

Thesis title: cAMP response element (CRE)-dependent regulation of aromatase in obesity and postmenopausal breast cancer

I would like to thank both examiners for their constructive comments and suggestions. I have made changes in the final draft of my thesis accordingly. Specific responses to each of the comments are provided below (examiner's comments in black and author's responses in blue).

Examiner: Professor CR Mendelson (University of Texas Southwestern Medical Center)

- 1) pp. 5, 39, 55 and possibly elsewhere – word 'principle' should be changed to 'principal.'

In agreement, the word "principle" in page 5, 39 and 55 has been changed to "principal".

- 2) p. 15, para 1 – Sentence beginning "DHEA is then converted to Δ^4 dione, which is aromatized by aromatase to yield oestrone by 3β -hydroxysteroid dehydrogenases" should be reworded to move "**by 3β -hydroxysteroid dehydrogenases/ketosteroid isomerases**" after 'converted,' for clarity.

I have now changed the original sentence "DHEA is then converted to Δ^4 -dione, which is aromatised by aromatase to yield oestrone by 3β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3β -HSDs/KSIs)" to the following "DHEA is then converted by 3β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3β -HSDs/KSIs) to Δ^4 -dione, which is aromatised by aromatase to yield oestrone".

- 3) ChIP assay description in manuscripts – It was not made clear what genomic regions were amplified and sequences of the primers that were used. Also, ChIP-qPCR (quantitative ChIP) is a preferred method of analysis to that utilized in all ChIP studies shown.

In agreement, the primer sequences used for PCR step in the ChIP assay were added to the materials and methods section in both chapter 2 and 3 manuscripts. I have also added the graphs showing the relative enrichment of CRTCs and HIF-1 α to the aromatase PII using qPCR data and performed the statistical analysis.

Manuscripts in chapter 2 and 3 are now accepted for publication after submitting my thesis to Monash University. So I have added the note in to general declaration (p. viii) and starting page of chapter 2 (p. 59) and 3 (p. 82) as below.

p. viii and p. 59 - Manuscript accepted for publication in Hormones and Cancer after first submitting of the thesis to University (March, 2013).

p. viii and p. 82 - Manuscript accepted for publication in Breast Cancer Research after first submitting of the thesis to University (April, 2013).

**cAMP response element (CRE)-dependent regulation of
aromatase in obesity and postmenopausal breast cancer**

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Submitted in the fulfilment of the requirements for the degree of

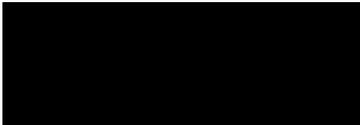
Doctor of Philosophy (Ph.D.)

December 2012

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Nirukshi Samarajeewa

TABLE OF CONTENTS

TABLE OF CONTENTS.....	i
LIST OF FIGURES AND TABLES	v
GENERAL DECLARATION	vii
PUBLICATIONS AND ABSTRACTS ARISING FROM THE THESIS.....	x
ACKNOWLEDGEMENTS.....	xiii
ABSTRACT.....	xv
LIST OF ABBREVIATIONS.....	xvii
INTRODUCTION	xxi
CHAPTER 1: LITERATURE REVIEW	1
1.1. Healthy breast versus cancerous breast	2
1.1.1. Functional anatomy and physiology of the human breast	2
1.1.2. Hormonal influences in developing breast cancer	4
1.1.3. Breast cancer incidence and mortality	7
1.1.4. Breast cancer causes and risk factors.....	9
5.1. Steroid hormones	12
1.2.1. Sources of oestrogens in premenopausal versus postmenopausal breast cancer.....	14
1.2.2. Steroid hormone biosynthesis or steroidogenesis.....	15
1.2.2.1. Steroidogenic acute regulator (StAR)	17
1.2.2.2. Cytochrome P450 enzymes	17
1.2.2.3.1. Cytochrome P450 side-chain cleavage (P450 _{scc} , <i>CYP11A1</i>).....	18
1.2.2.3.2. Cytochrome P450 17 α -hydroxylase/C17-20 lyase (P450 _{c17} , <i>CYP17A1</i>).....	19
1.2.2.3.3. Cytochrome P450 aromatase (P450 _{arom} , <i>CYP19A1</i>).....	19
1.2.2.3. Hydroxysteroid dehydrogenases (HSDs)	19
1.2.2.3.1. 3 β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3 β HSDs/KSIs)	20
1.2.2.3.2. 17 β -hydroxysteroid dehydrogenases (17 β -HSDs).....	21
1.3. Aromatase expression and breast cancer risk	21

1.3.1. The aromatase gene	23
1.3.2. Regulation of aromatase expression in breast cancer	25
1.4. CREB/ATF family members	28
1.4.1. CREB subgroup	31
1.4.1.1. CREB.....	31
1.4.1.2. CREM.....	32
1.4.1.3. ATF-1	33
1.4.2. CREB-BP1 subgroup.....	33
1.4.3. Role of CREB/ATF family members in regulating aromatase promoter II	35
1.5. CRTC family members.....	35
1.5.1. CRTC family and structure.....	35
1.5.1.1. CRTC1.....	37
1.5.1.2. CRTC2.....	37
1.5.1.3. CRTC3.....	37
1.5.2. CRTC co-activation of CREB	38
1.5.3. CRTC phosphorylation and subcellular localisation	38
1.5.4. AMPK-related family members and regulation of CRTC via upstream kinases.....	39
1.5.4.1. AMPKs	40
1.5.4.2. SIKs	42
1.5.5. Role of CRTCs in aromatase PII activation.....	42
1.6. Hypoxia inducible factors (HIFs) and CREB family members.....	43
1.6.1. Structure of HIFs	44
1.6.2. Regulation of HIFs.....	46
1.6.2.1. Regulation of HIF-1/2 α	47
1.6.2.2. Regulation of HIF-3 α	49
1.6.3. PGE ₂ -mediated regulation of HIF-1 α	50
1.6.4. HIF-1 α and aromatase expression	50
1.6.5. HIF-1/2 α in cancers	51
1.7. Treatment for oestrogen receptor positive breast cancer	52
1.7.1. Endocrine therapy	52

1.7.1.1. Tamoxifen.....	52
1.7.1.2. Aromatase inhibitors	53
1.7.2. Breast-specific inhibition of aromatase	54
1.7.2.1. Metformin.....	55
1.8. Summary	57
1.9. Hypothesis and objectives	58

CHAPTER 2: CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes..... 59

Declaration for thesis chapter 2	60
Summary of article.....	62
Introduction.....	65
Materials and methods	67
Results.....	70
Discussion.....	72
Acknowledgements.....	74
References.....	75
Figure legends.....	77
Figures	78

CHAPTER 3: HIF-1 α stimulates aromatase expression driven by prostaglandin E₂ in breast adipose stroma..... 82

Declaration for thesis chapter 3	83
Summary of article.....	86
Abstract.....	88
Introduction.....	89
Materials and methods	90
Results.....	95
Discussion.....	97
Conclusions.....	99
Acknowledgements.....	100
References.....	101

Figure legends.....	105
Figures	107
CHAPTER 4: Promoter-specific effects of metformin on aromatase transcript expression.	113
Declaration for thesis chapter 4	114
Summary of article.....	116
Abstract	118
Experimental	120
Results.....	121
Discussion	122
Acknowledgements.....	124
References.....	124
Figure Legends	127
Figures	128
CHAPTER 5: GENERAL DISCUSSION	130
5.1. Identification of novel aromatase PII regulators.....	132
5.2. Regulation of CRTCs and HIF-1 α by PGE ₂	133
5.3. Tissue-specific inhibition of aromatase and therapeutic implications.....	136
5.4. Future directions	138
5.5. Conclusions.....	139
APPENDIX 1.....	142
APPENDIX 2.....	155
REFERENCES	160

LIST OF FIGURES AND TABLES

CHAPTER 1: LITERATURE REVIEW

Figure 1:	Structure of lactating mammary glands.....	2
Figure 2:	Normal breast histological architecture at various stages.....	4
Figure 3:	Diagram of a breast duct, fibroblasts and adipocytes showing the principal location of aromatase in the fibroblasts.....	6
Figure 4:	Age-standardized breast cancer incidence and mortality per 100,000 by world area.....	8
Figure 5:	Simplified steroidogenesis pathway.....	16
Figure 6:	Aromatase expression in primary breast ASCs.....	22
Figure 7:	Mapping the promoters of the <i>CYP19A1</i> (P450arom) gene.....	24
Figure 8:	Epithelial – mesenchymal interactions regulating aromatase expression in the breast.....	26
Figure 9:	CREB/ATF family proteins.....	29
Figure 10:	Domain structure of the CRTC family of CREB co-activators, as exemplified by CRTC2.....	36
Figure 11:	Human tissue distribution of the TORC (CRTC) family.....	37
Figure 12:	Partial amino acid sequence (152-185) of human CRTC2 including the putative Ser171 phosphorylation site.....	41
Figure 13:	The structural domains of HIF-1/2/3 α and HIF-1 β /ARNT, and their known modulators.....	45
Table 1:	Overview of variables used in the risk models for breast cancer.....	10
Table 2:	The Breast Cancer Prevention Collaborative Group (BCPCG) prioritised breast cancer risk factors according to NIH priority score.....	11
Table 3:	Classes of steroids.....	13
Table 4:	Dimer formation between ATF/CREB family members (intrafamily dimerisation).....	30

CHAPTER 2: CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes

Figure 1: Role of CRTCs in aromatase PII activation.....78

Figure 2: Role of CRTCs and CREB1 in the regulation aromatase of PII activity.....79

Figure 3: Role of CREs in the activation of aromatase PII.....80

Figure 4: Effect of phosphorylating CRTC2 at Ser171 on its subcellular localization and the activation of aromatase PII in preadipocytes.....81

CHAPTER 3: HIF-1 α stimulates aromatase expression driven by prostaglandin E₂ in breast adipose stroma

Figure 1: Effect of PGE₂ on HIF-1 α expression and nuclear localization in primary human breast ASCs.....107

Figure 2: Role of HIF-1 α in aromatase PII activation.....108

Figure 3: HIF-1 α and aromatase expression in ASCs from tumor-bearing breast tissue compared to cancer-free using immunohistochemistry.....109

Figure 4: Model of the PGE₂-mediated regulation of aromatase expression by HIF-1 α in breast ASCs.....110

Supplementary Figure 1: Classification of ASCs for double immunohistochemistry.....111

Supplementary Table 1: Clinicopathological data of breast cancer patients.....112

CHAPTER 4: Promoter-specific effects of metformin on aromatase transcript expression

Figure 1: Effect of metformin on FSK/PMA-mediated aromatase expression in primary breast hASCs.....128

Figure 2: Effect of metformin on DEX/TNF α -mediated aromatase expression in primary human adipose stromal cells.....129

CHAPTER 5: General discussion

Figure 1: Model of the PGE₂-mediated regulation of aromatase expression by CRTCs and HIF-1 α in primary human breast ASCs.....140



Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) 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 one original paper published in peer reviewed journal and two unpublished manuscripts. Two additional conjointly authored publications appear in the appendices. The core theme of this thesis is the role of cAMP response element (CRE)-dependent regulation of aromatase in obesity and postmenopausal 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 of Medical Research through Department of Physiology under the supervision of Dr. Kristy Brown and Professor Evan Simpson.

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 two, three, four and appendices, my contribution to the work involved the following:

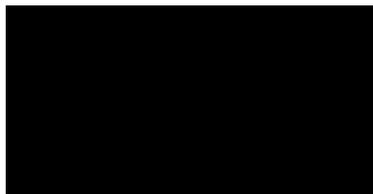
Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes.	Manuscript submitted to <i>Hormones and Cancer</i> (September, 2012) Manuscript accepted for publication in <i>Hormones and Cancer</i> after first submitting of the thesis to University (March, 2013)	(85% overall contribution) Performed laboratory work for all the figures, data analysis, prepared and drafted manuscript
3	HIF-1 α stimulates aromatase expression driven by prostaglandin E ₂ in breast adipose stroma.	Manuscript submitted to <i>Breast Cancer Research</i> (September, 2012) Manuscript accepted for publication in <i>Breast Cancer Research</i> after first submitting of the thesis to University (April, 2013)	(80% overall contribution) Performed laboratory work for all the figures, data analysis, prepared and drafted manuscript
4	Promoter-specific effects of metformin on aromatase transcript expression.	Published in <i>Steroids</i> (2011), 76(8): 768-71	(85% overall contribution) Performed laboratory work for all the figures, data

GENERAL DECLARATION

			analysis, prepared and drafted manuscript
Appendix 1	Endocrine-related cancers and the role of AMPK.	Published in Molecular and Cellular Endocrinology (2012)	25% contribution to writing
Appendix 2	CRTC2 (CREB regulated transcription coactivator 2)	Published in Atlas of Genetics and Cytogenetics in Oncology and Haematology (2010), 14(12): 1104-5	50% contribution to writing

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed



Date: 10/12/2012

PUBLICATIONS AND ABSTRACTS ARISING FROM THE THESIS

Publications

Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER & Brown KA, *HIF-1 α increases promoter II-driven aromatase expression in postmenopausal breast cancer*, Submitted to Breast Cancer Res, 2012 (Accepted for publication in Breast Cancer Res, 2013)

Samarajeewa NU, Docanto MM, Simpson ER & Brown KA, *CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes*, Submitted to Hormones and Cancer, 2012 (Accepted for publication in Hormones and Cancer, 2013)

Brown KA, **Samarajeewa NU** & Simpson ER, *Endocrine-related cancers and the role of AMPK*, Mol Cell Endocrinol, 2012 Jun 16 [Epub ahead of print]

Ham S, Meachem SJ, Choong CS, Charles AK, Baynam GS, Jones TW, **Samarajeewa NU**, Simpson ER & Brown KA, *Estrogen excess phenotype associated with loss of heterozygosity of the STK11 gene in the testis of two boys with Peutz Jeghers Syndrome*, Submitted to New Engl J Med, 2012

Samarajeewa NU, Ham S, Yang F, Simpson ER & Brown KA, *Promoter-specific effects of metformin on aromatase transcript expression*, Steroids, 2011; 76(8): 768-7

Brown KA & **Samarageewa NU**, *CRTC2 (CREB regulated transcription coactivator 2)*, Atlas Genet Cytogenet Oncol Haematol, 2010; 14(12): 1104-5

Oral Presentations

Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER & Brown KA, *Hypoxia inducible factor-1 α increases promoter II-*

driven aromatase expression in postmenopausal breast cancer, Endocrine Society of Australia (ESA), Gold Coast, Australia, 2012 - Novartis Junior Scientist award finalist

Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER & Brown KA, *Hypoxia inducible factor-1 α increases promoter II-driven aromatase expression in postmenopausal breast cancer*, Southern Health Research Week, Melbourne, Australia, 2012 - Early career investigator award finalist

Samarajeewa NU, Simpson ER & Brown KA, *Understanding the cAMP response element (CRE) dependent regulation of aromatase in postmenopausal breast cancer*, Tohoku Medical Society Invited Lectures, Sendai, Japan, 2012

Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER & Brown KA, *Hypoxia inducible factor-1 α increases promoter II-driven aromatase expression in obesity-related, postmenopausal breast cancer*, PHI Student Symposium, Melbourne, Australia 2012

Samarajeewa NU, Simpson ER & Brown KA, *HIF-1 α increases promoter II-driven aromatase expression in postmenopausal breast cancer*, PHI Student Symposium, Melbourne, Australia, 2011

Samarajeewa NU, Simpson ER & Brown KA, *Role of CRTC s in regulating PII-driven aromatase expression in breast cancer*. Biomed Link, St Vincent's Student Society, Melbourne, Australia, 2010 - Oral presentation award finalist

Samarajeewa NU, Simpson ER & Brown KA, *Role of CRTC s & HIF-1 α in regulating promoter II-driven aromatase expression in postmenopausal breast cancer*, PHI Student Symposium, Melbourne, Australia, 2010

Samarajeewa NU, Simpson ER & Brown KA, *Elucidating the role of CRTC co-activation of CREB in regulating promoter II-driven aromatase expression in human breast adipose stromal cells*, PHI Student Symposium, Melbourne, Australia, 2009

Samarajeewa NU, Simpson ER & Brown KA, *The role of AMPK-related family members in TORC2 co-activation of CREB, and their effect on local aromatase expression in postmenopausal breast cancer*, PHI Student Symposium, Melbourne, Australia, 2008 - Talk received a special commendation

Poster Presentations

Samarajeewa NU, Yang F, Docanto MM, Sakurai M, McNamara KM, Sasano H, Fox SB, Simpson ER & Brown KA, *Hypoxia inducible factor-1 α increases promoter II-driven aromatase expression in postmenopausal breast cancer*, Southern Health Research Week, Melbourne, Australia, 2012 - Poster award in Cancer Research

Samarajeewa NU, Simpson ER & Brown KA, *Elucidating the role of HIF-1 α in regulating promoter II-driven aromatase expression in postmenopausal breast cancer*, ENDO, Boston, USA, 2011

Samarajeewa NU, Simpson ER & Brown KA, *Elucidating the role of CRTC2 in regulating promoter II-driven aromatase expression in postmenopausal breast cancer*. ICHSHC 2010, Edinburgh, Scotland, 2010

Samarajeewa NU, Simpson ER & Brown KA, *Elucidating the role of CRTC2 in regulating promoter II-driven aromatase expression in postmenopausal breast cancer*, PhD student conference, Department of Biochemistry & Molecular Biology, Monash University, Melbourne, Australia, 2010

Samarajeewa NU, Simpson ER & Brown KA, *Phosphorylation of CRTC2 at Serine-171 Dictates Its Subcellular Localization and the Activation of Aromatase Promoter II in Preadipocytes*, ENDO, Washington, USA, 2009 - Presidential poster competition finalist

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ABSTRACT

The risk of breast cancer rises dramatically with advancing age, and recently obesity has also been recognised as a significant contributor. In postmenopausal women with breast cancer, the inappropriately elevated aromatase expression is governed by enhanced usage of the proximal promoters, PII and PI.3 which leads to an increased oestrogenic drive as a result of tumour-derived factors such as prostaglandin E₂ (PGE₂) in breast adipose stromal cells (ASCs). This increased expression of aromatase PII is largely regulated by the transcription factor cAMP response element (CRE) binding protein-1 (CREB1) which binds to proximal and distal CREs on PII. Our group has demonstrated that the CREB co-activator CRTC2 can bind and activate aromatase PII via CREs and that its subcellular localisation is regulated by PGE₂. There are three known members of the CRTC family (CRTC1-3) and the regulation of aromatase expression by other CRTC members is poorly understood. HIF-1 α is a master regulator of adaptive responses to hypoxia and found to be regulated by PGE₂ independent of hypoxia. We have identified a putative hypoxia response element (HRE) which overlaps with the proximal CRE of aromatase PII. However, the regulation of aromatase expression by HIF-1 α in breast cancer had not yet been characterised. Our group has previously demonstrated that the commonly used antidiabetic drug metformin inhibits aromatase expression in ASCs via the inhibition of PII activity. This study therefore aimed to characterise the role of CRTCs and HIF-1 α in the activation of aromatase PII, and to examine the effect of metformin on promoter-specific transcript expression in breast ASCs. The results indicated that PGE₂ causes nuclear translocation of all three CRTCs and HIF-1 α . Reporter assays demonstrated that basal PII activity is significantly increased with all CRTCs. FSK/PMA, to mimic PGE₂, results in a further significant increase in PII activity with all CRTCs. HIF-1 α significantly increases PII activity in the presence of PGE₂. Interestingly, CRTCs via both proximal and distal CREs, or HIF-1 α via the proximal CRE, act co-operatively with CREB1 to maximise PII activity. There is a significant increase in HIF-1 α positive ASCs in breast cancer patients compared to cancer-free women, and a positive association between HIF-1 α and aromatase expression. Metformin results in a dose-dependent inhibition of FSK/PMA-induced aromatase PII and PI.3 transcript expression and does not affect PI.4. In conclusion, this

study identifies CRTC₁ and HIF-1 α as modulators of aromatase PII, and metformin as a breast-specific inhibitor of aromatase in breast ASCs, thereby furthering our understanding of the complex nature of aromatase regulation in obesity-related, postmenopausal breast cancer.

LIST OF ABBREVIATIONS

17-OHP	17 α -hydroxyprogesterone
17-OH-Preg	17 α -hydroxypregnenolone
3 β -HSDs/KSIs	3 β -hydroxysteroid dehydrogenases/ketosteroid isomerases
17 β -HSD type I	17 β -hydroxysteroid dehydrogenases type I
Δ^4 -dione	Δ^4 -androstenedione
Δ^5 -diol	Androst-5-ene-3- β ,17 β -diol
aa	Amino acids
AIs	Aromatase inhibitors
AICAR	5-amino-imidazole-4-carboxamideriboside
AKRs	Aldo-keto reductases
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ARNT	Aryl Hydrocarbon Nuclear Translocators
ASCs	Adipose stromal cells
ATF	Activating transcription factor
ATP	Adenosine 5'-triphosphate
BMI	Body mass index
bZIP	Basic domain-leucine zipper
CBD	CREB binding domain
CBP	CREB-binding protein
CBS	Cystathionine- β -synthase
ChIP	Chromatin immunoprecipitation
CLS-B	Crown-like structures of the breast
COX-2	Cyclooxygenase-2
CRE	cAMP response element
CREB	cAMP response element binding protein
CRTC	CREB-regulated transcription co-activator
<i>CYP19A1</i> ,P450aro	Aromatase
CYPs	Cytochrome P450 family of monooxygenases
DEX	Dexamethasone

DFS	Disease-free survival
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulfate
DM2	Type 2 diabetes mellitus
DNA	Deoxyribonucleic acid
E2	Oestradiol
EGF	Epidermal growth factor
EP receptors	E prostanoid receptors
ER	Oestrogen receptor
ERR γ	Oestrogen-related receptor γ
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FSH	Follicle-stimulating hormone
FSK	Forskolin
GFP	Green fluorescent protein
HIF	Hypoxia inducible transcription factor
HREs	Hypoxia response elements
HSD	Hydroxysteroid dehydrogenases
ICER	Inducible cAMP early repressor
IMM	Inner mitochondrial membrane
IPAS	Inhibitory PAS domain protein
IR	Immunoreactivity
JNK	c-Jun N-terminal kinase
KID	Kinase inducible domain
LKB1	Liver kinase B1
LRH-1	Liver receptor homologue-1
MBD	Mammographic breast density
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
NES	Nuclear export sequence
NLS	Nuclear localisation sequence
OMM	Outer mitochondrial membrane

P	Promoter
P450c17, <i>CYP17A1</i>	Cytochrome P450 17 α -hydroxylase/C17-20 lyase
P450sc, <i>CYP11A1</i>	Cytochrome P450 side-chain cleavage
PAS	Per-aryl hydrocarbon receptor nuclear translocator-Sim
P-box	Phosphorylation box
PCR	Polymerase chain reaction
PGE ₂	Prostaglandin E ₂
PJS	Peutz-Jeghers Syndrome
PKA	Protein kinase A
PKC	Protein kinase C
PKC ϵ	Epsilon isoform of protein kinase C
PMA	Phorbol esters
PPAR γ	Peroxisome proliferator-activated receptor- γ
PR	Progesterone receptor
pVHL	von Hippel-Lindau protein
QPCR	Quantitative PCR
RNA	Ribonucleic acid
SAPK	Stress-activated protein kinase
SCP2	Sterol carrier protein 2
SDRs	Short-chain dehydrogenase reductases
SERM	Selective oestrogen receptor modulator
StAR	Steroidogenic acute regulator
START	StAR-related lipid transfer
SVC	Stromal-vascular cells
TAD-C	C-terminal transactivation domain
TAD-N	N-terminal transactivation domain
TLDU	Terminal duct lobular units
TNF α	Tumour necrosis factor- α
VEGF	Vascular endothelial growth factor

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in Australian women and is the second leading cause of cancer-related deaths in this population. Approximately 70% of postmenopausal breast cancers are oestrogen receptor (ER) positive, and this increased prevalence is correlated with elevated local levels of oestrogens (Huang et al., 2000, McCarty et al., 1983, Simpson et al., 2002). This is mainly due to an increase in aromatase expression within the breast itself as a result of the increased activation of proximal promoters, PII and PI.3 on the *CYP19A1* gene, in response to factors derived from the tumour such as prostaglandin E₂ (PGE₂) (Simpson and Brown, 2011, Agarwal et al., 1996a, Zhao et al., 1996a). One transcription factor shown to be involved in this process is cAMP response element (CRE) binding protein (CREB), which directly binds to PII and stimulates the expression of aromatase (Sofi et al., 2003). The regulation of aromatase is under complex tissue-specific control due to the unique organisation of its promoter region, which will be discussed more thoroughly in the following literature review.

Our group has demonstrated that the subcellular localisation of the CREB co-activator, CREB-regulated transcription co-activator 2 (CRTC2) is regulated by factors such as PGE₂ and this protein can directly activate PII (Brown et al., 2009). In addition, the phosphorylation of CRTC2 by adenosine monophosphate (AMP)-activated protein kinase (AMPK) results in its cytoplasmic retention via interaction with 14-3-3 proteins. The inhibition of AMPK activity was described as the mechanism by which PGE₂ mediated the CRTC2-dependent increase in aromatase expression within the breast (Screaton et al., 2004, Brown et al., 2009). The CRTC family consists of 3 members, namely CRTC1, CRTC2 and CRTC3 and they all share a highly conserved N-terminal coiled-coil domain that is known to interact with the bZIP domain of CREB (Altarejos and Montminy, 2011, Conkright et al., 2003). Since the role of CRTC1 and CRTC3 in regulating breast aromatase expression has not yet been extensively studied, the initial part of my project aimed to characterise the role of CRTC1 and CRTC3 in breast aromatase regulation.

Hypoxia inducible factor 1 (HIF-1), a heterodimer of HIF-1 α and HIF-1 β , acts as a key regulator of adaptive responses to hypoxia or low oxygen concentrations (Jiang et al., 1996, Yu et al., 1998, Wang et al., 1995). Under normoxic conditions (normal oxygen conditions), HIF-1 β is constitutively expressed whereas HIF-1 α expression is accompanied by its rapid degradation by the ubiquitin-proteasome system (Salceda and Caro, 1997). However, under hypoxic conditions, HIF-1 binds to hypoxia-response elements (HREs) and activates the expression of many target genes involved in glycolysis, angiogenesis and haematopoiesis (Semenza et al., 1996, Harris, 2002). Interestingly, PGE₂ has been shown to be involved in HIF-1 α protein stabilisation and nuclear translocation in PC-3ML human prostate cancer cells (Liu et al., 2002) and in HCT116 human colon carcinoma cells (Fukuda et al., 2003), independent of oxygen tension. Moreover, HIF-1 α has been shown to directly interact with coactivators of CREB, CBP and p300 (Arany et al., 1996) and also ATF2 (Choi et al., 2009). These findings warrant further investigation into the role of HIF-1 α in regulating aromatase expression in the context of postmenopausal breast cancer, as the second part of my project.

Aromatase inhibitors (AIs) and tamoxifen are now widely used for the treatment of postmenopausal breast cancer, and both classes effectively inhibit tumour growth by preventing oestrogen-mediated action. Nonetheless, third generation AIs have shown clear superiority compared to tamoxifen in disease-free survival (DFS) in the treatment of both metastatic and early breast cancer with comparatively less severe side-effects (Coombes et al., 2004, Howell et al., 2005, Forbes et al., 2008, Goss et al., 2003). However, AIs inhibit oestrogen biosynthesis globally within the body resulting in loss of oestrogenic effects that can be beneficial in some tissues including bone, brain and the cardiovascular system. Hence, the identification of new therapeutic strategies that inhibit aromatase specifically within the breast and protect other sites where oestrogen exerts protective effect, is highly desirable. The third part of my project aims to further characterise the action of metformin, which our group recently identified as an inhibitor of aromatase expression (Brown et al., 2010).

CHAPTER 1:

LITERATURE REVIEW

LITERATURE REVIEW

1.1. Healthy breast versus cancerous breast

1.1.1. Functional anatomy and physiology of the human breast

The adult female breast or mammary gland is thought to have developed from a modified sweat gland, part of the skin or integumentary system, and begins to develop as early as the fourth week of fetal development as a downgrowth from a thickened mammary ridge (milk line) of ectoderm along a line from the axilla to the inguinal region (Sinnatamby, 2006, Marieb and Hoehn, 2010). The development of the breast is stimulated by oestrogens produced during the menstrual cycle after puberty. However, the breast remains incompletely developed until pregnancy occurs, when large quantities of oestrogens secreted by the placenta, as well as progesterone, stimulate the complete development of lactating mammary glands (Figure 1) (Hall, 2011, Marieb and Hoehn, 2010). The growth of the ductal system is under the control of at least four other hormones including growth hormone, prolactin, adrenal glucocorticoids and insulin.

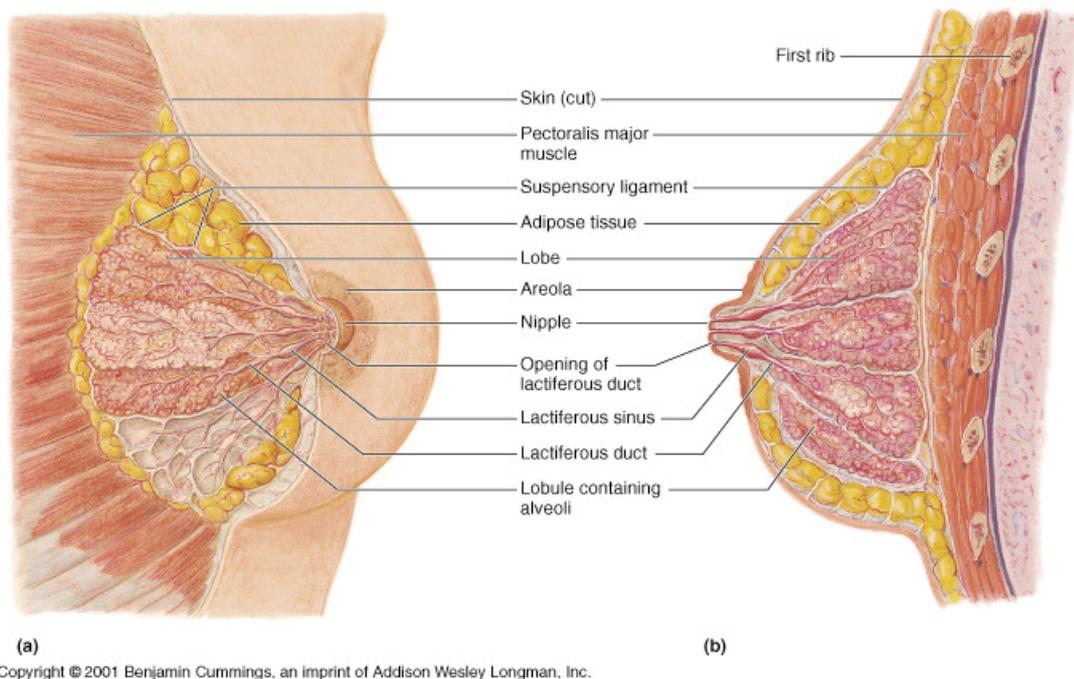


Figure1: Structure of lactating mammary glands (adapted from Marieb and Hoehn, 2010) . (a) Anterior view of a partially dissected breast. (b) Sagittal section of a breast.

Each breast is composed of skin, subcutaneous adipose tissue and breast tissue including parenchyma and stroma (Rubin and Strayer, 2012, Morris and Liberman, 2005). The parenchyma is divided into 15 to 20 lobes that radiate around the nipple. Up to 20 to 40 smaller units called lobules are found within the lobes and these contain 10 to 100 glandular alveoli or acini that produce milk when a woman is lactating. Each lobule also contains branching ducts that divide into subsegmental structures and terminate in the terminal duct lobular units (TDLU), consisting of the terminal duct and the acinus. The glandular tissue and ducts are surrounded by fat and connective tissue that arise from stromal tissue. This interlobular connective tissue forms suspensory ligaments that attach the breast to the underlying muscle fascia and to the overlying dermis, providing natural support for the breast. Alveolar glands pass the milk into the lactiferous ducts, which open to the outside at the nipple. Each lactiferous duct has a dilated region called a lactiferous sinus that stores milk during nursing. The stroma contains the adipose and connective tissue, blood vessels, lymphatics and nerves. Breast adipose tissue is primarily composed of mature adipocytes filled with lipid and stromal-vascular cells (SVC) such as adipose stromal cells (ASCs, also known as adipogenic progenitor cells, preadipocytes or fibroblasts), endothelial cells, pericytes and smooth muscle cells. ASCs are the main focus of my research project and reasons for this are discussed more thoroughly later on.

Interestingly, despite the effect of oestradiol (E2) and progesterone on physical development of the breast during pregnancy, they also exhibit an inhibitory effect on secretion of milk. The hormone prolactin produced by the mother's anterior pituitary gland stimulates milk secretion. Levels of prolactin rise gradually until parturition and attain concentrations 10 to 20 times that seen in nonpregnant women. However, after menopause, the loss of oestrogen stimulation results in progressive atrophy of lobes and ducts within the breast. The histological architecture of normal breast at various stages including adolescent, postpubertal, lactating and postmenopausal is presented in Figure 2.

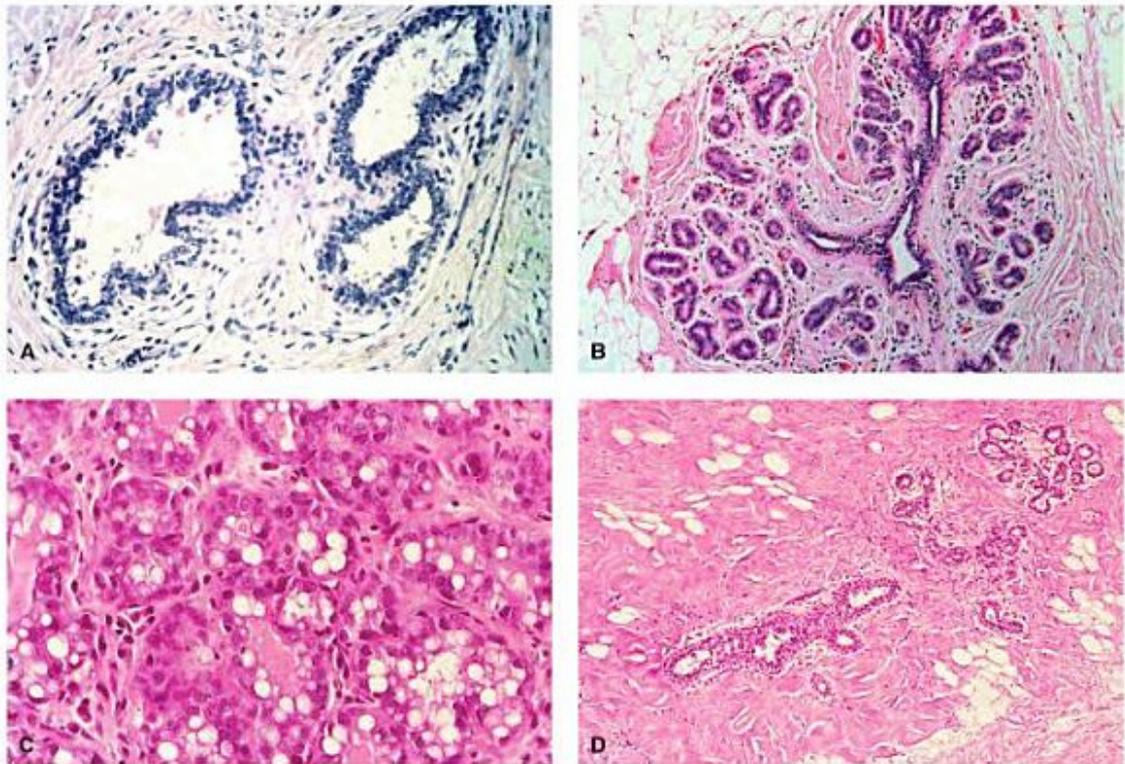


Figure 2: Normal breast histological architecture at various stages (adapted from Rubin and Strayer, 2012). (A) Adolescent breast. Large and intermediate-size ducts are seen within a dense fibrous stroma. No lobular units are present. (B) Postpubertal breast. The terminal duct lobular unit consists of small ductules arrayed around an intralobular duct. The two-cell-layered epithelium shows no secretory or mitotic activity. The intralobular stroma is dense and confluent with the interlobular stroma. (C) Lactating breast. The terminal duct lobular units are conspicuously enlarged, with inapparent interlobular and intralobular stroma. The individual terminal ducts, now termed acini, show prominent epithelial secretory activity (cytoplasmic vacuolization). The acinar lumina contain secretory material. (D) Postmenopausal breast. The terminal duct lobular units are absent. The remaining intermediate ducts and larger ducts are commonly dilated.

1.1.2. Hormonal influences in developing breast cancer

There is substantial evidence to conclude that high endogenous oestrogen levels are strongly associated with the increase risk of breast cancer (Bernstein and Ross, 1993, Key et al., 2002, Yue et al., 2003, Travis and Key, 2003). Several other hormones

including progesterone (Pike et al., 1993), prolactin (Clevenger et al., 2003) and testosterone (Lillie et al., 2003) can also contribute to breast cancer proliferation. Approximately 70% of postmenopausal breast cancers express oestrogen receptor- α (ER α) and are responsive to oestrogens (Huang et al., 2000, McCarty et al., 1983, Simpson et al., 2002). Moreover, breast epithelial cells from tumours have been shown to express significantly higher levels of ER α compared to normal (Ricketts et al., 1991).

In premenopausal women, the principal site of E2 formation is the ovary. E2 produced by ovaries passes into the circulation and acts on distal target organs such as the breast, uterus, bone and brain as a classical circulating hormone. However, in postmenopausal women, the ovaries cease to produce oestrogens due to exhaustion of the supply of ovarian follicles, and a number of extragonadal sites, including the adipose tissue, bone and brain become major E2 producing sites, where E2 now acts in a paracrine or intracrine manner (reviewed in Simpson and Brown, 2011). In adipose tissue, ASCs are the main cell type responsible for producing E2 rather than the lipid-filled adipocytes [44].

Along these lines, there is still controversy as to the source of oestrogen driving breast cancer risk in postmenopausal women. Two major competing theories have emerged; the first being that it is oestrogens produced locally within the breast that drives tumour growth and the second is that circulating oestrogens are taken up by breast tissue and drive tumour growth. Several epidemiological studies have supported the second tenet by showing a relationship between elevated plasma oestrogen levels and breast cancer risk in postmenopausal women (Berrino et al., 1996, Thomas et al., 1997, Toniolo et al., 1995). In addition, Haynes and colleagues have suggested that the plasma E2 binding to ER α rather than intratumoral oestrogen synthesis by aromatase or sulfatase is the major contributor to intratumoral E2 levels in postmenopausal breast cancer patients (Haynes et al., 2010).

A number of studies have also examined the concept that oestrogen produced locally in the breast governs breast cancer development (Santner et al., 1984, Gunnarsson et al., 2005). Larionov *et al.* have shown that both oestrone uptake from blood as well as local

biosynthesis occur in postmenopausal breast cancer tissues (Larionov et al., 2002). However, circulating levels of E2 in postmenopausal women are extremely low, 12pmol/L compared to 180pmol/L in premenopausal blood, as assessed by liquid chromatography-tandem mass spectrometry assays. A mechanism that would explain the observations behind both theories has recently been proposed by our group (Figure 3) (Simpson and Brown, 2011). It is proposed that oestrogens produced by the adipose tissue of the breast stimulate proliferation of breast epithelial cells locally within the breast. A fraction of oestrogens produced by adipose tissue would also pass into the bloodstream, and this will be again taken up by breast epithelial cells to enhance proliferation. Therefore, in this scheme, plasma oestrogens do not drive the increased breast cancer risk, but rather reflect the rate of oestrogen synthesis in breast adipose tissue.

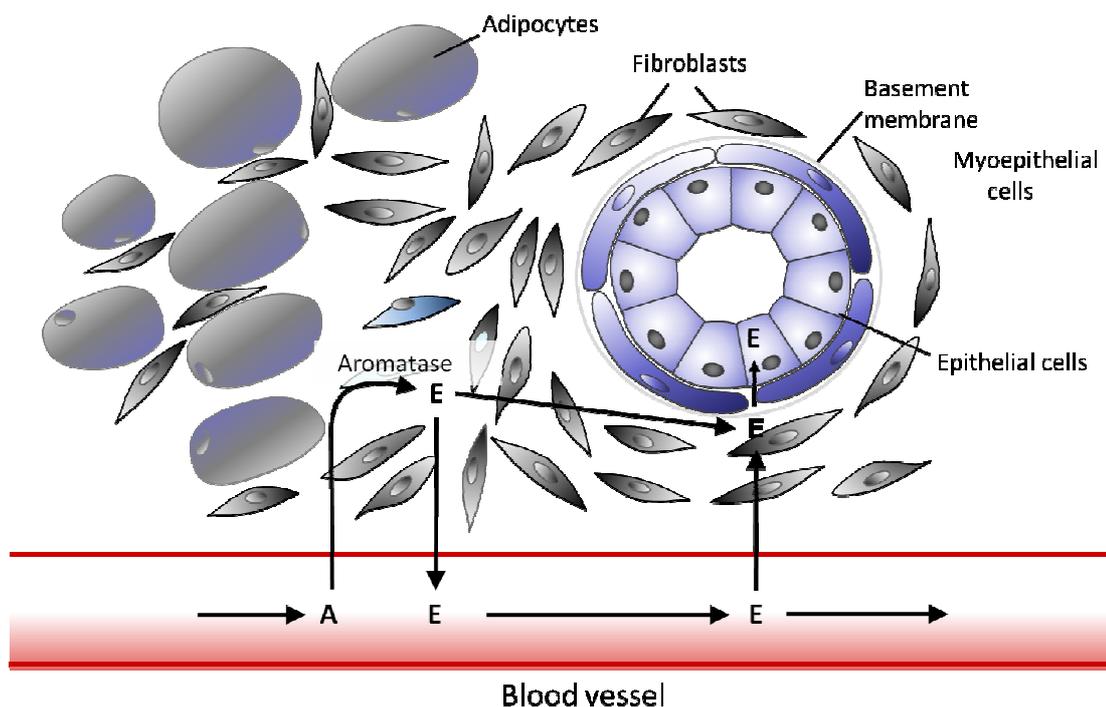


Figure 3: Diagram of a breast duct, fibroblasts and adipocytes showing the principal location of aromatase in the fibroblasts (adapted from Simpson and Brown, 2011). The pathways whereby oestrogen synthesised by these fibroblasts or ASCs enters the duct to stimulate epithelial proliferation are indicated. E=oestrogen, A=androgen

1.1.3. Breast cancer incidence and mortality

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in females worldwide, accounting for 22.9% (1.38 million) of the total new cancer cases and 13.7% (458,503) of the total cancer deaths in 2008 (World_Health_Ogranization-GLOBOCAN2008, 2008b). In general, breast cancer incidence and mortality rates remain highest in the economically developed countries compared with developing countries. Nonetheless, the burden of cancer continues to rise in economically developing countries as a result of lifestyle choices such as westernised diets, smoking and physical inactivity, as well as population aging and growth. Incidence rates for breast cancer have been shown to be high in Western, Northern and Southern Europe, Australia/New Zealand and North America; intermediate in South America, the Caribbean and Southern Africa; and low in Eastern and South-Central Asia, Melanesia, and Middle and Eastern Africa (Figure 4) (World_Health_Ogranization-GLOBOCAN2008, 2008a). Additionally, migrant studies have demonstrated that breast cancer incidence and mortality rates of migrants from low-risk countries to high-risk countries are gradually shifted towards a high-risk category over several successive generations (Kliwer and Smith, 1995, Ziegler et al., 1993). This suggests that the international variation in cancer rates is more likely due to environmental, lifestyle and cultural factors associated with specific geographical location, rather than genetic differences.

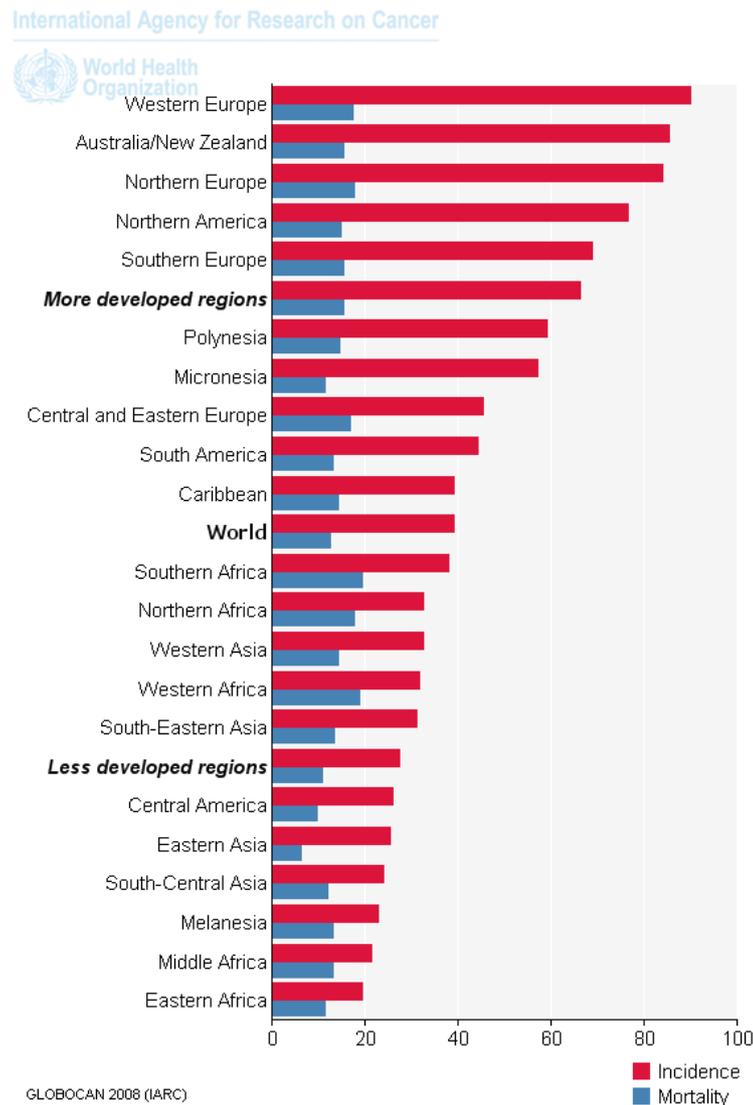


Figure 4: Age-standardized breast cancer incidence and mortality per 100,000 by world area (adapted from World Health Organization-GLOBOCAN2008, 2008a).

Breast cancer is also recognised as the most common form of cancer among Australian women, accounting for 27% of all cancer diagnoses in 2007 (Australian Government-Cancer Australia, 2011). The number of breast cancer cases diagnosed increased for females from 5291 in 1982 to 12,567 in 2007, and for males from 62 in 1982 to 103 in 2007. In terms of mortality, breast and lung cancer are the two leading causes of cancer-related deaths in Australian women, and 231 more deaths were reported for lung cancer

compared to breast cancer in 2007 (AustralianGovernment-CancerAustralia, 2011). There were 2,680 female and 26 male deaths from breast cancer in 2007.

1.1.4. Breast cancer causes and risk factors

Four types of valid models, namely Gail model, care model, log incidence model and Colditz model are currently used for breast cancer risk assessment (Table 1) (Stegeman and Bossuyt, 2012). These models incorporate age, body mass index (BMI), family history, alcohol consumption, plasma hormone levels, hormonal therapy, benign breast diseases, parity, reproductive and medical factors such as age at menarche and menopause, and number of breast biopsies as extensively validated risk assessment parameters. However, several other factors that are not included in these models can be used to enhance the power of risk assessment and identify women at very high risk of breast cancer. The Breast Cancer Prevention Collaborative Group (BCPCG) critically examined and prioritised new risk factors for an improved risk prediction model with potentially high predictive power (Santen et al., 2007). BCPCG prioritized these factors using NIH style priority score with 1 being the highest and 5 the lowest (Table 2), and further reviewed factors such as mammographic breast density, plasma hormone levels, history of menopausal hormone therapy (MHT) use, history of fracture and genetic components.

Table 1: Overview of variables used in the risk models for breast cancer (adapted from Stegeman and Bossuyt, 2012).

	Gail model 1 (Gail et al., 1989)	Gail model 2 (Costantino et al., 1999)	Care model (Gail et al., 2007)	Log incidence model (Rockhill et al., 2003)	Colditz, 2000 (Colditz et al., 2000)
<i>Demographic data</i>					
Age > 50	+	+	+		
Age				+	+
Number of biopsies	+	+	+		
<i>Hormone status</i>					
Age at menopause				+	+
Age at menarche	+	+	+	+	+
Oral oestrogen					+
Postmenopausal hormones (other than oestrogen)					+
Age first birth	+	+		+	+
<i>Other</i>					
Number of affected first degree relatives			+		
Parity				+	
Benign breast disease					+
Family history of breast cancer					+
BMI					+
Alcohol consumption					+

Difference between Gail model 1 and 2: model 2 contains age-specific breast cancer rates.

Table 2: The Breast Cancer Prevention Collaborative Group (BCPCG) prioritised breast cancer risk factors according to NIH priority score (adapted from Santen et al., 2007).

NIH priority score (1.0=highest, 5.0=lowest)	Risk factors
1.0 (high priority)	Gail Model parameters Quantitative Breast Density Free oestradiol plasma concentrations Parity (yes/no, number) Age at menopause
1.5	BMI Weight gain ages 20–50
2.0	Total oestrogen plasma concentrations (E1 + E2 + E1S) SHBG plasma concentrations Testosterone plasma concentrations Paternal history of breast cancer Second degree relatives with breast cancer Age of onset of breast cancer in family member Ovarian cancer and age of onset Familial history of breast cancer with identification whether unilateral or bilateral
2.5	Waist–hip ratio History of height loss since age 20 History of fracture in the past 5 years History of MHT * (no uniform consensus on this factor)
< 2.5	Alcohol use Years of breast feeding BRCA1 and 2 status (only 5% of population; expensive) Bone density Prolactin plasma concentrations IGF plasma concentrations Linear HT-E use alone Exercise Therapeutic abortion CHEK 2 MHT E + P or E alone ever/never Early for gestational dates High fat diet Oral contraceptive use Antioxidant use Oestradiol metabolite plasma concentrations

The risk of developing breast cancer increases greatly with age (Australian Government-Cancer Australia, 2011). Postmenopausal women have a higher risk of developing breast cancer than the younger population, and up to 70% of postmenopausal breast cancers are oestrogen receptor (ER α) positive. In 2007, about 24% of new breast cancer cases diagnosed were in women younger than 50 years, 51% in women aged 50-69, and 25% in women aged 70 and over in Australia. Women in Australia have a one in nine lifetime risk of developing breast cancer before the age of 85, and the average age of first diagnosis of breast cancer in women was 60 years in 2007. Moreover, the age-standardised incidence rate has increased from 80.8 in 1982 to 109.2 in 2007. However, the age-standardised rate of death has reduced from 30.8 deaths per 100,000 females in 1994 to 22.1 deaths per 100,000 females in 2007, a decrease of 27%.

This decline is largely due to recent advances in the treatment of breast cancer and early detection through mammography that is the current gold standard, which have led to the decline in breast cancer-associated mortality over the past decade (Institute of Medicine and Division of Earth and Life Studies. Committee on Technologies for the Early Detection of Breast Cancer, 2001). However, mammography-based screening is not yet feasible in developing countries due to high cost, therefore early detection strategies including public education and awareness of early signs and symptoms, screen process by clinical breast examination, and regular breast self-examination are promoted in these countries (Anderson et al., 2006, Yip et al., 2008). The best available strategies to reduce the risk of developing breast cancer include maintaining a healthy body weight, increasing physical activity and minimising alcohol intake (Kushi et al., 2006). A modern approach towards the better management of breast cancer has also emerged through the understanding of the cellular and molecular basis of the disease.

5.1. Steroid hormones

All steroid hormones have a complicated structure of fused rings and belong to a family of compounds known as terpenoids or terpenes (Norman and Litwack, 1997). Steroids are basically derived from a phenanthrene ring structure with a pentano ring attached, which results in the formation of completely hydrogenated form, cyclopentanoperhydrophenanthrene or the sterane ring structure. There are six families

of steroid hormones in mammalian systems that can be grouped according to their structural and hormonal basis, namely oestrogens, androgens, progestins, mineralocorticoids, glucocorticoids and vitamin D with its daughter metabolites. Moreover, bile acids form a seventh family of steroids due to their structural similarity to cholesterol (Table 3).

Table 3: Classes of steroids (Hall, 2011, Norman et al., 1993, Norman and Litwack, 1997).

Steroid class	Principal active steroid in humans	Number of carbon atoms	Parent ring structure	Major functions
Oestrogens	Oestradiol	18	Oestrane	Promotes growth and development of female reproductive system, female breasts, and female secondary sexual characteristics
Androgens	Testosterone	19	Androstane	Promotes development of male reproductive system and male secondary sexual characteristics
Progestins	Progesterone	21	Pregnane	Stimulates secretion of “uterine milk” by the uterine endometrial glands and promote development of secretory apparatus of breasts
Glucocorticoids	Cortisol	21	Pregnane	Has multiple metabolic functions for

				controlling metabolism of proteins, carbohydrates, and fats; also has anti-inflammatory effects
Mineralocorticoids	Aldosterone	21	Pregnane	Increases renal sodium reabsorption, potassium secretion, and hydrogen ion secretion
Vitamin D steroids	1,25-Dihydroxyvitamin D ₃	27	Cholestane	Stimulates intestinal Ca ²⁺ transport which involves rapid opening of Ca ²⁺ channels; effects on phospholipid metabolism in intestine, liver, parathyroid cells and kidney
Bile acids	Cholic acid	24	Cholane	Conjugates with other substances to form bile salts, which promote digestion and absorption of fats

1.2.1. Sources of oestrogens in premenopausal versus postmenopausal breast cancer

Oestrogen secretion by the ovaries ceases at menopause. Intracrine formation of oestrogens in peripheral tissues before menopause accounts for about 75%, and after menopause it is closer to 100% (Labrie et al., 2003). Moreover, plasma dehydroepiandrosterone-sulfate (DHEA-S) levels in postmenopausal women are found to be 1,000-10,000 times higher than E2 (Labrie et al., 2005).

In premenopausal women, the main source of oestrogens for breast cancer growth is the circulating oestrogens secreted from follicular granulosa cells of ovaries. Following menopause, the adrenal cortex continues to secrete C19 steroids including DHEA-S, dehydroepiandrosterone (DHEA) and Δ^4 -androstenedione (Δ^4 -dione). DHEA-S must be desulfated by steroid sulfatase in order to become physiologically active or to be utilised in carcinoma cells (Purohit et al., 2011, Reed et al., 2005). DHEA is then converted by 3β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3β -HSDs/KSIs) to Δ^4 -dione, which is aromatised by aromatase to yield oestrone. Oestrone can also be sulfoconjugated by oestrogen sulfotransferase, and subsequently cleaved by steroid sulfatase to free oestrone (Purohit et al., 2011, Reed et al., 2005). This free oestrone is further reduced to a more biologically potent oestrogen, E2 by 17β -hydroxysteroid dehydrogenases type I (17β -HSD type I), which contributes greatly to the growth and development of breast carcinoma cells (Miettinen et al., 1996, Luu The et al., 1989).

1.2.2. Steroid hormone biosynthesis or steroidogenesis

A number of different tissues including the adrenal gland, gonads, placenta, intestine, and central and peripheral nervous system contribute to the biosynthesis of a range of steroid hormones belonging to five classes (oestrogens, androgens, progestins, glucocorticoids and mineralocorticoids) from cholesterol, which acts as a common precursor to all steroid hormones (Figure 5) (Ghayee and Auchus, 2007, Payne and Hales, 2004, Mellon et al., 2004, Mellon, 2007, Mueller et al., 2007, Tsutsui et al., 2000). Members of the cytochrome P450 family of monooxygenases (CYPs), hydroxysteroid dehydrogenases (HSDs) and other accessory proteins are selectively expressed in these different tissues to ensure the tissue-specific production of steroid hormones. For the purpose of my project, the biosynthesis of oestrogens will be discussed in detail. All oestrogens are 18-carbon steroids and produced in the ovaries, both in the follicle and corpus luteum; and the fetal-placental unit in females. The testis also contributes to significant amounts of E2 in males. In both males and females, small quantities of oestrone are also produced by the adrenal cortex.

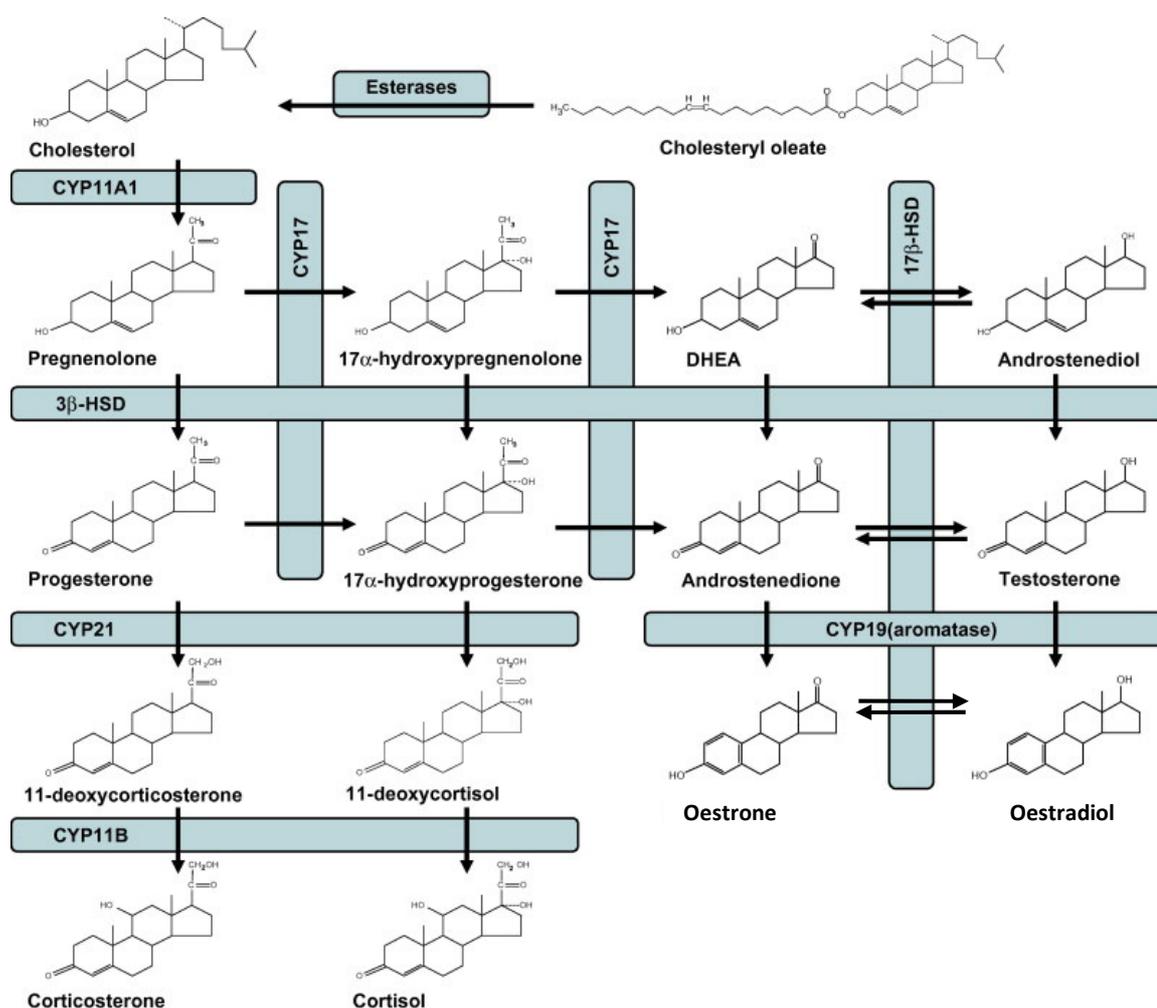


Figure 5: Simplified steroidogenesis pathway (adapted from Nishizato et al., 2010).

Once the lipoprotein-bound cholesterol enters a cell, it can be incorporated into the membranes of vesicles such as lysosomes, endosomes and peroxisomes that travel along cytoskeletal fibres, which can then be loaded into the outer mitochondrial membrane (OMM) (Soccio and Breslow, 2004, Hall and Almabobi, 1997, Hall, 1997). Otherwise, cholesterol can be rendered soluble by binding to specific proteins such as sterol carrier protein 2 (SCP2) (Gallegos et al., 2001), and a group of proteins termed StarD4, StarD5, and StarD6 which is proposed to have a role in non-vesicular intracellular cholesterol transport (Soccio and Breslow, 2003). The initial and rate-limiting step of steroid biosynthesis begins in the mitochondria where cholesterol is rapidly transferred from the OMM to the inner mitochondrial membrane (IMM) via steroidogenic acute

regulator (StAR) protein, where it can be targeted to the cholesterol side-chain cleavage enzyme (CYP11A1), and converted to soluble pregnenolone (Payne and Hales, 2004). Although it is now generally accepted that StAR is involved in the import of cholesterol, some evidence also supports the use of contact sites of OMM and IMM that bridge the two membranes (Thomson, 2003).

1.2.2.1. Steroidogenic acute regulator (StAR)

StAR is responsible for the mitochondrial transport of cholesterol from the OMM to IMM where *CYP11A1* is located (Miller, 1995). StAR is synthesised as a 37 kDa precursor that is rapidly imported into the mitochondria upon hormonal stimulation and processed to a mature 30 kDa form found in the mitochondrial matrix [77]. There are two major domains found within the StAR protein, the N-terminal mitochondrial targeting sequence of 25 amino acid residues, which is cleaved after its translocation into the mitochondria, and the C-terminal StAR-related lipid transfer (START) domain, which is biologically active and contains a hydrophobic tunnel hypothesised to be involved in cholesterol shuttling. It has been suggested that StAR undergoes a molten globule transformation under acidic conditions and this unfolding directs its action. Moreover, only newly synthesised StAR is functionally able to initiate steroidogenesis due to its rapid inactivation process.

1.2.2.2. Cytochrome P450 enzymes

Several steroidogenic enzymes are members of the cytochrome P450 group of oxidases (Norman and Litwack, 1997, Nebert and Gonzalez, 1987). This group of enzymes is responsible for oxidation of steroids, prostaglandins, fatty acids, biogenic amines, leukotrienes, pheromones and plant metabolites, and as well as chemical carcinogens, mutagens, many drugs and other environmental pollutants (Norman and Litwack, 1997, Nebert and Gonzalez, 1987). Catalysis by all cytochrome P450 enzymes uses molecular oxygen and electrons from NADPH or NADH ($R-H + O_2 + NADPH + H^+ \rightarrow R-OH + H_2O + NADP^+$) (Ghayee and Auchus, 2007, Omura, 2010). This family consists of different subclasses of enzymes, namely microsomal P450, mitochondrial P450, bacterial P450, P450-reductase fusion and P450nor (Omura, 2010).

The microsomal P450 enzymes are present in all eukaryotic organisms, and are tightly bound to the cytoplasmic surface of the smooth endoplasmic reticulum (Omura, 2010, Ghayee and Auchus, 2007). They use cytochrome P450-oxidoreductase with two flavins in order to oxidise NADPH and reduce P450 directly. Steroidogenic enzymes that belong to this subclass include *CYP17A1* (P450c17), *CYP21A2* (P450c21) and *CYP19A1* (aromatase, P450arom). The mitochondrial P450 enzymes are bound to the matrix-side surface of the inner mitochondrial membrane, and use adrenodoxin and adrenodoxin reductase, which are soluble in the matrix as their reductase system (Omura, 2010, Ghayee and Auchus, 2007). The flavoenzyme, adrenodoxin reductase oxidises NADPH and transfers electrons to adrenodoxin, a small iron sulfur protein, which in turn transfers these electrons to the P450. *CYP11A1* (P450_{scc}), *CYP11B1* (P450c11 β), *CYP11B2* (P450c11AS), and enzymes involved in vitamin D and bile acid metabolism are listed under mitochondrial P450 enzymes. The bacterial P450 enzymes and reducing system, ferredoxin and ferredoxin reductase are soluble in the cytoplasm (Omura, 2010). The bacterial P450 system is somewhat similar to the mitochondrial P450 system, but tends to utilise NADH rather than NADPH as their electron donor. The P450-reductase fusion enzymes are found in bacteria, and exist as fusion proteins of P450 and NADPH-dependent P450 reductase containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Omura, 2010). P450_{nor} with exceptional properties is found in the fungus in its soluble form, and accepts electrons from nitric oxide reduction directly from NADH without having any NADH-linked reductase or reducing system (Omura, 2010).

1.2.2.3.1. Cytochrome P450 side-chain cleavage (P450_{scc},
CYP11A1)

The first and key enzyme responsible for synthesis of all steroid hormones is the mitochondrial P450 enzyme, cytochrome P450 side-chain cleavage (P450_{scc}, *CYP11A1*) (Omura and Morohashi, 1995). It is responsible for the rate-limiting and hormonally regulated enzymatic conversion of 27-carbon cholesterol to 21-carbon pregnenolone via three distinct chemical reactions known as 20 α -hydroxylation, 22-hydroxylation and scission of the cholesterol side chain at the bond between carbon atoms 20 and 22 (Omura and Morohashi, 1995). This conversion occurs only in specific

cell types including testicular leydig cells, placental trophoblast cells, ovarian theca and corpus luteum cells, adrenal cortex cells, and certain neuronal cells in the brain (Ghayee and Auchus, 2007).

1.2.2.2.2. Cytochrome P450 17 α -hydroxylase/C17-20 lyase (P450c17, *CYP17A1*)

Cytochrome P450 17 α -hydroxylase/C17-20 lyase (P450c17, *CYP17A1*), expressed in the endoplasmic reticulum, is responsible for the two step conversion of 21-carbon steroids to 19-carbon steroids, which involves 17 α -hydroxylation and cleavage of the bond between carbon atoms 17 and 20. This enzyme catalyses the conversion of pregnenolone and progesterone to DHEA and androstenedione, respectively (Hall, 1991). In this two-step reaction, 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone exist as transient intermediates, and they are rapidly converted to their subsequent end products, DHEA and androstenedione.

1.2.2.2.3. Cytochrome P450 aromatase (P450arom, *CYP19A1*)

Aromatase, a microsomal member of the cytochrome P450 superfamily, is the enzyme responsible for the final and key step in the conversion of 19-carbon androgenic steroids to 18-carbon oestrogens (Nelson et al., 1993). This step is called an aromatisation reaction, which involves the conversion of the delta 4-3-one A-ring of C19 androgens to the corresponding phenolic A-ring characteristic of C18 oestrogens resulting in the loss of the angular methyl group as formic acid (Thompson and Siiteri, 1974). Androstenedione and testosterone serve as C19 substrates of aromatase that result in the formation of oestrone and E2, respectively. However, 16 α -hydroxyandrostenedione, a product from fetal 16 α -hydroxydehydroepiandrosterone sulphate, becomes the main substrate of aromatase in the human placenta, that gives rise to oestriol. The complex nature of the aromatase gene and its regulation will be discussed more thoroughly in section 1.3.

1.2.2.3. Hydroxysteroid dehydrogenases (HSDs)

The other major class of enzymes involved in steroidogenesis is the hydroxysteroid dehydrogenases (HSDs). HSDs exist in one or more isoforms, encoded by separate

genes with tissue specific expression. Hence, they play an essential role in the local production of steroid hormones in their target tissues. The concentration of every sex hormone is believed to be regulated by a pair of HSDs, which interconvert potent steroid hormones with their cognate inactive metabolites (Penning, 2003). In that case, one of the HSDs drives the reduction of a steroid ketone to a steroid alcohol as an NADPH-dependent ketosteroid reductase, and the other HSD is involved in the oxidation of a steroid alcohol to a steroid ketone as an NAD⁺-dependent hydroxysteroid oxidase. HSDs can be classified into two large gene superfamilies, the aldo-keto reductases (AKRs) (Bauman et al., 2004, Jez et al., 1997a, Jez et al., 1997b) and the short-chain dehydrogenase reductases (SDRs) (Jornvall et al., 1995, Peltoketo et al., 1999), which differ in their protein folds, stereochemistry of hydride transfer, kinetic, and catalytic mechanisms (Penning, 2011). The AKR gene superfamily consists of many of the 3 α -HSD and 20 α -HSD isoforms, whereas 11 β -HSDs and most of the 17 β -HSDs belong to the SDR family.

1.2.2.3.1. 3 β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3 β HSDs/KSIs)

3 β -hydroxysteroid dehydrogenases/ketosteroid isomerases (3 β HSDs/KSIs) catalyse the conversion of Δ^5 -3 β -hydroxysteroids to Δ^4 -3-ketosteroids as a dual-function enzyme, which is a crucial step in the biosynthesis of all active steroids including progesterone, glucocorticoids, mineralocorticoids, androgens and oestrogens (Labrie et al., 1992, Payne et al., 1997, Simard et al., 1996, Mason et al., 1997, Morel et al., 1997). They mainly utilise NAD⁺ as a cofactor to catalyse 3 β -hydroxysteroid dehydrogenation and $\Delta^5 \rightarrow \Delta^4$ -isomerisation of the steroid precursor pregnenolone, 17 α -hydroxypregnenolone (17-OH-Preg), DHEA and androst-5-ene-3- β ,17 β -diol (Δ^5 -diol) into progesterone, 17 α -hydroxyprogesterone (17-OHP), Δ^4 -dione and testosterone, respectively. 3 β -HSDs/KSIs are identified as membrane-bound enzymes, localised to the endoplasmic reticulum as well as the mitochondria, and exist in many isoforms (Keeney et al., 1993, Baker et al., 1999, McBride et al., 1999, Simard et al., 1991, Simard et al., 1993, Abbaszade et al., 1997). They have been found in steroidogenic tissues including placenta, adrenal cortex, ovary, and testis, as well as in other peripheral tissues, including the adipose tissue, breast, skin, lung, endometrium, prostate, liver, kidney, epididymis and brain (Labrie et

al., 1992, Payne et al., 1997, Simard et al., 1996, Mason et al., 1997, Morel et al., 1997, Labrie et al., 1991).

1.2.2.3.2. 17β -hydroxysteroid dehydrogenases (17β -HSDs)

17β -hydroxysteroid dehydrogenases (17β -HSDs) regulate the levels of active androgens and oestrogens in a tissue-specific manner by catalysing the bidirectional enzymatic reaction between biologically active and inactive sex steroids (Mindnich et al., 2004). They act on C17 as either oxidative 17β -HSDs by removing hydrogen to produce the inactive keto-form, or reductive 17β -HSDs result in the active hydroxyl-form by hydrogenation. There are currently fourteen multiple forms of 17β -HSDs, and type 1 and type 2 are the best characterised forms of the family (Nagasaki et al., 2009). 17β -HSDs should not be described as “isozymes”, as they are encoded by different non-homologous genes with distinct amino acid sequences revealing different subcellular localisation, substrate specificities, cofactor preference and tissue distribution. Moreover, they have been shown to be involved in both oxidation and reduction reactions *in vitro*, but they function as unidirectional enzymes *in vivo* (Luu-The et al., 1995, Blomquist, 1995).

1.3. Aromatase expression and breast cancer risk

In 1988, O'Neill and colleagues examined the variation in aromatase activity in the four quadrants of the breast, lower inner and outer, and upper inner and outer, during mastectomies performed on breast cancer patients (O'Neill et al., 1988). Aromatase activity was found to be significantly higher in the quadrants bearing the tumour compared to the tumour-free quadrants, and quadrants with lowest activity were never associated with the presence of tumour. Bulun and colleagues further extended the study by quantifying aromatase transcript expression in adipose tissue from four breast quadrants of mastectomy specimens, and reduction mammoplasty cancer-free specimens (Bulun et al., 1993). Highest aromatase transcript expression was strongly correlated with the quadrants bearing tumours as consistent with O'Neill and colleagues findings. However, no difference in aromatase expression was observed between various quadrants obtained from cancer-free specimens. Moreover, quadrants containing highest aromatase transcript levels showed a positive correlation with the

percentage of ASCs present. These two studies marked a significant milestone in the understanding of breast cancer biology, demonstrating increased aromatase transcript and activity, and also increased ASC to adipocyte ratio in adipose tissue obtained from breast cancer patients. These findings were further supported by other studies later on, showing enhanced aromatase transcript expression in the region of breast adjacent to a tumour and within the tumour itself (Harada et al., 1993, Agarwal et al., 1996a, Zhou et al., 1997, Irahara et al., 2006). Interestingly, Sasano and colleagues have demonstrated strong aromatase staining in the proliferative ASCs surrounding the breast tumour (Figure 6). Collectively, these data suggest that factors produced by the tumour, stimulate proliferation of ASCs and aromatase expression in these cells, and eventually lead to increased E2 production, which drives the growth and proliferation of breast tumour epithelium.

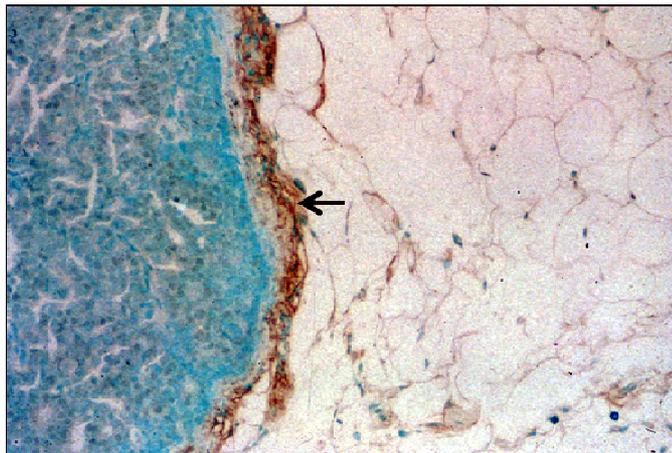


Figure 6: Aromatase expression in primary breast ASCs (Obtained directly from Prof. Hironobu Sasano, Japan). Increased aromatase staining (brown) is present in ASCs (indicated by arrow) adjacent to the tumour epithelial cells. Blue represents the hematoxylin nuclear counterstain.

Vachon and colleagues recently examined aromatase immunoreactivity (IR) in ultrasound-guided core biopsies obtained from dense and non-dense areas from the breasts of healthy women (Vachon et al., 2011), as mammographic breast density (MBD) is one of the strongest risk factors for breast cancer (McCormack et al., 2007).

In this study, overall mean aromatase IR was two-fold higher in dense compared to non-dense tissues in both stroma and epithelium, but not in the adipocytes. Interestingly, ASCs from dense tissue had a three-fold higher level of aromatase IR than in epithelial cells from dense tissues, suggesting a possible mechanism for MBD as a risk factor for breast cancer.

Obesity is a risk factor for developing hormone receptor positive, postmenopausal breast cancer, and increasing body mass index (BMI) is found to be associated with significant increase in oestrone, E2 and free E2 in postmenopausal women (Cleary and Grossmann, 2009, van Kruijsdijk et al., 2009). Morris and colleagues demonstrated that the presence of crown-like structures of the breast (CLS-B) that reflects the severity of inflammation, was correlated with BMI and adipocyte size (Morris et al., 2011). Moreover, aromatase transcript expression and activity were associated with both CLS-B index and BMI, but the correlation was much stronger with CLS-B index. Subbaramaiah and colleagues have further explored this phenomenon in the context of cyclooxygenase 2 (COX-2)-derived prostaglandin E₂ (PGE₂) (Subbaramaiah et al., 2012). In that case, PGE₂ was shown to stimulate the cAMP/PKA signal transduction pathway, resulting in increased aromatase transcript expression and activity and also progesterone receptor levels in breast tissues from overweight and obese women. Interestingly, CLS-B index was found to strongly correlate with these biological end points more so than BMI. These two studies suggest that the presence of the obesity→inflammation→aromatase axis as a novel target in reducing breast cancer risk in overweight and obese women.

1.3.1. The aromatase gene

The aromatase enzyme is encoded by the *CYP19A1* gene located on chromosome 15q21.2 within the human genome. The *CYP19A1* gene is approximately 123 kb long and contains a 30 kb coding region with nine coding exons (II-X) and a 93 kb regulatory region (Figure 7) (reviewed in Bulun et al., 2003, Bulun et al., 2004). The tissue-specific regulation of aromatase is governed by 10 distinct tissue-specific promoters, regulated by various hormones, second messengers and transcription factors, and mediates the expression of transcripts with unique 5'-untranslated regions. These

5'-ends of first exons are spliced into a common acceptor site upstream of the translational start site, hence the coding region is always the same regardless of the tissue site of aromatase expression. The three proximal promoters located within the 1 kb region upstream of the ATG translation start site in exon II, are PII (ovary / adipose tissue / breast cancer / endometriosis), PI.3 (adipose tissue / breast cancer) and PI.6 (bone). The remaining seven promoters are PI.2 (placenta-minor 1, ≈ 13 kb), PI.f (brain, ≈ 33 kb), PI.7 (endothelial cell / breast cancer, ≈ 36 kb), PI.5 (fetal tissues, ≈ 43 kb), PI.4 (bone / skin / adipose tissue, ≈ 73 kb), P2a (placental-minor 2, ≈ 78 kb) and PI.1 (placental-major, ≈ 93 kb).

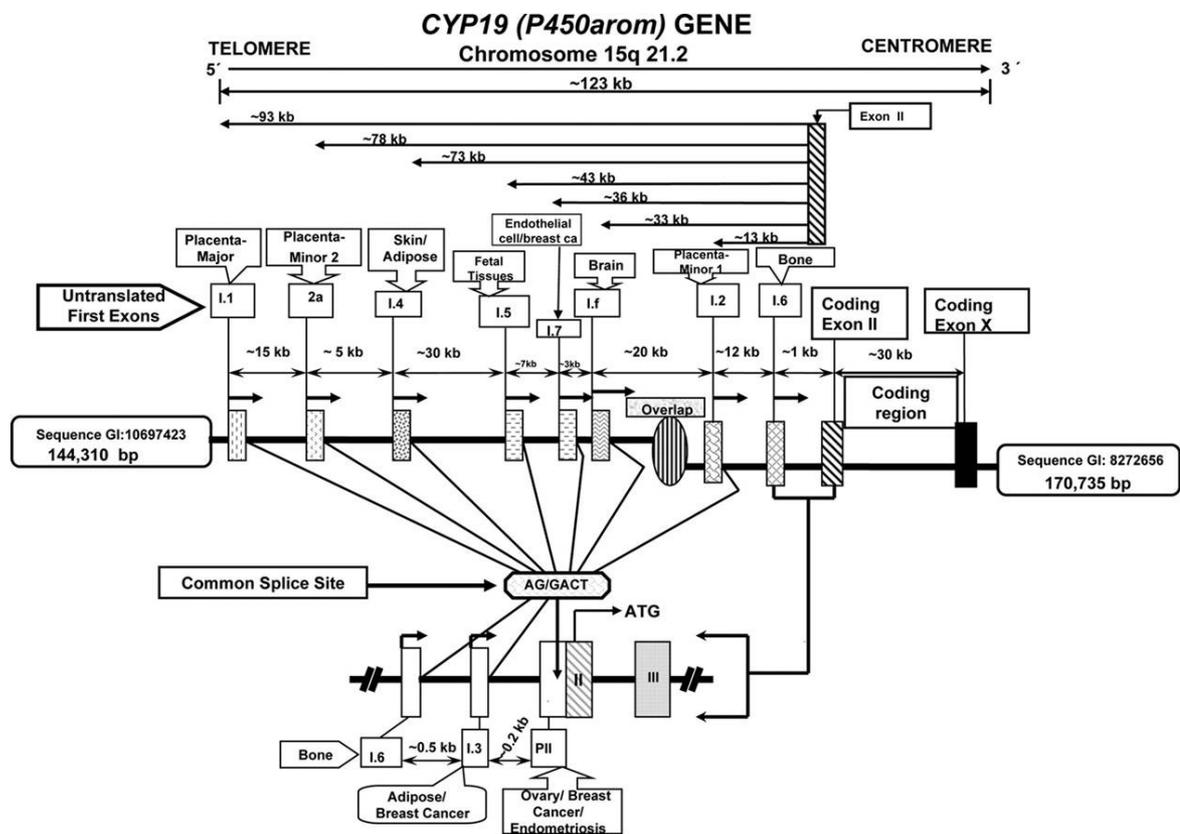


Figure 7: Mapping the promoters of the *CYP19A1* (P450arom) gene (adapted from Bulun et al., 2003). Model depicting location of each tissue-specific promoter of the P450arom gene in the chromosome 15q21.2 region. BLAST searches of various promoters and the coding region revealed alignment to distinct locations in two overlapping BAC clones (RP11-522G20 and RP11-108K3) of the chromosome 15q21.2

region. The distance of each promoter with respect to the first coding exon (exon II) was also determined. The major placental promoter I.1 is the most distally located (approximately 93-kb). Even though each tissue expresses a unique first exon or 5'-untranslated region (5'-UTR), by splicing into a highly promiscuous splice acceptor site (AG/GACT) of the exon II, the coding region and the translated protein product are identical in all the tissues.

Aromatase expression in the ovary is regulated in a time- and hormone-dependent manner via the proximal PII in response to cyclic AMP formed primarily through follicle-stimulating hormone (FSH) binding to the FSH receptor (Michael et al., 1995). In the placenta, the most distal promoter I.1 regulates aromatase expression (Mahendroo et al., 1991), and in human choriocarcinoma cells, PI.1-driven aromatase expression was found to be regulated by retinoids (Sun et al., 1998). Recent work by Kumar and Mendelson has demonstrated that PI.1 is regulated by the orphan nuclear receptor, oestrogen-related receptor γ (ERR γ), which is a HIF-1 α target gene (Kumar and Mendelson, 2011). The brain specific PI.f is regulated by the sex hormone progesterone, as demonstrated in mouse embryonic hypothalamic neurons, in a time-, concentration-, and ER α /progesterone receptor (PR) expression-dependent manner (Honda et al., 1994, Yilmaz et al., 2011). In bone osteoblasts, chondrocytes and osteoclasts, aromatase expression is primarily driven by the distal PI.4 (Shozu and Simpson, 1998, Oz et al., 2001). Moreover, in adipose tissue, PI.4 activation accounts for approximately half of aromatase transcript expression and is influenced by class I cytokines such as IL-6, IL-11 and TNF- α , in the presence of glucocorticoids, via the JAK-STAT (Janus kinase/signal transducer and activator of transcription) pathway as a second messenger system (Zhao et al., 1995a, Zhao et al., 1995b, Zhao et al., 1996b).

1.3.2. Regulation of aromatase expression in breast cancer

Aromatase expression in adipose tissue and ASCs in culture is mainly under the control of three promoters including PI.4, PII and PI.3 (Mahendroo et al., 1993, Zhou et al., 1996b). In cancer-free adipose tissue, the *CYP19A1* gene is expressed at low levels under the control of PI.4, which accounts for approximately half of transcripts, and PI.3/II, which account for the remainder (Agarwal et al., 1996b). The inappropriately

elevated aromatase activity due to increased *CYP19A1* gene expression in the stromal fraction of adipose tissue surrounding breast tumours is governed by the increased usage of proximal promoters, PII and PL3 and this is in response to an increase in cyclic AMP levels as a result of factors derived from the tumour, such as PGE₂ (Agarwal et al., 1996b, Zhao et al., 1996a) (Figure 8).

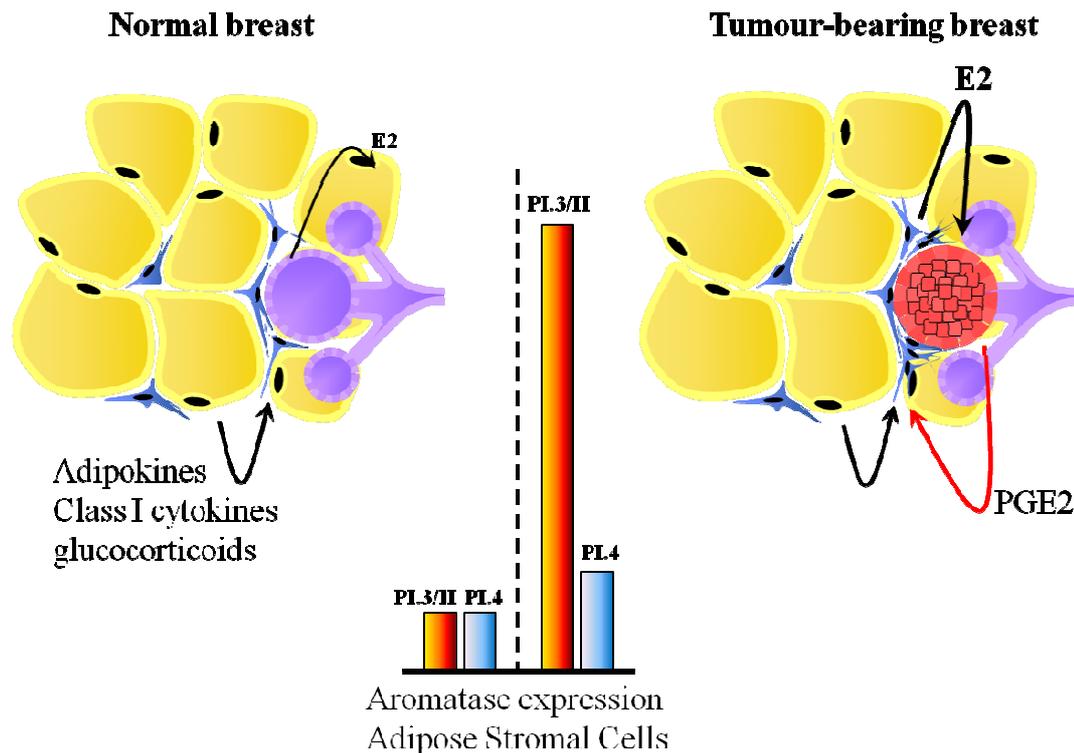


Figure 8: Epithelial – mesenchymal interactions regulating aromatase expression in the breast (adapted from Simpson and Brown, 2011). Inflammatory mediators such as PGE₂ produced by the tumorous epithelium stimulate the ASCs locally to increase aromatase expression. The resulting oestrogens in turn stimulate tumour cells to proliferate in a positive feed-on mechanism.

PGE₂ is mainly produced by cancerous epithelial cells, adipose fibroblasts, as well as macrophages infiltrating the tumour site (Zhao et al., 1997). The action of PGE₂ is mediated by the G-protein coupled transmembrane receptors, EP1 (Funk et al., 1993), EP2 (Regan et al., 1994), EP3 (Kunapuli et al., 1994, Yang et al., 1994) and EP4

(originally reported as EP2 subtype) (An et al., 1993, Bastien et al., 1994), which are involved in coordinating different signal transduction pathways. In human breast ASCs, PGE₂ induces aromatase activity and expression through binding to EP1 and EP2 receptor subtypes, which stimulate cAMP/PKA and PKC, respectively (Zhao et al., 1996a, Richards and Brueggemeier, 2003, Zhao et al., 1997). As a result, the action elicited by PGE₂ via activation of PKA and PKC pathways can be mimicked using both forskolin (FSK) and phorbol esters (PMA), which results in the maximal induction of PII-specific aromatase transcripts (Zhao et al., 1996a, Richards and Brueggemeier, 2003, Zhao et al., 1997). Interestingly, EP3 receptors have been shown to inhibit the PGE₂-mediated activation of aromatase in ASCs (Richards and Brueggemeier, 2003). Moreover, Subbaramaiah and colleagues have demonstrated that the EP2 and EP4 are the major receptor subtypes that are involved in PGE₂-induced aromatase overexpression using both receptor agonists and antagonists in visceral primary preadipocytes (Subbaramaiah et al., 2008).

The PGE₂-dependent induction of aromatase expression in breast ASCs also involves a nuclear receptor half-site (CAAGGTCA) found within the proximal PII, which is bound by liver receptor homologue-1 (LRH-1), an orphan member of the nuclear receptor superfamily (Clyne et al., 2002). LRH-1 is found to be expressed in breast adipose and breast cancer tissues, and is able to increase aromatase PII activity by more than 30-fold in response to FSK/PMA treatment in 3T3-L1 preadipocytes, transfected with LRH-1 (Clyne et al., 2002). Moreover, the differentiation process of cultured ASCs to adipocytes is associated with the time-dependent increase in peroxisome proliferator-activated receptor- γ (PPAR γ), and rapid loss of LRH-1 and aromatase expression (Clyne et al., 2002). Zhou and colleagues have demonstrated that LRH-1 transcripts are markedly elevated in adipose tissue adjacent to breast carcinoma compared to normal and breast carcinoma tissues, and the level of LRH-1 expression is strongly correlated with aromatase in these tissues (Zhou et al., 2005). In addition, exogenous LRH-1 in ASCs stimulates endogenous aromatase expression and its activity, and PII transcript (Zhou et al., 2005). Interestingly, PGE₂ has also been shown to increase LRH-1 transcript and aromatase expression in ASCs (Zhou et al., 2005).

1.4. CREB/ATF family members

CREB was first identified to interact with the cAMP responsive element (CRE) with the sequence of -TGACGTCA- on the somatostatin promoter in 1987 (Montminy and Bilezikjian, 1987). In that case, forskolin, an activator of adenylate cyclase, caused a 3-4-fold increase in the phosphorylation of CREB in PC12 pheochromocytoma cells. ATF was named in 1987 and found to interact with the E1A-inducible adenovirus promoters E2A, E3 and E4 with a common core sequence -CGTCA- (Lee et al., 1987). Interestingly, Lin *et al.* have demonstrated that ATF can bind and activate both E1A and cAMP-inducible promoters, and introduced a new consensus binding site for ATF as -TGACGT(C/A)(G/A)- (Lin and Green, 1988), which is similar to the CRE sequence.

The CREB/ATF family of proteins belong to the basic domain-leucine zipper (bZIP) superfamily and they are grouped into six subgroups called CREB, CRE-BP1, ATF3, ATF4, ATF6 and B-ATF (Figure 9) (reviewed in Vlahopoulos et al., 2008). The structure of CREB/ATF family members consists of a kinase inducible domain (KID) or phosphorylation box (P-box), which contains a conserved phosphorylation site, two glutamine rich domains, namely Q1 and Q2 that function as constitutive activators, and a highly conserved bZIP domain (reviewed in Servillo et al., 2002). This bZIP domain, an α -helical coiled coil structure with an adjacent basic domain is essential for dimerisation (Table 4) (reviewed in Vlahopoulos et al., 2008), DNA binding and coactivator interaction (Conkright et al., 2003).

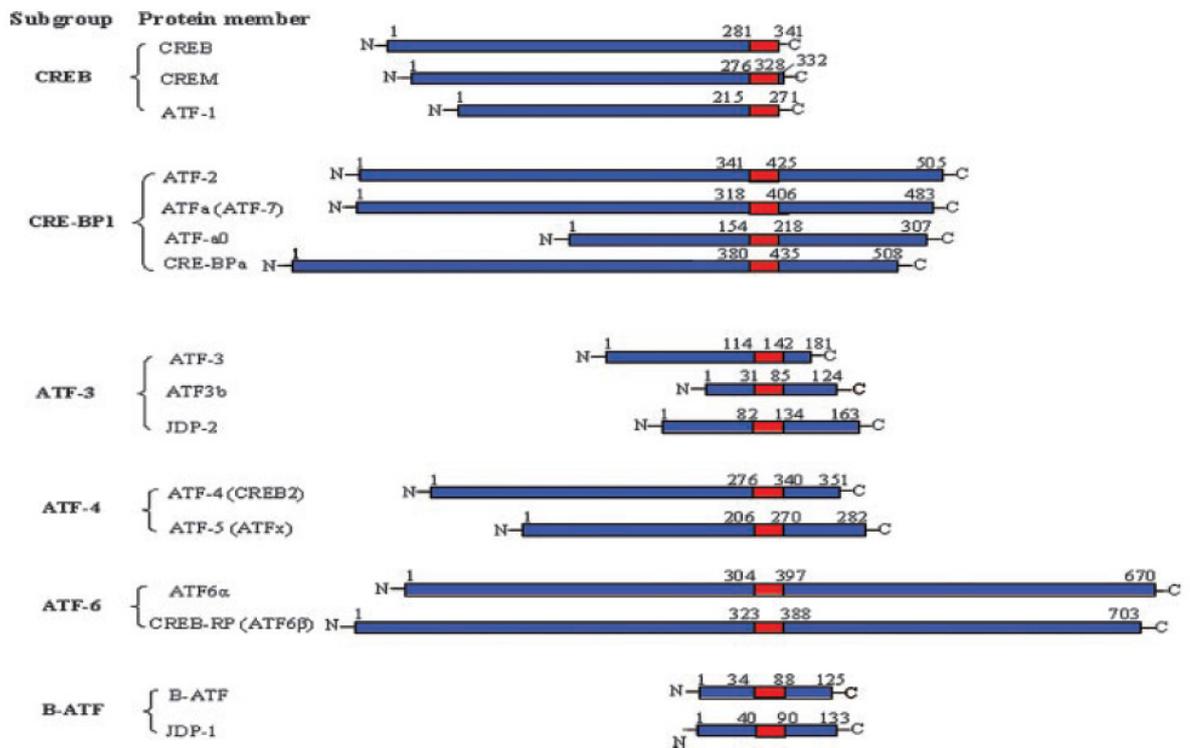


Figure 9: CREB/ATF family proteins (adapted from Vlahopoulos et al., 2008). The protein members can be grouped in six subgroups, according to their sequence similarity. The red boxes indicate the bZIP domain.

Table 4: Dimer formation between ATF/CREB family members (intrafamily dimerisation). (adapted from Vlahopoulos et al., 2008)

	ATF/CREB FAMILY MEMBERS															
	ATF-1	ATF-2	ATF-3	ATF3b	ATF-4	ATF-5	ATF6 α	ATF6 β	ATFa	ATF-a0	B-ATF	CREB	CREM	CRE-BPa	JDP-1	JDP-2
ATF-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATF-2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF-3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF3b	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF-4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF-5	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF6 α	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF6 β	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATFa	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ATF-a0	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B-ATF	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CREB	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CREM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CRE-BPa	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JDP-1	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JDP-2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: dimer formation.

-: dimer formation is not possible.

1.4.1. CREB subgroup

The CREB subgroup consists of CREB, CREM and ATF-1 proteins. CREB members exhibit more than 90% homology within their bZIP and core sequences in the KID, and approximately 70% homology overall. The activation of CREB, CREM and ATF-1 proteins is mediated through phosphorylation at a distinct serine residue via different signalling pathways (reviewed in Shaywitz and Greenberg, 1999). CREB is considered as the predominant form in many cell types and it is ubiquitously expressed in mice compared with CREM and ATF-1 (Bleckmann et al., 2002).

1.4.1.1. CREB

The CREB gene is located on human chromosome 2q34 (Pubmed gene ID: 1385). Alternative splicing of this gene results in three different transcript variants, namely isoform CREB-A (37kDa, 341 amino acids (aa)), isoform CREB-B (35kDa, 327aa), which lacks amino acids 88-101, and isoform 3 or htCREB (25kDa, 230 aa), which lacks amino acids 162-272 and is highly expressed in adult testis and sperm (UniProtKB/Swiss-Prot entry P16220). The CREB transactivation domain is bipartite, consisting of KID and constitutive (Q2) domains, activated by cAMP (Brindle et al., 1993, Quinn, 1993). The transactivation potential of CREB is largely mediated by phosphorylation at Ser133, located in KID at the N-terminus, by protein kinase A (PKA) upon stimulation with cAMP (Gonzalez and Montminy, 1989), whereas the glutamine-rich Q2 domain interaction with TFIID via TAF(II)130 promotes target gene expression (Nakajima et al., 1997, Felinski and Quinn, 1999, Saluja et al., 1998). Phosphorylation of CREB alters the affinity of its KID domain for the acceptor domain (KIX) of the histone acetylase coactivator paralogs CREB-binding protein (CBP) and p300 via a direct interaction, resulting in the improved transcription of CREB-target genes (Chrivia et al., 1993, Parker et al., 1996). This direct interaction is facilitated by the formation of a hydrogen bond between phospho-serine and Tyr-658 of KIX (Radhakrishnan et al., 1997), and also ion pair formation with Lys-662 of KIX (Parker et al., 1998). Moreover, a random coil to helix transition in KID is further stabilised in the CREB:CBP/p300 complex by specific hydrophobic interactions formed with residues lining a shallow groove in KIX (Parker et al., 1998, Radhakrishnan et al., 1999). Contradictory to Ser133 phosphorylation, phosphorylation of CREB at Ser142

inhibits CREB-mediated transcriptional activity via disrupting hydrophobic interactions between the α B helix of KID and the hydrophobic groove in KIX (Parker et al., 1998). Overexpression of CREB is found to be associated with breast cancer, prostate cancer, non-small-cell lung cancer and acute leukemia (Xiao et al., 2010). CREB transcript expression has been shown to be almost five times higher in adipose tissue of breasts containing a tumour as compared to normal breast adipose (Sofi et al., 2003). A study by Chhabra *et al.* reported that CREB is expressed in both tumour and normal breast adipose tissues, with higher levels in tumour tissues (Chhabra et al., 2007). CREB transcript expression is also found to be higher in ductal carcinoma as compared to lobular and other breast carcinoma, node-positive tumours compared to node-negative tumours, and patients with a poor prognosis and with metastasis compared to cancer-free patients (Chhabra et al., 2007). Moreover, breast cancer patients with higher CREB levels are shown to have significantly shorter disease-free survival (Chhabra et al., 2007).

1.4.1.2. CREM

CREM is located on human chromosome 10p11.21 (Pubmed gene ID: 1390). There are 22 isoforms produced by alternate promoter usage and alternative splicing (UniProtKB/Swiss-Prot entry Q03060). These isoforms can regulate gene expression by acting as either transcriptional activators or repressors. CREM is recognised as an important factor during spermatogenesis, contributing to round spermatid maturation arrest in idiopathically infertile men (reviewed in Behr and Weinbauer, 2001). The inducible cAMP early repressor (ICER) is the only inducible repressor of the class, acting as an endogenous repressor of CRE-mediated gene expression, due to lack of activation and kinase inducible domains (Servillo et al., 2002). It is the smallest transcription factor identified so far, binding to CREs, encoded by 108 or 120 aa with the predicted sizes of 12 kDa and 13.5 kDa (Molina et al., 1993). However, the presence of DNA-binding domains facilitates the formation of homo- or heterodimers and also interaction with CRE elements. ICER has been shown to play an important role in the regulation of circadian rhythm, neuroendocrine system and plasticity underlying epileptogenesis, fear memory, reaction to stress and possibly with other types of learning and memory (reviewd in Borlikova and Endo, 2009).

1.4.1.3. ATF-1

ATF-1 is located on human chromosome 12q21 (Pubmed gene ID: 466). It is a 29kDa protein with 271 aa (UniProtKB/Swiss-Prot entry P18846). ATF-1 has the ability to mediate transcriptional effects of PKA similar to CREB (Rehfuss et al., 1991). However, ATF-1 is found to be more responsive to cAMP than CREB due to its lower affinity for CREs under basal conditions (Rehfuss et al., 1991).

ATF-1 has been shown to act as a survival factor for human melanoma cells (Leslie and Bar-Eli, 2005) and also promotes tumour invasiveness in thyroid papillary carcinoma of the thyroid triggered by hepatocyte growth factor (Ghoneim et al., 2007). In contrast, higher ATF-1 expression is associated with significantly higher overall survival and progress free survival in patients with colorectal cancer (Huang et al., 2012).

1.4.2. CREB-BP1 subgroup

ATF-2 (CRE-BP1) is located on human chromosome 2q32 (Pubmed gene ID: 1386). There are two isoforms produced by alternative splicing, isoform 1 (55kDa, 505 aa) and isoform 2 (35kDa, 329 aa), which has a deletion in sequence 1-176 and substitutions in sequence 177-185 (UniProtKB/Swiss-Prot entry P15336). ATF2 remains inactive due to intermolecular interactions between its own bZIP and activation domains (Li and Green, 1996). In response to potential inducers, ATF-2 disrupts this interaction to become active by post-transcriptional modifications (Li and Green, 1996). The c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) subgroup of MAPK family (Livingstone et al., 1995, Gupta et al., 1995) and p38 (Raingeaud et al., 1995) phosphorylate ATF-2 on the same two closely spaced threonine residues, Thr69 and Thr71 within the N-terminal activation domain under stress conditions. Furthermore, there is a two-step mechanism involving two distinct Ras effector pathways that leads to transactivation of ATF-2 in human fibroblast cells in response to insulin, epidermal growth factor (EGF) and serum (Ouwens et al., 2002). The Raf-MEK-ERK pathway stimulates phosphorylation of ATF-2 at Thr71, whereas subsequent ATF-2 phosphorylation at Thr69 occurs via the Ral-RalGDS-Src-p38 pathway (Ouwens et al., 2002). Phosphorylated ATF2 can form homodimers or heterodimers with other members of the CREB/ATF and Fos/Jun families and then these dimers interact with

specific promoter regions, particularly with CREs to activate target genes (reviewed in Vlahopoulos et al., 2008).

ATF-2 has been shown to act as oncogene in different tumour types including breast, prostate, hepatic, melanoma, leukemias and nervous system, and also as a tumour suppressor in breast and non-malignant skin tumours (reviewed in Vlahopoulos et al., 2008). These opposing effects of ATF-2 are mediated via its subcellular localisation governed by the epsilon isoform of protein kinase C (PKC ϵ) (Lau et al., 2012). The nuclear accumulation of ATF-2 contributes to its oncogenic activities, whereas the mitochondrial localisation of ATF-2 determines its tumour suppressor functions. Phosphorylation of PKC ϵ at Ser729 directs ATF2 nuclear export and mitochondrial localisation upon genotoxic stress, which directs interaction of ATF-2 with the hexokinase-1 and voltage-dependent anion channel 1 (HK1:VDAC1) complex, followed by subsequent disruption of this complex, leading to increased mitochondrial permeability and promotion of apoptosis (Lau et al., 2012). In contrast, the high levels of PKC ϵ in melanoma cells directs nuclear localisation of ATF-2 via phosphorylation at Thr52, and is associated with resistance to genotoxic stress-induced cell death (Lau et al., 2012). Atf-2 heterozygous (Atf-2^{+/-}) mice were shown to be more susceptible to mammary tumour development and the median time (T₅₀) for tumour free survival was of about 90 weeks (Maekawa et al., 2007). In addition, loss of one copy of p53 in Atf-2^{+/-} mice resulted in higher rates of mammary tumour formation (Maekawa et al., 2008). Breast cancer cell lines have been widely used to study the effect of ATF-2 on breast cancer and a positive correlation of ATF-2 with proliferation, invasion, migration and resistance to DNA damaging agents has been demonstrated. In MCF-7 cells, treatment with E2 and spermine activate p38-mediated phosphorylation of ATF-2, leading to induction of cyclin D1 transcription by binding to the CRE of the cyclin D1 promoter and also an increase in cell proliferation (Lewis et al., 2005). In addition, p38-mediated activation of ATF-2 in MCF10A human breast epithelial cells leads to the induction of MMP-2 promoter activity and also invasive and migrative phenotypes (Song et al., 2006). Moreover, stable expression of ATF-2 in BT474 cells caused an increase in ATF-2 transcriptional activity and resistance to DNA damaging agents including, cisplatin, actinomycin D, MMS, and etoposide via JNK-dependent phosphorylation of

ATF-2 (Hayakawa et al., 2003). On the contrary, in MCF-7 cells, 3,3'-Diindolylmethane (DIM), a promising anticancer agent activates both JNK and p38 pathways, leading to phosphorylation of c-Jun and ATF-2, followed by subsequent binding of the homodimer or heterodimer of c-Jun/ATF-2 to the proximal regulatory element of the interferon-gamma (IFN γ) promoter, results in increased its transcription (Xue et al., 2005).

1.4.3. Role of CREB/ATF family members in regulating aromatase promoter II
In the presence of breast cancer, the activity of aromatase PII is largely regulated by the transcription factor CREB via cAMP-dependent mechanisms (Sofi et al., 2003). Sofi *et al.* showed that CREB binds to two distinct CRE-like sequences, namely CRE1 and CRE2 (approximately 80bp upstream of CRE1), within the PII region using 3T3-L1 mouse and primary human preadipocytes, and this interaction was increased in the presence of cAMP in 3T3-L1 preadipocytes (Sofi et al., 2003). A study by Chen *et al.* has demonstrated that PGE₂ causes an increase in phosphorylated ATF-2 binding to PI.3/II in breast ASCs after 15 min treatment, and that this increased interaction may contribute to the increased expression of aromatase (Chen et al., 2007a). Moreover, ICER, generated from an alternative intronic promoter of CREM and having cAMP inducibility via a cluster of four CREs in its promoter, was demonstrated to repress cAMP induced transcription strongly in a range of CRE-containing genes, including its own transcription via a negative autoregulatory mechanism (Molina et al., 1993). Interestingly, ICER has been shown to repress aromatase expression in rat granulosa cells of the ovary and R2C leydig cells (Morales et al., 2003).

1.5. CRTC family members

1.5.1. CRTC family and structure

CREs were identified in nearly 4000 promoters in the human genome and most have been shown to bind CREB *in vivo* (Zhang et al., 2005). The PKA-mediated phosphorylation of CREB at Ser133 upon activation of the cAMP pathway is not sufficient to stimulate the activation of all CREB-target genes (Zhang et al., 2005). A new family of CREB co-activators, termed CRTCs (CREB-regulated transcription co-activators) and previously known as TORCs (transducers of regulated CREB activity)

has recently been identified using high-throughput screens for modulators of CRE-luciferase reporter (Iourgenko et al., 2003, Conkright et al., 2003, Bittinger et al., 2004, Screatton et al., 2004). There are three members of the CRTC family, namely CRTC1, CRTC2 and CRTC3. All three members possess an N-terminal CREB binding domain (CBD), a central regulatory region (REG), a splicing domain (SD) and a C-terminal transactivation domain (TAD) (Figure 10) (reviewed in Altarejos and Montminy, 2011).

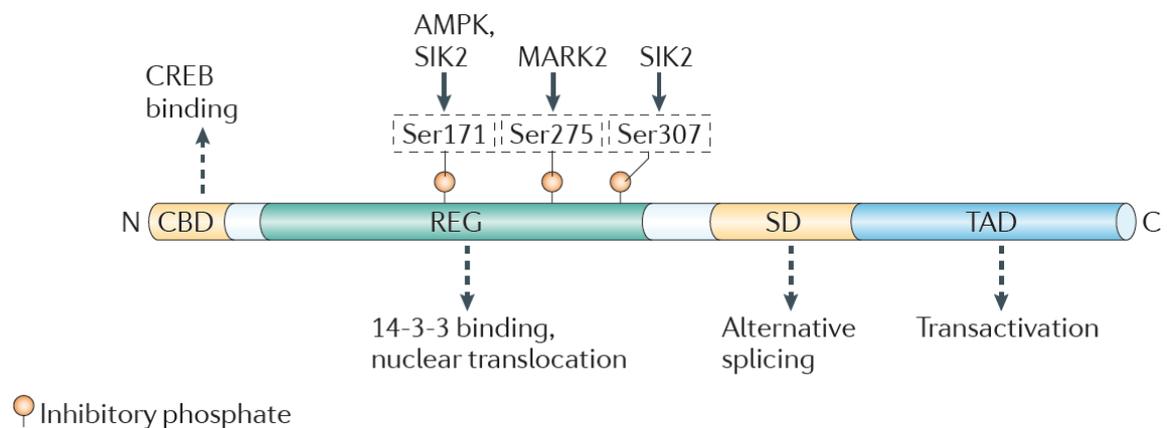


Figure 10: Domain structure of the CRTC family of CREB co-activators, as exemplified by CRTC2 (adapted from Altarejos and Montminy, 2011). CRTCs contain an N-terminal CREB binding domain (CBD), a central regulatory region (REG), a splicing domain (SD) and a C-terminal transactivation domain (TAD). CRTC phosphorylation at Ser171 (by AMPK and SIK2), Ser275 (by microtubule affinity-regulating kinase 2 (MARK2)) and Ser307 (by SIK2) promotes 14-3-3 protein binding and the cytoplasmic sequestration of CRTC2.

Gene profiling analysis has demonstrated that CRTCs are expressed at relatively low levels in most tissues (Conkright et al., 2003). However, CRTC1 is predominantly found to be in certain regions of the brain including prefrontal cortex and cerebellum, and CRTC2 and CRTC3 are particularly abundant in B and T lymphocytes (Conkright et al., 2003). A study by Wu *et al.* has also shown the relative human tissue distribution of CRTC family members (Figure 11) (Wu et al., 2006a).

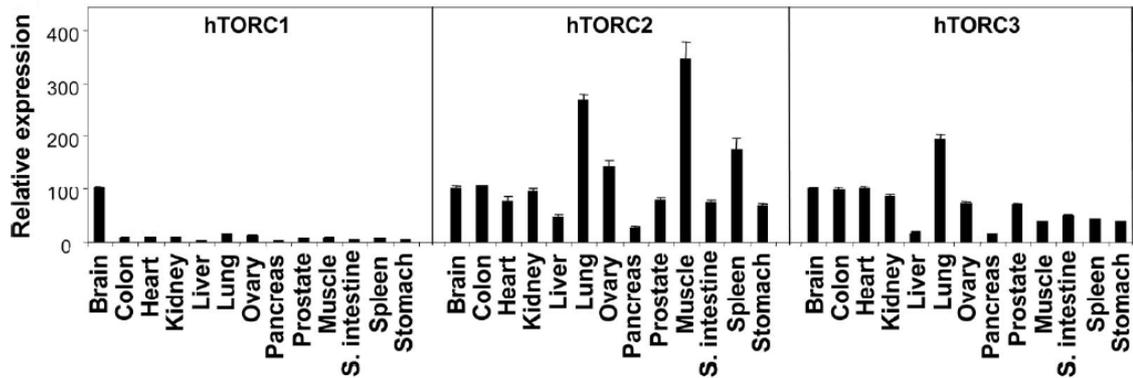


Figure 11: Human tissue distribution of the TORC (CRTC) family (adapted from Wu et al., 2006a). The mRNA expression levels of TORC1, 2 and 3 were measured by Q-PCR in a panel of human tissue cDNAs. The mRNA expression level of each TORC in brain is set at 100%. The mRNA expression levels in other tissues are relative to that of brain.

1.5.1.1. CRTC1

CRTC1 is located on human chromosome 19p13.11 (Pubmed gene ID: 23373). There are 3 isoforms produced by alternative splicing, namely isoform 1 (67kDa, 634 aa), which is the most commonly described, isoform 2 (69kDa, 650 aa), which has a 16-amino acid insertion at position 82 and isoform 3 (63kDa, 593 aa), which lacks the amino acids 475-503 and contains substitutions in amino acid sequence 580-634 (UniProtKB/Swiss-Prot entry Q6UUV9).

1.5.1.2. CRTC2

CRTC2 is located on human chromosome 1q21.3 (Pubmed gene ID: 200186). It is a 73kDa protein with 693 amino acids (UniProtKB/Swiss-Prot entry Q53ET0).

1.5.1.3. CRTC3

CRTC3 is located on human chromosome 15q26.1 (Pubmed gene ID: 64784). There are 2 isoforms produced by alternative splicing, namely isoform 1 (67kDa, 619 aa), which has been chosen as the canonical sequence, isoform 3 (67kDa, 618 aa), which has a deletion in amino acid 551 (UniProtKB/Swiss-Prot entry Q6UUV7).

1.5.2. CRTC co-activation of CREB

The highly conserved N-terminal coiled-coil domain of the CRTCs is known to interact with the bZip domain of CREB (Conkright et al., 2003). Conkright *et al.* have demonstrated that CRTCs can act as co-activators of CREB independent of its phosphorylation status at Ser133 (Conkright et al., 2003). In that case, CRTC1 overexpression in CREB^{-/-} mouse embryo fibroblasts resulted in an increase in CRE-reporter activity in cells transfected with wild-type or Ser133Ala mutant CREB that mimics the dephosphorylated state of CREB (Conkright et al., 2003). Interestingly, overexpression of CRTC1 and CRTC2 in HEK293T cells had no effect on phospho-CREB Ser133 levels (Conkright et al., 2003). Moreover, CRTCs increase the interaction with the TAF(II)130 component of TFIID, which directs an increase expression of CRE-target genes (Conkright et al., 2003).

1.5.3. CRTC phosphorylation and subcellular localisation

Both the nuclear localisation sequence (NLS) and nuclear export sequence (NES) motifs are found to be conserved within the three CRTC family members and CRTCs tend to shuttle in and out of the nucleus under resting conditions (Screaton et al., 2004). CRTC2 contains a NLS at amino acids 56-144 as well as two nuclear export sequences (NES1 and NES2) within the region of amino acids 145-320 (Screaton et al., 2004).

Interestingly, CRTC2 is mainly trapped in the cytoplasm under basal conditions through phosphorylation-dependent interactions with 14-3-3 proteins (Screaton et al., 2004). In contrast, CRTC3 is mostly sequestered in the nucleus under basal conditions and this is predicted to be due to the change of amino acid 282 from a phenylalanine (Phe) residue to a tyrosine (Tyr) residue in the NES1 of CRTC3 compared to CRTC1 and 2 in ATYB1 cells (human fibroblast cell line) (Screaton et al., 2004). This phenomenon was further supported by the observation that cytoplasmic retention was induced by the Tyr282Phe mutation of CRTC3 (Screaton et al., 2004). Nuclear translocation of the CRTC protein family through dephosphorylation or lack of phosphorylation is a critical and conserved step in the upregulation of CREB-target genes (Bittinger et al., 2004). Moreover, this is induced by increasing intracellular cAMP or calcium levels (Bittinger et al., 2004). Dephosphorylation of CRTC2 at Serine 171 (Ser171) and Serine 369

(Ser369) in response to cAMP agonists and calcium has been shown to drive its nuclear translocation by disrupting CRTC2:14-3-3 complexes (Screaton et al., 2004). Both sites, Ser171 and Ser369, are involved in binding to 14-3-3 protein, but Ser171 appears to have a more important role as the principal phosphoacceptor site (Screaton et al., 2004).

1.5.4. AMPK-related family members and regulation of CRTC via upstream kinases

AMPK-related family members are a unique subfamily of serine/threonine kinases, including AMPKs and Salt-inducible kinases (SIKs) which contribute to the phosphorylation of CRTC family members (Katoh et al., 2006, Koo et al., 2005, Mair et al., 2011, Screaton et al., 2004). Moreover, the tumour suppressor LKB1 encoded by human *STK11* gene (locus 19p13.3) has the ability to phosphorylate and activate 14 AMPK-related family members through their T-loop threonine residues, including the catalytic subunits of AMPK (AMPK α 1 and AMPK α 2), SIK, BRSK1 and 2 (brain-specific kinases 1 and 2), MARK1–4 (microtubule-affinityregulating kinases 1–4), NUAK1/ARK5 (AMPK-related kinase 5), NUAK2/SNARK [SNF1 (sucrose-non fermenting)/AMPKrelated kinase], QIK (Qin-induced kinase), QSK and SNRK (SNF-related kinase) (Thomson et al., 2008). Heterozygous germline mutations in LKB1 have been reported to lead to the hereditary Peutz-Jeghers Syndrome (PJS), which is associated with the development of benign gastrointestinal polyps and to the increased risk of developing malignant tumours in GI tract, breast and gynaecological organs (Giardiello et al., 2000). Somatic mutations in LKB1 have also been shown to be involved in the development of lung (Ji et al., 2007) and cervical cancer (Wingo et al., 2009). In addition, low LKB1 protein expression in human breast cancer samples is correlated with higher histological grade, larger tumour size, presence of lymph node metastasis, progesterone receptor status and shorter survival (Shen et al., 2002). Overexpression of LKB1 in MDA-MB-435 breast cancer cells, which lack the LKB1 gene, suppresses cell growth by G1 cell cycle arrest mediated by the up-regulation of cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} (Shen et al., 2002, Zhuang et al., 2006) and also inhibit tumour growth in the mammary fat pad, microvessel density and lung metastasis *in vivo* (Zhuang et al., 2006).

1.5.4.1. AMPKs

AMPK is a heterotrimeric enzyme composed of the catalytic subunit α and regulatory β and γ subunits (McGee and Hargreaves, 2008). Two α , two β and three γ subunits have been identified, resulting in 12 possible heterotrimeric combinations, together with splice variants promoting potential diversity within the AMPK family (McGee and Hargreaves, 2008). The α subunits consist of a kinase domain at the N-terminal region and as well as binding sites to the β and γ subunits in the C-terminal region. The β subunits tend to have 2 domains, namely a glycogen-binding domain in the central region and a C-terminal domain, which is involved in binding to the α and γ subunits (Hudson et al., 2003, Towler and Hardie, 2007). In addition, γ subunits contain variable N-terminal regions followed by 4 tandem repeats of approximately 60 amino acids termed cystathionine- β -synthase (CBS) motifs (Scott et al., 2004). Pairs of CBS motifs that form 2 domains are known as Bateman domains and act as sensors of cellular energy status by binding to AMP with higher affinity than ATP (Scott et al., 2004).

The AMPK system plays a major role in sensing cellular energy status in all eukaryotic cells. The activation of AMPK through a higher cellular AMP:ATP ratio causes an increase in energy production to ensure cellular survival through the catabolic ATP generating pathways, such as fatty acid oxidation and glucose uptake, and also limits energy utilisation by inhibiting cell growth, proliferation and biosynthetic pathways such as sterol and fatty acid synthesis (Hardie et al., 2006, McGee and Hargreaves, 2008). Studies undertaken in kidney cells have demonstrated that active AMPK can phosphorylate CRTC2, resulting in its cytoplasmic accumulation (Koo et al., 2005). Koo *et al.* have also demonstrated that activated AMPK phosphorylates wild-type but not Ser171Ala mutant of CRTC2 (mimicking a dephosphorylated state) in *in vitro* studies, even in the presence of a cAMP agonist (Koo et al., 2005). CRTC1 in *C. elegans* with conserved phosphorylation sites at Ser76 and Ser179 residing within 14-3-3-binding motifs, is shown to be a direct target of AMPK by demonstrating constitutive nuclear retention of CRTC1 mutants, Ser79Ala and Ser179Ala in response to nuclear export stimuli including *tax-6* RNAi and AAK-2 overexpression (Mair et al., 2011). The direct downstream targets of AMPK, including CRTCs, have specific amino acid sequences around the phosphorylated serine residue, notably, amino acids with a bulky

hydrophobic R-group (M,L,I,F,V) at P+4 and P-5 and at least one amino acid with a basic R-group (R,K,H) at either P-3 or P-4 (Figure 12) (Dale et al., 1995, Weekes et al., 1993). Moreover, synthetic peptide substrates containing a threonine in place of the major phosphorylation target residue Ser171, have also been identified as a reasonably good substrate for AMPK (Dale et al., 1995). Therefore, the substrate recognition motif of AMPK can be represented by M/L/I/F/V-(R/K/H,X)-X-X-S/T-X-X-X-M/L/I/F/V or Φ -(β X)-X-X-S/T-X-X-X- Φ (' indicates alternatives, commas indicate that the order of residues is not important, X indicates any kind of amino acid residue, Φ represents hydrophobic and β denotes basic residues) (Dale et al., 1995).

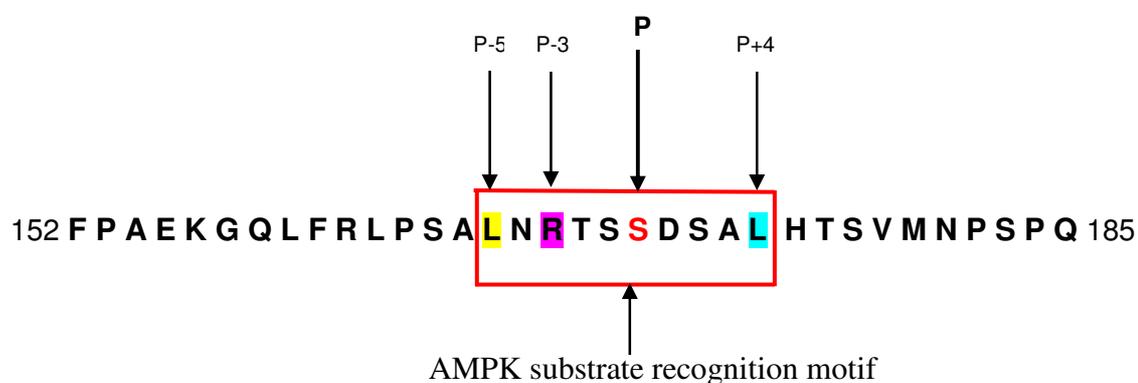


Figure 12: Partial amino acid sequence (152-185) of human CRTC2 including the putative Ser171 phosphorylation site. The major phosphorylation target Ser171 is shown by the arrow labelled “P” at the top and P-5, P-3 or P+4 refers to residues N or C terminal to the phosphorylated serine. P-5 and P+4 refer to the hydrophobic residue L and P-3 is the basic residue R.

LKB1 directly phosphorylates AMPK at Thr172 within the T loop region of the catalytic α subunit which leads to an increase in its kinase activity (Shaw et al., 2004). Komiya *et al* have also demonstrated that the somatic loss of LKB1 is correlated with dephosphorylation of endogenous CRTC1 and its increased nuclear localisation using LKB1-null (H2126 and H23) and wild-type (H2087 and H522) cancer cell lines (Komiya et al., 2009).

1.5.4.2. SIKs

Salt-inducible kinases (SIKs) are members of the AMPK-related family and present as three isoforms, namely SIK1, SIK2 and SIK3. In terms of the murine tissue distribution of SIKs, SIK1 is highly expressed in the adrenal cortex, regulating early phase of ACTH-signaling (Okamoto et al., 2004). SIK2 is an adipose-specific kinase, modulating early phase of insulin-signaling (Okamoto et al., 2004). SIK3 however, is ubiquitously expressed (Okamoto et al., 2004).

The phosphorylation motif of SIK is highly conserved amongst its three isoforms and SIK1 has the ability to phosphorylate all the CRTC2s except the Ser171Ala mutant of CRTC2, demonstrating that all CRTC2s act as substrates of SIK1 and the importance of the Ser171 residue of CRTC2 for its kinase-induced regulation (Katoh et al., 2006, Koo et al., 2005). SIK2 has also been shown to phosphorylate CRTC2 at Ser171, as well as promote the association of CRTC2 with 14-3-3 proteins (Screaton et al., 2004). Katoh *et al.* have demonstrated that LKB1 directly phosphorylates SIK1 at Thr182, resulting in its activation (Katoh et al., 2006).

1.5.5. Role of CRTC2s in aromatase PII activation

A study undertaken by Brown and colleagues, in breast ASCs, has demonstrated that FSK/PMA treatment, to mimic PGE₂, caused a significant reduction in LKB1 expression, as well as a decrease in AMPK phosphorylation at Thr172 (Brown et al., 2009). This study also demonstrated that activation of AMPK using the drug AICAR caused a significant reduction in aromatase expression in FSK/PMA-treated ASCs (Brown et al., 2009). Moreover, FSK/PMA-mediated repression of LKB1/AMPK led to the nuclear translocation of CRTC2, as well as the increased binding and activation of aromatase promoter PII (Brown et al., 2009). Importantly, mutation of the proximal CRE abolished the CRTC2-mediated induction of aromatase PII activity, confirming that CRTC2 acts primarily via CREB-dependent mechanisms (Brown et al., 2009).

1.6. Hypoxia inducible factors (HIFs) and CREB family members

Hypoxia is known to arise as a result of the imbalance between supply and consumption of oxygen (O_2), and it plays major roles in both tumour progression and resistance to therapy (reviewed in Harris, 2002, Vaupel and Mayer, 2007). A majority of healthy mammalian tissues experience 2-9% O_2 (on average 40 mm Hg) compared to 21% (150 mm Hg) of ambient air O_2 , while hypoxia is defined as $< 1\%$ O_2 and severe hypoxia or anoxia is defined as $< 0.1\%$ O_2 (Koh and Powis, 2012). Most solid tumours tend to have hypoxic regions because of the severe structural and functional abnormalities of tumour microvasculature. The hypoxia inducible factors (HIFs) act as master regulators of oxygen homeostasis in these tissues, which transactivate a large number of genes responsible for angiogenesis, anaerobic metabolism and resistance to apoptosis.

CREB has been shown to interact with three plasminogen activator inhibitor-1 (PAI-1) boxes, including hypoxia responsive element-2 (HRE2, E-box 2), E-box 4 and E-box 5 on the human PAI-1 promoter, and the sequence 5'-ACGT-3' of these boxes matches the core sequence of the CRE (5'-TGACGTCA-3') (Dimova et al., 2007). Arany *et al.* have demonstrated that HIF-1 α binds to transcriptional coactivators, CBP/p300, which also coactivate CREB during hypoxic conditions, and further reported that the cysteine/histidine-rich 1 (CH1) region of p300 is responsible for interacting with HIF-1 α (Arany et al., 1996).

Hypoxia has been shown to increase ATF2 expression and its phosphorylation (Choi et al., 2009). Furthermore, ATF2 has been identified as a regulator of HIF-1 α via direct interactions that lead to increased HIF-1 α protein stability and transcriptional activity, and that ATF2 inhibits the p53-mediated HIF-1 α degradation (Choi et al., 2009). Moreover, Choi *et al.* have demonstrated that the transcriptional complex of ATF2, HIF-1 α and coactivator CBP leads to the induction of phosphoenolpyruvate carboxykinase (PEPCK) transcription in response to hypoxia and retinoic acid (RA) signalling (Choi et al., 2005).

1.6.1. Structure of HIFs

There are three HIF- α genes in the human genome, namely HIF-1 α , HIF-2 α and HIF-3 α , and three HIF- β genes, also called Aryl Hydrocarbon Receptor Nuclear Translocators or ARNTs, including HIF-1 β (ARNT), HIF-2 β (ARNT2) and HIF-3 β (ARNT3). Both HIF- α and HIF- β belong to the Per-aryl hydrocarbon receptor nuclear translocator-Sim (PAS) group in the basic-helix-loop-helix (bHLH) family of transcription factors (Wang et al., 1995) (Figure 13). bHLH and PAS domains are involved in DNA binding and heterodimerization. The oxygen-dependent degradation (ODD) domain (\approx 200 amino acid residues) located in the central region of HIF- α is required for oxygen-dependent hydroxylation and degradation, and the N-terminal and C-terminal transactivation domains (TAD-N and TAD-C) are required for transcriptional activation.

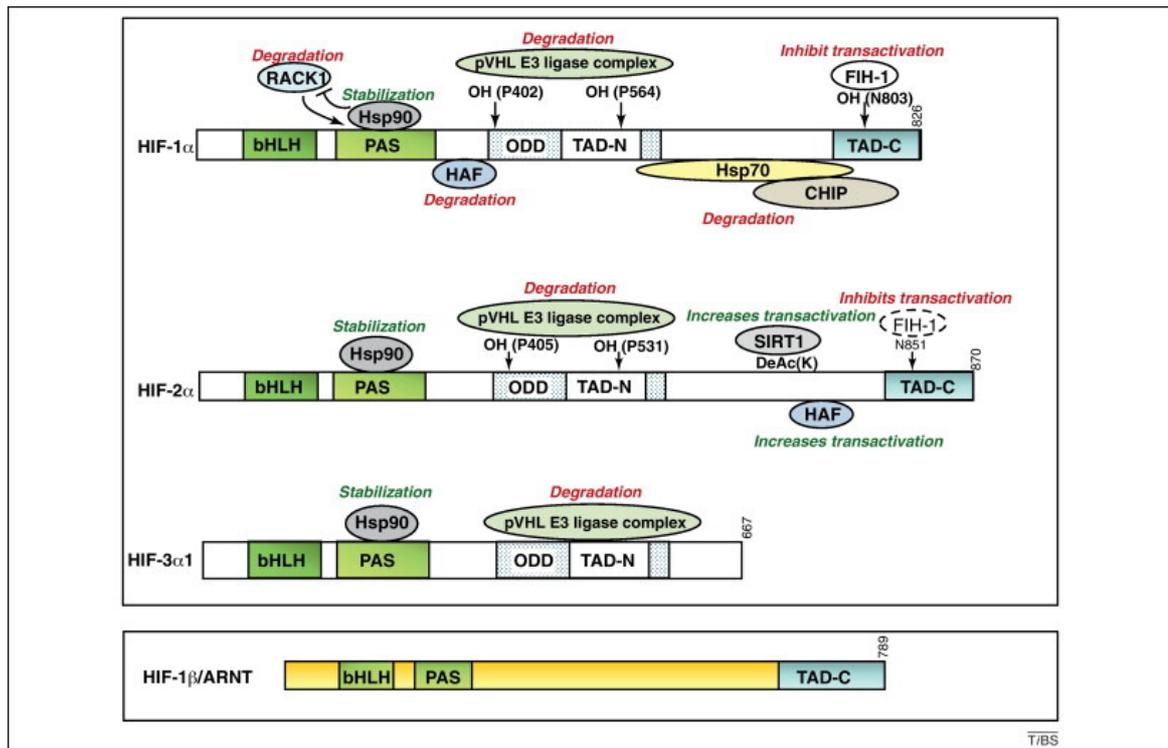


Figure 13: The structural domains of HIF-1/2/3 α and HIF-1 β /ARNT, and their known modulators (adapted from Koh and Powis, 2012). The binding domains of known modulators of HIF- α are depicted, along with the effects of these interactions on activity of the HIF transcriptional complex (red font indicates inhibitory interactions, green font indicates activating interactions). The von Hippel–Lindau protein (pVHL) E3 ligase complex regulates the oxygen-dependent degradation of all three major HIF- α subunits. Factor inhibiting HIF (FIH) hydroxylates HIF-2 α at a lower efficiency (broken oval) than HIF-1 α (unbroken oval). Receptor for activated protein kinase C 1 (RACK1) promotes the degradation of HIF-1 α when heat shock protein (Hsp)90 is inhibited, such as by Hsp90 inhibitors. The hypoxia-associated factor (HAF) selectively binds to HIF-1 α and HIF-2 α , mediating degradation and transactivation, respectively. Hsp70 promotes the binding of carboxyl terminus of Hsp70-interacting protein (CHIP) to HIF-1 α , resulting in HIF-1 α degradation. Sirtuin 1 (SIRT1) deacetylates HIF-2 α , resulting in activation.

One of three O₂-liable HIF- α subunits (HIF-1 α , HIF-2 α or HIF-3 α) and a HIF-1 β subunit together form HIF-1, HIF-2 and HIF-3 heterodimeric transcriptional complexes (Wang et al., 1995). HIF-1 α (MOP1, PASD8, bHLHe78), HIF-2 α (MOP2, PASD2, EPAS1, bHLHe73, HLF, ECYT4), HIF-3 α (MOP7, PASD7, IPAS, bHLHe17) and HIF-1 β (TANGO, bHLHe2, ARNT) are located on human chromosome 14q23.2 (Pubmed gene ID: 3091), 2p21-p16 (Pubmed gene ID: 2034), 19q13.32 (Pubmed gene ID: 64344) and 1q21 (Pubmed gene ID: 405), respectively. Alternative splicing of the HIF-1 α gene results in two different transcript variants, namely isoform 1 (93 kDa, 826 aa) and isoform 2 (83 kDa, 735 aa), which lacks amino acids 736-826 (UniProtKB/Swiss-Prot entry Q16665). HIF-2 α is a 96 kDa protein with 870 aa (UniProtKB/Swiss-Prot entry Q99814). HIF-3 α gene produces 7 isoforms by alternative splicing. HIF-3 α isoform 1 with canonical sequence is a 72 kDa protein with 669 aa (UniProtKB/Swiss-Prot entry Q9Y2N7). There are 3 isoforms produced by alternative splicing of the HIF-1 β gene, isoform 1 (87 kDa, 789 aa), isoform 2 (85 kDa, 774 aa) lacking amino acids 77-91 and isoform 3 (85 kDa, 775 aa) lacking amino acids 1-9 and 319-323 (UniProtKB/Swiss-Prot entry P27540).

1.6.2. Regulation of HIFs

The HIF system is known to present and be involved in the oxygen-sensing mechanism in the basal group of metazoan, *Trichoplax adhaerens* (Loenarz et al., 2011). HIF- α is one of the most significant factors induced by hypoxia or reduced oxygen availability and it shows an extremely short half-life of less than 5 minutes (Huang et al., 1996). Out of three major HIF- α subunits, HIF-1 α and HIF-2 α have been more extensively studied in regulating hypoxia-dependent gene expression. HIF-3 α which contains bHLH and PAS domains similar to HIF-1 α and HIF-2 α , but lacking TAD-C, has multiple splice variants (Jang et al., 2005, Maynard et al., 2007, Maynard et al., 2005). Interestingly, the inhibitory PAS domain protein (IPAS), which is a hypoxia inducible splicing variant of the HIF-3 α locus has been proposed to act as a negative regulator of HIF-1 α mediated gene expression (Makino et al., 2002, Makino et al., 2001). The regulation of HIF- α is mainly governed by posttranscriptional modifications such as hydroxylation, acetylation and phosphorylation (reviewed in Semenza, 2003, Koh and Powis, 2012). HIF- α is continuously synthesised and degraded under normoxic conditions via the

ubiquitin-proteasome system, and HIF-1 β is continuously expressed independent of oxygen levels (Salceda and Caro, 1997).

1.6.2.1. Regulation of HIF-1/2 α

HIF-1 α has been shown to be active during the acute phase of hypoxia (<0.1% O₂) and HIF-2 α plays a major role later during chronic phases of hypoxia (<5% O₂) (Holmquist-Mengelbier et al., 2006, Koh et al., 2011). Both isoforms have 48% overall amino acid identity and similar protein structure. However, they have distinct target genes and mechanisms of regulation. The difference between HIF-1 α and HIF-2 α is present mostly within the TAD-N domain, which is responsible for the distinct target gene regulation by these isoforms (Hu et al., 2007). The TAD-C is homologous between the two and regulates expression of common target genes (Hu et al., 2007). Apart from many common genes regulated by these members, HIF-1 α preferentially or even specifically induces genes involved in glycolytic pathway (Wang et al., 2005, Hu et al., 2003), and HIF-2 α regulates genes important for tumour growth, cell cycle progression and maintaining stem cell pluripotency such as c-Myc (Gordan et al., 2007) and Oct-4 (Covello et al., 2006). The targeted disruption of both genes in mice leads to embryonic lethality, but they show differential phenotypes. HIF-1 α ^{-/-} mice show severe vascular deformities and embryonic lethality at embryonic day (E) 10.5 (Kotch et al., 1999). HIF-2 α ^{-/-} embryos display severe vascular defects and abnormal lung maturation, and mostly die between E9.5 and E13.5, but sometimes survive postnatally and die from fetal respiratory distress syndrome (RDS) due to insufficient surfactant production by alveolar type 2 cells (Compornolle et al., 2002, Peng et al., 2000).

Under normoxic conditions, the ODD domain of HIF-1/2 α controls its degradation (Huang et al., 1998). Furthermore, Huang *et al.* have demonstrated that HIF-1 α without its entire ODD domain generates stable HIF-1 α and is capable of heterodimerisation, DNA binding and transactivation independent of oxygen availability (Huang et al., 1998). The rapid degradation of HIF-1/2 α under normoxia involves hydroxylation of two proline residues Pro564 (Yu et al., 2001, Ivan et al., 2001, Jaakkola et al., 2001) and Pro402 (Masson et al., 2001) in HIF-1 α and Pro531 and Pro405 in HIF-2 α (Koh and Powis, 2012)) within the ODD domain. Each site contains a common motif

LXXLAP (where A is alanine, L is leucine, P is proline and X is any amino acid), hydroxylated by three mammalian prolyl-hydroxylases, namely PHD1, 2 and 3, in a reaction requiring oxygen, ferrous ions (Fe^{2+}) and 2-oxoglutarate (Bruick and McKnight, 2001, Epstein et al., 2001). However, PHD2 has been shown to be the most critical form for HIF-1 α hydroxylation as demonstrated by Berra *et al.* (Berra et al., 2003), as knocking down PHD2 with short interfering RNA was sufficient to stabilise and activate HIF-1 α under normoxic conditions in the human cells examined, including CAL51 (breast cancer cell line), RCC4/pVHL (RCC4 stably transfected with pCDNA3pVHL), WM9 (melanoma cell line) and FHN (primary fibroblasts). Hydroxylated HIF-1/2 α then interacts with the von Hippel-Lindau protein (pVHL) (Maxwell et al., 1999). pVHL, as part of a multisubunit ubiquitin E3 ligase complex (VHLE3), which shows homology to the SCF (Skp-1-Cdc53/Cullin-F-box) class of E3 ligases (Lisztwan et al., 1999), tags the α -subunit with polyubiquitin allowing recognition by the proteasome and subsequent degradation (Maxwell et al., 1999, Cockman et al., 2000, Kamura et al., 2000). Another hydroxylation mechanism of HIF-1 α at Asn803 and HIF-2 α at Asn851 in the TAD-C via asparagine hydroxylase under normoxic conditions prevents the activation of HIF target genes by blocking recruitment of transcriptional coactivators, CBP and p300 (Lando et al., 2002b). Lando *et al.* have demonstrated that inhibitors of Fe^{2+} and 2-oxoglutarate-dependent dioxygenases can prevent hydroxylation of Asn803 and Asn851 promoting TAD-C to interact with p300 and induce transcription (Lando et al., 2002b). Furthermore, replacement of Asn by Ala resulted in strong transcriptional activity via TAD-C/p300 interaction, suggesting that maximal induction of HIF-1/2 α depends on the abolition of both Pro and Asn hydroxylation during normoxic conditions. Interestingly, studies have revealed that the asparagine hydroxylase is the same as factor inhibiting HIF (FIH) shown to catalyse the hydroxylation of HIF-1 α at Asn803 and corresponding Asn within HIF-2 α (Koivunen et al., 2004, Hewitson et al., 2002, Lando et al., 2002a). However, HIF-1 α is hydroxylated more efficiently than HIF-2 α by FIH due to presence of valine closer to hydroxylated Asp in HIF-1 α in contrast to alanine in HIF-2 α (Bracken et al., 2006). FIH has also been shown to interact with pVHL and recruit histone deacetylases (HDACS) to HIF-1 α , which eventually results in inhibition of HIF-1 α transactivation (Mahon et al., 2001). Besides hydroxylation, acetylation of HIF-1 α at Lys532 by the acetyltransferase ARD1

also directs pVHL binding and HIF-1 α ubiquitination that targets HIF-1 α for proteasomal degradation (Jeong et al., 2002). The receptor of activated protein C kinase (RACK1) is shown to compete with HSP90 for binding to PAS and promotes HIF-1 α degradation in an O₂-independent manner (Liu and Semenza, 2007).

Under hypoxic conditions, the degradation of HIF-1/2 α is inhibited resulting in its rapid accumulation and nuclear translocation followed by subsequent dimerisation with HIF-1 β . This active HIF-1/2 complex binds to hypoxia responsive elements (HREs) containing the 5'-RCGTG-3' sequence (Semenza et al., 1996, Mole et al., 2009) with other transcriptional factors such as CBP/p300 (Arany et al., 1996), resulting in transcriptional activation of hypoxia regulated genes.

1.6.2.2. Regulation of HIF-3 α

Very little is known about HIF-3 α concerning two other HIF- α isoforms. HIF-3 α has high similarity with HIF-1 α and HIF-2 α in the bHLH and PAS domains, but it lacks the TAD-C domain (Gu et al., 1998). The first study to define the role of HIF-3 α in mice demonstrated that IPAS, a splice variant of HIF-3 α has no endogenous transactivation function but shows dominant negative regulation of HIF-mediated gene expression (Makino et al., 2001). In that case, IPAS is predominantly expressed in mice in Purkinje cells of the cerebellum and in corneal epithelium of the eye, and is involved in negative regulation of angiogenesis and maintenance of an avascular phenotype. The HIF-3 α transcript was also observed in heart and lung of hypoxia-induced mice, demonstrating a feedback inhibition of adaptive responses to hypoxia in these specific tissues (Makino et al., 2002). The human HIF-3 α was first characterised in 2001 and shown to suppress hypoxia-inducible HIF-mediated gene expression in the human kidney (Hara et al., 2001). To date there are several HIF-3 α isoforms generated by alternative mRNA splicing (Jang et al., 2005, Maynard et al., 2007, Maynard et al., 2005). Augstein *et al.* study revealed for the first time that HIF-3 α is expressed in human endothelial cells (EC) and vascular smooth muscle cells (VSMC) (Augstein et al., 2011). Overexpression of the endogenously expressed isoforms HIF-3 α 2 and HIF-3 α 3 in EC and VSMC demonstrated that these isoforms are negative regulators able to prevent the overactivation of the HIF pathway.

1.6.3. PGE₂-mediated regulation of HIF-1 α

Interestingly, PGE₂ has been shown to stabilise HIF-1 α protein under both normoxic and hypoxic conditions. Research in PC-3ML human prostate cancer cells (Liu et al., 2002) and in HCT116 human colon carcinoma cells (Fukuda et al., 2003) has demonstrated that PGE₂ and hypoxia act both independently and synergistically to increase HIF-1 α protein levels, but with no effect on HIF-1 α mRNA expression, suggesting that the increase in HIF-1 α protein is due to either increased protein synthesis and/or decreased protein degradation. Liu *et al.* and Fukuda *et al.* have further demonstrated the time-dependent nuclear accumulation of HIF-1 α in response to PGE₂ (Fukuda et al., 2003, Liu et al., 2002), and Fukuda *et al.* have shown that concentrations of PGE₂ ranging from 1 μ M to 100 μ M result in the dose-dependent increase in HIF-1 α protein levels (Fukuda et al., 2003). The PGE₂ induced upregulation of HIF-1 α is mediated through the EP2 and EP4 receptor activation in PC-3ML cells (Liu et al., 2002) and only EP1 receptor activation in HCT116 cells (Fukuda et al., 2003). Several other studies have also demonstrated increased HIF-1 α protein expression in the human gastric adenocarcinoma cell line, AGS (Huang et al., 2005) and an increase in both HIF-1 α mRNA and protein via the EP2 receptor in human endometrium (Critchley et al., 2006) in response to PGE₂. Furthermore, the COX-2 inhibitor NS398 has been shown to decrease the expression of HIF-1 α protein and mRNA in COX-2 positive PC-3 prostate cancer cells and COX-2 negative HCT116 colon cancer cells, and to accelerate HIF-1 α degradation by increasing ubiquitination and promoting the clearance of ubiquitinated protein (Zhong et al., 2004).

1.6.4. HIF-1 α and aromatase expression

Interestingly, the regulation of aromatase by HIF-1 α in the context of placental aromatase regulation has been studied and shown to be inhibitory via distal placental-specific promoter I.1 on the *hCYP19* gene (Kumar and Mendelson, 2011). The oestrogen-related receptor γ (ERR γ), which is upregulated in the human trophoblast during syncytiotrophoblast differentiation, binds to PI.1 and acts synergistically with ER α to stimulate placental aromatase expression through a nuclear receptor element (NRE) at -183 bp (Kumar and Mendelson, 2011). In this study, it was demonstrated that hypoxia leads to the HIF-1 α -dependent downregulation of ERR γ , which is crucial for

oxygen-mediated induction of aromatase expression, and hence inhibition of aromatase expression (Kumar and Mendelson, 2011).

1.6.5. HIF-1/2 α in cancers

Overexpression of HIF-1 α is detected in many different types of human cancers (Zhong et al., 1999, Talks et al., 2000). Zhong *et al.* have shown that HIF-1 α was overexpressed in 13/19 tumour types including colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas using 179 clinical tumour specimens compared to normal tissues, and for the first time, they have reported that HIF-1 α may play an important role in propagation of human cancers (Zhong et al., 1999). HIF-1 α expression in these tumour specimens was correlated with aberrant p53 accumulation and cell proliferation (Zhong et al., 1999). HIF-1 α was also overexpressed in preneoplastic lesions in breast, colon, and prostate but not in benign tumours in breast and uterus (Zhong et al., 1999). Interestingly, 29% of primary breast cancers and 69% of breast cancer metastases also showed HIF-1 α overexpression (Zhong et al., 1999). Yamamoto *et al.* have demonstrated that 63 out of 171 invasive breast cancer cases express HIF-1 α , and this expression is positively correlated with tumour size, lymph node metastasis, tumour stage, histological grade, hormone receptor negativity (oestrogen and progesterone), human epidermal growth factor receptor-2 (HER2), VEGF, COX-2, p53 and Ki67 labelling index (Yamamoto et al., 2008). Furthermore, increased HIF-1 α levels were significantly associated with shorter disease-free survival (SDF) and overall survival (OS), compared to HIF-1 α negative patients according to univariate survival analysis (Yamamoto et al., 2008). Interestingly, HIF-1 α is recognised as an independent prognostic factor in multivariate analysis of DFS and OS (Yamamoto et al., 2008).

Talks *et al.* have demonstrated, by examining 57 cases, that both HIF-1 α and HIF-2 α are expressed in human tumours, including bladder, breast, colon, glial, hepatocellular, ovarian, pancreatic, prostate, and renal, and also very low level expression of both proteins in normal tissues (Talks et al., 2000). However, HIF-2 α was shown to be expressed at higher levels in bone marrow macrophages regardless of the tumour or normal tissues (Talks et al., 2000). A few other studies have also demonstrated that elevated HIF-2 α is associated with poor prognosis in renal cell carcinoma, glioblastoma

and non-small cell lung cancer (Franovic et al., 2009, Holmquist-Mengelbier et al., 2006).

According to previous studies, HIF-1 α is now emerging as a potential target for cancer therapy (reviewed in Melillo, 2007, Semenza, 2003). However, identification of HIF-1 α inhibitors and designing unbiased clinical trials to assess the drug standard and evaluate their efficacy is very challenging. The currently available HIF-1 α inhibitors targeting its mechanism of action including mRNA expression (EZN-2968, aminoflavone), protein translation (topotecan, EZN-2208, digoxin, PX-478), protein degradation (galdanamycin, histone deacetylase inhibitors), DNA binding (echinomycin, doxorubicin, daunorubicin) and transcriptional activity (bortezomib) have been widely described (reviewed in Onnis et al., 2009).

1.7. Treatment for oestrogen receptor positive breast cancer

1.7.1. Endocrine therapy

Two major endocrine therapies including tamoxifen and aromatase inhibitors (AIs) are considered to be highly effective in treating oestrogen receptor positive breast cancers at present, regardless of their side effects. Tamoxifen is a selective oestrogen receptor modulator (SERM), which can act as either an ER antagonist or a partial ER agonist depending on its target tissue (Dutertre and Smith, 2000). AIs block the action of the aromatase enzyme and thus prevent the conversion of androgens into oestrogens.

1.7.1.1. Tamoxifen

Tamoxifen (previously known as compound ICI46474), a non-steroidal, triphenylethylene-based antioestrogen first synthesized in 1993, has been used to treat early and advanced breast cancer following surgery, chemotherapy, radiation or combinations of treatment (Clemons et al., 2002, Jaiyesimi et al., 1995). In the breast, tamoxifen acts via inhibiting the binding of E2 to ER in a competitive manner, which results in blocking the G₁ phase of the cell cycle. Meta-analysis of randomized trials after 5 years adjuvant tamoxifen treatment demonstrates a significant 15-year risk reduction in early breast cancer recurrence and mortality (Davies et al., 2011). Other benefits associated with the use of tamoxifen include the prevention of bone loss in

postmenopausal women at increased risk of developing osteoporosis (Powles et al., 1996), significantly reduced levels of total serum cholesterol, LDL cholesterol and lipoprotein (Love et al., 1994), and a substantial reduction in cardiovascular morbidity (Rutqvist and Mattsson, 1993). However, several adverse effects are known to be associated with tamoxifen use, such as significant loss of bone mineral density in premenopausal women, endometrial cancer, thromboembolism, deep vein thrombosis, stroke, cataract formation and cataract surgery, and as well as short term side effects including hot flushes, irregular menses, vaginal bleeding or discharge, pruritus vulvae, and oedema (Clemons et al., 2002, Powles et al., 1996). More severe side-effects associated with longer-term tamoxifen use have led to limiting adjuvant use to 5 years (Fisher et al., 2001).

1.7.1.2. Aromatase inhibitors

The concept of using aromatase inhibitors (AI) was first developed for the treatment of oestrogen-dependent processes in the 1960s. The first generation aromatase inhibitors, aminoglutethimide and testololactone were discovered nearly 30 years ago (Santen, 2002, Santen and Harvey, 1999). Testololactone wasn't successful as a potent inhibitor; conversely aminoglutethimide was able to block several P450-mediated enzymatic reactions. However, the major drawback of aminoglutethimide in the clinical settings was more severe side effects associated with its use (Santen et al., 1990, Smith et al., 1982). Hence, second and third generation AIs were developed with greater potency compared to first generation AIs.

Third generation AIs consist of two groups, namely steroidal inhibitors, including exemestane (Evans et al., 1992), and nonsteroidal inhibitors, including anastrozole (Yates et al., 1996) and letrozole (Lipton et al., 1995). These inhibitors, exemestane (Coombes et al., 2004), anastrozole (Howell et al., 2005, Forbes et al., 2008) and letrozole (Goss et al., 2003), have been proven superior in reducing breast cancer recurrence compared to tamoxifen, which improves disease-free survival in postmenopausal women. The incidence of severe adverse events such as endometrial cancer and thromboembolic events is also significantly lower with AIs compared to tamoxifen (Mouridsen, 2006). Hence, AIs are gradually replacing tamoxifen as a gold

standard for adjuvant endocrine therapy in treating breast cancer. Interestingly, current guidelines from the USA recommend incorporating AIs at some point during adjuvant treatment, either as up-front therapy or as sequential treatment after tamoxifen for postmenopausal women with hormone receptor-positive breast cancer (Burstein et al., 2010). However, toxicity profiles for AIs include hot flushes, arthralgia and myalgia commonly associated with osteoporosis (Mouridsen, 2006, Gaillard and Stearns, 2011, Muslimani et al., 2009), adverse skin reactions such as erythema nodosum, subacute cutaneous lupus erythematosus, cutaneous rashes, vasculitis (Santoro et al., 2011), cardiovascular events (Bundred, 2005), cognitive defects (Bender et al., 2007) and mood disturbances (Rocha-Cadman et al., 2012).

Letrozole has been found to be a more potent suppressor of whole-body aromatisation and oestrogen levels compared to anastrozole (Geisler et al., 2002, Geisler et al., 2008). The ALIQUOT (Anastrozole vs Letrozole, an Investigation of Quality Of Life and Tolerability) study by Dixon and colleagues has also demonstrated that letrozole suppresses plasma E2 and oestrone sulfate levels to a greater extent than anastrozole (Dixon et al., 2008). They have further extended the ALIQUOT study to evaluate the relationship between suppression of plasma oestrogen levels and BMI in postmenopausal women with early ER-positive breast cancer (Folkerd et al., 2012). Results indicated that the baseline values of E2 and oestrone sulphate are significantly correlated with BMI, and obese women tend to have less suppression of both oestrogens compared to leaner women (Folkerd et al., 2012). Moreover, suppression of E2 and oestrone sulfate is greater with letrozole compared to anastrozole in all weight groups, and the relationship between levels of both oestrogens and BMI is only significant with letrozole treatment (Folkerd et al., 2012).

1.7.2. Breast-specific inhibition of aromatase

As indicated above, third generation aromatase inhibitors are proving superior to tamoxifen in the adjuvant and neoadjuvant settings. Their use, however, results in global inhibition of aromatase within the body and as a result, oestrogen biosynthesis is also inhibited in other body sites besides the breast, such as bone, brain and the cardiovascular system where oestrogens play an important role. In postmenopausal

women, when the ovaries cease to make E2, the principal site of oestrogen formation, which drives the breast cancer growth is primarily the breast. Hence, identification of new drugs that specifically target breast-specific PII would be able to inhibit aromatase expression only within the breast while leaving other sites untouched. Recently, our group has shown that metformin has the ability to inhibit PII-specific aromatase expression in breast ASCs (Brown et al., 2010).

1.7.2.1. Metformin

Metformin is a widely prescribed oral anti-diabetic drug, which has been used for treating patients with type 2 diabetes mellitus (DM2) for many years. Metformin acts primarily by activating AMPK (Zhou et al., 2001), which is regulated by upstream protein kinase LKB1 (Hawley et al., 2003, Lizcano et al., 2004). In observational studies, metformin use is associated with reduced breast cancer risk in patients with diabetes, demonstrating the possible utility of metformin in the management and prevention of breast cancer, and the need for large scale clinical trials to assess the efficacy of metformin in breast cancer patients (Chlebowski et al., 2012, Cazzaniga et al., 2009, Evans et al., 2005, Decensi et al., 2010, Bosco et al., 2011). Interestingly, there are extensive pre-clinical data showing the anticancer effects of metformin on breast cancer. Metformin was found to act as a growth inhibitor rather than an insulin sensitiser in MCF-7 human breast cancer cells, and this metformin-induced growth inhibition could be rescued using siRNA against AMPK (Zakikhani et al., 2006). Further, the metformin-mediated AMPK activation resulted in inhibition of translation initiation and the mammalian target of rapamycin (mTOR) pathway, providing a possible mechanism for its effects on cancer cell growth (Dowling et al., 2007). Phoenix and colleagues have demonstrated that metformin almost completely repressed cell proliferation in ER-positive MCF-7 and T47D, but was less efficient in ER-negative MDA-MB-231 and MDA-MB-435 human breast cancer cell lines (Phoenix et al., 2009). In that case, metformin induced a significant increase in VEGF expression in MDA-MB-435 cells but not MCF-7 cells in an AMPK-dependent manner. Furthermore, MDA-MB-435 xenograft tumours showed increased tumour growth, viability and angiogenesis in response to metformin treatment. On the contrary, nude mice with tumour xenografts of the triple negative cell line MDA-MB-231 showed significant

reductions in tumour growth and cell proliferation with metformin treatment (Liu et al., 2009). Metformin has also been shown to be involved in killing cancer stem cells in four genetically different types of breast cancers using MCF-7, SKBR3, MDA-MB-486 and MCF10A ER-*Src* cells (Hirsch et al., 2009). Cancer stem cells and non-stem transformed cells were defined as CD44^{high}/CD24^{low} and CD44^{low}/CD24^{high}, respectively. Interestingly, combined treatments of metformin and chemotherapeutic agent, doxorubicin, killed both cancer stem cells and non-stem cancer cells in culture, and reduced tumour growth and prolonged remission in a xenograft mouse model. Moreover, metformin was able to reduce the expression of the cancer stem cell marker OCT4 in E2 and endocrine-disrupting chemical 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treated 3-dimensional MCF-7 mammospheres, which represents a human breast cancer stem cell population (Jung et al., 2011). A recent study demonstrated that metformin kills and radiosensitizes cancer cells, and both metformin and irradiation can activate AMPK and suppress mTOR and its downstream targets S6K1 and 4EBP1, which is an important signalling pathway for cancer cell proliferation and survival (Song et al., 2012). Our group has previously examined the effect of metformin, via activation of AMPK, in regulating aromatase expression in breast ASCs (Brown et al., 2010). Metformin significantly downregulated the FSK/PMA-mediated aromatase expression at concentrations of 10 μ M and 50 μ M, increased phosphorylation of AMPK at Thr172 and LKB1 protein levels at 50 μ M, and increased LKB1 promoter activity at 10 μ M and 50 μ M in ASCs. Furthermore, metformin prevented the FSK/PMA-mediated nuclear translocation of CRTC2 at 50 μ M, and it was further confirmed by reporter assays showing a significant reduction in FSK/PMA-mediated aromatase PII activity at 20 μ M and 50 μ M. Hence, metformin inhibits aromatase expression by limiting CREB transactivation and it appears that the actions of metformin on aromatase expression may be promoter-specific.

1.8. Summary

Aromatase is the key enzyme for oestrogen biosynthesis. The baseline level of aromatase in normal breast adipose tissue is maintained primarily through promoter I.4, whereas PGE₂ secreted from malignant breast epithelial cells stimulates aromatase expression via promoter II, leading to increased local concentrations of oestrogens. Aromatase inhibitors, the current most effective endocrine treatment for breast cancer indiscriminately abolish oestrogen synthesis in all tissues, resulting in major side-effects throughout the body. Hence, understanding the cellular and molecular basis of the disease will allow aromatase to be targeted specifically within the breast. Considering the important role of aromatase in breast cancer development and progression, it will be of great interest to elucidate the exact mechanisms whereby CRTC family members and HIF-1 α regulate its expression in ASCs. This may provide an opportunity to develop novel therapeutic strategies, as was discovered for metformin, for breast cancer treatment with significantly fewer side-effects, that specifically target pathways to PII activation in ASCs.

1.9. Hypothesis and objectives

Despite the demonstrated role of CRTC2 in the activation of aromatase expression in ASCs, the role of other CRTC family members and HIF-1 α in regulating PII-driven aromatase expression is not yet characterised. The overall hypothesis of this thesis is that in postmenopausal women, the activation of aromatase PII in the context of breast cancer is regulated by various transcription factors such as CRTC family members and HIF-1 α in response to tumour derived inflammatory mediator, PGE₂. This thesis is also focused on further elucidating the effect of metformin on breast-specific inhibition of aromatase. The specific objectives of this thesis were therefore;

- 1) To examine the regulation of CRTC family members and HIF-1 α in breast ASCs in response to the tumour-derived factor, PGE₂.
- 2) To determine the role of CRTC family members and HIF-1 α in regulating aromatase PII activity via CREs.
- 3) To determine the effect of CREB1 on CRTC family members and HIF-1 α -mediated aromatase PII activity.
- 4) To examine the relationship between HIF-1 α and aromatase expression in tissues from breast cancer patients and cancer-free women.
- 5) To examine the effect of metformin on endogenous PII-, PI.3- and PI.4-specific transcript expression in breast ASCs.

CHAPTER 2:

CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes.

Manuscript submitted to Hormones and Cancer (September, 2012)

Manuscript accepted for publication in Hormones and Cancer after first submitting of the thesis to University (March, 2013)



Declaration for thesis chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed laboratory work for all the figures, data analysis, prepared and drafted manuscript	85%

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
Maria M. Docanto	Contributed to laboratory work for figure 1C (ChIP repeat)	
Evan R. Simpson	Involved in conception of certain aspects of project, manuscript revision	
Kristy A. Brown	Involved in conception of project and supervision, manuscript revision	

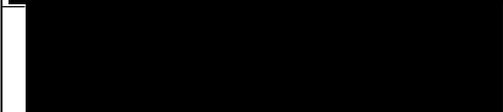
Candidate's Signature		Date 10/12/2012
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Declaration by co-authors

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 of Medical Research (PHIMR), Clayton, VIC, Australia**

Signature 1		Date 13/12/2012
Signature 2		11/12/2012
Signature 3		18/12/2012

Summary of article

Summary:

- The CRTC family consists of three members (CRTC1, CRTC2 and CRTC3). This study examined the contribution of CRTC family members in regulating aromatase expression in the context of postmenopausal breast cancer.
- Results from ChIP, reporter assays and immunofluorescence/confocal imaging demonstrated that FSK/PMA increases binding to aromatase PII and its activity in CREs dependent manner with all three CRTCs, consistent with their nuclear translocation in response to PGE₂ treatment. Moreover, CREB1 shows an additive effect with CRTCs on activating aromatase PII.

Work's contribution to the advancement of science:

- This work demonstrates for the first time that all three CRTC family members stimulate PII-driven aromatase expression in response to PGE₂ in human breast ASCs.
- Overall, CRTCs are identified as possible therapeutic targets for treating obesity-related, postmenopausal breast cancer.

Title

CREB-regulated transcription co-activator family stimulates promoter II-driven aromatase expression in preadipocytes.

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Abstract

The dramatically increased prevalence of breast cancer after menopause is of great concern and is correlated with elevated local levels of estrogens. This is mainly due to an increase in aromatase expression driven by its proximal promoter II (PII). We have previously demonstrated that the CREB co-activator, CRTC2 binds directly to PII and stimulates its activity via mechanisms involving LKB1-AMPK in response to PGE₂. There are three members of the CRTC family (CRTC1-3) and this study aims to characterise the role of other CRTCs on the activation of aromatase PII. The expression and subcellular localization of CRTCs examined in preadipocytes using QPCR and immunofluorescence. Under basal conditions, CRTC1 expression is the lowest and CRTC3 transcripts present at higher levels. Basally, CRTCs are cytoplasmic and PGE₂ causes their nuclear translocation. Reporter assays and ChIP performed to assess the effect of CRTCs on PII activity and binding. The basal PII activity is significantly increased with all CRTCs. FSK/PMA, to mimic PGE₂, results in a further significant increase in PII activity with all CRTCs, as consistent with ChIP data showing an increased binding of CRTCs to PII with FSK/PMA. Interestingly, CRTCs and CREB1 act co-operatively to maximise PII activity and both CREs are essential for the maximal induction of PII activity by CRTCs. Phosphorylation of CRTC2 at its AMPK target site, Ser171, dictates its subcellular localization and the activation of aromatase PII in preadipocytes. In conclusion, this study demonstrated that the overexpression of aromatase in breast preadipocytes involves more than one CRTC.

Keywords

CRTC, prostaglandin E₂, aromatase, breast cancer, adipose stromal cells

Introduction

The incidence of breast cancer cases increases dramatically with advancing age, and 70% of postmenopausal breast cancers are estrogen receptor (ER) positive. This is largely due to an increase in locally produced estrogens within the breast adipose via the enhanced expression of aromatase. This occurs as a result of the promoter switching from the distal promoter I.4 to the alternative proximal promoters II and I.3 on the *CYP19A1* gene in response to tumor-derived factors such as prostaglandin E₂ (PGE₂) [reviewed in 1]. The activity of aromatase PII is largely regulated by the transcription factor CREB1 via cAMP-dependent mechanisms [2]. CREB1 has been shown to bind to two distinct CRE-like sequences, namely CRE1 and CRE2 (approximately 80bp upstream of CRE1), within the PII region of the *CYP19A1* gene using the mouse 3T3-L1 preadipocyte cell line and primary human preadipocytes, and this interaction is increased in the presence of cAMP in 3T3-L1 preadipocytes [2]. The transactivation potential of CREB1 is largely mediated by phosphorylation at Ser133, located in the kinase inducible domain, by protein kinase A (PKA) upon stimulation with cAMP [3]. Obesity is a well-defined risk factor for developing breast cancer [4, 5] and body mass index (BMI) greater than 30 is found to be associated with a two-fold increased risk of breast cancer. Higher estrogen production in postmenopausal women has been shown to account for this obesity-associated breast cancer risk [6]. Interestingly, a recent study has also demonstrated that the high levels of PGE₂ produced from inflamed breast tissues from overweight/obese women correlate with both aromatase expression and activity [7].

Nevertheless, the PKA-mediated phosphorylation of CREB1 at Ser133 is not sufficient to stimulate the activation of all CREB-target genes [8]. A family of CREB co-activators, termed CRTCs (CREB-regulated transcription co-activators) and previously known as TORCs (transducers of regulated CREB activity) has recently been identified using high-throughput screens for modulators of CRE-luciferase reporters [9-12]. There are three members of CRTC family, namely CRTC1, CRTC2 and CRTC3. All three members possess an N-terminal CREB binding domain (CBD), a central regulatory region (REG), a splicing domain (SD) and a C-terminal transactivation domain (TAD) [reviewed in 13]. The highly conserved N-terminal coiled-coil domain of the CRTCs is

known to interact with the bZip domain of CREB [10]. Furthermore, CRTCs have shown to act as co-activators of CREB independent of its phosphorylation status at Ser133 [10]. CRTCs also demonstrate their ability to increase the interaction with the TAF(II)130 component of TFIID, which directs an increase expression of CRE-target genes [10].

Nuclear translocation of CRTC proteins through their lack of phosphorylation or dephosphorylation is a critical and conserved step in the upregulation of CREB-target genes [11]. Moreover, this is induced by increasing intracellular cAMP or calcium levels [11]. Both the nuclear localization sequence (NLS) and nuclear export sequence (NES) motifs are found to be conserved within the three CRTC family members and they tend to shuttle in and out of the nucleus under resting conditions [12]. CRTC2 has been previously shown to be mainly sequestered in the cytoplasm under basal conditions through phosphorylation-dependent interactions with 14-3-3 proteins [12]. In that case, dephosphorylation of CRTC2 at Serine171 (Ser171) in response to cAMP agonists and calcium drives its nuclear translocation by disrupting CRTC2:14-3-3 complexes [12]. AMP-activated protein kinase (AMPK), which acts as a master regulator of energy homeostasis, and salt-inducible kinase (SIK) have been shown to directly phosphorylate CRTC family members [12, 14-16]. Studies undertaken in kidney cells have demonstrated that active AMPK can phosphorylate CRTC2 at Ser171, resulting in its cytoplasmic accumulation [15]. CRTC1 in *C. elegans*, with conserved phosphorylation sites at Ser76 and Ser179, has also been shown to be a direct target of AMPK [16]. SIK1 has the ability to phosphorylate all the CRTCs [14, 15], while other studies have shown that SIK2 can phosphorylate CRTC2 at Ser171 and promote its association with 14-3-3 proteins [12].

We have previously demonstrated that the existence of a relationship between CRTC2 and PII-driven aromatase expression in primary human breast preadipocytes, in the context of post-menopausal breast cancer [17]. In that case, FSK/PMA-mediated repression of LKB1/AMPK led to the nuclear translocation of CRTC2, as well as the increased binding and activation of aromatase promoter PII. Importantly, mutation of the proximal CRE abolished the CRTC2-mediated induction of aromatase promoter PII

activity, confirming that CRTC2 acts primarily via CREB-dependent mechanisms. However, it remains to be clarified as to whether other CRTCs play a role in regulating PII-driven aromatase expression. This study aimed to characterize the role of CRTCs on the activation of aromatase PII in response to tumor-derived and obesity-related factor, PGE₂.

Materials and methods

Plasmids. The *CYP19A1* PII-516 luciferase reporter plasmid, which contains 502bp (-516 to -14) of the proximal promoter PII was generated as previously described [18]. The pCMV.CREB1 plasmid was purchased from Promega (USA). The Flag tagged CRTC-pcDNA vectors were obtained from Mark Montminy from Salk Institute, generated as described previously [10].

Mutagenic primers for CRE mutants of PII luciferase reporter plasmid and phosphorylation site mutants of CRTC2 were designed using Agilent Technologies's web-based QuickChange Primer Design Application available at website <https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15>. Designed primer sequences were proxy CRE mut PII F:

5'-TTTGGCTTTCAATTGGGAATGGAATTCACCTCTACCCACTCAAGGGC-3',
proxy CRE mut PII R: 5'-GCCCTTGAGTGGGTAGAGTGAATTCCATTCCCAATTGAAAGCCAAA-3', distal
CRE mut PII F: 5'-CCTAAACAAAACCTGCTGATGGATTCACAAAATGACTCCACCTCT-3', distal
CRE mut PII R: 5'-AGAGGTGGAGTCATTTTGTGAATCCATCAGCAGGTTTTGTTTAGG-3', CRTC2
S171A F: 5'-CACTTAACAGGACAAGCGCTGACTCTGCTCTTCAC-3', CRTC2
S171A R: 5'-GTGAAGAGCAGAGTCAGCGCTTGTCCTGTAAAGTG-3', CRTC2
S171D F: 5'-TGC ACTTAACAGGACAAGCGATGACTCTGCTCTTCACACA-3'
and CRTC2 S171D R: 5'-TGTGTGAAGAGCAGAGTCATCGCTTGTCCTGTAAAGTGCA-3'. Site directed mutagenesis (SDM) was performed using QuickChange[®] II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer's instructions. Briefly,

thermal cycling was used to generate a mutant plasmid containing staggered nicks using two synthetic primers both containing desired mutation. The product was then treated with DpnI endonuclease to digest parental methylated and hemimethylated DNA. Following digestion, the nicked vector containing the desired mutations was transformed into XL1-blue supercompetent cells.

Cell culture: Primary human breast preadipocytes were isolated after collagenase treatment of whole subcutaneous adipose tissue obtained from women undergoing reduction mammoplasty and cultured in Waymouth's medium (Invitrogen, USA), as previously described [19]. The studies have been approved by Southern Health Human Research Ethics Committee B. COS-7 (African green monkey kidney preadipocyte-like cell line) and 3T3-L1 (mouse preadipocyte cell line) cells were obtained from ATCC, USA and grown in Dulbecco's modified eagle medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin at 37⁰C in 5% CO₂, as recommended. Before treatments, cells were serum starved for 24 hours in DMEM containing 0.1% bovine serum albumin (BSA), 50 U/ml penicillin and 50 µg/ml streptomycin. Treatments included prostaglandin E₂ (PGE₂, 1µM), forskolin (FSK, 25µM)/phorbol 12-myristate 13-acetate (PMA, 4nM) and 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, 0.5mM; an AMPK activator), purchased from Sigma-Aldrich (USA).

Reverse transcription and quantitative PCR (QPCR). Total RNA was extracted from cultured primary human breast preadipocytes in 6-well plates using the RNeasy Mini Kit (QIAGEN, Germany), treated with DNaseI (Ambion, USA) and quantified using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). 0.3 to 1µg of RNA was reverse transcribed using AMV RT Kit using oligo-dT primer (Promega, USA) as directed by the manufacturer. cDNAs were amplified on the LightCycler using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Germany). Quantification of human CRT1, CRT2, CRT3 and housekeeping gene, β-actin transcripts was carried out using primers hCRT1 F: 5'-CAGTCCCAGGAATGGAAGAG-3', hCRT1 R: 5'-GCAGACGGGAAGATGTTGAT-3', hCRT2 F: 5'-TGA CTTCAACCTGGGGAATC-3', hCRT2 R: 5'-GTGGGTCAAGTTCTGGTGGT-

3', hCRTC3 F: 5'-ACAACGTGTGGGAGACCAAGG-3', hCRTC3 R: 5'-GTGTTCAAGGTCCCAAGAA-3', β -actin F: 5'-TGCGTGACATTAAGGAGAAG-3' and β -actin R: 5'-GCTCGTAGCTCTTCTCCA -3'. Cycling conditions were one cycle at 95⁰C for 10 min followed by 40 cycles of 95⁰C for 10 sec, 59⁰C for 5 sec and 72⁰C for 6 sec for CRTC1 and CRTC3, 40 cycles of 95⁰C for 10 sec, 59⁰C for 6 sec and 72⁰C for 6 sec for CRTC2 and 30 cycles of 95⁰C for 10 sec, 59⁰C for 5 sec and 72⁰C for 10 sec for β -actin. All the samples were quantified using standards of known concentrations and corrected for abundance with the housekeeping gene β -actin.

Immunofluorescence and confocal imaging. The subcellular localization of endogenously expressed CRTC proteins in primary human breast preadipocytes was visualized using immunofluorescence and confocal microscopy, as previously described (Brown et al., 2010). The cells were grown on coverslips and treated with PGE₂ for 24 hours. Immunofluorescence was performed using CRTC1 (2501, 1/200 dilution), CRTC2 (3826, 1/200 dilution) and CRTC3 (2768, 1/200 dilution) antibodies from Cell Signaling Technology, USA and lamin B1+B2 antibody (1/1000 dilution, Abcam, USA) for nuclear stain and visualized using secondary antibodies, alexa fluor-546 (red) and -488 (green) from Invitrogen (USA), respectively, using confocal microscopy (Olympus Optical Co Ltd, Japan). In order to visualize the subcellular localization of phosphorylation site mutants of CRTC2, 3T3-L1 cells were transfected with 2 μ g of GFP-tagged CRTC2 mutants using Amaxa Cell line Nucleofector kit V, program T-030 (Lonza Cologne GmbH, Germany) as directed by manufacturer.

Chromatin immunoprecipitation (ChIP). ChIP was performed on primary human breast preadipocytes treated with FSK/PMA for 45 min, to examine the endogenous binding of CRTCs to aromatase PII using the ChIP-IT express kit (Active Motif, USA) according to manufacturer's guide with minor modifications (Brown et al., 2009). Briefly, cells were fixed in PBS containing 1% formaldehyde, followed by termination of reaction by adding glycine. Isolated chromatin was then sheared using sonication at 20% amplitude, 7 times for 30 sec pulses. Immunoprecipitation performed overnight with CRTC1 (sc-46270X, Santa Cruz Biotechnology, USA), CRTC2 (sc-46272X, Santa Cruz Biotechnology, USA), CRTC3 (2768, Cell Signaling Technology, USA)

antibodies and IgG as a control. Immunoprecipitated and reverse cross-linked chromatin was analysed by QPCR using primers flanking the CREs of *CYP19A1* PII (PII-ChIP-F: 5'-TTTCCACACTACCGTTGGCCG-3' and PII-ChIP-R: 5'-GGCAATCTTCTTCCCTTGAAGC-3'), and the reaction was stopped within the linear range of amplification. The products were then run on agarose gel and visualized by ethidium bromide staining.

Reporter gene assays. COS-7 cells, grown in 6-well plates were transfected with either 470 ng of the wild type *CYP19A1* PII-516 luciferase reporter construct, or proximal, distal or double CRE mutated *CYP19A1* PII-516 luciferase reporter constructs, together with 35 ng of CRTCs-pcDNA or CREB1-pcDNA. 3T3-L1 cells were transfected using Cell line Nucleofector kit V, program T-030 (Lonza Cologne GmbH, Germany), with 1 µg of *CYP19A1* PII-516 luciferase reporter construct and 1µg of wild type (wt) or phosphorylation site mutant of CRTC2, as directed by the manufacturer. β-galactosidase control vector was co-transfected into COS-7 cells. Transfected cells were serum starved for 24 hours, followed by 24 hours treatment with FSK/PMA. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's guide and data was normalized to β-galactosidase activity in COS-7 cells, and total protein using Pierce BCA protein assay kit (Thermo Scientific, USA) in 3T3-L1 cells.

Statistical analysis. All data are reported as mean ± standard error (SE) and analyzed by student's t test of group means using GraphPad Prism version 5.00 (GraphPad, La Jolla, CA, USA). Statistical significance was defined as *, P<0.05; **, P≤0.005; ***, P≤0.0005, ****, P≤0.0001.

Results

CRTCs bind to and activate aromatase PII in primary breast preadipocytes.

Relative levels of CRTC transcripts and the effect of PGE₂ on transcript expression of CRTCs were examined in primary breast preadipocytes. Under basal conditions, CRTC3 was expressed at higher levels than CRTC2 followed by CRTC1 (Fig. 1A). CRTC2 mRNA was significantly decreased in response to PGE₂, and no change

observed for CRTC1 and CRTC3 with the treatment (Fig. 1A). The subcellular localization of endogenously expressed CRTCs was examined using immunofluorescence and confocal microscopy in primary breast preadipocytes after treatment with PGE₂. Under resting conditions, all the CRTCs were mainly found within the cytoplasm and most of CRTC1 being localized in the perinuclear area. Interestingly, with PGE₂ treatment, all three CRTCs were translocated to the nucleus (Fig. 1B). To demonstrate the interaction between CRTCs at the aromatase PII, Chromatin immunoprecipitation was utilized (Fig. 1C). Treatment with FSK/PMA, a mimetic of PGE₂, resulted in an increase binding of three CRTCs to aromatase PII compared to vehicle control, consistent with the nuclear import of CRTCs in the presence of PGE₂. In order to assess the effect of the CRTCs on aromatase PII activity, reporter assays were carried out in COS-7 cells (Fig. 1D). Cells transfected with each CRTC resulted in an increase in PII activity compared to pcDNA control vector under resting conditions. Moreover, treatment with FSK/PMA caused a further significant increase in PII activity with all three CRTCs, and CRTC2 had the highest fold-induction of PII activity with the treatment compared to CRTC1 and CRTC3, although CRTC3 showed the highest induction.

CREB1 shows an additive effect with CRTCs on aromatase PII activity. In order to determine the combined effect of CREB1 and CRTCs on aromatase PII activity, reporter assays were performed in COS-7 cells (Fig. 2). Results demonstrated that the cells transfected with CREB1 led to a significant increase in PII activity, and the effect of each CRTC family member on the CREB1-mediated aromatase PII activity was additive.

Both CREs on aromatase PII contribute to the maximal induction by CRTCs. To determine the contribution of each CRE to the CRTC-mediated activity of aromatase PII, reporter assays were performed using CRE-mutant reporter constructs in COS-7 cells (Fig. 3). There was a significant reduction in promoter activity in cells transfected with CRTC constructs and proximal CRE-mutated or distal-CRE mutated PII reporter constructs compared to wt PII reporter construct in response FSK/PMA. Interestingly, the effect on PII activity was further reduced significantly in cells transfected with

double CRE-mutated PII reporter construct compared to cells transfected with CRTCs constructs and proximal or distal CRE-mutated PII reporter constructs with FSK/PMA treatment, suggesting that both CREs are essential for the maximal induction of PII activity with CRTCs.

Phosphorylation of CRTC2 at Serine 171 dictates its subcellular localization and the activation of aromatase promoter II in preadipocytes. The effect of mutating CRTC2 at Ser171 (AMPK phosphorylation site) on its subcellular localization (Fig. 4A) and on the activation of PII (Fig. 4B) was examined in 3T3-L1 preadipocytes using immunofluorescence/confocal imaging and reporter assays, respectively. Results demonstrated that the CRTC2-GFP fusion protein was mainly localized in the cytoplasm under resting conditions, however FSK/PMA treatment, resulted in its nuclear translocation. The S171A mutant, that mimics a dephosphorylated state, was nuclear under both resting conditions and after AICAR treatment, which stimulates AMPK activity. Conversely, the S171D mutant, that mimics a phosphorylated state, was cytoplasmic under both resting conditions and after FSK/PMA treatment. PII luciferase assays revealed that treatment of wt CRTC2-transfected cells with FSK/PMA led to a significant increase in PII activity. Interestingly, transfection with the S171A mutant in the absence of FSK/PMA resulted in PII activity similar to that observed in cells transfected with wt CRTC2 treated with FSK/PMA. Conversely, the S171D mutant was unable to cause an increase in PII activity, even in the presence of FSK/PMA.

Discussion

This study provides evidence that all three CRTC family members can activate aromatase PII in a CRE-dependent manner, together with CREB1, in the context of postmenopausal breast cancer. Recent advances in the treatment of breast cancer using aromatase inhibitors (AIs) have led to the decline in breast cancer-associated mortality, however, inhibition of aromatase throughout the body leads to unwanted side-effects. Understanding the cellular and molecular mechanisms behind the PII-specific expression of aromatase will allow estrogen production to be targeted specifically within the breast, thereby circumventing any currently observed side-effects associated with AIs. Therefore, this study identifies the CRTC-CREB interaction as a potential

therapeutic target for the treatment of obesity-related, estrogen-dependent, postmenopausal breast cancer.

The expression and regulation of CRTCs in preadipocytes were previously not well characterized. In this study, we demonstrate that CRTC3 transcript is highly abundant and CRTC1 is relatively low. In response to PGE₂, known to be elevated within breast tissue in obesity and cancer, CRTC2 transcripts were significantly reduced, but no significant effect on CRTC1 and CRTC3 transcript expression was detected. This could be due to negative feedback mechanisms on CRTC2 transcripts via the activation of CREB1 expression in response to PGE₂ and further studies will be required to elucidate the mechanisms behind this observation. Moreover, FSK/PMA, to mimic PGE₂, results in an increase binding of each CRTC to aromatase PII, and also an increase in PII activity in preadipocytes, as consistent with the nuclear translocation of all three CRTCs with PGE₂. This is consistent with the previous findings by Wang *et al.* demonstrating that CRTC2 is recruited to the CREs on the PEPCK promoter with FSK treatment [21].

CREB1 is found to be overexpressed in breast cancer, prostate cancer, non-small-cell lung cancer and acute leukemia [22]. CREB1 transcript expression has been shown to be almost five times higher in adipose tissue of breasts containing a tumor as compared to normal breast adipose [2]. Study by Chhabra *et al.* reported that CREB1 is expressed in both tumor and normal breast adipose tissues, with higher levels in tumor tissues [23]. CREB1 transcript expression is also found to be higher in ductal carcinoma as compared to lobular and other breast carcinoma, node-positive tumors compared to node-negative tumors, and patients with a poor prognosis and with metastasis compared to cancer-free patients [23]. Moreover, breast cancer patients with higher CREB1 levels are shown to have significantly shorter disease-free survival [23]. In the current study, we have demonstrated for the first time that CREB1 and each CRTC act additively to induce aromatase PII. Given the importance of overexpression of CREB1 in cancer development and progression, this additive effect of CREB1 and CRTCs helps to further clarify the mechanism of action and the effects behind it.

Proximal and distal CREs within aromatase PII have been previously reported to play a pivotal role in CREB-mediated PII activation [2]. Interestingly, the current report shows that both proximal and distal CREs are essential for the maximal induction of aromatase PII using reporter assays with mutated proximal and distal CRE sites. Moreover, mutational analysis and reporter assays revealed that the phosphorylation of CRTC2 at Ser171 dictates its subcellular localization and directly affects its ability to activate aromatase PII. Taken with previous results demonstrating that PGE₂ leads to a decrease in AMPK activity and as a consequence the increased nuclear localization of CRTC2 [17], this study identifies the LKB1/AMPK/CRTC2 pathway as a potential target for the breast cancer treatment. Moreover, the overexpression of LKB1 has been shown to be associated with cytoplasmic sequestration of CRTC2 with FSK/PMA treatment, and knockdown of LKB1 resulted in the nuclear translocation of CRTC2 [17].

In summary, we have identified that all three members of the CRTC family contribute to an increase in PII activity, in conjugation with CREB1, in a CRE-dependent manner, suggesting a critical role for CRTCs in regulating aromatase expression in human breast preadipocytes. However, additional studies are required to test the possibility of targeting CRTC-CREB interactions to treat and possibly to prevent obesity-related, postmenopausal breast cancer.

Acknowledgements

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Figure legends

Fig. 1 Role of CRTCs in aromatase PII activation. (A) Relative abundance of CRTC transcripts in primary breast preadipocytes with and without PGE₂ treatment. (B) Confocal images of immunofluorescence on endogenous CRTC proteins (green) in primary breast preadipocytes with and without PGE₂ treatment, lamin B1+B2 nuclear stain in red. (C) Chromatin immunoprecipitation (ChIP) showing endogenous binding of CRTCs to aromatase PII in human breast preadipocytes with and without FSK/PMA treatment. (D) Reporter assays demonstrating the effect of CRTCs on aromatase PII activity with and without FSK/PMA treatment. vc: vehicle control, RLU: relative luciferase units, β -gal: β -galactosidase activity. Mean \pm SEM, n=3 for QPCR and reporter assays, confocal images are representative of the majority of cells examined, scale bar represents 20.0 μ m, all experiments repeated twice.

Fig. 2 Role of CRTCs and CREB1 in the regulation of aromatase PII activity. Reporter assays demonstrating the effect of CREB1 with (A) CRTC1, (B) CRTC2 and (C) CRTC3 on aromatase PII activity with or without FSK/PMA treatment. RLU: relative luciferase units, β -gal: β -galactosidase activity. Mean \pm SEM, n=3, repeated twice.

Fig. 3 Role of CREs in the activation of aromatase PII. Reporter assays demonstrating the effect of proximal and distal CREs with (A) CRTC1, (B) CRTC2 and (C) CRTC3 on aromatase PII activity with or without FSK/PMA treatment. RLU: relative luciferase units, β -gal: β -galactosidase activity. Mean \pm SEM, n=3, repeated twice.

Fig. 4 Effect of phosphorylating CRTC2 at Ser171 on its subcellular localization and the activation of aromatase PII in preadipocytes. (A) Confocal images on overexpressed GFP-tagged wt, S171A and S171D of CRTC2 (green) and immunofluorescence on lamin B1+B2 nuclear stain in red, with or without FSK/PMA or AICAR treatment. (B) Reporter assays demonstrating the effect of wt, S171A and S171D of CRTC2 on aromatase PII activity with or without FSK/PMA treatment. vc: vehicle control, RLU: relative luciferase units, β -gal: β -galactosidase activity. Mean \pm SEM, n=3 for reporter assays, confocal images are representative of the majority of cells examined, scale bar represents 50.0 μ m, all experiments repeated twice.

Figures

Fig. 1

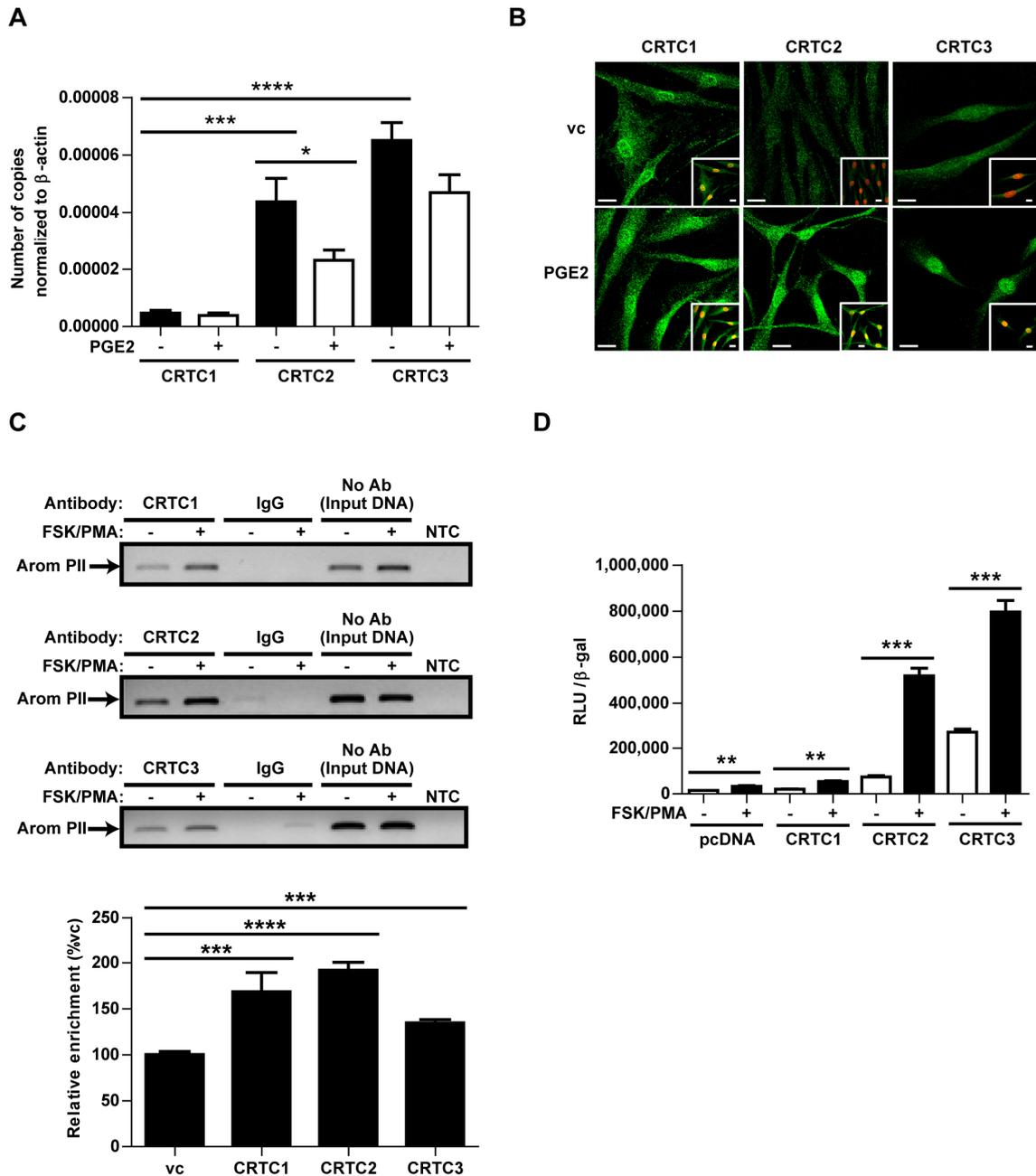


Fig. 2

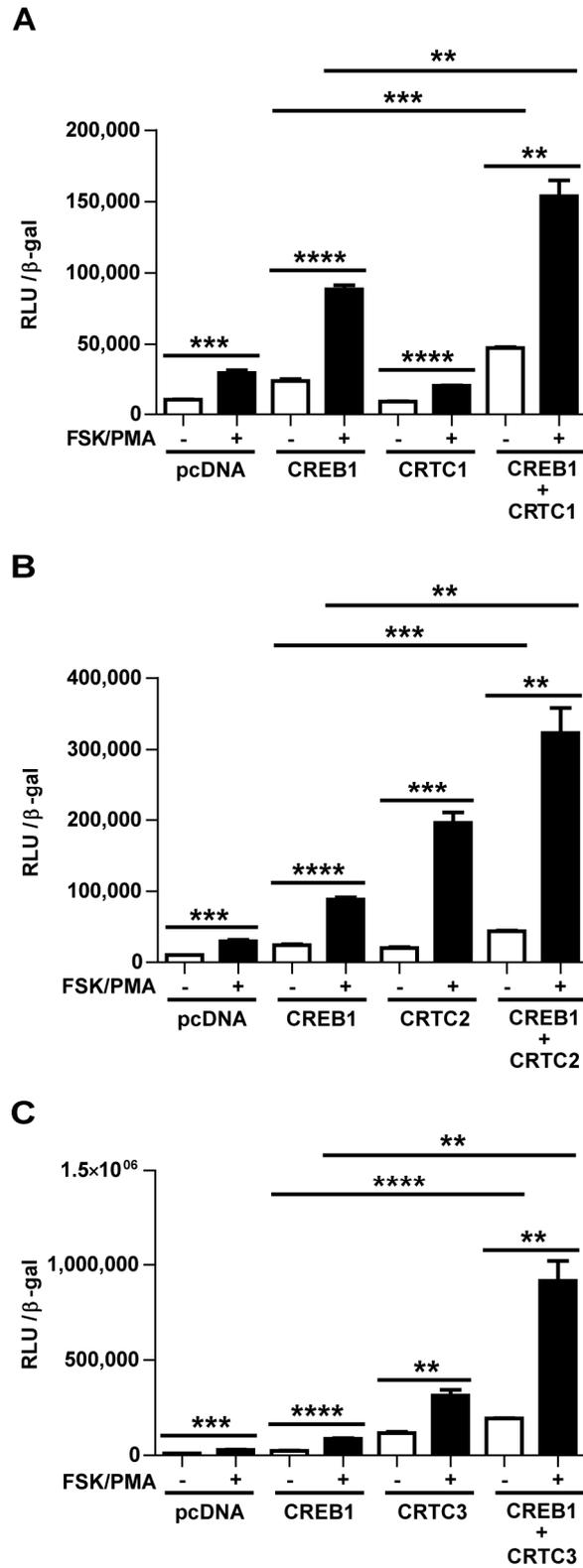


Fig. 3

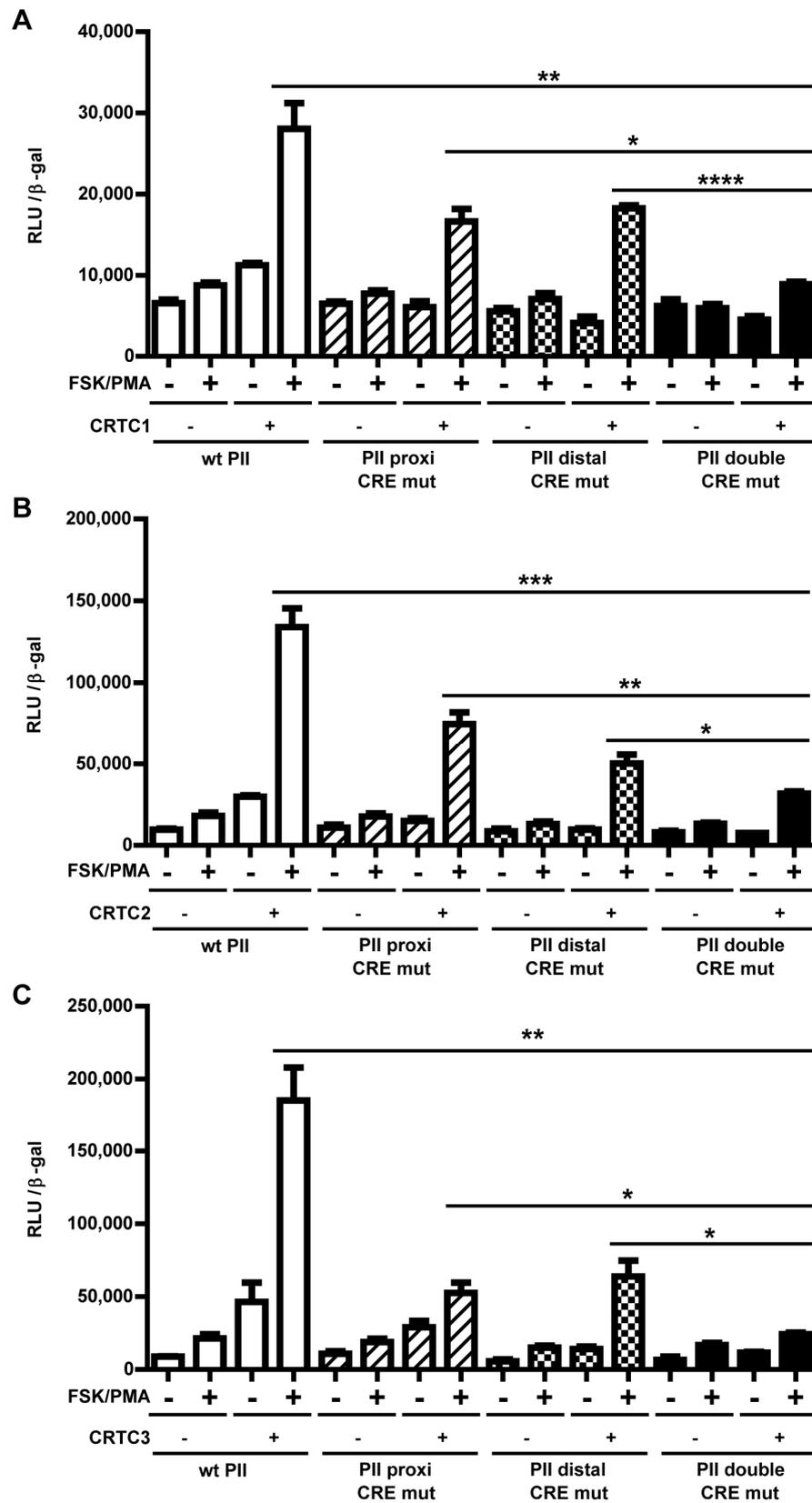
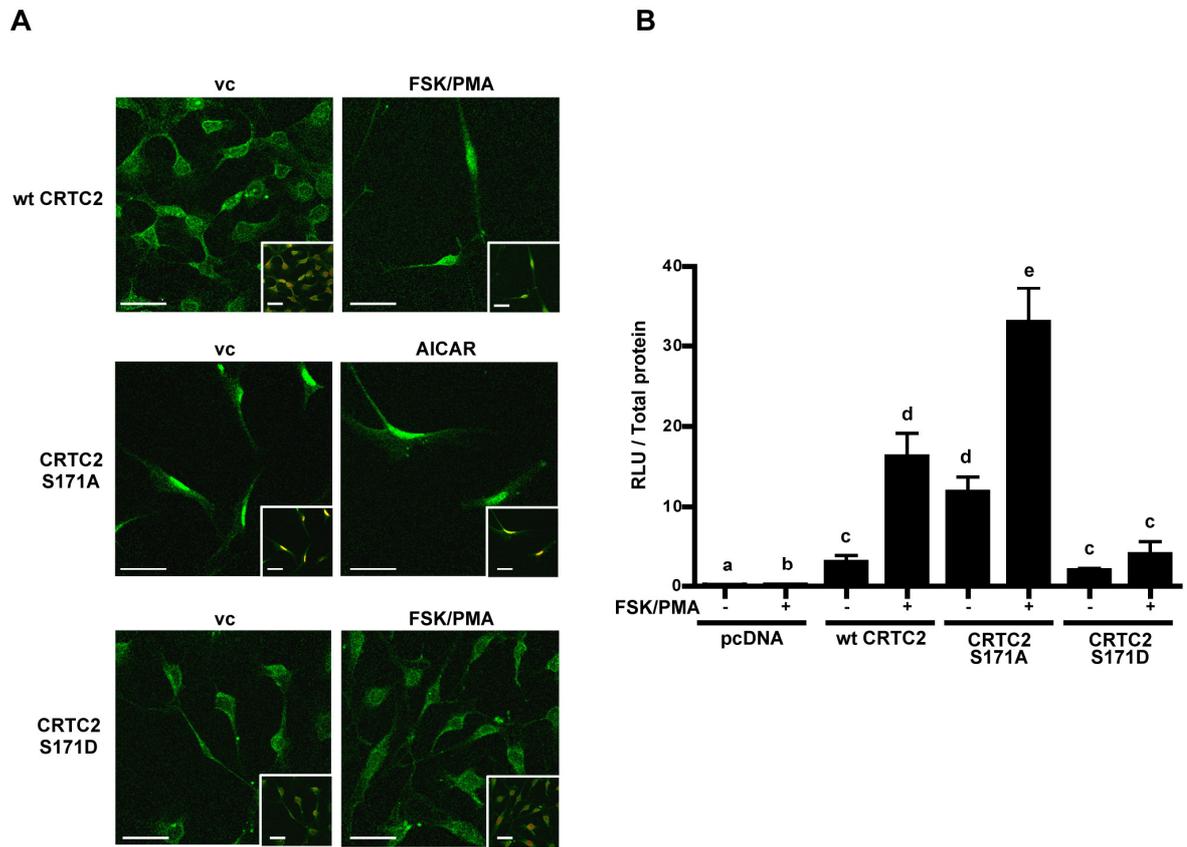


Fig. 4



CHAPTER 3:

HIF-1 α stimulates aromatase expression driven by prostaglandin E₂ in breast adipose stroma.

Manuscript submitted to Breast Cancer Research (September, 2012)

*Manuscript accepted for publication in Breast Cancer Research after first submitting
of the thesis to University (April, 2013)*



Declaration for thesis chapter 3

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 (%)
Performed laboratory work for all the figures, data analysis, prepared and drafted 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
Fangyuan Yang	Contributed to laboratory work for Figure 1 (high content screening) and counting immunostained slides	
Maria M. Docanto	Contributed to laboratory work for Figure 1 (QPCR)	
Minako Sakurai	Contributed to counting immunostained slides	2%
Keely M.	Contributed to counting immunostained	

McNamara	slides	
Hironobu Sasano	Provided FFPE sections of breast cancer patients and their data, manuscript revision	
Stephen B. Fox	Assisted with HIF-1 α staining, manuscript revision	
Evan R. Simpson	Involved in conception of project, manuscript revision	
Kristy A. Brown	Involved in conception of project and supervision, manuscript revision, establishment of collaboration, assisted with counting immunostained slides	

Candidate's Signature		Date 10/12/2012
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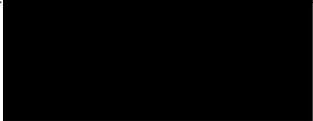
Declaration by co-authors

The undersigned hereby certify that:

- (7) 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.
- (8) 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;
- (9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (10) there are no other authors of the publication according to these criteria;

- (11) 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
- (12) 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 of Medical Research (PHIMR), Clayton, VIC, Australia**

Signature 1		Date 11/12/2012
Signature 2		13/12/2012
Signature 3		12/12/2012
Signature 4		12/12/2012
Signature 5		10/12/2012
Signature 6		13/12/2012
Signature 7		11/12/2012
Signature 8		18/12/2012

Summary of article

Summary:

- This study examined the regulation of aromatase expression via HIF-1 α under normoxic conditions in the context of postmenopausal breast cancer.
- The regulation of PII-driven aromatase expression in human breast ASCs was studied with the treatment of PGE₂. Results from RT-PCR, Western blot, immunofluorescence/confocal imaging and ChIP revealed that PGE₂ stimulates HIF-1 α transcript and protein expression, nuclear localisation and binding to aromatase PII in breast ASCs. Reporter assays demonstrated that HIF-1 α contributes to the maximal induction of aromatase PII in a proximal CRE dependent manner and acts cooperatively with CREB1.
- Immunohistochemical data confirmed *in vitro* data showing a significant increase in HIF-1 α positive ASCs in tissues obtained from breast cancer patients compared to cancer free women, and a positive association between HIF-1 α and aromatase expression.

Work's contribution to the advancement of science:

- This work demonstrates for the first time that HIF-1 α stimulates PII-driven aromatase expression in response to PGE₂ in breast ASCs under normal oxygen tension.
- Overall, HIF-1 α is identified as a potential therapeutic target for treating obesity-related, postmenopausal breast cancer.

Title

HIF-1 α stimulates aromatase expression driven by prostaglandin E₂ in breast adipose stroma.

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Running Title

HIF-1 α stimulates aromatase in breast cancer.

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Total number of figures and tables: 4 Figures + Supplementary Figure and Table

Abstract

Introduction: The majority of postmenopausal breast cancers are estrogen-dependent. Tumor-derived factors such as prostaglandin E₂ (PGE₂) stimulate CREB1 binding to cAMP response elements (CREs) on aromatase promoter II (PII), leading to the increased expression of aromatase and biosynthesis of estrogens within human breast adipose stromal cells (ASCs). Hypoxia inducible factor-1 α (HIF-1 α), a key mediator of cellular adaptation to low oxygen levels, is emerging as a novel prognostic marker in breast cancer. We have identified the presence of a consensus HIF-1 α binding motif overlapping with the proximal CRE of aromatase PII. However, the regulation of aromatase expression by HIF-1 α in breast cancer has not been characterized. This study aimed to characterize the role of HIF-1 α in the activation of aromatase PII.

Methods: HIF-1 α expression and localization were examined in human breast ASCs using QPCR, Western blotting, immunofluorescence and high content screening. Reporter assays and CHIP were performed to assess the effect of HIF-1 α on PII activity and binding. Treatments included PGE₂ or DMOG (HIF-1 α stabilizer). Double immunohistochemistry for HIF-1 α and aromatase was performed on tissues obtained from breast cancer and cancer-free patients.

Results: Results indicate that PGE₂ increases HIF-1 α transcript and protein expression, nuclear localization and binding to aromatase PII in human breast ASCs. Reporter assays demonstrate that HIF-1 α significantly increases PII activity in the presence of DMOG and/or PGE₂, and HIF-1 α and CREB1 act co-operatively on PII. There is a significant increase in HIF-1 α positive ASCs in breast cancer patients compared to cancer-free women, and a positive association between HIF-1 α and aromatase expression.

Conclusions: This study is the first to identify HIF-1 α as a modulator of PII-driven aromatase expression in human breast tumor-associated stroma and provides a novel mechanism for estrogen regulation in obesity-related, post-menopausal breast cancer. Together with our on-going studies on the role of AMP-activated protein kinase

(AMPK) in the regulation of breast aromatase, this work provides another link between dysregulated metabolism and breast cancer.

Keywords

HIF-1 α / prostaglandin E₂ / aromatase / breast cancer / adipose stromal cells

Introduction

Epidemiological studies indicate that the proportion of estrogen-dependent breast cancer cases is dramatically increased in postmenopausal women and this, despite low levels of estrogens found in the circulation. Postmenopausally, breast cancer risk also increases with obesity [1]. After menopause, when the ovaries no longer produce measurable amounts of estrogens, an increase in locally produced estrogens within the tumor and surrounding adipose tissue is believed to drive tumor growth via the action of markedly high levels of aromatase enzyme [reviewed in 2]. The enhanced local expression of aromatase within the breast is mediated via promoter switching from distal promoter I.4 to the alternative proximal promoter II (PII) on the *CYP19A1* gene, in response to inflammatory mediators derived from the tumor, such as prostaglandin E₂ (PGE₂) [3, 4]. A recent study demonstrated that PGE₂ is also increased in breast tissues from overweight and obese women and is associated with higher aromatase transcript expression [5]. One of the transcription factors shown to be involved in this process is cAMP response element (CRE) binding protein-1 (CREB1) which binds to proximal and distal CREs on PII, and stimulates the expression of aromatase [6]. CREB1-coactivators including CRTC2 [7], CBP [8] and p300 [9] are also known to regulate PII-driven aromatase expression.

Many breast cancers are associated with heterogeneously distributed hypoxic tissue areas within the tumor mass [10] and hypoxia inducible factor-1 α (HIF-1 α) is found to be a key mediator of hypoxia-mediated tumor responses [reviewed in 11]. Previous studies have demonstrated that HIF-1 α is a novel prognostic marker in determining the aggressive phenotype of breast cancer [12, 13] and is emerging as a potential target for cancer treatment [14, 15]. HIF-1 consists of 2 subunits, namely HIF1- α and HIF-1 β which belong to the basic-helix-loop-helix (bHLH) protein family containing a per-aryl

hydrocarbon receptor nuclear translocator-sim (PAS) domain [16]. HIF-1 β is continuously expressed and HIF-1 α is continuously synthesized and degraded under normoxic conditions mainly through ubiquitin-proteasome dependent pathways after hydroxylation by prolyl-hydroxylases (PHDs) [17, 18]. Under hypoxic conditions, HIF-1 is stabilized and binds to core hypoxia response elements (HREs) containing the 5'-RCGTG-3' sequence [19] with other transcription factors such as CBP/p300 via its CH1 domain [20], which results in the transcriptional activation of hypoxia-regulated genes including vascular endothelial growth factor (VEGF), known to promote angiogenesis [reviewed in 21]. In PC-3ML human prostate cancer cells [22] and in HCT116 human colon carcinoma cells [23], it was demonstrated that PGE₂ and hypoxia act both independently and synergistically to increase HIF-1 α protein levels, and further demonstrated the time-dependent nuclear accumulation of HIF-1 α in response to PGE₂.

We have identified a putative HRE which overlaps with the proximal CRE of aromatase PII. These findings led us to hypothesize that HIF-1 α may have a role in regulating aromatase expression in response to the tumor-derived and obesity-associated factor, PGE₂, in breast ASCs.

Materials and methods

Plasmids. The *CYP19A1* PII-516 luciferase reporter plasmid, which contains 502bp (-516 to -14) of the proximal promoter PII was generated as previously described [24]. The HA-HIF-1 α -pcDNA vector (Addgene plasmid 18949) was generated as previously described [25]. The pCMV.CREB1 plasmid was purchased from Promega (USA).

Human tissue, cell culture. Primary human breast ASCs were isolated by collagenase digestion of subcutaneous adipose tissue from women undergoing reduction mammoplasty and cultured in Waymouth's medium (Invitrogen, USA), as previously described [26]. The studies have been approved by Southern Health Human Research Ethics Committee B (#B00109). MCF-7 cells (human breast adenocarcinoma cell line) were cultured in dulbecco's modified eagle medium (Invitrogen, USA). Before treatments, cells were serum starved for 24 hours in medium containing 0.1% bovine serum albumin. Treatments included prostaglandin E₂ (PGE₂, 1 μ M) and

dimethyloxalglycine (DMOG, 100 μ M; prolyl-4-hydroxylase inhibitor which stabilizes HIF-1 α) purchased from Sigma-Aldrich (USA). Sections of formalin-fixed and paraffin-embedded breast tissues from 10 Japanese breast cancer patients (IDC, invasive ductal carcinoma and DCIS: ductal carcinoma *in situ*), with differing hormone receptor status and grade (Supplementary Table 1), and 10 cancer-free women were used for double immunohistochemistry studies. Japanese female patients with IDC and DCIS were obtained from St. Luke's International Hospital (Tokyo). The informed consent being obtained from these patients before surgery and the research protocols were approved by the ethics committee at St Luke's International Hospital (2010-509). All the clinical data were retrieved from relevant patient's file and the histological grade was independently evaluated.

Nuclear extraction and Western blot analysis. Primary breast ASCs were cultured on 10cm plates and treated as described above. Nuclear extracts were obtained as described in Abcam technical website (www.abcam.com/technical) with minor modifications, including addition of cobalt chloride (1mM) and protease inhibitor cocktail tablet-complete mini from Roche (Germany). BCA protein assay (Thermo Scientific, USA) was performed to quantify protein amount according to manufacturer's instructions. 10 μ g of nuclear protein diluted in loading buffer containing β -mercaptoethanol, was run on 10% denaturing polyacrylamide gel and transferred to nitrocellulose membrane. HIF-1 α and histone H3 protein levels were detected using HIF-1 α (sc-10790; 1/200 dilution, Santa Cruz Biotechnology, USA), histone H3 (ab1791; 1/10,000 dilution; Abcam, USA) and Alexa Fluor 700 goat anti-rabbit secondary (1/10,000 dilution, Invitrogen, USA) antibodies using the Odyssey infrared imaging system (LI-COR Biosciences, USA). The intensity of the bands detected from Western blotting was quantified using densitometric analysis.

Reverse transcription and quantitative PCR (QPCR). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany) and 0.3 to 1 μ g of RNA was reverse transcribed using AMV RT Kit using oligo-dT primer (Promega, USA) as directed by the manufacturer. DNA was digested using the DNA-free DNase Treatment and Removal Kit (Ambion, USA). QPCR was performed on the LightCycler using

LightCycler FastStart DNA Master SYBR Green I kit (Roche, Germany). Quantification of human HIF-1 α and housekeeping gene, β -actin transcripts was carried out using primers hHIF-1 α F: 5'-GTACCCTAACTAGCCGAGGAAGAA-3', hHIF-1 α R: 5'-GTGAATGTGGCCTGTGCAGT-3', β -actin F: 5'- TGC GTGACATTAAGGAGAAG-3' and β -actin R: 5'- GCTCGTAGCTCTTCTCCA -3'. Cycling conditions for HIF-1 α and β -actin were one cycle at 95⁰C for 10 min followed by 40 cycles of 95⁰C for 10 sec, 59⁰C for 6 sec and 72⁰C for 4 sec, and 30 cycles of 95⁰C for 10 sec, 59⁰C for 5 sec and 72⁰C for 10 sec, respectively. All the samples were quantified using the standards of known concentrations and corrected for abundance with the housekeeping gene β -actin.

Immunofluorescence and confocal imaging. HIF-1 α protein was visualized in primary breast ASCs using immunofluorescence and confocal microscopy. ASCs were plated onto coverslips and cultured until they reached ~70% confluency. Cells were serum-starved overnight and treated for 24hrs. Immunofluorescence was performed as previously described [27], using HIF-1 α antibody (sc-10790; 1/2000 dilution, Santa Cruz Biotechnology, USA) and lamin B1+B2 antibody (1/1000 dilution, Abcam, USA) for nuclear stain and visualized using alexa fluor-546 (red) and -488 (green) from Invitrogen (USA), respectively, using confocal microscopy (Olympus Optical Co Ltd, Japan).

High content screening. Primary breast ASCs cells were plated in 96-well plates at a density of 6,000 cells per well. One day after plating, the cells were serum-starved for 24 hours and treated with PGE₂ for 6 hours. Fixation was carried out in ice-cold methanol for 40 min at -20⁰C, followed by 2 \times PBS washes. Cells were blocked in 0.5% BSA/PBS for 30 min and incubated with HIF-1 α antibody (sc-10790; 1/2,000 dilution in 0.5% BSA/PBS, Santa Cruz Biotechnology, USA) overnight at 4⁰C with gentle rocking. After 2 \times PBS washes, Alexa fluor 488 goat anti-rabbit secondary antibody (1/1,000 dilution, Invitrogen, USA) and Hoechst nuclear counterstain (1/5,000 dilution) in 0.5% BSA/PBS were applied for 90 min. In order to quantitate nuclear fluorescence, images were captured on an ArrayScan VTI instrument (Thermo Fisher Scientific, USA) and analyzed using Cellomics software and the Compartmental Analysis Bioapplication. The analysis algorithm used Hoechst fluorescence to define a mask used

to measure nuclear fluorescence. Threshold for nuclear staining was determined by assessing nuclear intensity in negative control samples and set to two standard deviations. Cells with nuclear HIF-1 α pixel intensity ≥ 230 were considered HIF-1 α positive.

Chromatin immunoprecipitation (ChIP). Primary breast ASCs treated with PGE₂ or DMOG for 45 min were used for ChIP to examine endogenous binding of HIF-1 α to aromatase PII using the ChIP-IT express kit (Active Motif, USA) as directed by the manufacturer with minor modifications [7]. Briefly, cells were fixed using 1% formaldehyde to cross-link and preserve endogenous protein-DNA interactions. The DNA was then sheared into small fragments using sonication at 20% amplitude, 7 times for 30 sec pulses. Specific protein-DNA complexes were immunoprecipitated using HIF-1 α antibody (Santa Cruz Biotechnology, USA), IgG or water as controls. QPCR was then performed using primers flanking the CREs of *CYP19A1* PII (PII-ChIP-F: 5'-TTCCACACTACCGTTGGCCG-3' and PII-ChIP-R: 5'-GGCAATCTTCTTCCCTTGAAGC-3'), and normalized to input DNA [7].

Reporter gene assays. MCF-7 cells were transfected with wild type or proximal CRE mutated *CYP19A1* PII-516 luciferase construct, with/without human HIF-1 α -pcDNA (Addgene, USA) and/or human CREB1-pcDNA or HIF-1 α siRNA (sc-35561, Santa Cruz Biotechnology, USA), using the cell line nucleofector kit V, program E-014 (Lonza Cologne GmbH, Germany), according to the manufacturer's instructions. β -galactosidase was co-transfected and used as a transfection control. After transfection, cells were serum-starved and treated with PGE₂ and/or DMOG for 24 hours. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's protocol and data was normalized to β -galactosidase activity.

Double immunohistochemistry (IHC). Formalin-fixed paraffin-embedded tissue sections from breast cancer and cancer-free patients were dewaxed in xylene and were rehydrated through descending concentrations of ethanol solutions to distilled H₂O. Tissue sections were incubated in 10% horse serum in CAS-block (Invitrogen, USA) for

30 min. Previously characterized aromatase mouse monoclonal primary antibody 677 (1/250 dilution from 2.6 mg/ml stock in 0.5% BSA/PBS, [28]) was added to the slides and incubated overnight at 4⁰C. After washing in PBS, biotinylated universal secondary antibody (1/200 dilution, Vectastain Universal ABC-AP kit, Vector laboratories, USA) was applied for 30 min and then incubated with Vectastain ABC-AP reagent for 30 min. Alkaline phosphatase substrate (Vector blue alkaline phosphate substrate kit, Vector Laboratories, USA) was added until desired cytoplasmic blue stain intensity developed and the reaction stopped with distilled H₂O.

Slides were then subjected to antigen retrieval in an autoclave, at 121⁰C for 5 min in Tris EDTA pH 9 (10 mM Tris base, 1mM EDTA). After cooling for 30 min, sections were washed in PBS and incubated in 10% horse serum in CAS-block (Invitrogen, USA) for 30 min. The HIF-1 α primary antibody (sc-10790; 1/250 dilution in 0.5% BSA/PBS, Santa Cruz Biotechnology, USA) was added to the slides and incubated overnight at 4⁰C. After washing in PBS, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 minutes. Anti-rabbit IgG secondary antibody (1/1,000 dilution, Vectastain ABC-AP kit-rabbit IgG, Vector laboratories, USA) was applied for 30 min and then incubated with Vectastain ABC-AP reagent for 30 min. Slides were then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, USA) until desired nuclear brown stain intensity developed and the reaction stopped with distilled H₂O. Finally, the sections were mounted with fluorsave reagent (Calbiochem, USA).

Double immunohistochemically stained slides for HIF-1 α and aromatase were evaluated independently by two observers (N.U.S. and M.S. or N.U.S. and K.A.B.). The ASCs were examined using systematic random sampling on a stereology microscope (Olympus, Japan) with the aid of CAST-Grid version 1.60 and categorized into four groups including HIF-1 α positive and aromatase positive, HIF-1 α positive and aromatase negative, HIF-1 α negative and aromatase positive, and HIF-1 α negative and aromatase negative (Supplementary Figure 1).

Statistical analysis. All data were expressed as mean \pm standard error (SE) and analyzed by student's t test of group means. Statistical significance was defined as *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$, ****, $P \leq 0.0001$. Data analysis was performed using GraphPad Prism version 5.00.

Results

PGE₂ increases HIF-1 α expression and nuclear localization in primary human breast ASCs. The effect of PGE₂ on HIF-1 α transcript and protein expression was examined in primary breast ASCs. HIF-1 α mRNA was significantly increased with PGE₂ treatment (Figure 1A). Moreover, Western blotting demonstrated that PGE₂ also caused a significant increase in HIF-1 α protein abundance in the nucleus (Figure 1B). The subcellular localization of endogenously expressed HIF-1 α was examined using immunofluorescence and confocal microscopy after treating with PGE₂ and /or DMOG. Results demonstrated that punctate staining for HIF-1 α appears in the nucleus after PGE₂ treatment (Figure 1C, top right) compared to cytoplasmic localization under basal conditions (Figure 1C, top left). Furthermore, DMOG (Figure 1C, bottom left) and DMOG with PGE₂ (Figure 1C, bottom right) resulted in more intense staining for HIF-1 α in the nucleus. Results from high content screening also demonstrated that the percentage cells positive for HIF-1 α in the nucleus (Figure 1D) was significantly increased in response to PGE₂ treatment.

HIF-1 α binds to and activates aromatase PII in primary breast ASCs. PROMO 3.0 software analysis was used to determine a putative binding site of HIF-1 α on aromatase PII [29, 30]. Results demonstrated that the predicted HRE overlaps with the proximal CRE of aromatase PII (Figure 2A). The endogenous binding of HIF-1 α to aromatase PII was evaluated by ChIP. Treatment with either PGE₂ or DMOG resulted in increased binding of HIF-1 α to aromatase PII compared to vehicle control (Figure 2B).

To determine the effect of HIF-1 α on aromatase PII activity, luciferase reporter assays were performed in MCF-7 cells, transfected with HIF-1 α and wild type or proximal CRE mutated *CYP19A1* PII-516 luciferase reporter constructs and treated with DMOG. Cells transfected with HIF-1 α treated with DMOG and DMOG alone showed a

significant increase in promoter II activity, and this HIF-1 α /DMOG-mediated effect on promoter II activity was completely abolished using a proximal CRE-mutated PII reporter construct (Figure 2C). As the putative HRE overlaps with the proximal CRE on aromatase PII, experiments were performed to determine whether HIF-1 α acted cooperatively or competitively with CREB1. MCF-7 cells were transfected with HIF-1 α and/or CREB1 together with the *CYP19A1* PII-516 luciferase reporter construct, and then treated with DMOG and/or PGE₂. PII activity was significantly increased in HIF-1 α transfected cells in the presence of PGE₂ or DMOG, and treatment with both PGE₂ and DMOG led to a further significant increase in PII activity (Figure 2D). Interestingly, cells transfected with both HIF-1 α and CREB1 showed a further increase in PII activity ($P \leq 0.005$) with both PGE₂ and DMOG compared to cells transfected with either HIF-1 α or CREB1 alone (Figure 2D). Hence, HIF-1 α and CREB1 act cooperatively to increase PII activity. Additional reporter assays were also performed to examine requirement for HIF-1 α for the PGE₂-mediated increase in PII activity. MCF-7 cells transfected with HIF-1 α siRNA showed a significant reduction in aromatase PII activity (Figure 2E; black bars) and the PGE₂-mediated effect on PII via HIF-1 α was suppressed (Figure 2E; white bars).

HIF-1 α is increased in ASCs from tumor-bearing breast tissues compared to cancer free breast tissue. Double immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) tissues from breast cancer patients and cancer-free women. Results demonstrated that there is a significant increase in the percentage of HIF-1 α positive ASCs in breast cancer patients compared to cancer-free women (Figure 3A). Interestingly, ASCs which were HIF-1 α and aromatase double-positive, and which were single-positive for HIF-1 α or aromatase, were significantly increased in tumor patients compared to cancer-free women (Figure 3B). Furthermore, double negative ASCs were significantly decreased in tumor cases compared to normal (Figure 3B) and the percentage of cells either double-positive or double-negative was significantly higher than single-positive cells (analysis not shown), suggesting a positive association between HIF-1 α and aromatase expression.

Discussion

In this study, novel evidence is provided for the regulation of HIF-1 α and its role in regulating aromatase expression in adipose stromal cells in the context of obesity and breast cancer. Namely, that HIF-1 α expression and nuclear localization are increased in response to PGE₂ and stimulate the promoter II-driven expression of aromatase in breast ASCs via binding to the proximal CRE (Figure 4).

HIF-1 α has been shown to be overexpressed in many different types of tumors including those of the ovary, prostate and breast [31]. However, the expression and regulation of HIF-1 α in cancer-associated adipose stromal cells is less well characterized. Here, we demonstrate that HIF-1 α transcript, protein expression and protein nuclear localization are increased in breast ASCs in response to the tumour-derived factor PGE₂. Consistent with these observations, we also demonstrate that in clinical samples, the number of HIF-1 α positive ASCs is increased in breast cancer patient samples compared to cancer-free breast tissue. Despite the limited number of patient samples examined and these being from women of differing age, tumor hormone receptor status, and tumor grade, the dramatic increase observed was highly conserved amongst all patients. This may be attributable to the majority of breast tumors overexpressing COX-2 and secreting high levels of PGE₂ [32, 33]. Our results are consistent with observations demonstrating that PGE₂ causes the stabilization of HIF-1 α independent of hypoxia in PC-3ML human prostate cancer cells [22], HCT116 human colon carcinoma cells [23] and AGS gastric carcinoma cells [34].

The PGE₂-induced upregulation of HIF-1 α has been shown to be mediated through EP2 and EP4 receptor activation in PC-3ML cells [22] and the EP1 receptor alone in HCT116 cells [23]. In human embryonic kidney cells expressing the human EP1 receptor, PGE₂ has been shown to upregulate HIF-1 α protein expression in a time-dependent manner under normoxic conditions [35]. Interestingly, the expression of EP receptors has been demonstrated in breast ASCs and the regulation of aromatase by PGE₂ in these cells has been shown to be dependent on activation the EP1 and EP2 receptors [4, 36]. These data suggest that aromatase upregulation via EP receptor activation may involve the induction of HIF-1 α . Indeed, HIF-1 α increases the activity of

aromatase PII, and the increased nuclear localization and punctuate appearance of HIF-1 α in response to PGE₂ is also associated with the increased binding of HIF-1 α to aromatase PII via the proximal CRE. The regulation of aromatase by HIF-1 α has also recently been examined in the context of placental aromatase regulation. In that case, aromatase expression is mediated by distal placental-specific promoter I.1 and is dependent on binding of estrogen-related receptor γ (ERR γ) to the promoter [37]. Hypoxia has been shown to cause the HIF-1 α -dependent downregulation of ERR γ [37]. Contrary to our findings, the HIF-1 α -mediated effects on PI.1 are inhibitory and appear to be indirect.

Promoter analysis revealed that a putative core HRE sequence is present on the antisense strand of aromatase PII and overlaps with the proximal CRE, suggesting that HIF-1 α may interact with CREB1 in ASCs. Previous studies have demonstrated that CREB1 binds to the HRE in the plasminogen activator inhibitor-1 (PAI-1) promoter [38] and HIF-1 α can also interact with ATF2/CBP/p300 [39-41]. There is a significant elevation in CREB1 transcript expression in breast tumor tissues compared to non-neoplastic breast tissues and this is positively associated with poor prognosis, metastatic disease and nodal involvement [42, 43]. Interestingly, the present study demonstrates that HIF-1 α alone can stimulate aromatase promoter PII and that it also acts cooperatively with CREB1 to increase aromatase PII activity.

The local biosynthesis of estrogens from breast ASCs is considered a key mediator of tumor cell growth in postmenopausal breast cancer, and increased PII activity accounts for the majority of transcripts detected [3]. We have observed that the majority of ASCs in the tumor-bearing tissue are either double-positive or double-negative for HIF-1 α and aromatase expression, suggesting an association between the two proteins. These findings support our in vitro data demonstrating that HIF-1 α directly stimulates aromatase expression.

Collectively, the results obtained in this study show for the first time that HIF-1 α activates PII-driven aromatase expression in breast ASCs in response to PGE₂, independent of oxygen availability. Hence, this specific association is likely to be an

important mechanism for the regulation of estrogen biosynthesis in obesity-related, postmenopausal breast cancer. Third-generation aromatase inhibitors (AIs) are currently the most effective treatment and have been shown to be superior to tamoxifen for hormone receptor positive postmenopausal breast cancer as aromatase catalyses the conversion of circulating androgenic precursors to estrogens [44-46]. However, many women cease use of AIs due to increasingly severe side-effects associated with their use [47-49]. Currently, small molecule inhibitors of HIF-1 α are being tested in the clinical setting [reviewed in 50]. We believe that better understanding of the regulation of aromatase PII will allow us to target aromatase expression specifically within the breast, leaving sites such as the bone, brain and heart, where estrogens have beneficial effects, unaffected.

Conclusions

This study demonstrates that HIF-1 α , a master regulator of oxygen homeostasis, stimulates PII-driven aromatase expression in human breast ASCs with other transcription factors including CREB1 in response to tumor-derived and obesity-associated inflammatory mediator PGE₂. Our findings of HIF-1 α in tumour-associated breast stroma implicate its potential as a therapeutic target in obesity-related, postmenopausal breast cancer.

Abbreviations

HIF-1 α : hypoxia inducible factor 1 alpha; ASC: adipose stromal cells; PGE₂: Prostaglandin E₂, CREs: cAMP response elements; CREB1: CRE binding protein 1; PII: promoter II, AMPK: AMP-activated protein kinase, bHLH: basic-helix-loop-helix; PAS: per-aryl hydrocarbon receptor nuclear translocator-sim; PHDs: prolyl-hydroxylases; HREs: hypoxia response elements; VEGF: vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

NUS and KAB designed all the experiments. NUS conducted all the experiments, with the help from FY, MMD, MS, KMM, HS and SBF. The paper was written by NUS, and KAB, ERS, SBF and HS were involved in manuscript revision. KAB and ERS contributed to the conception of project.

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Figure legends

Figure 1: Effect of PGE₂ on HIF-1 α expression and nuclear localization in primary human breast ASCs. PGE₂ caused a significant increase in HIF-1 α transcript (A) and nuclear protein expression (B). Confocal microscopy demonstrated that HIF-1 α (green) is mainly perinuclear in breast ASCs under basal conditions (C, top left) and that PGE₂ stimulates the translocation of HIF-1 α to the nucleus (C, top right). Treatment with DMOG (C, bottom left) and DMOG with PGE₂ (C, bottom right) caused a much higher HIF-1 α staining in the nucleus. The merged lamin B1+B2 nuclear stain (red) and HIF-1 α are found as insets at the bottom right of each image. The percentage of cells positive for nuclear HIF-1 α was also shown to be significantly increased with PGE₂ treatment (D). vc = vehicle control, Mean \pm SEM, n=3, repeated twice. Confocal images are representative of the majority of cells examined.

Figure 2: Role of HIF-1 α in aromatase PII activation. (A) A putative HRE (*italic*) was found to overlap with the proximal CRE (*boxed*) of aromatase PII. (B) ChIP demonstrated that PGE₂ and DMOG stimulated the endogenous binding of HIF-1 α to aromatase PII. (C) Reporter assays demonstrated that mutation of the proximal CRE of aromatase PII inhibited the HIF-1 α /DMOG-mediated effect on PII activity. (D) PII activity was significantly increased in HIF-1 α transfected cells, treated with DMOG and PGE₂, and co-transfection with CREB1 resulted in a further increase in PII activity compared to cells transfected with either HIF-1 α or CREB1 alone. (E) HIF-1 α siRNA significantly reduced PII activity and suppressed the PGE₂-mediated effect on PII activity. RLU: relative luciferase units, β -gal: β -galactosidase activity. Mean \pm SEM, n=3 for reporter assays, all experiments repeated twice.

Figure 3: HIF-1 α and aromatase expression in ASCs from tumor-bearing breast tissue compared to cancer-free using immunohistochemistry. (A) Percentage of HIF-1 α positive ASCs from tumor-bearing breast tissue was significantly increased compared to tissue from cancer-free women. (B) Double-positive and single-positive

ASCs for HIF-1 α and aromatase were shown to be significantly increased in tumor-bearing compared to cancer-free tissues. Double negative adipose stromal cells for HIF-1 α and aromatase were significantly reduced in tumor-bearing compared to cancer-free tissues. n = 10 for tumour-bearing breast tissue; n = 10/cancer-free breast tissue.

Figure 4: Model of the PGE₂-mediated regulation of aromatase expression by HIF-1 α in breast ASCs. Tumor-derived factor PGE₂ increases HIF-1 α transcript expression and nuclear localization. HIF-1 α dimerizes with HIF-1 β and then translocates to nucleus where it interacts with proximal CRE of aromatase PII. HIF-1 α together with CREB1, CRTCs, CBP and p300 act to increase the PII-driven expression of aromatase.

Supplementary Figure 1: Classification of ASCs for double immunohistochemistry. (A, B; *arrows*) HIF-1 α and aromatase double positive cells. (C, D; *arrows*) HIF-1 α positive and aromatase negative cells. (E, F; *filled arrows*) HIF-1 α negative and aromatase positive cells. (E, *empty arrow*) HIF-1 α positive and aromatase positive cell. (F, *empty arrow*) HIF-1 α and aromatase double negative cell. Blue color in the cytoplasm as a result of Vector blue colorimetric reaction represents aromatase immunoreactivity while brown color in the nuclei as a result of DAB colorimetric reaction represents HIF-1 α immunoreactivity.

Figures

Figure 1

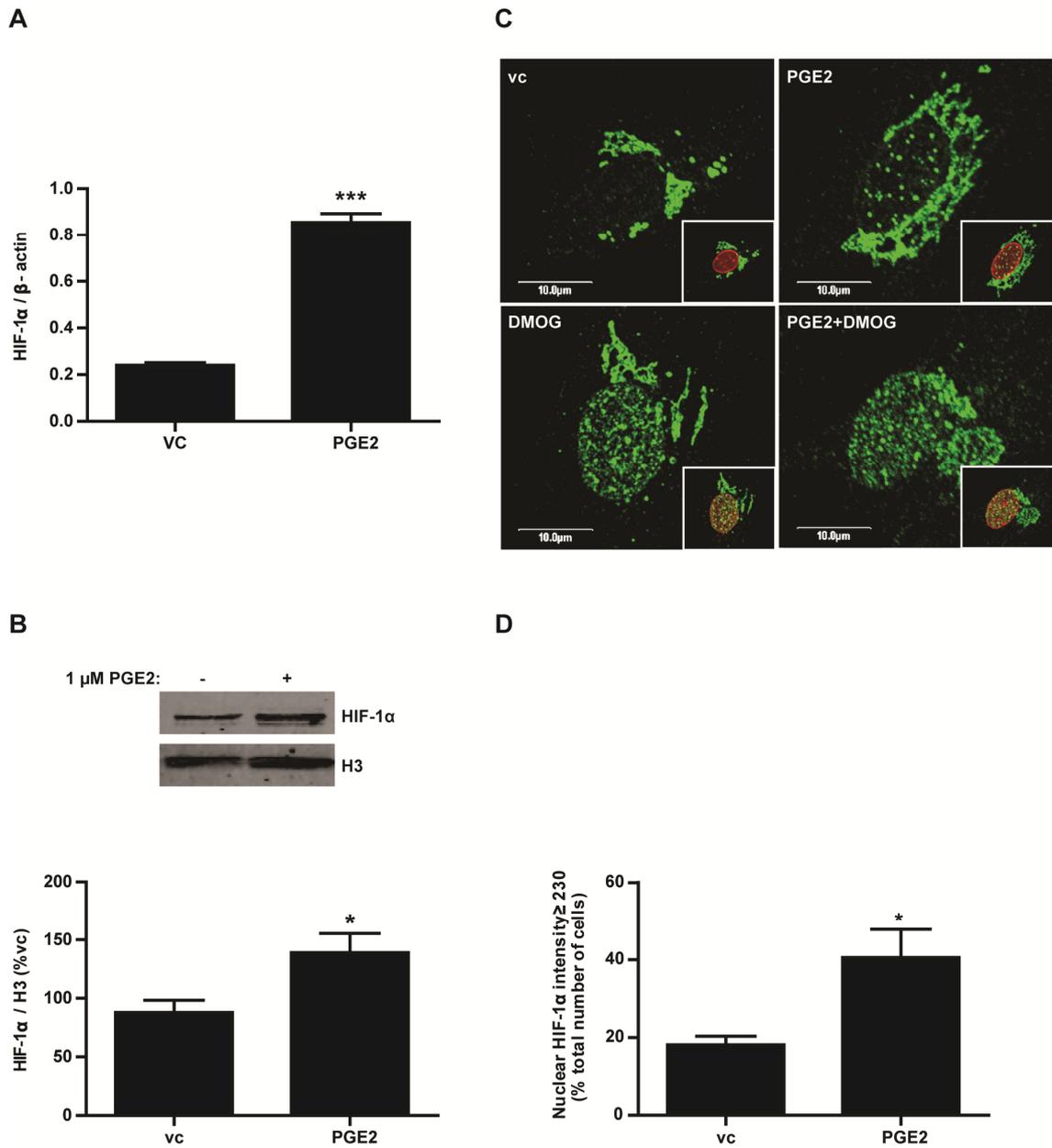


Figure 2

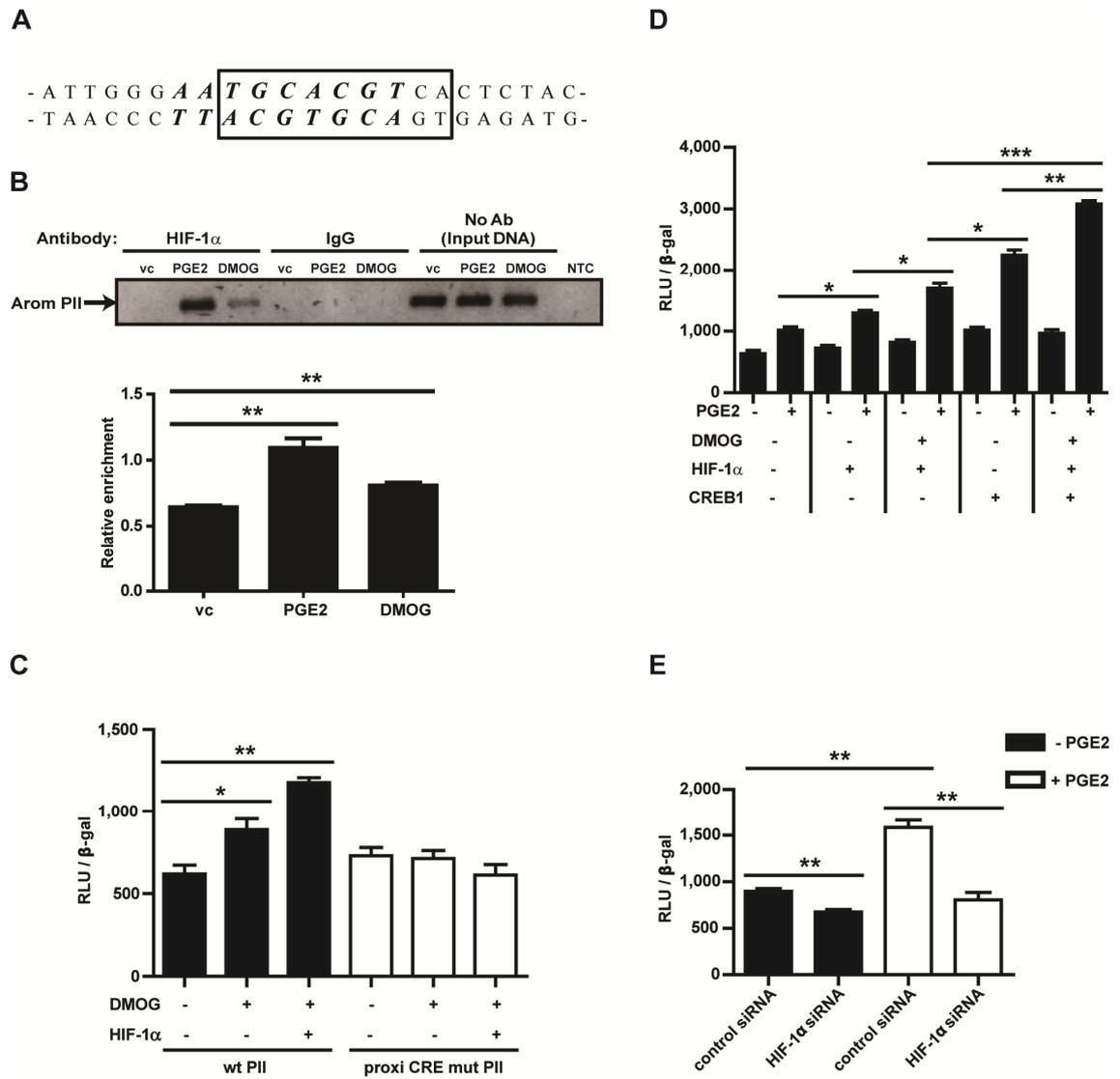


Figure 3

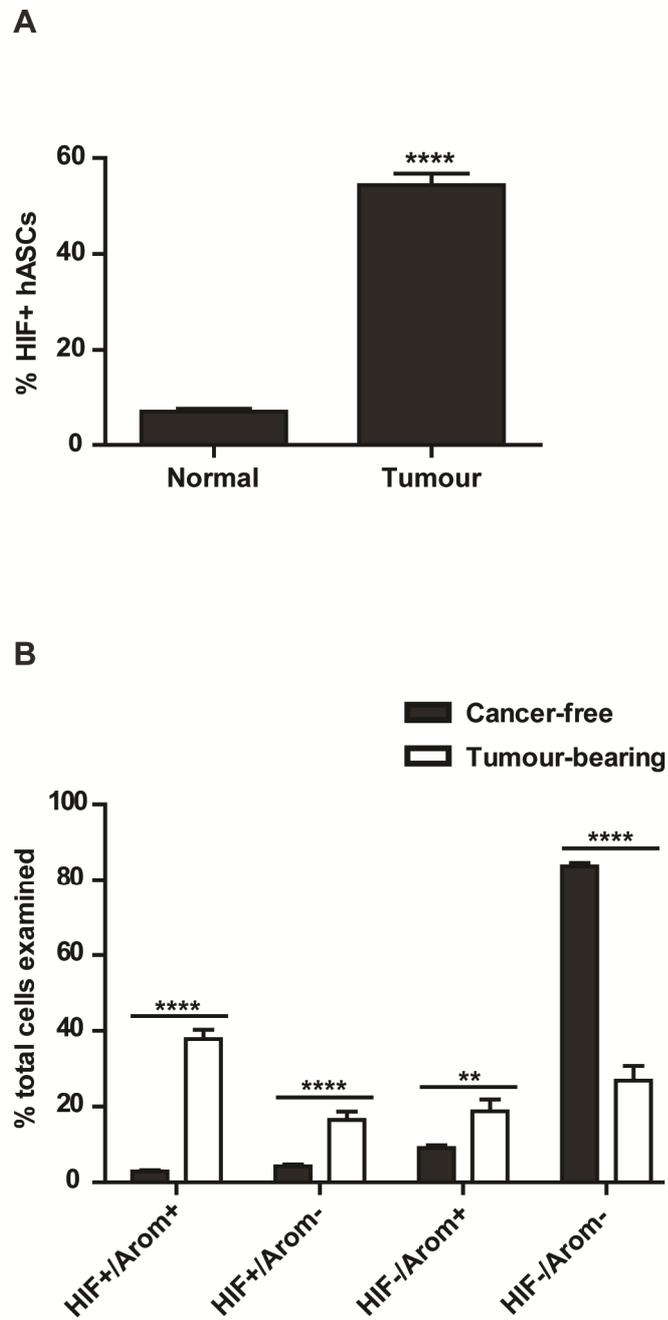
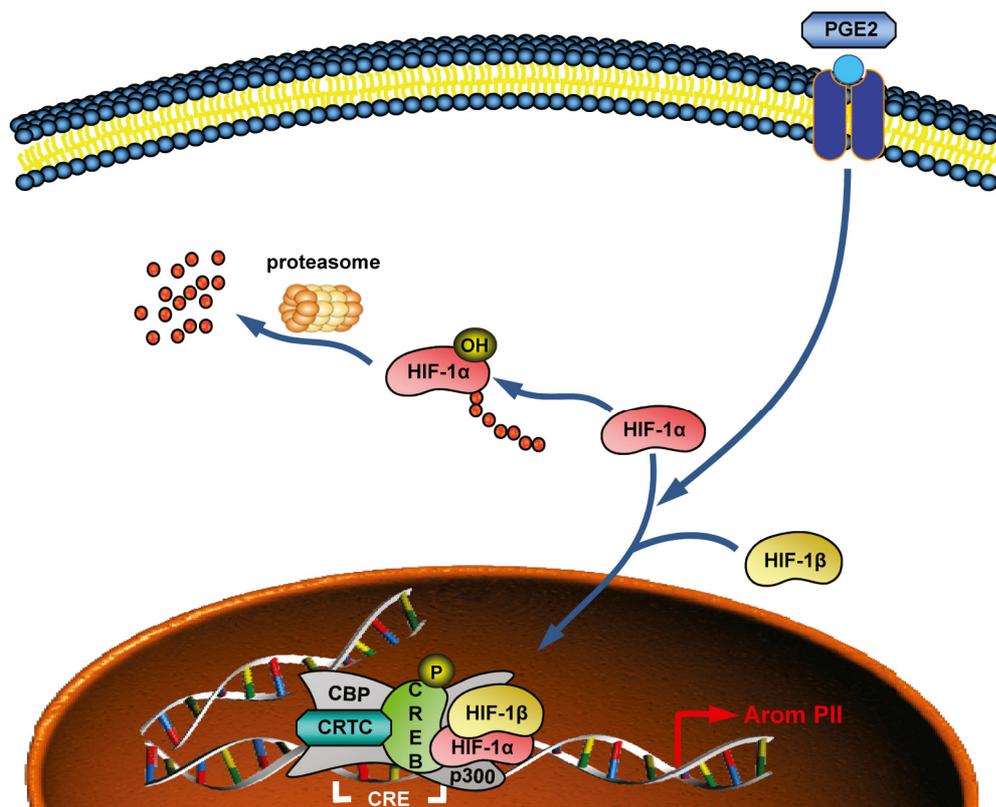
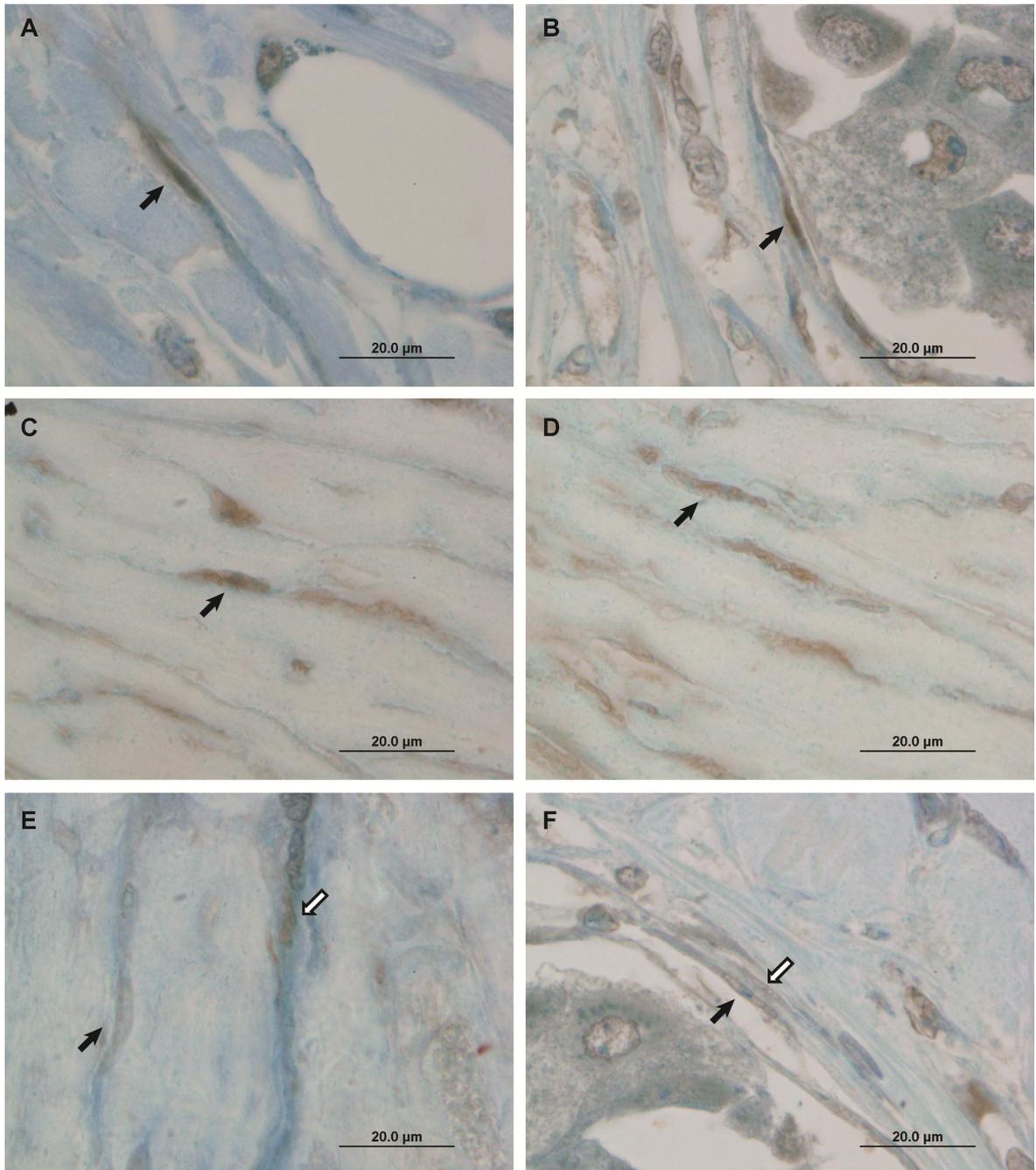


Figure 4



Supplementary Figure 1



Supplementary Table 1: Clinicopathological data of breast cancer patients.

	Age	Tumor type	Nuclear grade	Nottingham's histological grade	ER (Allred score)	PgR (Allred score)	HER2 (Allred score)
1	69	DCIS	II	N/A	8	6	1
2	61	IDC, papillotubular carcinoma	I	N/A	8	8	0
3	36	IDC, solid-tubular carcinoma	III	N/A	0	0	1
4	75	IDC, solid-tubular carcinoma	III	N/A	8	5	1
5	66	DCIS	I	N/A	8	7	1
6	57	IDC, scirrhous carcinoma *	III	III	8	7	2
7	76	IDC, scirrhous carcinoma	I	II	8	7	0
8	55	IDC, scirrhous carcinoma *	I	II	8	3	1
9	60	IDC, scirrhous carcinoma *	II	II	8	7	0
10	59	IDC, scirrhous carcinoma *	III	III	4	0	3

DCIS: ductal carcinoma in situ; IDC: invasive ductal carcinoma; * post-chemotherapy;
N/A: not available

CHAPTER 4:

Promoter-specific effects of metformin on aromatase transcript expression.

Published in Steroids (2011), 76(8): 768-71.



Declaration for thesis chapter 4

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 (%)
Performed laboratory work for all the figures, data analysis, prepared and drafted manuscript	85%

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
Seungmin Ham	Contributed to data analysis	2%
Fangyuan Yang	Provided assistance in laboratory work	
Evan R. Simpson	Involved in conception of certain aspects of project	
Kristy A. Brown	Involved in conception of project, manuscript preparation and drafting	

**Candidate's
Signature**

A black rectangular box redacting the candidate's signature.

Date
10/12/2012

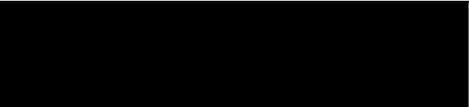
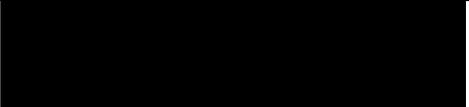
Declaration by co-authors

The undersigned hereby certify that:

- (13) 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.
- (14) 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;
- (15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (16) there are no other authors of the publication according to these criteria;
- (17) 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
- (18) 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 of Medical Research (PHIMR), Clayton, VIC, Australia
--

Signature 1		Date 13/12/2012
Signature 2		11/12/2012
Signature 3		11/12/2012
Signature 4		18/12/2012

Summary of article

Summary:

- This study extended previous findings by examining the effect of metformin on endogenous PII-, PI.3- and PI.4-specific transcript expression in primary breast ASCs.
- PCR for promoter-specific transcript expression confirmed that metformin causes a significant dose-dependent decrease in total aromatase expression as a result of decreased expression of PII- and PI.3-specific transcripts. In addition, metformin has no effect on the DEX/TNF α -mediated aromatase expression.

Work's contribution to the advancement of science:

- This work demonstrates for the first time that the inhibition of aromatase expression by metformin is due to decreases in both PII- and PI.3-specific transcripts expression.
- Overall, understanding the role of metformin in inhibiting PII/PI.3-driven aromatase expression offers a therapeutic advantage over the use of aromatase inhibitors.

Title

Promoter-specific effects of metformin on aromatase transcript expression.

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Abstract

Phase III aromatase inhibitors (AIs) are proving successful in the treatment of hormone-dependent postmenopausal breast cancer. Side-effects associated with total body aromatase inhibition have prompted new research into the development of breast-specific AIs. The identification of tissue- and disease-specific usage of aromatase promoters has made the inhibition of aromatase at the transcriptional level an interesting approach. We have previously demonstrated that AMPK-activating drugs, including metformin, were potent inhibitors of aromatase expression in primary human breast adipose stromal cells (hASCs). This study examines the promoter-specific effects of metformin on inhibiting aromatase expression in hASCs. Tumour-associated promoters PII/PI.3 were activated using forskolin (FSK)/phorbol ester (PMA), whereas normal adipose associated promoter PI.4 was activated using dexamethasone (DEX)/tumour necrosis factor- α (TNF α). Results demonstrate that metformin significantly decreased the FSK/PMA-, but not the DEX/TNF α -mediated expression of total aromatase at concentrations of 10, 20, and 50 μ M ($P \leq 0.05$). Using PCR to amplify promoter-specific transcripts of aromatase, it appears that the inhibition of the FSK/PMA-mediated expression of aromatase is due to decreases in PII/PI.3-specific transcripts, whereas no effect of metformin is observed on any promoter-specific transcript, including PI.4, in DEX/ TNF α -treated hASCs. This report therefore supports the hypothesis that metformin would act as a breast-specific inhibitor of aromatase expression in the context of postmenopausal breast cancer.

Keywords: Aromatase, metformin, promoter-specific, breast cancer, AMPK, adipose stromal cell

Introduction

Endocrine therapy involving aromatase inhibitors (AIs) is current best practice for estrogen receptor-positive breast cancer treatment, reflecting their superiority to available selective estrogen receptor modulators alone [reviewed in 1]. Their efficacy in suppressing estrogen biosynthesis via the inhibition of aromatase activity throughout the body often leads the development of side-effects similar to those observed in cases of estrogen deficiency, namely arthralgia, bone loss and possible cardiovascular and neuro-cognitive defects [reviewed in 2]. Recent efforts to develop next generation AIs have focussed on drug discovery aimed at selectively inhibiting aromatase within the breast. The targeted inhibition of aromatase is possible due to the unique nature of the *CYP19A1* gene, which encodes aromatase and is regulated by a number of tissue-specific promoters [3]. In the postmenopausal woman, when the ovaries have ceased to produce significant amounts of estrogens, the organ which produces the highest levels of estrogens is the adipose tissue.

Normal adipose maintains readily measurable levels of aromatase expression predominantly via the paracrine and autocrine actions of glucocorticoids and class I cytokines. Notably, within breast and other adipose depots approximately half of total aromatase transcript expression is driven by promoter PI.4, whereas the other half is composed of PI.3- and PII-specific transcripts [4]. This ratio dramatically changes in breast adipose adjacent to tumour tissue, where aromatase levels are 4 times higher than in normal adipose and the majority of aromatase transcripts are those derived from PII and PI.3 [5]. Inflammatory factors produced by the tumour, including prostaglandin E₂ (PGE₂), have been identified as key mediators of aromatase expression in the adipose stromal cells of the tumour-bearing breast [6]. Many transcription factors have been implicated in the PGE₂-dependent regulation of aromatase PII/I.3, including the liver receptor homolog-1 (LRH-1) [7], CREB [8, 9], and more recently, the CREB co-activator CRTC2 (CREB regulated transcription coactivator 2) [10]. Interestingly, a direct negative regulator of CRTC2 activity is AMP-activated protein kinase (AMPK) and we and others have demonstrated that drugs which activate AMPK, such as AICAR and metformin potently inhibit aromatase expression in human breast adipose stromal cells (hASCs) [11], luteinised granulosa cells [12] and endometriotic stromal cells [13].

Metformin is the most commonly prescribed anti-diabetic drug and is known to cause few side-effects. Recent epidemiological evidence also suggests that metformin may lower cancer risk and reduce the incidence of cancer-associated death in diabetic patients treated with the drug [14]. This study aimed to examine the effect of metformin treatment on endogenous PII-, PI.3- and PI.4-specific transcript expression in primary hASCs in the presence of forskolin (FSK)/phorbol ester (PMA), to mimic PGE₂, but also in the presence of the glucocorticoid dexamethasone (DEX)/tumour necrosis factor- α (TNF α).

Experimental

Human tissue, cell culture and treatments

Primary human breast adipose stromal cells (hASCs) were isolated from human breast tissue obtained after breast reduction surgery and cultured as previously described [15]. The studies presented herein have been approved by Southern Health Human Research Ethics Committee B and all subjects have given informed consent. Prior to treatment, cells were serum-starved for 24 hours. Cells were then treated for 24 hours with experimental agents, which included 25 μ M forskolin (FSK; Sigma-Aldrich Pty Ltd, Sydney, Australia), 4 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Pty Ltd, Sydney, Australia), 250 nM dexamethasone (DEX; Sigma-Aldrich Pty Ltd, Sydney, Australia), 5 ng/ml tumour necrosis factor- α (TNF α ; Sigma-Aldrich Pty Ltd, Sydney, Australia) and 10, 20 and 50 μ M 1,1-dimethylbiguanide hydrochloride (metformin; Sigma-Aldrich Pty Ltd, Sydney, Australia).

RNA extraction, quantitative and qualitative PCR

Total RNA was extracted from hASCs using the QIAshredder and RNeasy Mini kits (Qiagen Pty Ltd., Doncaster, Australia) and reverse-transcription was performed using AMV RT and oligo(dT) primers (Promega Corp., Sydney, Australia) as directed by the manufacturer. Briefly, 450 ng RNA was incubated with 0.5 μ g oligo(dT) primers at 65⁰C for 5 min, and RT reaction was incubated at 37⁰C for 1 h. Real-time PCR amplifications were performed on the LightCycler using Fast Start Master SYBR Green 1 (Roche Diagnostics Australia Pty Ltd., Castle Hill, Australia). Quantification of total human aromatase mRNA was performed using primers RT-7: 5'-

TTGGAAATGCTGAACCCGAT-3', RT-8: 5'-CAGGAATCTGCCGTGGGGAT-3', and quantification of 18S transcript expression was performed using primers 18S-F: 5'-CGGCTACCACATCCAAGGAA-3' and 18S-R: 5'-GCTGGAATTACCGCGGCT-3'. Cycling conditions were as previously described [11]. Experimental samples were quantified by comparison with standards of known concentrations. All samples were normalized to 18S transcript levels. Qualitative PCR to examine expression of promoter-specific transcripts was performed using previously characterised primer pairs [16]. Briefly, promoter-specific primer pairs were used to amplify promoter-specific transcripts using GoTaq Green (Promega Corp., Sydney, Australia). Cycling conditions were 95°C for 2 min followed by 30 cycles at 95°C for 30 sec, T_m (melting temperature) for 30 sec, 72°C for 30 sec and a final elongation step at 72°C for 5 minutes, where T_m was 56°C, 53°C and 50°C for PII-, PI.3- and PI.4-specific transcript expression, respectively. The number of cycles was determined to be within the linear phase of amplification of the product. Products were analysed on a 1% agarose gel and visualised using ethidium bromide under UV light.

Statistics

All experiments were performed twice with n = 3 for each experiment. Results are presented as mean ± SEM. Statistical analyses were performed using two-tailed student t-tests and metformin-dependent effects were analysed by one-way ANOVA followed by Dunnett's multiple comparison test. Results that were statistically significant were labelled with different letters. GraphPad Prism version 3.00 was used.

Results

Effect of metformin on forskolin/PMA-mediated aromatase expression

The expression of aromatase in FSK/PMA-treated primary breast hASCs was quantified using real-time PCR. Results demonstrate that metformin significantly inhibits aromatase expression at concentrations of 10, 20 and 50 µM (Figure 1A). This is reflected in the changes in expression observed for promoter-specific transcripts for PII and PI.3 (Figure 1B). As previously reported, levels of PI.4-derived transcripts remains low/undetectable in the absence of glucocorticoids (Figure 1B).

Effect of metformin on dexamethasone/TNF α -mediated aromatase expression

Primary breast hASCs were treated with 250 nM dexamethasone (DEX) and 5 ng/ml TNF α and the effect of metformin was quantified using real-time PCR. Metformin had no effect on DEX/TNF α -mediated aromatase expression (Figure 2A), and no effect on PI.4-specific transcript expression (Figure 2B). Expression of PII/I.3-specific transcripts was low/undetectable under all experimental conditions (Figure 2B).

Discussion

The majority of postmenopausal breast cancers are estrogen receptor positive [17]. In the postmenopausal woman, when the ovaries have ceased to actively synthesize estrogens, the adipose becomes the predominant source of estrogen biosynthesis where aromatization of androgens correlates with adipose tissue mass [18]. Evidence also suggests that estrogen formation within the breast is that which supports tumour growth [19-21].

Our results demonstrate that micromolar concentrations of metformin, which are pharmacologically relevant, decrease the FSK/PMA-mediated expression of total aromatase within primary human adipose stromal cells (hASCs), consistent with previous findings in cells of the breast [11], ovary [12] and endometrium [13]. Our findings also demonstrate that the effect of metformin on total aromatase expression is a direct result of the decrease in expression of PII/I.3-specific transcripts. Many factors known to inhibit cAMP-mediated aromatase expression also tend to increase glucocorticoid-dependent activation of aromatase PI.4. Notably, insulin and IGF-1 inhibit the stimulatory effect of cAMP on aromatase activity, but potentiate the actions of dexamethasone [22]. Prolactin has also been shown to potentiate the effect of glucocorticoids, having little effect on its own [23]. Our results demonstrate a dose-dependent inhibition of aromatase PII/I.3, with no effect on the glucocorticoid-mediated expression of aromatase indicating a mechanism of action which is separate from those discussed above.

Considering the important relative contribution of PII/I.3-specific transcripts to total aromatase expression compared to PI.4 in tumour-bearing breast adipose, this study

describes metformin as a selective inhibitor of breast aromatase expression. In addition to inhibiting aromatase, metformin and other AMPK-activating drugs are currently under investigation for their anti-proliferative effects via inhibition of ATP-utilising pathways, such as those involved in protein biosynthesis (mediated by mTOR), fatty acid synthesis, as well as through its insulin sensitising effects [reviewed in 24]. Agonists of PPAR γ , such as rosiglitazone and troglitazone, are potent anti-diabetics which induce adipocyte differentiation by activating a specific set of genes. These agonists have also been shown to inhibit aromatase expression in hASCs via decreasing activity of PI.4 [25]. Moreover, PPAR γ also increases adiponectin expression and inhibits leptin, known to inhibit and increase aromatase expression, respectively [10]. Furthermore, the tolerability to metformin and other anti-diabetics compared to AIs may be an important factor in driving future studies to evaluate their use in the clinical setting. Of interest, diabetic patients treated with metformin are at lower risk of sustaining bone fractures than untreated patients and than patients using PPAR γ agonists [26].

Nonetheless, metformin effects on mediators of aromatase expression other than those examined in this study, including insulin, class I cytokines, prolactin, as well as those known to increase PII-derived transcript expression such as catecholamines [27] and leptin need to be evaluated. Moreover, it is important to bear in mind that in addition to the stromal cell compartment of the breast, aromatase expression has also previously been detected in carcinoma or parenchymal cells [reviewed in 28]. Aromatase expression in these cells is mainly under the control of PI.3, however, the mechanisms regulating its expression appear to be different than that in hASCs. Namely, activation of cAMP-dependent pathways causes weak activation of PI.3 in breast cancer epithelial cells compared to hASCs [7, 29, 30]. Furthermore, it was demonstrated that ERR α bound to aromatase PI.3 in the breast cancer cell line SK-BR-3 and activated its expression but had no effect on the promoter in hASCs [31]. Further studies are therefore necessary to evaluate the effect of metformin on promoter-specific expression of aromatase in the breast tumour and adipose. Ultimately, adequately designed clinical trials will determine the efficacy of metformin to treat and possibly prevent postmenopausal breast, in combination or as an alternative to current endocrine therapy.

Acknowledgements

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Figure Legends

Figure 1

Effect of metformin on FSK/PMA-mediated aromatase expression in primary breast hASCs. Metformin caused a significant dose-dependent decrease in the forskolin (FSK; 25 μ M)/phorbol ester (PMA; 4 nM)-mediated total aromatase expression (A), PII- and PI.3-specific transcripts (B) and had no effect on PI.4-specific transcript expression (B). Single bands on right panel demonstrate amplification of known standard performed alongside experimental samples. n = 3; 2 separate experiments; mean \pm SEM. Statistically different values are labelled with different letters.

Figure 2

Effect of metformin on DEX/TNF α -mediated aromatase expression in primary human adipose stromal cells. Metformin had no effect on the dexamethasone (DEX; 250 nM)/tumour necrosis factor- α (TNF α ; 5 ng/ml)-mediated total aromatase expression (A). No change in PI.4-specific transcripts was observed in response to metformin in DEX/TNF α treated cells (B). PII/I.3-derived transcripts were low/undetectable under all experimental conditions (B). Single bands on right panel demonstrate amplification of known standard performed alongside experimental samples. n = 3; 2 separate experiments; mean \pm SEM. Statistically different values are labelled with different letters.

Figures

Figure 1

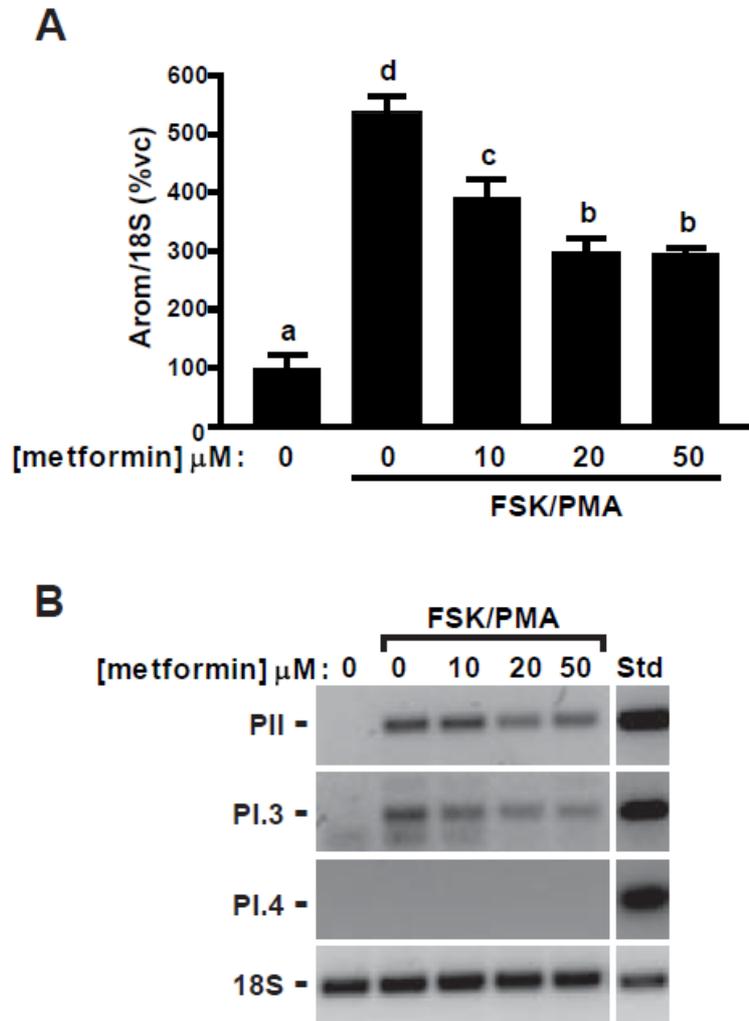
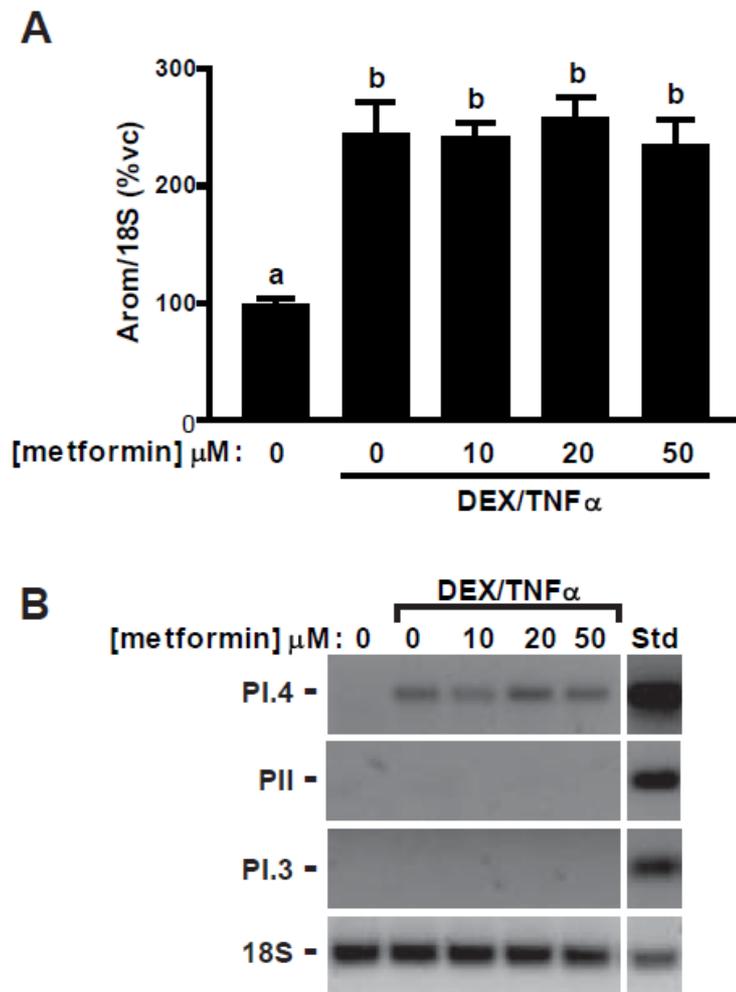


Figure 2



CHAPTER 5:

GENERAL DISCUSSION

GENERAL DISCUSSION

Breast cancer is the most common form of cancer in women worldwide. Although current therapy efficiently combats primary tumours in more than 90% of women with hormone receptor positive cancers, these treatments are also associated with side effects that impact compliance. The need is therefore before us to identify alternatives to these therapies, which will be possible with a better understanding of molecular mechanisms behind the disease.

Age and BMI are important risk factors for breast cancer. Oestrogens play a vital role in the development and progression of postmenopausal breast cancer. In premenopausal women, the main source of oestrogens is the ovary, but following menopause peripheral tissues, predominantly the adipose, convert circulating adrenal androgens into oestrogens in a process that requires a number of specific enzymes including steroid sulfatase, 3β -HSDs/KSIs, 17β -HSDs and aromatase in breast adipose tissues. Aromatase is the key enzyme which catalyses the final step in oestrogen biosynthesis, and exhibits a tissue specific expression profile as a result of a number of alternative non-coding first exons or promoters within the *CYP19A1* gene.

Currently available endocrine strategies for the treatment of ER+ postmenopausal breast cancer include the selective oestrogen receptor modulator tamoxifen or aromatase inhibitors. However, these current treatment options have significant side effects as oestrogens also serve as protective factors in tissues such as the bone, brain and the vasculature. In obese postmenopausal women or in postmenopausal women with breast cancer, the promoter responsible for the elevated local breast aromatase expression and therefore high oestrogen levels is the adipose specific promoter II. This promoter requires a specific region of DNA, which encompasses a cAMP response element (CRE) for its activation by obesity-related and tumour-derived factors including PGE_2 . Hence, better understanding the regulation of aromatase PII, which is the main focus of this thesis, will lead to the identification of novel therapeutic strategies to allow aromatase to be targeted specifically within the breast, with significantly fewer side effects compared to currently available endocrine therapies.

The overall hypothesis of this thesis is that transcription factors including CRTCs and HIF-1 α are involved in the regulation of PII-driven aromatase expression in primary human breast ASCs in response to PGE₂, and that signaling pathways involving these transcription factors could be targeted to inhibit aromatase in a breast-specific manner. Hence, this thesis expands on our existing knowledge and sheds light on future directions for the field.

5.1. Identification of novel aromatase PII regulators

The maximal induction of aromatase promoter PII by PGE₂ is dependent on two cAMP response elements found -211 to -203 bp (CRE2, proximal) and -292 to -285 bp (CRE1, distal) upstream of the transcription start site (Sofi et al., 2003). These regions of DNA have previously been shown to bind CREB1 (Sofi et al., 2003) and CREB family members, including ATF-2 (Chen et al., 2007a). CREB1 is also known to interact with co-activators such as CBP/p300 to enhance PII-driven aromatase expression (Zhou et al., 2002, Subbaramaiah et al., 2008). Previous studies have suggested that CREB1 requires additional coactivators, including the CREB-regulated transcription coactivators (CRTCs), to stimulate target gene expression (Zhang et al., 2005). More recently, our laboratory has identified CRTC2 as a novel coactivator of CREB1 in the context of aromatase PII regulation (Brown et al., 2009). In the present study, both CRTC1 and CRTC3 were also examined and the role of CRTCs in binding to and regulating aromatase PII activity was elucidated. All three CRTCs bound to PII in breast ASCs and were capable of inducing aromatase promoter activity, yet CRTC2 and CRTC3 transcripts were expressed at higher levels and had more potent effects on PII activation. Previous studies have also demonstrated that all three CRTC isoforms induce peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) transcription and its promoter activity in a CREB1-dependent manner (Wu et al., 2006b). Interestingly, PGC-1 α has also been shown to stimulate aromatase expression in breast ASCs as a transcriptional coactivator of LRH-1 (Safi et al., 2005). Therefore, the roles of CRTCs to regulate PII may go beyond the direct interaction described in the current thesis. Upon closer examination of the region of DNA -213 to -203 bp upstream of the transcription start site, we also identified a conserved hypoxia response element (HRE), which overlaps with the proximal CRE on the antisense strand of

aromatase PII, suggesting a role for HIF-1 α in mediating CREB1-dependent aromatase expression. A previous study has described the interaction of HIF-1 α with CBP/p300 to increase the expression of hypoxia regulated genes (Arany et al., 1996). Indeed, HIF-1 α stimulates aromatase PII activity, independent of oxygen availability.

Moreover, our studies revealed that CREB1 acts co-operatively with CRTCs and HIF-1 α to induce aromatase expression, suggesting that the maximal induction of PII-driven aromatase expression is dependent on CREB1 and its transcriptional activators, CRTCs and HIF-1 α . Additional studies using reporter constructs with mutated distal and proximal CREs have demonstrated that CRTCs act through both proximal and distal CREs of aromatase PII, whereas HIF-1 α only acts via the proximal CRE, which includes the core HRE. Hence, the simultaneous interaction of these novel inducers of aromatase expression including CRTCs and HIF-1 α with CREB1, CBP and p300 may contribute to the transcriptional synergy that leads to maximise the PII-driven aromatase expression in ER+ postmenopausal breast cancer.

5.2. Regulation of CRTCs and HIF-1 α by PGE₂

Tumour-derived, obesity-related inflammatory factor PGE₂ stimulates aromatase expression in human breast ASCs, endometriosis-derived stromal cells and rat granulosa cells, predominantly via activation of promoter PII. This occurs as a result of activation of one or more of four receptors, EP1-4. EP2 and 4 are coupled to adenylate cyclase and cAMP formation, whereas EP1 is linked to phospholipase C, DAG formation and activation of protein kinase C (PKC). This leads to the regulation of a number of transcription factors, including CREB1 (Zhao et al., 1996a, Cai et al., 2007, Chen et al., 2007b, Noble et al., 1997, Lu et al., 2006). Little was known, however, of the role of PGE₂ to regulate CRTCs and HIF-1 α expression and activity in ASCs. We have shown that PGE₂ causes the nuclear translocation of all three CRTCs, as well as HIF-1 α , which is associated with an increase in their binding to aromatase PII in breast ASCs. Furthermore, the effect of all three CRTCs and HIF-1 α to increase PII activity was potentiated in response to PGE₂ or the PGE₂ mimetic, FSK/PMA. Consistent with our data, previous studies have also demonstrated that PGE₂ is able to stabilise HIF-1 α and

promote its nuclear translocation under normoxic conditions in human prostate, colon and gastric adenocarcinoma cancer cells (Huang et al., 2005, Liu et al., 2002, Fukuda et al., 2003). Our work, however, is the first to demonstrate transcriptional regulation of CRTCs in response to PGE₂.

CRTCs show differential tissue expression patterns in humans (Wu et al., 2006a) and subcellular distribution in human ATYB1 ASCs, HIT and MIN6 cells (Screaton et al., 2004). Moreover, our laboratory has previously shown that treatment of breast ASCs with the PGE₂ mimetic FSK/PMA results in the nuclear translocation of CRTC2 (Brown et al., 2009). Another interesting report using pancreatic islet cells has demonstrated that FSK induced translocation of CRTC2 and its cytoplasmic sequestration under basal conditions via phosphorylation dependent interaction with 14-3-3 protein (Screaton et al., 2004). AMPK has previously been found to stimulate phosphorylation of CRTC2 at Ser171 which leads to the formation of a complex with 14-3-3 proteins and as a consequence, causes their inhibition (Screaton et al., 2004). Further extending our previous work into the regulation of aromatase via CRTC2 in breast ASCs and also given the importance of Ser171 in the complex formation between CRTC2 and 14-3-3 proteins, the present study aimed to demonstrate that phosphorylation of CRTC2 at Ser171 dictates its subcellular localisation and directly affects the activation of aromatase PII in ASCs. This was accomplished using S171A and S171D mutants of CRTC2, which mimic the dephosphorylated and phosphorylated states, respectively. Our results show that the CRTC2 phosphorylation mimetic (S171D) is found in the cytoplasm under basal conditions and in cells treated with FSK/PMA. Consistent with these results, we also demonstrate that treatment of S171D-transfected cells with FSK/PMA significantly reduces the fold-induction of PII activity. Conversely, transfection of the dephosphorylation mimetic of CRTC2 (S171A) significantly induces PII activity under basal conditions and is associated with nuclear localisation of the CRTC2 protein. These data suggest that phosphorylation of CRTCs by AMPK will dictate their activity towards aromatase regulation and may serve as a novel therapeutic target.

Locally advanced solid tumours tend to develop heterogeneously distributed hypoxic tissue areas within the tumour mass (Vaupel and Mayer, 2005). However, the degree of oxygen availability in the adipose stroma is not yet clearly understood. Healthy breast ASCs are known to secrete a collagenous extra-cellular matrix with well defined and centrally located blood vessels (Kim et al., 2005). On the other hand, less well organised breast cancer cells with tumour angiogenesis producing poorly defined blood vessels intermingle with the stromal element (Kim et al., 2005). The present study aimed to characterise the role of HIF-1 α , a master regulator of hypoxic responses in the regulation of PII-driven aromatase expression in human breast ASCs independent of oxygen availability. The rationale for taking this approach, as opposed to examining hypoxia-mediated HIF effects, was also supported by the fact that all cytochrome P450 enzymes including aromatase require molecular oxygen and electrons from NADPH or NADH for their catalytic activity (Ghayee and Auchus, 2007, Omura, 2010), and the relevance of aromatase induction under hypoxic conditions remains to be elucidated. Nonetheless, our *in vitro* findings support a role for HIF-1 α to regulate aromatase expression under normoxic conditions. Our studies were also expanded upon using breast tissues obtained from breast cancer patients and healthy women. Here, we demonstrate that the percentage of HIF-1 α positive ASCs is significantly increased in tissues obtained from breast cancer patients compared to healthy women, and there is also a positive association between HIF-1 α and aromatase staining in breast ASCs.

The work presented in chapter 2 and 3 has suggested that the elevated aromatase expression in tumour and obesity associated ASCs is associated with increased expression of nuclear CRTCs and HIF-1 α in response to PGE₂. This study also identifies the CRTCs/HIF-1 α -CREB1 interactions as potential targets for the treatment of oestrogen-dependent, obesity-related postmenopausal breast cancer, as nuclear entry of CRTCs/HIF-1 α is an important step in the CREB1 target gene activation. Overall, these findings will enable novel treatment strategies based on the modulation of these factors to overcome therapeutic resistance and lead to improved results of hormonal therapy.

5.3. Tissue-specific inhibition of aromatase and therapeutic implications

The currently available endocrine therapies for breast cancer act principally either by blocking oestrogen action at the receptor level using SERMs such as tamoxifen or blocking oestrogen production with aromatase inhibitors. Aromatase inhibitors, superior to tamoxifen when examining recurrence rates and cancer-associated mortality, are now widely used in the treatment of postmenopausal women with early stage and metastatic ER+ breast cancer (Coombes et al., 2004, Howell et al., 2005, Forbes et al., 2008, Goss et al., 2003). However, the global inhibition of aromatase via inhibiting the catalytic activity of aromatase limits their use due to a number of undesirable side effects including hot flushes, arthralgia, myalgia, adverse skin reactions, mood disturbances, cardiovascular events and cognitive defects (Mouridsen, 2006, Gaillard and Stearns, 2011, Muslimani et al., 2009, Santoro et al., 2011, Bundred, 2005, Bender et al., 2007, Rocha-Cadman et al., 2012). Hence, the solution is to find a way to inhibit aromatase expression specifically within the breast. This can be accomplished in principle due to the fact that aromatase expression is regulated via tissue-specific promoters.

Total aromatase transcript expression in adipose tissue is mainly governed by PI.4, which accounts for approximately half of transcripts and the other half is driven by the activation of PII and PI.3 (Agarwal et al., 1997). However, in breast cancer associated adipose tissue, total aromatase transcript expression is shown to be 4-fold higher than normal adipose and the majority of transcripts are derived from the coordinated activation of PII and PI.3 (Zhou et al., 1996a). Interestingly, our laboratory has recently demonstrated that metformin, which has been used for the treatment of type 2 diabetes for many years, inhibits aromatase expression through activation of AMPK in breast ASCs (Brown et al., 2010). This inhibition occurs at concentrations ranging from 10 μ M to 50 μ M which are present in the plasma of women who are given metformin for the treatment of diabetes and polycystic ovary syndrome. Moreover, treatment with metformin prevents the FSK/PMA-mediated nuclear entry of CRTC2, thus blocking its ability to coactivate CREB1 and subsequent stimulation of aromatase expression. The aim of the work in Chapter 4 was to extend these findings to examine the effect of metformin on endogenous PII-, PI.3- and PI.4-specific transcript expression in breast ASCs in response to FSK/PMA and also DEX/TNF α . The present study demonstrated

that the inhibition of FSK/PMA-mediated expression of aromatase with metformin treatment is a direct result of the decrease in endogenous PII and PI.3-specific transcripts in breast ASCs (Samarajeewa et al., 2011). In addition, treatment of metformin had no effect on the DEX/TNF α -mediated expression of aromatase, and specifically, had no effect on PI.4, which is the main promoter utilised by bone to drive aromatase expression.

The effect of metformin to reduce aromatase expression via PII- and PI.3-specific transcripts compared to PI.4 warrants its use in treating ER+ postmenopausal breast cancer. Metformin is also inexpensive and has relatively fewer side effects compared to third generation aromatase inhibitors and tamoxifen. A number of observational studies have already demonstrated the ability of metformin to reduce the risk of prostate, colon and breast cancers (Cazzaniga et al., 2009, Evans et al., 2005). Nevertheless, there is a need for randomized, double-blind, placebo-controlled trials to assess the efficacy and toxicity profile of metformin in the management of breast cancer. Following on from observational and preclinical studies, several clinical trials, including window of opportunity studies, phase II neoadjuvant randomized trials and phase I-II trials in metastatic disease, are currently underway or have recently been approved to test the efficacy of metformin in treating patients with breast cancer (ClinicalTrials.gov, Gonzalez-Angulo and Meric-Bernstam, 2010, Martin-Castillo et al., 2010, Cazzaniga et al., 2009). A phase III randomized trial of metformin versus placebo in early stage breast cancer by the National Cancer Institute of Canada (NCIC) clinical trials group is currently recruiting participants (ClinicalTrials.gov). This study is primarily looking at the invasive disease-free survival of patients treated with metformin versus placebo who will receive oral metformin or placebo for up to 5 years in the absence of disease progression or unacceptable toxicity, and also looking at the overall survival, distant disease-free survival, breast cancer-free interval, long-term clinical and laboratory safety, changes in BMI, relevant medical endpoints (new diabetes, cardiovascular hospitalizations), health-related quality of life and correlative science outcome, as secondary outcome measures. Our laboratory has also initiated two clinical studies currently at the recruitment phase. The first, a pilot study examining the effect of 4-week metformin treatment on aromatase expression in breast tissue from healthy weight

mid-life women scheduled to undergo breast reduction surgery. The second, a window-of-opportunity study whereby postmenopausal women diagnosed with early stage, invasive breast cancer will be given metformin for two weeks, followed by randomisation to either aromatase inhibitors alone or a combination of metformin and aromatase inhibitors. Aromatase expression will be evaluated prior to and after metformin alone and at primary surgery. It is expected that these studies will allow us to determine the efficacy of metformin to inhibit aromatase *in vivo*, which may then lead to a reduction in the need for currently used aromatase inhibitors.

5.4. Future directions

Breast cancer is the most common malignancy to occur in women. Geographical variation in the incidence of breast cancer has also been observed around the world. Breast cancer is becoming the leading cause of cancer-related death in females worldwide and the second most common cause of cancer death in Australian women. Medical research over the past several decades has resulted in numerous advances in the treatment of breast cancer; however the heterogeneous nature of this disease presents unique management challenges. This thesis describes how aromatase PII is regulated by CRTCs and HIF-1 α , and the possibility of developing novel breast-specific anti-oestrogen therapies as safer treatment options superior to currently available therapies.

It is impossible to use mice as models to study the regulation of aromatase in the context of oestrogen-dependent breast cancers due to the differences in *cyp19a1* gene organisation and a lack of dependence on mammary steroidogenesis in mice. Hence, primary human breast ASCs utilised in the current studies offer a powerful model to study novel regulators of aromatase within the breast. Future directions will involve the study of newly identified regulators of aromatase, CRTCs and HIF-1 α , in tissue micro arrays (TMAs). This will allow high throughput analysis of multiple specimens at the same time, obtained from large cohort of breast cancer patients with varying menopausal status, BMI, tumour type, ER status and tumour grade compared to healthy women. This will allow us to strengthen our *in-vitro* as well as preliminary *in-vivo* findings. In order to investigate the prognostic impact of these factors, clinical data including disease free survival, lymph node status and tumour size will be counted.

Furthermore, dissecting stromal fractions of tissues obtained from both cancer patients and healthy women using laser capture microdissection will enable improved interpretation of complex data.

Despite the studies looking at the inhibition of breast-specific PII driven aromatase expression in ASCs [23, 24], the actual use of metformin to suppress aromatase action in the clinical setting is largely uncharacterised. Hence, the completion of currently established clinical trials by our laboratory to test the efficacy of metformin to treat postmenopausal breast cancer will solidify the hypothesis that aromatase can be inhibited in a breast-specific manner and will justify its use for the treatment of hormone receptor positive breast cancer.

5.5. Conclusions

In conclusion, the results presented herein demonstrated that CRTCs and HIF-1 α stimulate PII-driven aromatase expression in primary human breast ASCs in response to the tumour-derived, obesity-related inflammatory factor, PGE₂ (Figure 1). The importance of CREB1, together with CRTCs and HIF-1 α , in driving maximal induction of aromatase adds significant insight into breast aromatase regulation. Moreover, discovering the action of metformin to inhibit aromatase expression via decreasing the PII- and I.3-specific transcripts has greatly broadened our concepts of its role in breast cancer therapy.

Prevention, diagnosis and management of breast cancer are all continually evolving at present. As this study reveals novel insights into the roles of CRTCs and HIF-1 α in regulating breast aromatase expression in ASCs, it will be possible to develop novel inhibitors of aromatase expression, which are specific to the breast, or biomarkers that will further improve risk assessment and therapy for individual patients. However, further extensive research and clinical trials will be required before they can be considered for tissue-specific inhibition of aromatase. The treatment of breast cancer continues to be based on surgery, chemotherapy, radiation therapy and endocrine therapy, and each approach remains a mainstay in the overall treatment plan. The future holds promise of further reductions in risk by more targeted therapies, better risk

predictors of disease development or progression through biomarkers and improved diagnostic accuracy, which eventually lead to improved quality of life and improved survival.

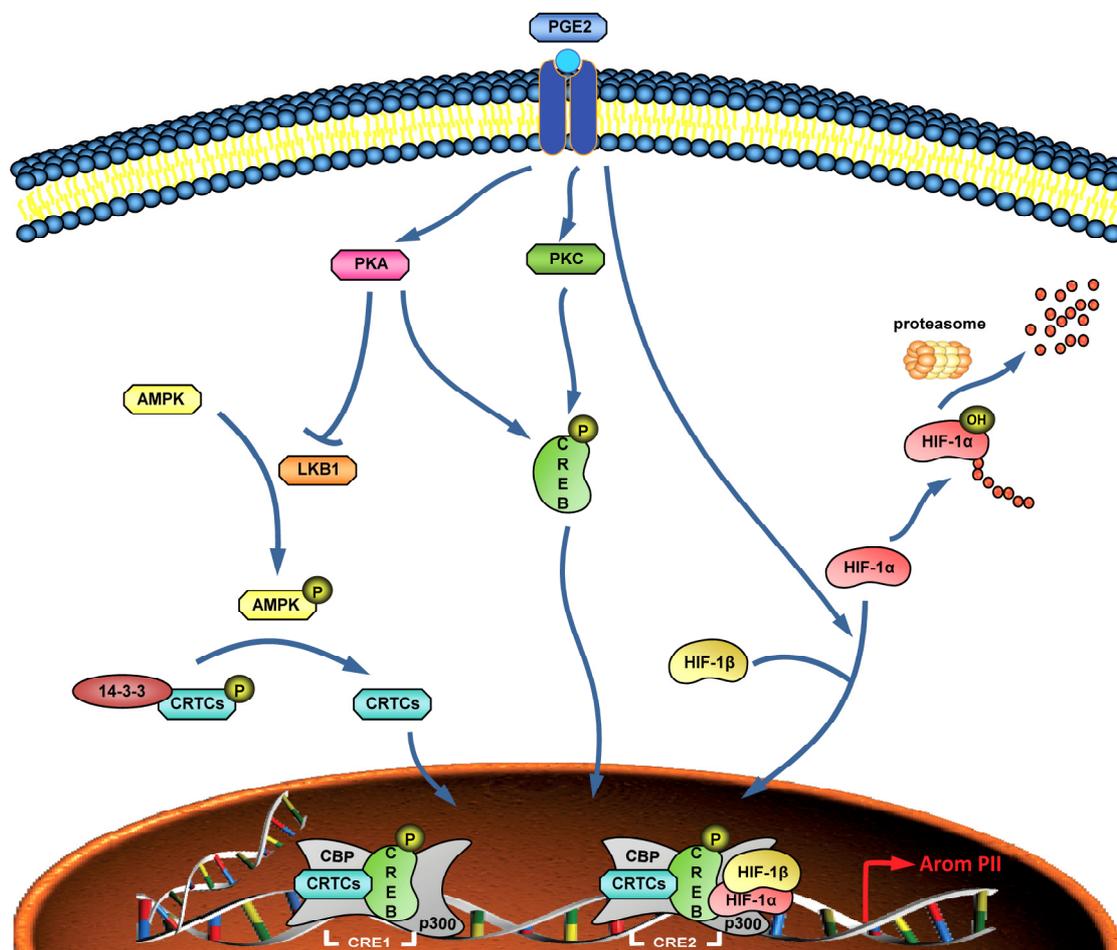


Figure 1: Model of the PGE₂-mediated regulation of aromatase expression by CRTCs and HIF-1 α in primary human breast ASCs. Tumour-derived, obesity-related inflammatory factor PGE₂ activates both PKA and PKC signaling pathways. LKB1 expression and activity are downregulated leading to a decrease in AMPK activity and nuclear entry of CRTCs. In addition, activation of PKA and PKC leads to the phosphorylation of CREB1, its nuclear entry followed by binding to CRE1 and CRE2, and co-activation by CRTCs resulting in the induction of aromatase expression. PGE₂ stimulates HIF-1 α transcript expression and stabilisation under normoxic

conditions. HIF-1 α dimerises with HIF-1 β and then translocates to nucleus where it interacts with CRE2 of aromatase PII. CRTCs and HIF-1 α act to increase PII-driven aromatase expression together with CREB1, CBP and p300.

AMPK, Adenosine monophosphate-activated protein kinase; CBP, CREB-binding protein; CRE1, cAMP response element 1 or distal cAMP response element; CRE2, cAMP response element 2 or proximal cAMP response element; CREB1, cAMP-response element binding protein 1; CRTCs, CREB-regulated transcription co-activators; HIF-1 α , Hypoxia inducible factor 1 α ; HIF-1 β , Hypoxia inducible factor 1 β , LKB1, Liver kinase B1; P, Phosphorylated ; PGE₂, Prostaglandin E₂; PKA, Protein kinase A; PKC, Protein kinase C.

APPENDIX 1



Declaration for Appendix 1

Declaration by candidate

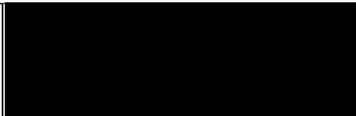
In the case of Appendix 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Manuscript preparation	25%

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
Kristy A. Brown	Manuscript preparation	
Evan R. Simpson	Manuscript preparation	

**Candidate's
Signature**

	Date 10/12/2012
---	---------------------------

Declaration by co-authors

The undersigned hereby certify that:

- (19) 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.
- (20) 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;
- (21) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (22) there are no other authors of the publication according to these criteria;
- (23) 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
- (24) 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 of Medical Research (PHIMR), Clayton, VIC, Australia

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Review

Endocrine-related cancers and the role of AMPK

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ABSTRACT

AMP-activated protein kinase (AMPK) is a master regulator of energy homeostasis involved in the regulation of a number of physiological processes including β -oxidation of fatty acids, lipogenesis, protein and cholesterol synthesis, as well as cell cycle inhibition and apoptosis. Important changes to these processes are known to occur in cancer due to changes in AMPK activity within cancer cells and in the periphery. This review aims to present findings relating to the role and regulation of AMPK in endocrine-related cancers. Obesity is a known risk factor for many types of cancers and a number of endocrine factors, including adipokines and steroid hormones, are regulated by and regulate AMPK. A clear role for AMPK in breast cancer is evident from the already impressive body of work published to date. However, information pertaining to its role in prostate cancer is still contentious, and future work should unravel the intricacies behind its role to inhibit, in some cases, and stimulate cancer growth in others. This review also presents data relating to the role of AMPK in cancers of the endometrium, ovary and colon, and discusses the possible use of AMPK-activating drugs including metformin for the treatment of all endocrine-related cancers.

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Contents

1. Introduction	00
2. AMPK and breast cancer	00
2.1. Endocrine regulators	00
2.2. Metformin	00
2.3. Other pharmacological regulators of AMPK	00
3. AMPK in prostate cancer	00
3.1. Endocrine regulators	00
3.2. Metformin	00
3.3. Other pharmacological regulators of AMPK	00
4. AMPK in endometrial and ovarian cancer	00
4.1. Endocrine regulators	00
4.2. Metformin	00
5. AMPK in colon cancer	00
5.1. Endocrine regulators	00
5.2. Metformin	00
5.3. Other pharmacological regulators of AMPK	00
6. Conclusions	00
Acknowledgements	00
References	00

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K.A. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

1. Introduction

Interest in the role and regulation of AMPK in cancer has steadily increased in the last decade. AMP-activated protein kinase (AMPK) is a master regulator of energy homeostasis which senses whole-body and cellular changes in nutrient availability. AMPK is a heterotrimeric enzyme composed of three subunits, namely α , β and γ . Multiple isoforms of each subunit have been identified, specifically two α subunits, two β subunits and three γ subunits [reviewed in Steinberg and Kemp (2009)]. Each isoform is encoded by a distinct gene and multiple transcript variants for each isoform, with alternative transcription start sites or splice sites, have been identified. These subunits are expressed and activated in a tissue-specific manner. Although expression patterns are well characterised in animal models, less is known of the tissue distribution in humans and more importantly with regards to this review, in cancer. Nevertheless, one report describes that the main AMPK active complex found in the MCF7 breast cancer cell line is the $\alpha 1\beta 1\gamma 1$ heterotrimer (Sanli and et al., 2012).

Activation of AMPK in response to energy deficiencies leads to the inhibition of pathways of energy utilisation and stimulation of pathways of energy production (Towler and Hardie, 2007). This is largely mediated via the direct binding of AMP or ADP to the regulatory γ subunit of AMPK which leads to conformational changes and as a consequence causes the phosphorylation of the catalytic α subunit of AMPK at Thr172 (Oakhill and et al., 2011). While phosphorylation of the α subunit at Thr172 is considered the major activating post-translational modification of AMPK, multiple other phosphorylation sites exist on the α and β subunits [reviewed in Steinberg and Kemp (2009)]. For example, the protein kinase A-dependent phosphorylation of Ser485/491 on the α subunit of AMPK has been shown to lead to inhibition of AMPK activity (Hurley and et al., 2006). Liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase β (CaMKK β) have been shown to act as upstream kinases to AMPK. Nonetheless, it is believed that the majority of AMPK signals are governed by LKB1 in the muscle (Thomson and et al., 2007), the liver (Shaw and et al., 2005), and that LKB1 is required to maintain normal AMPK-signalling in adipocytes (Gormand and et al., 2011). LKB1, a tumour suppressor, is mutated in the majority of cases of the autosomal dominant inherited Peutz–Jeghers Syndrome (PJS) (Hemminki and et al., 1998). PJS patients tend to develop hyperpigmented macules of the oral mucosa and intestinal hamartomatous polyps, and are predisposed to developing a number of cancers including those of the gastrointestinal tract, and other epithelial malignancies including those of the breast (Alessi and et al., 2006). Sporadic mutations in LKB1 have also been reported in lung, pancreatic and biliary cancers, as well as in melanomas [reviewed in Hezel and Bardeesy (2008)].

The malignant phenotype of cancer cells is characterised by an increase in lipid production, DNA and protein synthesis, and cell proliferation and migration, and AMPK has been shown to be a key regulator of these events. The role of AMPK to regulate fatty acid and cholesterol synthesis is well characterised. It directly phosphorylates and inhibits acetyl CoA carboxylase (ACC) and HMG-CoA reductase, and as a consequence limits *de novo* synthesis of fatty acids and cholesterol, respectively [reviewed in Luo and et al. (2010)]. The tumour suppressor proteins tuberous sclerosis complex (TSC) 1 and 2, also known as hamartin and tuberlin respectively, are known substrates of AMPK and TSC2 is a negative regulator of mammalian target of rapamycin (mTOR) (Mak and Yeung, 2004). Many sporadic cancers are associated with mutations in TSC1 and TSC2, including those of the breast, prostate, endometrium, colon and lung. Nutrients and growth factors interplay to regulate mTOR activity which complexes with raptor, PRAS40

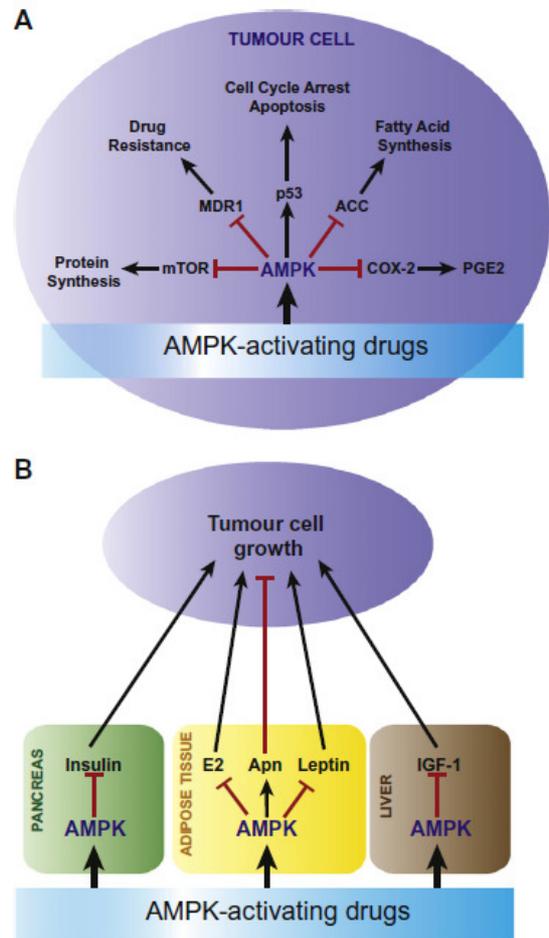


Fig. 1. The direct and indirect roles of AMPK in cancer. An overview of the role of AMPK in regulating processes of importance in cancer cells is depicted in (A). This is largely based on data currently available from breast cancer studies, as the role of AMPK in prostate cancer remains contentious and little is known about its role in other endocrine-related tumours. Briefly, direct effects of AMPK to inhibit tumour growth involves decreasing the activity and/or expression of proteins involved in protein synthesis (mTOR), drug resistance (MDR1), fatty acid synthesis (ACC) and PGE2 synthesis (COX-2), and by increasing the activity of the tumour suppressor p53. AMPK can also affect tumour growth via indirect mechanisms (B). It inhibits the secretion of insulin and IGF-1, known to be mitogenic in cancer, as well as the biosynthesis of oestrogens (E2) and the secretion of leptin, known to increase cancer cell proliferation and affect energy utilisation. Conversely, AMPK stimulates adiponectin (Apn) secretion, known to inhibit tumour cell growth. Taken together, this suggests that AMPK-activating drugs, including metformin, would inhibit tumour growth by targeting tumour cells directly and indirectly.

and mLST8 to form mTORC1 or with rictor, mSIN1, PRR5/Protor, mLST8 to form mTORC2 which unlike mTORC1, does not rely on growth factors for its regulation [reviewed by Shaw (2009)]. mTORC1 is known to control angiogenesis, cell growth and metabolism, whereas mTORC2 phosphorylates a number of kinases including Akt, SGK and PKC family members. Interestingly, AMPK also plays an important role in the regulation of the tumour suppressor p53 [reviewed in Luo and et al. (2010)], whereby activation of AMPK leads to the upregulation of p53 and its phosphorylation

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K.A. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

3

Table 1
Effect of endocrine and plant-derived factors on AMPK activity in endocrine-related cancers.

Endocrine and plant-derived factors	Effect on AMPK activity	Type of cancer; cell type
<i>Endocrine factors</i>		
17 β -oestradiol	Negative	BC: MCF-7 (Brown et al., 2011)
Adiponectin	Positive	BC: MCF-7, T47D, MDA-MB-231 (Taliaferro-Smith and et al., 2009)
	Positive	Primary human breast ASCs (Brown and et al., 2009)
	Positive	PC: PTEN-deficient LNCaP (Barb and et al., 2007)
	Positive	EC: KLE and RL-95 (Moon and et al., 2011)
	Positive	CC: HCT116, HT29, LoVo (Kim and et al., 2010b; Sugiyama and et al., 2009), HT-29 (Zakikhani and et al., 2008)
Androgen deprivation	Positive	PC: LNCaP (Chhipa and et al., 2011)
CCL2	Negative	PC: PC3, DU145, C4-2B (Zakikhani and et al., 2008)
FSH	Negative	Primary rat granulosa cells (Kayampilly and Menon, 2009)
Glucose deprivation	Positive	PC: C4-2 (Chhipa and et al., 2010), DU145 (Yun and et al., 2005; Jung and et al., 2008)
IGF-1	Negative	CC: HCT-8 (Luo and et al., 2011)
Leptin	Negative	Primary human breast ASCs (Brown and et al., 2009)
Oxygen deprivation	Positive	PC: LNCaP (Chhipa and et al., 2011)
PGE2	Negative	Primary human breast ASCs (Brown and et al., 2009)
Prolactin	Positive	BC: MDA-MB-231 (Linher-Melville and et al., 2011)
Resistin	Negative	Bovine and rat granulosa cells (Maillard and et al., 2011)
<i>Plant-derived factors</i>		
20(S)-ginsenoside Rg3	Positive	CC: HT-29 (Yuan and et al., 2010)
Alternol	Negative	PC: C4-2 (Yeung and et al., 2011)
	Positive	Prostate cells: RWPE-1 (Yeung and et al., 2011)
Anthocyanins	Positive	CC: HT-29 (Lee and et al., 2010)
Capsaicin	Positive	CC: HT-29 (Kim and et al., 2007)
Catechin	Positive	CC: HT-29 (Park and et al., 2009)
Compound K	Positive	BC: MCF-7 (Kim and et al., 2010)
	Positive	CC: HT-29 (Kim do and et al., 2009)
Curcumin	Positive	BC: MCF-7 (Lee and et al., 2009)
	Positive	CC: HT-29 (Lee and et al., 2009)
EGCG	Positive	CC: HT-29 (Hwang and et al., 2007)
Genistein	Positive	PC: DU145 (Park and et al., 2010)
p-HPEA-EDA	Positive	CC: HT-29 (Khanal and et al., 2011)
Pitavastatin	Positive	CC: mouse colonic preneoplastic lesions (Yasuda and et al., 2010)
Puerarin	Positive	BC: MCF-7 (Hien and et al., 2010)
Quercetin	Positive	BC: MCF-7 (Lee and et al., 2009)
	Positive	CC: HT-29 (Lee and et al., 2009)
Resveratrol	Positive	BC: MCF-7, MDA-MB-231 (Lin and et al., 2010)
	Positive	CC: HT-29 (Hwang and et al., 2007)
Rhizochalin	Positive	CC: HT-29 (Khanal and et al., 2011)

BC: Breast cancer; PC: Prostate cancer; EC: Endometrial cancer; CC: Colon cancer; ASCs: adipose stromal cells.

at Ser15. Conversely, p53 has also been shown to increase the expression of the $\beta 1$ subunit of AMPK (Feng and et al., 2007).

Stemming from its role as a master regulator of energy homeostasis, investigators have sought to understand how changes in AMPK may be associated with the increased tumorigenicity of cancer cells via direct effects on cell metabolism and protein synthesis, but also via indirect actions involving endocrine mediators such as insulin and oestrogens (Fig. 1). Moreover, regulation of AMPK by endocrine factors in tumour cells and within the periphery impacts tumour growth and development (Table 1). The following review aims to provide the reader with a comprehensive view of findings with regards to the role of AMPK in endocrine-related cancers of the breast, prostate, endometrium, ovary, and colon.

2. AMPK and breast cancer

Of all endocrine-related cancers, the role of AMPK in breast cancer is best characterised. Nonetheless, few studies have examined the expression and activity of AMPK in clinical breast cancer specimens. One study by Hadad et al. demonstrated using immunohistochemistry that phosphorylated AMPK was highly expressed in normal breast epithelium and that expression was significantly reduced in primary breast cancer samples (Hadad et al., 2009). Moreover, AMPK signalling was also decreased as phosphorylated acetyl

CoA carboxylase (ACC) correlated with phosphorylation of AMPK (Thr172; pAMPK) signals. The loss of signal for pAMPK was also associated with higher histological grade and axillary node metastasis. No association between pAMPK and hormone receptor status was demonstrated. This was somewhat unexpected as LKB1 expression in breast cancer cell lines appeared to be dependent on ER-status. Namely, MCF-7 cells, which are ER-positive, express LKB1, whereas ER-negative cell lines, such as MDA-MB-435 and MDA-MB-231, have a reduced LKB1 expression (Shen and et al., 2005). Nonetheless, it was recently demonstrated that LKB1 expression and AMPK phosphorylation are decreased in response to oestradiol in MCF-7 cells (Brown et al., 2011), providing a molecular explanation behind the clinical findings showing that LKB1 and pAMPK are low in breast cancer irrespective of ER expression (Hadad et al., 2009; Brown et al., 2011). Single-nucleotide polymorphism (SNP) analysis of genes which encode the $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ subunits of AMPK was performed in order to identify associations with breast cancer risk (Campa and et al., 2011). Despite a large number of cases (1340 breast cancer and 2536 controls), no association was found. Interestingly, a SNP was discovered within the third intron of the *PRKAG2* gene, which encodes for the $\gamma 2$ subunit of AMPK, and this was associated with an increased risk of ER-negative breast cancer (Liu and et al., 2009). Despite the SNP being found in a *bona fide* p53 binding site, this gene did not appear to be regulated by p53 in the cell lines examined.

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2.1. Endocrine regulators

Obesity is a known risk factor for breast cancer [reviewed in Brown and Simpson (2010)]. In particular, postmenopausal women with a body mass index (BMI) of 30, categorised as obese, are two times more likely to develop breast cancer when compared to their healthy weight counterpart (Rinaldi and et al., 2006; Carmichael, 2006; van den Brandt et al., 2000; La Vecchia and et al., 1997). The menopausal transition is associated with increased body weight (Lovejoy and et al., 2009; Poehlman and et al., 1995) and increased breast cancer risk (Simpson, 2003). Moreover, the majority of obesity-related postmenopausal breast cancers are oestrogen receptor positive [reviewed in Althuis and et al. (2004), Suzuki and et al. (2006), Yang and et al. (2011)]. As oestrogens are no longer produced in postmenopausal ovaries, this suggests that locally produced oestrogens and/or other endocrine factors are affecting tumour growth within the breast (Harvie and et al., 2003; Rose and et al., 2004). Data relating to the relationship between obesity and premenopausal breast cancer has been conflicting. Obesity was once believed to be associated with a decreased risk of breast cancer in younger women, however, a number of studies now indicate that a BMI > 30 increases the risk of hormone receptor negative tumours in women prior to menopause (Daling and et al., 2001; Cotterchio and et al., 2003; Fagherazzi and et al., 2011), indicating that these tumour cells are not dependent on oestrogens for proliferation but rather, may rely more heavily on insulin, insulin-like growth factor-1 (IGF-1) and adipose-derived factors for growth. Interestingly, weight gain prior to menopause, particularly from 30 years of age to menopause, has been associated with an increased risk of developing breast cancer after menopause (Howell and et al., 2009). Adiponectin, reduced in obesity, has previously been shown to activate AMPK in a number of cell types, including hepatocytes, myocytes and adipocytes, and it is known to inhibit the proliferation and metastasis of breast cancer cells. In the ER-positive MCF-7 and T47D cells, adiponectin treatment leads to an increase in AMPK activity and ACC phosphorylation, and inhibition of p70S6 kinase, which is dependent on LKB1 (Taliaferro-Smith and et al., 2009). In MDA-MB-231 cells, adiponectin has been shown to activate AMPK, dependent on LKB1 (Taliaferro-Smith and et al., 2009), and as a result to stimulate dephosphorylation of Akt by increasing protein phosphatase 2A activity (Kim and et al., 2009). Moreover, the peptide adiponectin receptor agonist ADP 355 has been shown to increase AMPK and inhibit the growth of orthotopic human breast cancer xenografts (Otvos and et al., 2011). Reports predominantly suggest an anti-proliferative role for AMPK in breast cancer. Nonetheless, a few studies have suggested opposing mechanisms. Linher-Melville et al. demonstrated that prolactin, known to stimulate breast cancer cell proliferation, stimulated carnitine palmitoyltransferase 1A (CPT1A) expression and activity in MDA-MB-231 cells, and consistent with this, caused the increased phosphorylation of AMPK at Thr172 (Linher-Melville and et al., 2011). The maintained effect of prolactin to stimulate CPT1A activity in cells where LKB1 or AMPK are knocked-down suggests that other mechanisms may be involved in regulating β -oxidation of fatty acids in these cells, therefore bypassing the need for AMPK activation and possibly providing an explanation for the seemingly disparate effects of AMPK on breast cancer cell proliferation.

In addition to direct effects, indirect effects of AMPK on cancer cell growth have been reported. Breast cancer cells are largely dependent on endocrine and locally produced factors for proliferation, including those produced by pre- and mature adipocytes. In the context of breast cancer, silencing of AMPK leads to the increased secretion of IL-6 and IL-8 by lipopolysaccharide-treated adipocytes, which in turn increases MCF-7 cell proliferation (Grisouard and et al., 2011). Moreover, the majority of postmeno-

pausal breast cancers are oestrogen-dependent and evidence suggests that it is the oestrogens produced locally within the breast that are responsible for the increased proliferation of cancer cells [reviewed in Simpson and Brown (2011)]. Interestingly, AMPK has been shown to be a negative regulator of aromatase, the enzyme responsible for the biosynthesis of oestrogens, in human breast adipose stromal cells (Brown and et al., 2009). Results demonstrate that AMPK prevents the nuclear translocation of the CREB-coactivator CRTC2, required for activation of the breast-specific promoter of aromatase. Moreover, the tumour-derived inflammatory factor PGE2 and the adipokine leptin, cause the downregulation of LKB1 expression and AMPK phosphorylation at Thr172, whereas PGE2 causes an increase in phosphorylation of AMPK at the inhibitory Ser485/491, thereby leading to the activation of aromatase transcription and providing an explanation for the increased biosynthesis of oestrogens in obesity and breast cancer. Furthermore, activation of AMPK using adiponectin or AICAR (5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside) led to a decrease in stimulated aromatase expression in these cells, suggesting that AMPK-activators may be useful as next-generation aromatase inhibitors.

2.2. Metformin

Due to the known effect of the anti-diabetic drug metformin to activate AMPK, and reported decreases in cancer incidence in diabetic patients treated with metformin (Beck and Scheen, 2010), extensive studies have been undertaken to determine whether this commonly prescribed drug may be useful for the treatment of endocrine-related cancers. A number of clinical trials have been performed or are currently underway to explore the utility of metformin for cancer patients [reviewed in Belda-Iniesta and et al. (2011)]. Of interest, a pre-operative, window-of-opportunity, randomised trial, whereby non-diabetic women with operable invasive breast cancer were given metformin for 2 weeks, saw a significant decrease in the proliferation marker Ki67, despite insulin levels remaining stable (Hadad et al., 2011). Moreover, treatment was associated with, amongst others, changes in TNFR1 signaling, phosphodiesterase 3B, and cell cycle inhibitors. Genome-wide studies in breast cancer cells have also identified a number of AMPK targets inhibited after metformin treatment (Oliveras-Ferreras and et al., 2009). Of note are inhibitory effects on ribosomal proteins and mitosis-related gene family members, including kinesins, tubulins, histones, auroras and polo-like kinases.

Metformin has also been shown to inhibit the proliferation of breast, prostate, ovarian, colon and pancreatic cancer cells (Buzza and et al., 2007; Zhuang and Miskimins, 2008; Ben Sahra and et al., 2008; Phoenix and et al., 2009), and the effect of metformin on specific breast cancer subtypes has been explored. In LKB1-expressing breast cancer cells, including MCF-7 cells, metformin treatment leads to the activation of AMPK and the inhibition of the cell cycle via the release of sequestered CDK inhibitors, p27Kip1 and p21Cip1 (Zhuang and Miskimins, 2008). Moreover, metformin treatment was shown to cause a 30% decrease in global protein synthesis (Dowling and et al., 2007). This was attributed to a decrease in translation initiation as a consequence of mTOR inhibition. LKB1 expression was shown to be required as LKB1-deficient MDA-MB-231 cells were unaffected by metformin treatment. Zakikhani et al. found that in contrast to rapamycin, which acts directly on mTOR, metformin treatment decreased Akt activation and caused the AMPK-dependent phosphorylation of IRS-1 at inhibitory Ser789 (Zakikhani and et al., 2010).

In rats, metformin was shown to reduce palpable mammary tumour incidence, multiplicity and burden (Zhu and et al., 2011). However, these studies were performed using relatively high doses

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KA. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

5

of metformin (1.0%/0.25%). Lower doses did not inhibit carcinogenesis despite lowering plasma insulin. The authors hypothesise that poor responses may be due to alterations in the expression of transporters OCT-1 and OCT-3 thereby limiting bio-availability of the drug. Contradicting findings by Liu et al. demonstrate that metformin has specific effects on triple negative breast cancer cells both *in vitro* and *in vivo* (Liu and et al., 2009). This study demonstrates that metformin treatment led to the inhibition of cell proliferation and colony formation, as well as the induction of apoptosis. Moreover, nude mice with MDA-MB-231 xenografts showed a significant decrease in tumour growth in response to metformin treatment.

The action of metformin to reduce insulin and IGF-1 signalling suggests that metformin may also inhibit tumour growth via indirect actions [reviewed in Jalving and et al. (2010)]. In an orthotopic model of ER-negative breast cancer, treatment with metformin caused an increase in VEGF expression and intratumoral microvascular density (Phoenix and et al., 2009). Nonetheless, treatment was also associated with a decrease in systemic IGF-1 and inhibition of tumour cell proliferation. Moreover, metformin significantly inhibited the expression of aromatase in human breast adipose stromal cells at micromolar concentrations, similar to the concentrations present in the blood of women treated with metformin (Brown and et al., 2010). This was associated with the increased expression of LKB1 and phosphorylation of AMPK at Thr172, and with the decreased nuclear translocation of CRT2. The effects of metformin on aromatase expression were also shown to be promoter-specific, implying that treatment may inhibit oestrogen production specifically within the breast and thereby prevent side-effects associated with current endocrine therapy (Samarajeewa and et al., 2011).

Metformin has also been shown to have growth inhibitory effects in tamoxifen-resistant and long term oestrogen-deprived breast cancer cells (Berstein and et al., 2011) and metformin also decreased the expression of the multidrug resistance protein 1 (MDR1) (Kim and et al., 2011), implying that metformin may in fact overcome resistance and sensitise cancer cells to other treatments to prevent the emergence of a drug resistance phenotype.

2.3. Other pharmacological regulators of AMPK

Considering the beneficial effect of metformin for cancer treatment and prevention in the majority of the studies described, it is not unexpected that other AMPK-activators are being developed. Lee et al. have described that the AMPK-activator OSU-53 significantly inhibits the viability and clonogenic growth of triple negative breast cancer cells *in vitro* and *in vivo*, leaving the non-malignant MCF-10A cells unaffected (Lee and et al., 2011). This study demonstrated that the drug had inhibitory effects on mTOR signalling, lipogenesis, and it suppressed biosynthesis and increased oxidation of fatty acids. It also caused a 47–49% decrease in the growth of MDA-MB-231 cells in tumour-bearing mice. Interestingly, the action of AICAR the commonly used AMPK-activator to stimulate AMPK activity is dependent on its conversion to ZMP, an AMP mimetic. Use of methotrexate, to inhibit metabolism of ZMP, potentiates the effects of AICAR to stimulate the anti-proliferative effects of AMPK in cancer cells (Beckers and et al., 2006).

Limiting energy availability using 2-deoxyglucose also leads to an increase in AMPK activity in MDA-MB-468 breast cancer cells, an effect accompanied by a decrease in Akt phosphorylation (Jiang and et al., 2008). Consistent with these changes, phosphorylation of ACC is increased and mTOR is inhibited. It has also been demonstrated that AMPK can mediate the pro-apoptotic signal elicited by E2F1, and that signalling is impaired in tumour cells due to the PI3 K-dependent downregulation of the AMPK α 2 subunit (Hallstrom and et al., 2008). Chemopreventive anti-cancer agents have

been suggested to promote G1-to-S phase cell cycle arrest by, amongst other mechanisms, increasing AMPK-TSC signalling, inhibiting mTOR and upregulating transcription and translation initiation of p27 (Kip1) (Eto, 2010, 2006).

Interestingly, the isoflavonoid puerarin, a major isoflavonoid derived from the Chinese medical herb *Radix puerariae*, has similar effects to metformin to inhibit MDR1 expression and increase the activity of AMPK, and the phosphorylation of ACC and GSK-3 β (Hien and et al., 2010). Moreover, the saponin Compound K, isolated from ginseng, has been shown to suppress cancer cell growth and induce apoptosis. In MCF-7 cells, these effects were dependent on AMPK activation which led to a decrease in COX-2 expression and PGE2 production, and an increase in ROS (Kim and et al., 2010). Inhibition of cell proliferation as a consequence of AMPK-dependent COX-2 downregulation has been examined in response to various agents, including metformin, curcumin (principal curcuminoid of the spice turmeric), 5-fluorouracil, epigallocatechin gallate (EGCG; most abundant catechin in tea), selenium, genistein (isoflavone found in a number of plants), capsaicin (active component of chili peppers), p-HPEA-EDA (a phenolic compound of virgin olive oil), catechin (component of green tea extract), and quercetin (flavonoid found in fruits, vegetables, leaves and grains), whereby activation of AMPK has been shown to be associated with a decrease in COX-2 expression and suppression of growth of breast and colon cancer cells, and ovarian granulosa cells (Park and et al., 2009; Lee and et al., 2009; Khanal and et al., 2011; Kim and et al., 2007; Hwang and et al., 2006, 2007, 2005, 2009; Elia and et al., 2006). Interestingly, resveratrol, a compound found largely in the skins of red grapes, has also been shown to inhibit cell proliferation of breast cancer cells in an AMPK-dependent manner and independent of hormone receptor status (Lin and et al., 2010).

3. AMPK in prostate cancer

The role of AMPK in prostate cancer remains poorly characterised, as there are many discrepancies in the literature. Namely, a number of findings suggest that AMPK promotes tumour growth, whereas an almost equal number propose that it has cell cycle inhibitory properties. These conflicting results may resolve themselves with a better understanding of the possibly differing role of AMPK in tumour initiation, growth and progression.

Little is known of the expression and activity of AMPK in prostate cancer clinical samples. Nonetheless, prostate cancer development has been shown to be associated with an increase in *de novo* lipogenesis within the tumorous epithelium (Zadra and et al., 2010). Considering that *de novo* lipogenesis is often associated with decreased AMPK activity, this study contrasts findings from 2009, where Park et al. demonstrated that 40% of human prostate cancer specimens examined had elevated phosphorylated ACC, a direct measure of AMPK activity, and which results in inhibition of lipogenesis (Park and et al., 2009). Moreover, CaMKK β , a known upstream AMPK kinase, is increased in prostate cancers and as a result AMPK phosphorylation is also increased, and inhibition of this signalling axis has been shown to block androgen-mediated cell migration and invasion (Frigo and et al., 2011).

AMPK phosphorylation at Thr172 is detectable in a number of prostate cancer cell lines, including the androgen-sensitive LNCaP cells and the androgen-independent C4-2B, CWR22Rv1, DU-145 and PC-3 cells (Park and et al., 2009). Moreover, knockdown or pharmacological inhibition of AMPK in CWR22Rv1 or LNCaP cells significantly decreases cell viability and number. Signalling of the pro-apoptotic Fas death receptor is impaired in DU-145 cells, despite high cell-surface expression of wt Fas, and due to the increased activity of AMPK (Jung and et al., 2009).

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6

K.A. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

Despite this, a number of studies have also demonstrated AMPK to be inhibitory of tumour cell growth. Notably, introducing a dominant negative mutant of AMPK or silencing its expression has been shown to increase cell proliferation, migration and anchorage-independent growth in C4-2 cells (Zhou and et al., 2009). Furthermore, a number of AMPK downstream targets have been implicated in the suppression of tumour cell growth and its activation leads to the increased expression of lipopolysaccharide-induced tumour necrosis factor (TNF) α factor (LITAF) and TNF superfamily member 15 (TNFSF15), two proteins which inhibit proliferation and anchorage-independent growth (Zhou and et al., 2011).

3.1. Endocrine regulators

Changes in nutrient availability occur as a result of tumour growth and the role of AMPK in prostate energy sensing has been explored. The reliance of prostate cancer cells on *de novo* purine synthesis is emphasised in LNCaP cells whereby inhibition of purine synthesis using the antifolate reagent AG2034 leads to an increase in AMPK activity, with and without hypoxanthine, and as a result leads to the increased expression of p53, p21, p27 and p16 and cell senescence (Obajimi and et al., 2009). In contrast, non-tumorigenic RWPE-1 cells favour salvage pathways to maintain ATP pools and AG2034 has no effect on AMPK in the presence of hypoxanthine. Interestingly, it was also shown that glucose deprivation, known to increase relative levels of AMP/ATP and as a result activate AMPK, has more profound effects on AMPK in androgen-independent prostate cancer cell lines compared to androgen-dependent ones, where AMPK activation is associated with increased cell survival (Chhipa and et al., 2010). In androgen-dependent prostate cancer, surgical or medical castration leads to decreased androgen and oxygen levels, and leads to nutrient depletion in prostate tumour tissue. Androgen and oxygen deprivation of LNCaP cells leads to an increase in AMPK activity associated with an increase in the pro-autophagy protein beclin (Chhipa and et al., 2011).

The link between AMPK and HIF1 α is well established in prostate cancer cells. Glucose deprivation in DU145 cells has been shown to be associated with an increase in ROS and HIF1 α activity and consequently, with the increased expression of VEGF, GLUT1 and PFKFB3 transcripts (Yun and et al., 2005; Jung and et al., 2008). HIF1 α is stabilised in human prostate cancer DU145 cells under hypoxic conditions or treated with cobalt chloride (CoCl₂), and this is preceded by the activation of AMPK.

Conversely, the cytokine CCL2, elevated in prostate cancers, has been shown to promote PC3 cell survival by inducing mTOR signalling (Roca and et al., 2008). In 2009, the same group demonstrated that CCL2 inhibited AMPK phosphorylation at Thr172 in serum starved PC3, DU145 and C4-2B cells, resulting in the hyperactivation of mTORC1, independent of Akt (Roca and et al., 2009). Studies examining the effect of adiponectin on AMPK in prostate cancer are few. One study demonstrated that adiponectin significantly decreased PC3 cell proliferation and that this was associated with a reduction in mTOR phosphorylation at Ser2448 (Zakikhani and et al., 2008). Consistent with these findings, treatment was also associated with a decrease in phosphorylation of mTOR targets p70S6 kinase and ribosomal protein S6. Interestingly, a study by Barb et al. demonstrated that although adiponectin did activate AMPK in PTEN-deficient LNCaP cells, it also increased the activity of mTOR and the expression of its downstream targets p70 S6 kinase and ribosomal protein S6 (Barb and et al., 2007). This counterintuitive result was hypothesised to be due to the effect of adiponectin to increase PI3K/Akt signalling and the authors suggest that the anti-proliferative effect of adiponectin may be cell-type specific.

3.2. Metformin

The combination of metformin and 2-deoxyglucose has been shown to induce apoptosis in LNCaP prostate cancer cells in an AMPK-dependent manner (Ben Sahra and et al., 2010). Metformin also inhibited the 2-deoxyglucose-induced autophagy and as a result inhibited beclin-1 expression and caused cell death. Curiously, the high concentrations used (1–5 mM) in these experiments caused 50% of the LNCaP cells to die, but resulted in less than 25% cell death in the normal prostate epithelial cell line P69 suggesting that metformin may have cell-specific effects (Ben Sahra and et al., 2008). *In vivo*, LNCaP xenograft-bearing mice treated with metformin orally and intraperitoneally resulted in a 50% and 35% reduction in tumour growth, respectively.

3.3. Other pharmacological regulators of AMPK

AICAR has been shown to decrease the proliferation and viability of a number of cancer cell types, including the androgen-insensitive PC3, DU145 and C4-2 prostate cancer cells and androgen-dependent LNCaP cells (Zhou and et al., 2009; Xiang and et al., 2004; Isebaert and et al., 2011). Treatment of prostate cancer cells with AICAR is associated with an increase in ROS and the expression of the cell cycle inhibitor p21, induction of apoptosis, and a decrease in the concentration of malonyl CoA, a fatty acid synthesis intermediate.

Interestingly, treatment of prostate cancer cells with alternol, a fermentation product purified from a microorganism found in the bark of yew trees, leads to inhibition of AMPK phosphorylation and a decrease in cell viability (Yeung and et al., 2011). It has also been suggested that the lower incidence of prostate cancer in Asian countries may be due to the higher consumption of soy products containing isoflavones. In DU145 prostate cancer cells, the isoflavone genistein significantly decreased ROS levels, a mechanism shown to be primarily dependent on AMPK activation (Park and et al., 2010). Moreover, the environmentally toxic metal salt vanadate, known to cause tumour formation in animals and accumulation of HIF1 α , also causes the activation of AMPK in DU145 cells. This activation was shown to be required for the vanadate-induced expression of HIF1 α (Hwang and et al., 2004). The same group has also demonstrated a role for AMPK in the arsenite and cobalt regulation of VEGF (Lee and et al., 2006). Blockade of AMPK activity decreases the hypoxia-induced secretion of VEGF and glucose uptake (Lee and et al., 2003).

Despite many interesting findings, the role of AMPK in prostate cancer formation, growth and progression remains poorly defined and contentious. Conflicting results may be attributable to a changing role of AMPK in different disease states and acute effects of AMPK modulation may differ substantially from chronic effects seen in the physiological/pathological state. Much work remains to be done to fully understand the complex role of AMPK in prostate cancer.

4. AMPK in endometrial and ovarian cancer

The role of AMPK in ovarian and endometrial cancer has been explored, however, much less is known of its role in other female reproductive cancers. This section will predominantly present data pertaining to the ovary and the endometrium.

In ovarian carcinoma samples, decreased AMPK expression is correlated with higher tumour grade and associated with poorer prognosis (Buckendahl and et al., 2011). Moreover, glucose concentrations are higher in AMPK-deficient tumours indicating a deregulation of AMPK-dependent energy metabolism.

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4.1. Endocrine regulators

AMPK has been shown to be expressed in rat and chicken granulosa cells and AMPK activation is associated with the inhibition of follicle stimulating hormone (FSH) and IGF-1-stimulated progesterone production (Tosca and et al., 2006, 2005). Moreover, proliferation of granulosa cells in response to FSH is dependent on inhibition of AMPK, via the decreased phosphorylation of AMPK at Thr172 and the increased phosphorylation at Ser485/491, thereby leading to an increase in cyclin D2 and decrease in p27 kip expression (Kayampilly and Menon, 2009). These findings were shown to be dependent on Akt activity. Resistin also affects granulosa cell proliferation in the presence and absence of IGF-1, and was shown to inhibit AMPK signalling (Maillard and et al., 2011).

A major risk factor for endometrial cancer is obesity, and low adiponectin levels have been demonstrated to be an independent risk factor for endometrial cancer in epidemiological studies [reviewed in Paz-Filho and et al. (2011)]. Using the endometrial cancer cell lines KLE and RL-95, it was demonstrated that adiponectin inhibited cell proliferation, colony formation, adhesion and invasion in an adiponectin receptor-dependent fashion (Moon and et al., 2011). In the same study, the authors demonstrate that adiponectin increased the phosphorylation of LKB1 and AMPK, and that knockdown of LKB1 decreased or abolished the effect of adiponectin to inhibit cell proliferation, colony formation, cell adhesion and/or invasion.

4.2. Metformin

Progesterone, via actions on the progesterone receptor (PR), has anti-proliferative effects on endometrial cancer cells. However, resistance often occurs due to the downregulation of the PR. AMPK has been shown to increase PR expression in metformin-treated cells, where metformin also synergised with medroxyprogesterone acetate to inhibit cell proliferation (Xie and et al., 2011). Metformin is routinely used to treat the reproductive and insulin resistant phenotype observed in polycystic ovary syndrome and has been shown to decrease inducible nitric oxide synthase in the ovary of hyperandrogenised mice (Elia and et al., 2006), and activation of insulin and IGF pathways has been implicated in tumour growth. Consistent with the effect of metformin to decrease steroidogenesis and proliferation in response to FSH/IGF in bovine granulosa cells (Tosca and et al., 2010, 2007). Moreover, treatment was shown to stimulate phosphorylation of AMPK in the presence of insulin and stimulate lactate production in human granulosa cells (Richardson and et al., 2009; Pellatt and et al., 2011).

5. AMPK in colon cancer

There may be debate as to whether or not colon cancer should be considered an endocrine-related disease. However, its association with obesity and the metabolic syndrome suggests that adipose-derived factors may play an important role in mediating its occurrence. This section will therefore present data which describe the regulation of AMPK in colon cancer and explores the mechanisms which may underlie its role as a regulator of tumorigenesis.

In clinical samples, phosphorylated AMPK expression was not associated with survival. However, its expression in pMAPK3/1-positive cases was associated with increased colorectal cancer-specific survival with an adjusted HR of 0.42 (95% confidence interval, 0.24–0.74) (Baba and et al., 2010).

The role of AMPK in mediating pro-apoptotic effects in colon cancer cells has been explored. Namely, activation of AMPK using

AICAR, in combination with either TRAIL or TNF α , leads to the enhanced activity of caspase-8, -9 and -3, and leads to a decrease in mitochondrial membrane potential, and as a consequence sensitises cells to apoptosis (Su and et al., 2007). This effect was shown to be dependent on p53 activity and to involve p38 and c-Jun NH(2)-terminal kinase. The effect of AMPK to inhibit cell growth appears to be dependent on p53-status. Activation of AMPK in p53(–/–) HT116 cells, and not p53(+/+) HT116 cells, leads to increased apoptosis both *in vivo* and *in vitro* (Buzzai and et al., 2007), whereas activation of AMPK in p53(+/+) cells, and not p53(–/–) cells, results in increased autophagy. By contrast AMPK has been shown to antagonise pro-apoptotic ERK signals in response to glucose deprivation via p53-dependent increased expression of dual-specificity phosphatases 1 & 2, thereby providing a mechanism whereby a tumour cell may survive under energy stress (Kim and et al., 2010a).

5.1. Endocrine regulators

Adiponectin decreases colon cancer cell growth *in vitro* via activation of the adiponectin receptors AdipoR1 and AdipoR2 (Kim and et al., 2010b; Sugiyama and et al., 2009). This is accompanied by an increase in AMPK phosphorylation and the suppression of mTOR signalling, as well as with the increased expression of cell cycle inhibitor proteins p21Cip1 and p27Kip1. The reduction in mTOR activation by adiponectin was also shown to require AMPK activation in HT-29 colon cancer cells (Zakikhani and et al., 2008). In animal models, loss of adiponectin has been shown to increase intestinal polyp formation 3.2-fold, and azoxymethane-induced tumour formation was increased 71% (Mutoh and et al., 2011). These effects were accompanied by a significant decrease in AMPK phosphorylation in intestinal epithelial cells of adiponectin-knock-out mice compared to wild-type.

IGF-1 is a known mitogenic agent in a number of cancers [reviewed in Annunziata and et al. (2011)]. Effects of IGF-1 to increase lipogenesis in HCT-8 colon cancer cells have been shown to occur as a result of decreased ATM/AMPK signalling with consequent decreases in ACC phosphorylation (Luo and et al., 2011). This study also demonstrated a cooperative effect with ERK signalling whereby stimulation with IGF-1 led to an ERK1/2-dependent stimulation of ACC α expression. Interestingly, IGF-1 had no effect on HCT-8 cell proliferation. AMPK has also been speculated to play a role in melatonin-induced autophagy in colon cancer cells (Motilva and et al., 2011), however, more studies are required to confirm these hypotheses.

5.2. Metformin

In colon cancer xenografts, metformin was shown to selectively inhibit p53-null cell proliferation (Buzzai and et al., 2007). Moreover, metformin suppresses the high-fat diet induced growth of MC38 colon cancer cells in mouse xenografts (Algire and et al., 2010) and prevents the azoxymethane-induced formation of colorectal aberrant crypt foci by activating AMPK (Hosono and et al., 2010). Phenformin has been shown to be a more potent inhibitor of colon cancer cell growth than metformin (Lea and et al., 2011). In the same study, combination of phenformin and 2-deoxyglucose was shown to prevent the phenformin-induced acidification of media but enhance the growth inhibitory effects.

5.3. Other pharmacological regulators of AMPK

The lipophilic statin pitavastatin prevents the azoxymethane-induced preneoplastic lesion formation within the colon by increasing AMPK phosphorylation and decreasing the expression of TNF α , IL-6, IL-18 and COX-2 (Yasuda and et al., 2010). Toxic

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8

K.A. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

levels of hydrogen peroxide lead to the activation of AMPK in HT-29 colon cancer cells, an effect associated with a decrease in COX-2 expression and an increase in cell apoptosis (Park and et al., 2006). Chemopreventative and chemotherapeutic agents, including naturally-derived products such as rhizochalin, compound K and 20(S)-ginsenoside Rg3, induce apoptosis in colon cancer cells via activation of AMPK (Lee and et al., 2010; Kim do and et al., 2009; Yuan and et al., 2010; Khanal and et al., 2011). HT-29 cells treated with rhizochalin and its derivatives displayed increased PARP activation, DNA fragmentation and apoptosis, and consistent with AMPK activation, had decreased mTOR-p70S6 kinase-ERK signaling. Anthocyanins, natural non-toxic food colouring agents which have recently received attention due to their antioxidant and anti-carcinogenic properties, inhibit colon cancer cell growth *in vivo* and *in vitro*, dependent on AMPK (Lee and et al., 2010). Resveratrol, in combination with etoposide, has also been shown to cause AMPK activation in HT-29 colon cancer cells (Hwang and et al., 2007). This was associated with inhibition of cell growth, induction of apoptosis and an increase in ROS.

6. Conclusions

A growing body of evidence suggests an important role of AMPK in preventing and inhibiting tumour cell growth. Nevertheless, some evidence indicates that AMPK may in fact be pro-proliferative in certain disease states. Conflicting studies need particular attention in order to understand the intricacies behind metabolic control of tumour growth in relation to tumour type and stage of the disease, especially when so much attention has been given to AMPK-activating drugs as potential anti-cancer agents. Notwithstanding, reports are accumulating to support the use of drugs like metformin to treat and possibly prevent cancer and whose actions not only target tumour cell growth via inhibition of *in situ* protein synthesis and cell cycle, but also inhibit endocrine and intracrine factors, such as insulin, IGF-I and oestrogens, which are known to support tumour growth.

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KA. Brown et al./Molecular and Cellular Endocrinology xxx (2012) xxx–xxx

9

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10

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APPENDIX 2



Declaration for Appendix 2

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 (%)
Manuscript preparation	50%

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
Kristy A. Brown	Manuscript preparation	

Candidate's
Signature

	Date 10/12/2012
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Declaration by co-authors

The undersigned hereby certify that:

- (25) 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.
- (26) 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;
- (27) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
- (28) there are no other authors of the publication according to these criteria;
- (29) 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
- (30) 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 of Medical Research (PHIMR), Clayton,
VIC, Australia**

Signature 1

	Date 18/12/2012
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Gene Section

Mini Review

CRTC2 (CREB regulated transcription coactivator 2)

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Identity

Other names: TORC2, RP11-422P24.6

HGNC (Hugo): CRTC2

Location: 1q21.3

DNA/RNA

Description

10,893 bases; on minus strand.

Includes 14 exons.

Transcription

Transcript measures 2598 bp with a 2082 bp coding sequence.

Protein

Description

693 amino acids; 73,302 Da.

Expression

Particularly abundant in B and T lymphocytes. Higher levels were also seen in muscle, lung, spleen, ovary and breast. Lower expressions found in brain, colon, heart, kidney, prostate, small intestine and stomach, with significantly lowest expression in liver and pancreas.

Localisation

Phosphorylation of CRTC2 triggers the phosphorylation-dependent binding to 14-3-3 proteins, and hence sequestration of CRTC2 in the cytosol thereby preventing its nuclear translocation and the activation of CREB. Proteins known to phosphorylate CRTC2 at Ser171 include AMP-

activated protein kinase (AMPK) and the salt-inducible kinases (SIKs). Dephosphorylated CRTC2 readily translocates to the nucleus. CRTC2 contains a nuclear

localisation sequence (NLS) at amino acids 56-144 as well as two nuclear export sequences (NES1 and NES2) within the region of amino acids 145-320.

Function

Transcriptional coactivator for CREB (cAMP-responsive element binding protein). The highly conserved N-terminal coiled-coil domain of the CRTC2 interacts with the bZip domain of CREB which activates both consensus and variant cAMP response element (CRE) sites, leading to activation of CREB target gene expression. CRTC2 responds to stimulation by cAMP, calcium, fasting hormones, G protein-coupled receptors, and AMPK/SIKs.

Implicated in

Peutz-Jeghers syndrome

Note

Peutz-Jeghers syndrome (PJS) is an autosomal-dominant genetic disorder that is characterised by an increased risk of developing malignant tumours. Most of the identified mutations in the LKB1 gene are localised to the catalytic kinase domain so that it is thought that PJS results from loss of LKB1 kinase activity. The silencing of LKB1, leads to the decreased activity of AMPK and SIK and leads to the increased nuclear translocation and activity of CRTC2.

Disease

Gastrointestinal polyps and cancers including esophagus, stomach, small intestine, colon, pancreas, lung, testes, breast, uterus, ovary and cervix.

Oestrogen-receptor (ER) positive breast cancer

Note

The increased prevalence of oestrogen-dependent, postmenopausal breast cancers is correlated with

elevated local levels of oestrogens as a result of an increase in cytochrome P450 aromatase expression within the adipose stromal (hAS) cells surrounding the breast tumour - aromatase is the enzyme responsible for the conversion of androgens to oestrogens. This is governed by promoter switching from the distal promoter I.4 to the proximal promoter PII on the CYP19A1 gene, that encodes aromatase, in response to factors derived from the tumour such as prostaglandin E2 (PGE2). Interestingly, the LKB1/ AMPK pathway has been shown to inhibit aromatase expression via the cytoplasmic sequestration of CRTC2. However, PGE2 inhibits LKB1/AMPK signaling, leading to the nuclear translocation of CRTC2 and its enhanced binding and activation of aromatase promoter PII in hAS cells. Furthermore, the adipokine leptin, produced at higher levels in obesity, has been shown to cause an increase in CRTC2 nuclear translocation and consequently, in aromatase expression.

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