied between normal and cancer-associated stroma, although no correlation could be drawn with corresponding expression levels.

4. Discussion

The data from this study present an analysis of the methylation status of the *EP2* promoter region in the context of established breast cancer cell lines, *in vitro* cultured BAFs and clinically isolated breast cancer-associated stroma. Strong evidence was established for epigenetic regulation of *EP2* expression in the cell lines, where an inverse correlation was observed between percentage methylation across the bisulphite sequenced region and mRNA expression in the respective cell line. This data was reinforced by DNA methylation inhibition by 5aza causing an increase in mRNA expression in the hypermethylated cell line MDA-MB-231. An examination of breast cancer cell lines not only established a trend for *EP2* methylation and expression in a diverse range of breast cancer contexts, but also allows us to examine effects of autocrine PGE2 signalling on tumour epithelial cells. Indeed, it has been shown that treatment of epithelial cell lines MDA-MB-231, MCF7 and MCF10A stimulates expression of aromatase via PI.3/PII [25], indicating that not only is the EP2 methylation status important in epithelial cells, but also targetting these as well as cancer-associated fibroblasts is important in anti-oestrogen therapies.

Our findings in established breast cancer cell lines are consistent with a previously published study examining a large number of genes in response to inhibition of DNA methylation in breast cancer

cells [33]. They found that *EP2* was amongst the genes upregulated upon 5aza treatment in MDA-MB-231 cells, but not amongst genes found to be upregulated upon the same treatment in MCF7 cells. Our sodium bisulphite sequencing results indicated that the *EP2* promoter in both these cell types is heavily methylated, corresponding to a low expression level of *EP2* mRNA. Given that our demethylation data only results in upregulation of *EP2* in one cell type, MDA-MB-231 cells consistent with the aforementioned study, there are likely to be further upstream factor that regulate *EP2* also under epigenetic regulation that are present in one cell type and not the other. This is supported by our finding that 5aza treatment of T47D cells increases *EP2* expression despite the promoter region being natively hypomethylated, indicating that epigenetic regulation is likely at the level of other upstream factors. msRE-PCR results confirmed that there was a degree of demethylation occurring in the *EP2* promoter in 5aza treated cells, and enforces that *EP2* promoter methylation levels are not indicative of expression levels. Our results from the methylation profiling of the *EP2* promoter in other breast cancer cell lines HS578t (ER−) and T47D (ER+) show that the inverse correlation with mRNA expression does not hold true for all cell types, a finding that reflects in our studies on clinical breast stroma which do not show correlation between EP2 methylation and expression. These findings would therefore suggest that ER+ and ER− breast cancers are genetically and epigenetically diversified such that *EP2* expression is differentially regulated.

Whilst our study only examined changes in the DNA methylation profile, other epigenetic processes such as histone acetylation may also contribute to expression levels. Indeed, it has been demonstrated in a cohort of non small cell lung cancer samples that histone acetylation at least in part regulates EP2 expression levels [32]. This was also supported by our findings in the breast cancer cell lines through the use of the histone deactylated inhibitor TSA, indicating cooperative regulation through both DNA methylation and histone modifications, a regulatory feature common in many genes. Our examination of DNA methylation and histone acetylation as regulatory factors in *EP2* expression presents the finding that their respective influence is varied among the different cell lines. In the ER− cell line MDA-MB-231, TSA alone had no effect on expression levels, but appeared to enhance the effect of 5aza in upregulating *EP2* mRNA. HS578t cells (ER−) however showed modest but significant increases in *EP2* expression with 5aza treatment, and this was not increased by combination treatment with TSA. Both MCF7 (ER+) and MCF10A (normal epithelial) only showed increases in *EP2* expression when both agents where used in combination. T47D cells (ER+) appear to be heavily influenced by histone modifications, with robust upregulation of *EP2* seen upon TSA treatment. Taken together, these results indicate that both DNA methylation and histone acetylation are critical in regulating *EP2* expression levels, and that ER status does not appear to be a determining factor in the relative influence of both forms of epigenetic regulation.

Additional epigenetic, genetic and post-translational mechanisms may also be differentially utilised in breast cancer subtypes to explain the discrepancies in correlation between methylation and expression of *EP2* that we observed. Transcription factors as well as repressors or activators at the genomic level may undergo differential regulation in tumour cells to alter *EP2* transcriptional levels. Whilst we examined the influence of histone acetylation in this study, other forms of histone modifications, including histone methylation, may be critical. Finally, post-translational modifications may affect the final availability of the receptor on the cell surface. A thorough examination of genetic as well as protein regulators of *EP2* expression would be necessary to assess at what stage in the regulatory process any clear distinctions between ER+ and ER− breast cancer cell types can be identified.

We have shown that in normal human BAFs expression of *EP2* mRNA is present at basal levels, even though hypermethylation of the promoter is evidenced through msRE-PCR analysis. Although 5aza treatment demethylates *EP2* and results in an increase in expression levels, there is no evidence that this is being directly mediated solely by changes in the *EP2* promoter methylation status and not through upstream factors. Accordingly, our study of clinical samples of normal and matching tumour-associated stroma isolated by LCM demonstrated that although differences in *EP2* mRNA levels could be measured, these changes could not be attributed to direct changes in *EP2* promoter DNA methylation. This suggests that factors regulating *EP2* expression may themselves be under epigenetic regulation, and it is the change in their respective methylation status that is driving increases in EP2 expression. Alternatively, methylation in other parts of the *EP2* promoter which may be less CpG-rich may be interfering with transcription factor binding. Interestingly, lower expression levels of EP2 were detected in some tumour-associated stromal samples compared to matching normal stroma. This was evident in two of three triple negative samples and two of three IDC samples. Whether or not this is indicative of a particular breast cancer subtype remains to be determined, as a larger cohort of samples would be required for analysis.

The EP2 receptor is one of a family of four receptors known to mediate PGE2 signalling. Whilst our results indicate that *EP2* promoter methylation may not be directly affecting expression levels within the stroma, there may be some involvement of the other EP receptors in the upstream epigenetic mechanisms regulating *CYP19A1* expression. Expression of *EP1* and *EP3* have previously been reported in relation to breast cancer, with a recent study linking EP1 receptor expression to a positive prognosis [34], and EP3 shown to regulate aromatase activity in disease-free adipose stromal cells [35]. Additionally, EP4 is shown to be critical to PGE2 signalling in an ER+ tumour context [14]. Promoter analysis of the regulatory region for each of the encoding genes reveals no defined CpG island in the *EP3* promoter, however both *EP1* and *EP4* contain substantial regions of high CpG percentage (data not shown). To date, there has been no published literature examining the methylation status of either the *EP1* or *EP4* promoter region or its relation to cancer progression. A further study looking at the expression levels and methylation status of both of these EP receptors in the context of ER+ breast cancer may be pertinent in order to establish a clearer picture on which specific upstream factors are contributing to the epigenetic regulation of *CYP19A1* expression.

To expand on the results of this present study, it would be interesting to investigate at what stage of tumour progression changes in *EP2* expression levels can first be detected. Although it does not appear that direct DNA methylation changes are responsible for this effect, upstream factors affecting its expression may indeed be under epigenetic regulation. Previous work has shown that, like genetic mutations, global epigenetic changes are cumulative and that certain genes display a loss or gain of methylation in a stage-specific manner. This has been demonstrated for ovarian cancer [36], gastric cancer [37], acute lymphoblastic leukaemia [38], and oesophageal adenocarcinoma [39]. Epigenetic signatures in certain genes can be used not only as a prognostic indicator, but also to diagnose a stage of disease and to predict response to particular therapies [40]. Hypermethylation of genes has already been associated with high grade breast cancer metastasis to the brain, bone and lungs [41], although *EP2* was not amongst those examined. Clarification of stage specific *EP2* expression changes coupled with information on the CpG methylation status of its regulatory factors may in future serve as a diagnostic indicator.

In summary, our findings demonstrate a role for the EP2 receptor in the upstream epigenetic regulation of *CYP19A1* expression and indicate that therapeutic targeting of epigenetic processes may serve as an ER+ breast cancer treatment.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2012.07.007.

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Appendix 5: Melatonin suppresses aromatase expression and activity in breast cancer associated fibroblasts

Research article published in *Breast Cancer Research and Treatment*

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Original research article written in collaboration with Kevin Knower, Evan Simpson, Colin Clyne as well as colleagues in Japan. My contribution was to the study was 10%, and I performed experiments for Figure 2 and reviewed the finished manuscript. This work was completed during my Ph.D. candidature and continues the broad theme of understanding oestrogen regulation in breast cancer.

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BRIEF REPORT

Melatonin suppresses aromatase expression and activity in breast cancer associated fibroblasts

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Abstract The main biological active substance secreted by the pineal gland, melatonin (MLT), counteracts the effects of estrogens in breast cancer via exerting a number of its own oncostatic properties. Recent studies of postmenopausal women have identified that the major metabolite of MLT is statistically significantly associated with a lower risk of developing breast cancer. While MLT production decreases with age, breast cancer risk, however, increases with age and obesity. We hypothesize that MLT inhibits estrogen production in breast adipose fibroblasts (BAFs), the main local source of estrogen in breast tumors of postmenopausal women, by inhibiting transcription of the *CYP19A1* gene that encodes the key enzyme aromatase. Normal BAFs were cultured from women undergoing breast reduction surgery, while breast cancer-associated fibroblasts (CAFs) were isolated from three women with estrogen receptor (ER) positive invasive ductal carcinomas. *MTNR1A* and *MTNR1B* receptor expression and *CYP19A1* mRNA expression following MLT treatments were determined by qRT-PCR. BAFs express the G-protein coupled MLT receptors *MTNR1A* and *MTNR1B* with elevated levels of *MTNR1A* found in CAFs. Treatment of BAFs and CAFs with MLT resulted in significant suppression of *CYP19A1* transcription and aromatase activity at pharmacological, physiological and sub-physiological concentrations. MLT suppression occurred through promoter-specific PI.4-, PI.3- and PII-derived *CYP19A1* mRNA. Stimulation of *CYP19A1* PII-mRNA and aromatase activity by prostaglandin E_2 (PGE_2) were significantly attenuated by physiological doses of MLT. Lower levels of MLT in aging women may increase the risk of progressing ER-positive breast cancer through a decreased ability to suppress *CYP19A1* expression and subsequent local estrogen production in BAFs/CAFs.

Keywords Melatonin · Aromatase · Postmenopause · Breast cancer · Epigenetic · Prostaglandin E_2

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Introduction

Breast cancer is a classical model of hormone-dependent malignancy. Two-thirds of breast cancer occurs in postmenopausal women, where ovaries have ceased to be functional and changes in the hormonal milieu are associated with an increase in total adiposity and breast cancer risk. Aromatase P450, encoded by the gene *CYP19A1*, is the key enzyme catalysing the synthesis of estrogens from circulating C_{19} steroids [1]. In postmenopausal women, cancer-associated fibroblasts (CAFs, also referred to as breast adipose fibroblasts (BAFs) in the absence of a tumor) of the stromal compartment adjacent to a tumor become the major source of local estrogen production, coinciding with an increase in *CYP19A1* expression via the tissue-specific promoters PI.4, PI.3 and PII in these cells

[2]. The stimulation of these tissue-specific promoters occurs through paracrine signals, such as prostaglandin E_2 (PGE_2) (PI.3/II) and class I cytokines and glucocorticoids (PI.4), derived from neighboring tumor epithelial cells and infiltrating macrophages [3, 4]. The development of breast-specific aromatase inhibitors (AIs) and suppressers of *CYP19A1* gene expression is therefore a critical aspect in inhibiting local estrogen production in breast cancer and is at the frontline of therapeutic care.

The main biological active substance secreted by the pineal gland, melatonin (MLT), can counteract the effects of estrogens in breast cancer through a number of its own oncostatic properties and signaling mechanisms [5–7]. MLT hormone is secreted in response to darkness and, as such, levels rise and fall throughout the day. Evidence from clinical studies has led to the hypothesis that increased risk for breast cancer among women is closely related to circulating MLT levels. For example, light-induced inhibition of MLT secretion increases breast cancer risks, e.g. within flight attendants and night-shift workers; conversely, totally blind women also have the lowest incidence of breast cancer [8]. A recent cohort study of postmenopausal women has also revealed that an increased concentration of urinary 6-sulfatoxymelatonin (aMT6s), the major metabolite of MLT, is statistically significantly associated with a lower risk of developing breast cancer [9]. These observations are further supported by the fact that MLT production gradually declines with advancing age, with MLT levels reported to be low in elderly individuals, associated with ER-positive breast cancer and linked with enhanced tumor growth in rats [10–12].

Since surrounding BAFs are the major source of local estrogen production in postmenopausal women, it is possible that many of the protective properties of MLT are exerted upon these cells but lost with increasing age. Physiological concentrations of MLT have been shown to reduce aromatase expression and activity in the ER-positive breast cancer derived cell line MCF-7 [13, 14]. However, it is acknowledged that CAFs, not epithelial cells, are the abundant source of elevated *CYP19A1* mRNA in postmenopausal breast cancer [15]. In this study we show that MLT suppresses *CYP19A1* mRNA expression and aromatase activity in normal BAFs and CAFs obtained from women with ER-positive invasive ductal carcinoma (IDC).

Materials and methods

Patient information

Subcutaneous adipose tissue was obtained from Caucasian cancer-free women at the time of reduction mammoplasty approved by the Southern Health Human Ethics Research Committee at Prince Henry's Institute. Specimens of IDC of the breast were obtained from Japanese female patients at Tohoku University Hospital and Tohoku Kosai Hospital. Relevant clinical data were retrieved from the review of the patients' files. The histologic grade of each specimen was independently evaluated. The ethics committees at Tohoku University School of Medicine and Tohoku Kosai Hospital approved the research protocols (2004-144, 2005-068, and 2006-042, respectively), with informed consent being obtained from these patients before surgery in each institution.

Cell culture

Human breast adipose fibroblasts were isolated by collagenase digestion and cultured as previously described [16]. Where BAFs were used as a comparison to other cell types, averaged data were obtained from BAFs isolated from three independent women. Cell line MCF-7 (ATCC No. HTB-22) was subcultured according to supplier's procedures. For treatments, BAFs grown to ~60% confluence were initially starved in serum-free media containing 0.1% BSA for 24 h. MLT was prepared in dimethyl sulfoxide (DMSO) at described concentrations and cells were treated in serum-free media for 90 min. To stimulate promoter-specific *CYP19A1* transcription, serum-free media containing PGE_2 (1 μM) was added to cells for 24 h to stimulate PII. In all treatments, equal amounts of vehicle alone (the agent in which the compound was dissolved) were used as a control.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Mini kit (QIAGEN). Following DNAse treatment, first strand cDNA synthesis from a minimum of 200 ng of total RNA was performed using AMVRT (Promega) primed by random hexamers. Semi-quantitative RT-PCR of *MTNR1A* and *MTNR1B* receptors was performed with the primer pairs for *MTNR1A*—(Fwd) 5′-GCCACAGTCTCAAGTA CGACA-3′ (Rev) 5′-CTGGAGAACCAGGATCCATAT-3′ and *MTNR1B*—(Fwd) 5′-TGCCTCATCTGGCTCCTCAC-3′ (Rev) 5′-TAGGGAGGAGGAAGTGGATG-3′. Quantitative RT-PCR (qRT-PCR) of total *CYP19A1* mRNA was performed with SYBR Green detection methodologies with Roche LightCycler Systems using the primer pair termed RT-7 (Fwd) 5′-TTGGAAATGCTGAACCCGAT-3′ and RT-8 (Rev) 5′-CAGGAATCTGCCGTGGGGAT-3′; or the ABI 7900HT Sequence Detection System using (Fwd) 5′-CACATCCTCAATACCAGGTCC-3′ and (Rev) 5′-CA GAGATCCAGACTCGCATG-3′ and probe 5′-6-FAM-CC CTCATCTCCCACGGCAGATTCC-TAMRA-3′. PII-driven transcripts were quantitated with Roche LightCycler

using (Fwd) 5′-GCAACAGGAGCTATAGAT-3′ and RT-8; or the ABI 7900HT using (Fwd) 5′-GCAACAGGAGCTAT AGATGAA-3′ and ExonII (Rev) 5′-AGGCACGATGCT GGTGATG-3′ and probe 5′-6-FAM-TGCCCCCTCTGA GGTCAAGGAACA-TAMRA-3′. In combination with ExonII (Rev)–PI.4 (Fwd) 5′-GTAGAACGTGACCAAC TGGAG-3′ and probe 5′-6-FAM-ATGGGCTGACCAGTG CCAGGGACC-TAMRA-3′; PI.3 (Fwd) 5′-GTCTTGCC TAAATGTCTGATCAC-3′ and probe 5′-6-FAM-TGCCCC CTCTGAGGTCAAGGAACAC-TAMRA-3′ were quantitated with the ABI 7900HT. qRT-PCR amplification of *18S* was conducted with SYBR Green detection methodologies using (Fwd) 5′-CGGCTACCACATCCAAGGA-3′ and (Rev) 5′-GCTGGAATTACCGCGGCT-3′.

Aromatase activity assays

BAFs and CAFs were grown to ~60% confluence and then serum starved overnight in phenol-red free medium containing 0.1% BSA. After serum starvation, cells were treated with experimental agents at the concentrations indicated for 24 h. Endogenous aromatase activity was measured by the detection of 3HOH released during aromatization of [1ß-3H] androstenedione, as previously described [16].

Statistical analysis

Data were analyzed by one-way ANOVA followed by Newman–Keuls Multiple Comparison test. Statistical analysis was performed with GraphPad Prism Software.

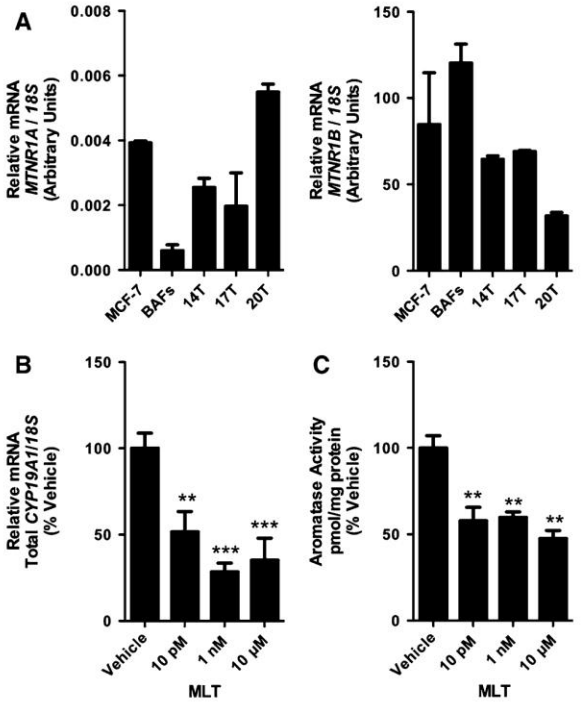

Fig. 1 MLT suppresses *CYP19A1* mRNA expression and activity in BAFs. **a** qRT-PCR of *MTNR1A* and *MTNR1B* in cDNA from BAFs and MCF-7 cells. cDNA from primary CAF lines 14T, 17T and 20T (Table 1) were also analyzed. **b** qRT-PCR of total *CYP19A1* mRNA in BAFs treated with final concentrations of 10 pM, 1 nM and 10 µM of MLT for 90 min. **c** Aromatase activity in BAFs following 24 h treatments of MLT. Data obtained from two to three independent experiments. Error *bars* represent standard error of means. Data was analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test (**$p < 0.01$, ***$p < 0.001$)

Results

MLT acts through its G-protein-coupled receptors, MT1 and MT2, located at the plasma membrane. Initially, expression of encoding genes *MTNR1A* (MT1) and *MTNR1B* (MT2) was assessed in BAFs by qRT-PCR. BAFs and MCF-7 (positive control) cells expressed both receptors, albeit to differing levels, with *MTNR1A* levels predominantly lower in BAFs compared to MCF-7 cells (Fig. 1a). Following confirmation of MLT receptor expression, to determine whether MLT altered *CYP19A1* mRNA expression, pharmacological (10 µM), physiological (1 nM) and sub-physiological (10 pM) concentrations of MLT were used to treat BAFs. In each instance, a significant suppression of total *CYP19A1* mRNA levels was observed when compared to vehicle alone (100%); 51% (10 pM), 28% (1 nM), and 35% (10 µM) (Fig. 1b). Following the observed suppression of *CYP19A1* mRNA, aromatase activity assays were performed to measure the production of estrogen by measuring the transfer of tritium at the 1ß position of [1ß-3H]androstenedione (1ß-AE) to water. In comparison to vehicle alone (100%), significant decreases in aromatase activity were observed in BAFs treated with MLT—57% (10 pM), 60% (1 nM) and 48% (10 µM) (Fig. 1c).

Expression of *CYP19A1* in BAFs is under the control of the tissue-specific promoters PI.4, PI.3 and PII, with PI.4 being the predominant transcript present in disease-free cells. To ascertain if the effects of MLT is promoter specific, exon-specific qRT-PCR was performed for each promoter following MLT treatment. Physiological concentrations of MLT (1 nM) significantly decreased expression of all three promoters when compared to vehicle (100%); PI.4 (34%), PI.3 (48%) and PII (42%) (Fig. 2a). The secretion of PGE_2 by breast tumor epithelial cells is associated with the increased tumor PII-derived transcriptional activity in surrounding BAFs through both cAMP/PKA- and PKC-dependent signaling and the

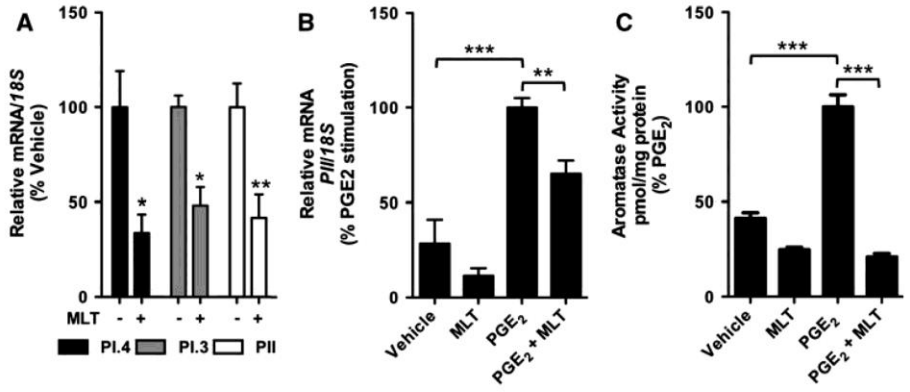

Fig. 2 Suppressive effects of MLT on promoter-specific *CYP19A1* expression and activity in BAFs. **a** Exon-specific qRT-PCR of *CYP19A1* promoters PI.4, PI.3 and PII following MLT (1 nM) treatment. **b** Exon-specific qRT-PCR of PII-derived mRNA following PGE_2 (1 μM) stimulation alone or in combination with MLT (1 nM). **c** Aromatase activity following PGE_2 (1 μM) stimulation alone or in combination with MLT (1 nM). Data obtained from three independent experiments. Error *bars* represent standard error of means. Data was analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

activation of a number of transcription factors [3]. As MLT is known to decrease intracellular concentrations of cAMP [14], we tested the ability of MLT to suppress PGE_2 PII-stimulation in BAFs. PGE_2 increased PII-derived *CYP19A1* mRNA ~3.5-fold compared to vehicle (Fig. 2b). In the presence of physiological concentrations of MLT, however, the PII-stimulation was significantly decreased (65%) compared to PGE_2 alone treatment (100%) (Fig. 2b). Likewise, PGE_2 stimulated aromatase activity ~2-fold, a process that is also significantly reduced following co-treatment with MLT (21%) (Fig. 2c).

As MLT was found to suppress *CYP19A1* expression in BAFs isolated from normal women, we extended these observations to CAFs isolated from women with ER-positive IDC (Table 1). Three tumor primary cell lines, two from postmenopausal women (Patient ID: 17T and 20T) and one from premenopausal (Patient ID: 14T) were initially screened for aromatase activity levels. The three CAF lines showed significant higher levels of aromatase activity, upwards of ~2-fold in the case of 14T and 20T, compared to disease-free BAFs (Fig. 3). *MTNR1A* and *MTNR1B* receptor expression was determined by qRT-PCR. All three CAF lines maintained MLT receptor expression; however, our findings demonstrated higher levels of *MTNR1A* and lower levels of *MTNR1B* mRNA in the three CAF lines compared to disease-free BAFs (Fig. 1a). Physiological doses of MLT were used to treat CAFs and total *CYP19A1* mRNA levels and aromatase activity assessed. When compared to vehicle alone treatments (100%), each CAF had significantly reduced levels of total *CYP19A1* mRNA following MLT treatment; 14T (37%), 17T (29%) and 20T (43%) (Fig. 4a). This reduction also conferred into a significant decrease in aromatase activity compared to vehicle (100%); 14T (22%), 17T (41%) and 20T (37%).

Discussion

Multiple lines of in vivo and in vitro evidence show that the MLT oncostatic actions in breast cancer are primarily through its effects on hormone-dependent tumors through its ability to interact with estrogen signaling pathways [5]. In tumor epithelial cells, these interactions have shown to modulate estrogen receptor actions as well as the enzymes responsible for estrogen biosynthesis [17]. Crosstalk within the IDC breast tumor microenvironment results in the elevated levels of aromatase expression in neighboring adipose fibroblasts and the main source of local estrogen in postmenopausal women. In this study we demonstrate MLT oncostatic actions on normal BAFs and CAFs isolated from IDCs, two of which were postmenopausal, to down-regulate aromatase mRNA expression at physiological concentrations. Both BAFs and CAFs predominately express the *MTNR1A* receptor, which may correlate to the binding of MLT to MT1 as seen as one of the first steps in aromatase antagonism in epithelial cells [18].

Understanding the promoter-specific control of *CYP19A1* expression in BAFs is fundamental for creating modes of antagonism. While PI.4 is elevated in the presence of a tumor, PI.3 and PII make up 80–90% of *CYP19A1*-derived transcripts also up-regulated, as such, much work has been carried out to understand this regulatory pathway as a means to decrease aromatase expression [19]. In this study, we show that MLT actions on BAFs are not promoter-specific and suppress each of the three promoters expressed. This data correlates with what is observed in MCF-7 cells despite the subtle differences in modes of PII transcriptional activation by alternative transcription factors in the two different cell types [14, 20]. Elevated levels of PI.4 in CAFs are due to elevated levels of class I cytokines and glucocorticoids within the tumor

Breast Cancer Res Treat (2012) 132:765–771

Table 1 The clinicopathological information of the three cases of breast cancer in which stromal cells were obtained

Sample ID	Age	Sex	Histopathological diagnosis	Nottingham's histological grade (tubular formation, nuclear atypia, mitosis)	TNM	ER	PgR	HER2
14T	47	F	Invasive ductal carcinoma, scirrhous carcinoma	Grade II (3, 2, 1)	pT1c, pN0, cM0, G2, Stage I	Positive (PS5 + IS3 = TS8, almost 100%)	Positive (PS5 + IS3 = TS8, almost 100%)	Negative (score 0)
17T	76	F	Invasive ductal carcinoma, scirrhous carcinoma	Grade II (3, 2, 1)	pT4b, pN1, cM0, G2, Stage IIIB	Positive (PS5 + IS3 = TS8, 100%)	Positive (PS4 + IS3 = TS7, 40%)	Negative (score 0)
20T	69	F	Invasive ductal carcinoma, scirrhous carcinoma	Grade II (2, 3, 1)	pT4b, pN0, cM0, G1, Stage IIIB	Positive (PS5 + IS3 = TS8, almost 100%)	Positive (PS5 + IS3 = TS8, almost 100%)	Negative (score 0)

Histological grade was determined according to Robbins et al. [28] and each score represents that of tubular formation, nuclear atypia and mitosis. ER and PR immunoreactivity was evaluated according to Allred et al. [29]. HER2 immunoreactivity was evaluated based on the CAP-ASCO guideline [30]

PS proportional score, *IS* intensity score, *TS* total score

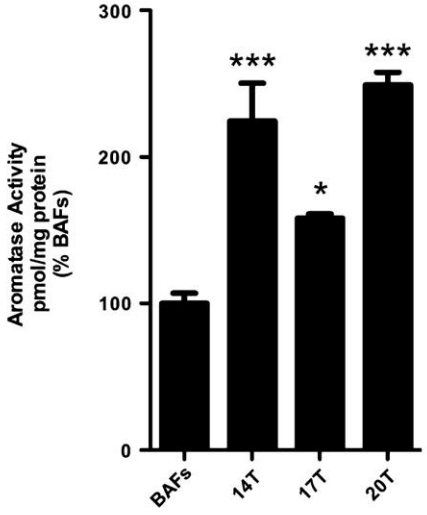

Fig. 3 Aromatase activity is elevated in 14T, 17T and 20T CAF lines. Data obtained from triplicate experiments. Error *bars* represent standard error of means. Data was analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test (*$p < 0.05$, ***$p < 0.001$)

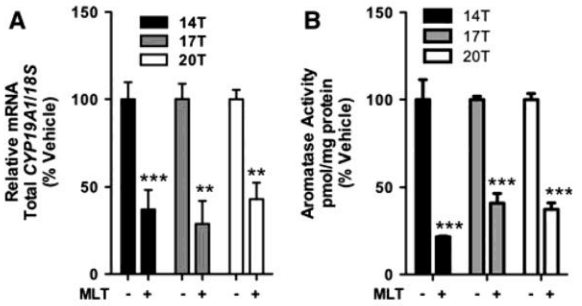

Fig. 4 MLT suppresses aromatase expression and activity in CAFs. **a** qRT-PCR of total *CYP19A1* mRNA from 14T, 17T and 20T CAF cell lines treated with MLT (1 nM) for 90 min. **b** Aromatase activity following 24-h treatment with MLT (1 nM). Data obtained from three experiments. Error *bars* represent standard error of means. Data was analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test (**$p < 0.01$, ***$p < 0.001$)

microenvironment. Interestingly, MLT can repress dexamethasone induced transcriptional activation in MCF-7 cells and reduce proinflammatory cytokines such as TNF-α and IL-6 [21, 22]. Factors such as these are critical for PI.4-driven *CYP19A1* expression and may serve as two possible modes of MLT PI.4 inhibition in BAFs.

The dramatic elevation of PI.3/PII transcripts in CAFs is a direct consequence of increases in cellular cAMP levels through PGE_2 secreted by tumor epithelial cells. By modeling this in BAFs, we show that this PII-driven stimulation by PGE_2 can be inhibited by MLT. Our data are consistent with previous observations that MLT can down-regulate

cAMP levels and downstream targets in different cell types including MCF-7 cells [14]. While elevated PGE_2 in the microenvironment, driven by increases in *COX-2* expression in tumor epithelial cells, acts on CAFs predominantly in a paracrine manner, up-regulation of *COX-2* in the stroma may also serve as a mechanism of PGE_2 autocrine action to stimulate PII. However, whereas MLT was shown to down-regulate *COX-2* expression in MCF-7 cells [14], our observations suggest that this is not the case in CAFs (data not shown).

The breast is delicately sensitive to interactions within the tumor microenvironment and has thus emerged as an important therapeutic target, with anti-stromal therapies now being added to the armament of anti-cancer weapons. The ability of MLT to suppress aromatase expression in multiple cell types within the breast tumor microenvironment, including CAFs as shown in this study, adds to the growing evidence supporting this non-toxic hormone to be used in the treatment of postmenopausal estrogen-dependent breast cancer. Not only can MLT disrupt the phenotype of aromatase expressing mouse fibroblasts [23], the suppressive effects of MLT on aromatase activity can also be enhanced when used in combination with the AI aminoglutethimide in in vitro [24] and in vivo studies [25]. Furthermore, in combination with metformin, an AMPK-activator that inhibits aromatase expression in BAFs [26], MLT has shown to suppress mammary tumor growth in HER-2/neu transgenic mice [27]. The potential of metformin and melatonin used together to inhibit aromatase expression in the breast is yet to be tested and warrants further investigation.

In summary, our findings demonstrate a negative effect of MLT on aromatase expression in CAFs and reinforces the importance that circulating MLT levels may have in the protection against ER-receptor positive breast cancer.

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Conflict of interest The authors declare that they have no conflicts of interest.

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COMPOSITION OF BUFFERS AND SOLUTIONS

B-galactosidase assay buffer

200mM sodium phosphate buffer (pH 7.3)
2mM $MgCl_2$
100mM B-Mercapoethanol
1.33mg/ml ONPG

ChIP lysis buffer

1% SDS
10mM EDTA (pH 8.0)
50mM Tris (pH 8.0)

EMSA gel shift buffer 5X

100mM HEPES pH 8.0
50% glycerol
250mM KCl
5mM EDTA
H_2O to 5ml

EMSA TBE sample buffer

10mM Tris-HCl (pH 7.6)
0.03% bromophenol blue
0.03% xylene cyanol FF
60mM EDTA
H_2O to 5ml

EMSA running gel (for 50ml gel)

30% acrylamide	10ml
0.5x TBE	25ml
MilliQ H2O	15ml
10% APS	400µl
TEMED	50µl

Orange G loading buffer 6X

Orange G	0.05g
Ficoll	2g
0.5M EDTA	0.4ml
H_2O	to 10ml

TBE 10X

Tris base	108g
Boric acid	55g
EDTA	7.5g
H_20	to 1 litre

LIST OF OLIGONUCLEOTIDES

Gene name	**Application**	**Primer Sequence**
Egr1	qRT-PCR	F 5'- CAGTGGCCTAGTGAGCATGA-3' R 5'- CCGCAAGTGGATCTTGGTAT-3'
Egr2	qRT-PCR	F 5'- TTGACCAGATGAACGGAGTG-3' R 5'- GTTGAAGCTGGGGAAGTGAC-3'
Egr3	qRT-PCR	F 5'- CAATCTGTACCCCGAGGAGA-3' R 5'- GGAAGGAGCCGGAGTAAGAG-3'
Egr4	qRT-PCR	F 5'- CGCGCTCCTCGTCAAGTC-3' R 5'- GCTCAAGAAGTCGCCTGCTC-3'
18s	qRT-PCR	F 5'- CGGCTACCACATCCAAGGA-3' R 5'- GCTGGAATTACCGCGGCT-3'
B-Actin	qRT-PCR	F 5'- TGCGTGACATTGCGTGACATTAAGGAGAAG-3' R 5'- GCTCGTAGCTCTTCTCCA-3'
CYP19A1 (RT7/RT8)	qRT-PCR	F 5'- TTGGAAATGCTGAACCCGAT-3' R 5'- CAGGAATCTGCCGTGGGGAT-3'
TNFR1	qRT-PCR	F 5'- TCAGTCCCGTGCCCAGTTCCACCTT-3' R 5'- CTGAAGGGGGTTGGGGATGGGGTC-3'
GREB1	qRT-PCR	F 5'- GTGGTAGCCGAGTGGACAAT-3' R 5'- AAACCCGTCTGTGGTACAGC-3'
PI.4	qRT-PCR	F 5- GTAGAACGTGACCAACTGG-3' R 5'- CACCCGGTTGTAGTAGTTGCAGGCACTGCC-3'
Egr1	Cloning	F 5'- GATGATGGATCCGCCACCATGGACTACAAAGACGA TGACGACAAGATGGCCGCGGCCAAGGCCG -3' R 5'- TCCTCGAATTCTTAGCAAATTTCAAT -3'
Egr2	Cloning	F 5'- GATGATGGATCCGCCACCATGGACTACAAAG ACGATGACGACAAGATGATGACCGCCAAGGCC-3' R 5'- TCCTCGAATTCTCAAGGTGTCCGGGTCCG-3'
Egr3	Cloning	F 5'- GATGATGGATCCGCCACCATGGACTACAAAGACGA TGACGACAAGATGACCGGCAAACTCGCCG-3' R 5'- TCCTCGAATTCTCAGGCGCAGGTGGTGAC-3'

Egr4	Cloning	F 5'- GATGATGGATCCGCCACCATGGACTACAAAGACGATG ACGACAAGATGCTCCACCTTAGCGAGTTTTCCG-3' R 5'- GAAGAAGAATTCTCAGAGAGAAGCGAA-3'
PI.4 Egr ChIP	ChIP PCR	F 5'- ATGACCAACCAAGACTAAGAG-3' R 5'- CAGTTGGTCACGTTCTACTTGG-3'
MMP9 Egr ChIP	ChIP PCR	F 5'- AGGCTGCTACTGTCCCCTTT-3' R 5'- CTCCCTGACAGCCTTCTTTG-3'
PI.4 EMSA	EMSA probe	AAACTGGCTCCTGGCTCCAAGTAGAACGTGACCAACTGGA
Egr positive	EMSA probe	GGATCCAGCGGGGGCGAGCGGGGGCGA
Egr negative	EMSA probe	GGATCCAGCTAGGGCGAGCTAGGGCGA

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