Hormonal Regulation of the Anticoagulant Protein S

Quintin Hughes

**Supervisors Prof. Jennet Harvey, Dr. Janelle Staton
and Dr. Mark Watson**

**This thesis is a requirement for a postgraduate Doctorate of
Philosophy undertaken at the University of Western Australia, School
of Surgery and Pathology**

November 2007

PREFACE

This thesis 'Hormonal Regulation of the Anticoagulant Protein S' is a true representation of my own research. All experiments were conducted by myself in the Department of Haematology and the Research Centre, both situated at Royal Perth Hospital, Perth, Western Australia, under the supervision of Dr. Janelle Staton, with the following exceptions. Figures 3.7C and 3.8B were performed by Vanessa Atkinson. The pregnancy cohort DNA obtained from King Edward Hospital detailed in Section 2.1 was extracted by Dr. Anne Bardon while the associated levels of free, total and functional Protein S were determined by Leesa Ivey, Jim Thom and Grace Gilmore.

Data presented in this thesis is to the best of my knowledge and belief original, except where acknowledge in the text, and has not been submitted, either whole or in part, for a degree at this or any other university.

Quintin W. Hughes

April 2007

ABSTRACT

Every year thousands of individuals suffer from thrombotic related complications that in some cases can be fatal and every year millions of women take some form of hormonal contraceptive. In some cases, there is a cause and effect relationship between the two as users of the combined oral contraceptive pill have an increased risk of developing a thrombotic event. Increased circulating levels of oestrogen cause a prothrombotic shift in the coagulation cascade resulting from upregulation of several procoagulant proteins and a decrease of key anticoagulant proteins. One of the most oestrogen sensitive anticoagulants is Protein S (PS), a product of the PROS1 gene. PS acts as a cofactor to activated protein C (aPC) and the PS-aPC complex serves to downregulate clot formation by deactivating the tenase and prothrombinase complexes via proteolytic cleavage of activated factors VIII and V, respectively. As such, low PS levels are associated with an increased risk of developing thrombotic disorders such as pulmonary embolism, stroke or coronary thrombosis and deep vein thrombosis. During pregnancy when oestrogen levels increase, a steady decline in PS is evident in the early weeks of gestation and continues to decrease to below the normal range in the 2nd trimester, remaining there until post-partum. In addition, reduced free and total PS levels are observed in users of the combined oral contraceptive (COC) pill that contains an oestrogen and a progestin. Interestingly, users of 3rd generation COCs have significantly greater reductions of PS than do 2nd generation COC users. The difference between the two forms is the type of progestin, not the oestrogen, which is predominantly ethinyl oestradiol in the majority of commercially available preparations. At present, a mechanism to describe the relationship between oestrogen and/or progesterone associated with the observed *in vivo* changes in the levels of PS has not been identified. The aim of this thesis was to define the molecular mechanisms involved in the regulation of PS expression by oestrogen and progesterone. In this study, a Combined Single-stranded conformational analysis and Heteroduplex Analysis (CSHA)

methodology was optimised for screening both PROS1 DNA and mRNA for the detection of mutations. Two patient cohorts were screened, the first comprised patients with abnormally low PS levels while the second was a pregnancy cohort consisting of normal controls and women who experienced greater than normal decreases in their PS levels during gestation. Several mutations were detected including four novel mutations; T101A and G340C both within the PROS1 coding region, g-175a located in the 5' untranslated region and a three nucleotide deletion mapping to intron A. The g-175a variant is of particular interest because its location in the 5'UTR could potentially disrupt binding of transcriptional factors and hormone receptor complexes affecting regulation of the PROS1 promoter. Two other previously described variants were also detected in the 5'UTR, t-397c and c-407t. These variants underwent further investigation as both mapped to a potential progesterone response element (PRE) identified using *in silico* analysis. In addition, a putative oestrogen response element (ERE) was identified at positions -526 to -538 within the 5'UTR. The effects of these elements were investigated in transfection experiments. A 950bp fragment of the PROS1 5'UTR was inserted upstream from an enhanced green fluorescence protein (EGFP) reporter gene. The resultant plasmid, denoted pPMTR, was used for transient transfections of the liver and breast carcinoma cell lines, HuH-7 and MCF-7, respectively. Post-transfection treatment with 17 β -oestradiol and a plasmid expressing oestrogen receptor alpha reduced activity of the PROS1 promoter by up to 35% when the MCF-7 line was used. The response was stronger (reduction of 46%) in the HuH-7 cell line. Conversely, PROS1 promoter activity increased (up by 25%) when the pPMTR vector was transiently transfected into the MCF-7 cells treated with progesterone. The response was reversed in the presence of the progestin receptor antagonist RU486 (activity equivalent to no progesterone control). The level of increased activity was lower when the experiments were repeated in the HuH-7 cell line (17% increased activity in the

presence of progesterone). Subsequent investigation identified a mechanism involving the B-isoform of the progesterone receptor (PR-B) binding to the postulated PRE containing the t-397c and c-407t variants. Introduction of one of the variants, c-407t, via site directed mutagenesis, disrupted PR-B binding, reducing the progestin mediated upregulation of the PROS1 promoter. In addition, an electromobility shift assay was performed which established PR-B specific binding to the PRE and further demonstrated that incorporation of the c-407t variant reduced binding affinity. This suggested that the area spanning c-407t is involved in direct binding of the progesterone/PR-B complex. Surprisingly, assessment of different progestin isoforms found the level of transcriptional upregulation varied greatly with medroxyprogesterone eliciting a response more than double the effect observed for progesterone. This may explain why users of 3rd generation COCs display a greater reduction in circulating PS levels compared to 2nd generation users. To investigate potential PS interactions with other proteins that could be hormonally regulated, a yeast-2-hybrid (Y-2-H) screen was performed using the PS molecule as a 'bait' against molecules derived from liver and bone marrow cDNA libraries. A clone that contained a portion of another haemostatic protein, Protein Z (PZ) was isolated and confirmed via sequencing. As no full length PZ clones were identified, a second Y-2-H screen was performed once again using the PS molecule as bait and the PZ molecule as the fish. Interaction between the two proteins was shown to be possible via the successful growth of colonies on triple knock out selective media and by positive β -galactosidase activity.

PUBLICATIONS

Prizes

2004 ASTH Medal

2005 MRF Young Scientific Investigator Award

2006 CBSM Oral Presentation Prize

Runner up 2006 ASTH Medal

Complete list of publications

- Oral Presentation- The 6th Combined Annual Scientific Meeting of the Haematology Society of Australia and New Zealand, the Australian and New Zealand Society of Blood Transfusion, and the Australasian Society of Thrombosis and Haemostasis (HAA) Annual Scientific Meeting, Melbourne, Australia. October 17-20th 2004.
- Oral Presentation- Australian Society of Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia. June 7th 2005.
- Poster Presentation- XXth Congress of the International Society of Thrombosis and Haemostasis (ISTH), Sydney, Australia. August 16-18th 2005.
- Oral Presentation- The 15th Annual Combined Biological Sciences Meeting (CBSM), Perth, Australia. August 26th 2005.
- Oral Presentation- Medical Research Foundation (MRF) Young Investigators Day, Perth, Australia. September 1st 2005.
- Oral Presentation- The 7th Combined Annual Scientific Meeting of the Haematology Society of Australia and New Zealand, the Australian and New Zealand Society of Blood Transfusion, and the Australasian Society of Thrombosis and Haemostasis (HAA) Annual Scientific Meeting, Sydney, Australia. October 16-19th 2005.
- Oral Presentation- Medical Research Foundation (MRF) Young Investigators Day, Perth, Australia. November 9th 2005.

- Oral Presentation- University Department of Medicine Seminar, Perth, Australia. 25th May 2006.
- Oral Presentation- Australian Society of Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia. June 9th 2006.
- Oral Presentation- 11th Congress of the European Hematology Association (EHA), Amsterdam, The Netherlands. June 15-18th 2006.
- Oral Presentation- Royal Perth Hospital Department of Haematology Breakfast Meeting, Perth, Australia. June 27th 2006.
- Oral Presentation- The 16th Annual Combined Biological Sciences Meeting (CBSM), Perth, Australia. August 18th 2006.
- Oral Presentation- University of Western Australia School of Surgery and Pathology Seminar, Perth, Australia. September 29th 2006.
- Oral Presentation- The Combined Annual Scientific Meeting of the Haematology Society of Australia and New Zealand, the Australian and New Zealand Society of Blood Transfusion, and the Australasian Society of Thrombosis and Haemostasis (HAA) Annual Scientific Meeting, Hobart, Australia. October 15-18th 2006.

Key findings of Chapter 4 have been published in the Journal of Thrombosis and Haemostasis-

- Hughes, Q., Watson, M., Cole, V., Sayer, M., Baker, R. and Staton, J. (2007) Up-regulation of Protein S by progestins. *Journal of Thrombosis and Haemostasis*. 5(11) 2243–2249.

TABLE OF CONTENTS

TITLE	I
PREFACE.....	II
ABSTRACT	III
PUBLICATIONS	VI
TABLE OF CONTENTS.....	VIII
ACKNOWLEDGEMENTS.....	XI
ABBREVIATIONS	XIV
CHAPTER 1 LITERATURE REVIEW.....	1
1.1 INTRODUCTION	2
1.2 THROMBOSIS	2
1.3 THE COAGULATION CASCADE	4
1.4 THE PROTEIN C PATHWAY	5
1.5 PROTEIN S	8
1.6 THE ORAL CONTRACEPTIVE PILL	12
1.7 PREGNANCY	15
1.8 HORMONE REPLACEMENT THERAPY	16
1.9 OESTROGEN RECEPTOR BIOLOGY.....	17
1.10 PROGESTERONE RECEPTOR BIOLOGY.....	21
1.11 THE EFFECT OF HORMONES ON HAEMOSTATIC PARAMETERS	23
1.12 THE EFFECT OF HORMONES ON PROTEIN S.....	25
1.13 PROTEIN S GENETICS.....	26
1.14 AIMS.....	28
CHAPTER 2 MATERIALS AND METHODS.....	30
2.1 PATIENTS AND CONTROLS	31
2.2 DNA AND RNA EXTRACTION	34

2.3	COMBINED SSCA AND HETERODUPLEX ANALYSIS (CSHA).....	34
2.4	SILVER STAINING	35
2.5	ISOLATION OF EXON 5 FRAGMENT.....	35
2.6	CELL CULTURE.....	36
2.7	PLASMID CONSTRUCTION FOR TRANSIENT TRANSFECTION STUDIES.....	36
2.8	TRANSIENT TRANSFECTION STUDIES.....	38
2.9	NUCLEAR PROTEIN EXTRACTION	39
2.10	ELECTROMOBILITY SHIFT ASSAYS	39
2.11	PLASMID CONSTRUCTION FOR YEAST-2-HYBRID	40
2.12	CONFIRMATION OF VECTOR SEQUENCE.....	41
2.13	YEAST-TWO-HYBRID SYSTEM	41
2.14	DATA AND STATISTICAL ANALYSIS.....	43
CHAPTER 3 MUTATION ANALYSIS OF THE PROS1 GENE.....		44
3.1	INTRODUCTION.....	45
3.1.1	<i>Sensitivity of CSHA Compared to Other Detection Methodologies</i>	<i>48</i>
3.2	RESULTS.....	49
3.2.1	<i>Development of the Method</i>	<i>49</i>
3.2.2	<i>Mutations Identified in the Coding Region</i>	<i>55</i>
3.2.3	<i>Mutations Identified in the 5' Untranslated Region</i>	<i>59</i>
3.2.4	<i>Results of mRNA Analysis</i>	<i>59</i>
3.3	DISCUSSION	63
CHAPTER 4 ANALYSIS OF THE PROS1 PROMOTER.....		71
4.1	INTRODUCTION.....	72
4.2	RESULTS.....	75
4.2.1	<i>ERα downregulates the PROS1 promoter but not via the ERE consensus sequence.....</i>	<i>75</i>
4.2.2	<i>Progesterone upregulates PROS1 promoter activity</i>	<i>80</i>
4.2.3	<i>Identification of the PR binding domain within the PROS1 promoter</i>	<i>81</i>
4.2.4	<i>Differential responses to different types of progestins</i>	<i>82</i>
4.2.5	<i>Differential response to glucocorticoid.....</i>	<i>82</i>

4.3	DISCUSSION	91
CHAPTER 5 IDENTIFICATION OF NOVEL PROTEIN S BINDING PARTNERS		99
5.1	INTRODUCTION	100
5.2	THE YEAST-2-HYBRID SYSTEM.....	101
5.2.1	<i>Limitations of the Yeast Two Hybrid Screen.....</i>	<i>104</i>
5.2.2	<i>MatchMaker Yeast-2-Hybrid System</i>	<i>104</i>
5.3	RESULTS	106
5.4	DISCUSSION	110
5.4.1	<i>Follow up Studies required to confirm the PS/PZ Interaction</i>	<i>113</i>
CHAPTER 6 GENERAL DISCUSSION.....		114
REFERENCES		119
APPENDICES.....		137

ACKNOWLEDGEMENTS

First, let me say that Royal Perth Hospital is a great place to do a PhD. So many friendly and helpful people, it's hard to know where to start, but for me, the heart of it is the Research Centre Mob. With people like Marian Sturm, Kay Hudson, Kath Shaw and all the CTTWA crew, how could you ever possibly be glum? Thanks to you all for all your help and advice. Long live the tearoom!

The Research Centre isn't the only source of great people, it seems to me the whole of Level 2 North Block is full of genuine people who are happy offer advice or reagents and provide you with the benefits of their many years of accumulated knowledge. Jim Thom and Grace Gilmore in Coagulation Research; Melinda Higgins, Kerry Stoner, Rom Kruger and John O'Reilly in Haematology; Jacky Bental, Mark Thomas and Diana Azzam in Anatomical Pathology; Ken Robinson and Amanda Hooper in Biochemistry and all the helpful staff in Immunology.

A special thanks goes to all the people directly associated with my project. Thanks to Ross Baker for supporting my project and importantly for allowing me the freedom to pursue concepts and ideas. My supervisor Janelle Staton has provided the best form of supervision for someone like myself and has tirelessly assisted me throughout my candidature. I am indebted to her and Melissa Sayer, both of whom have three small children and other commitments to contend with, but have always found time for my project.

My other co-supervisor Mark Watson has been invaluable in his provision of advice, reagents and equipment. A genuine good bloke if ever I've meet one. This thesis would

read quite differently I'm sure if it hadn't been for your input, especially early on in the piece. Thanks Mark.

I would also like to thank my principal supervisor Prof. Jennet Harvey and the School of Surgery and Pathology (particularly Cora D'Souza) for all their help and support throughout my candidature. I will also take this opportunity to thank Prof. Bob Mead of Murdoch University for the support he gave me as a mature age student in my undergraduate days, all those years ago.

A heartfelt thanks to my lab partner Vanessa Cole (now Atkinson!!), who has put up with my insanity and has helped me in countless ways throughout the past few years. A better fellow scientist I could not imagine. Ta and once again, congratulations Mrs. Atkinson!! I extend the same level of appreciation to Julianna Degenaar too, arguably the best RA in the history of science! Thanks heaps Jules!

I will also use this opportunity to thank other past lab buddies too, including Kate Maslen and Leesa Ivey who provided me with important background on the pregnancy cohort, including providing the serum PS levels data.

Thanks to Dr Evan Ingley from the MRF for his help with the Yeast-2-Hybrid work and for providing the pBTM116 vector and FGR clone. Thanks also to other members of the MRF including Dr Andrew Redfern for providing the oestrogen receptor expression vector and Dr Esme Hatchell for supplying me with reagents and advice.

Other researchers I would like to thank for providing me with vital reagents and helpful advice are Dr Yolanda Espinosa-Parrilla and Dr Nuria Sala for providing the PS DNA positive controls referred to in Chapter 3. Thanks to Prof. Christine Clarke, Prof. Pierre

Chambon and Prof. Tom Ratajczak for providing the progesterone and glucocorticoid expression vectors.

Lastly I would like to thank the people in my personal life. My mother who has, for some unknown reason, always held an unwavering belief in me and my uncle Will and Aunty Ida, who similarly also seem to hold me in high regard? My mates who are more than willing to have a drink (or three) with me when called upon, which of course is very rarely.....

And the biggest thanks goes to Natasha Buzzacott and her beautiful daughter (Queen of the Cheeky Monkeys), Maya. Both of you supported me in many varied and different ways throughout my candidature and I cannot thank you enough. My favourite way to sort out a perplexing problem was to go for a long ride around the lake with The Little One and be instantly reminded that at its core, life should be enjoyed. I will always cherish the time we had together.

ABBREVIATIONS

3'UTR	3' Untranslated Region
3AT	3-Aminotriazole
5'UTR	5' Untranslated Region
Ab	Antibody
aF	Activated Factor
AP-1	Activator Protein 1
aPC	Activated Protein C
aPC-R	Activated Protein C Receptor
AT	Antithrombin
ATP	Adenosine Tri-phosphate
C4bBP	Complement-4b Binding Protein
CMV	Cytomegalovirus
COC	Combined Oral Contraceptive
CORT	Corticosterone
CSHA	Combined Single stranded Conformational and Heteroduplex Analysis
dHPLC	Denaturing High Performance Liquid Chromatography
DMEM	Dulbeccos Modified Eagles Medium
DsRed2	Red Fluorescent Protein
DVT	Deep Vein Thrombosis
E ₂	17 β -Oestradiol
EDTA	Ethylene diamine tetra acetic acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene glycol tetra acetic acid
EMSA	Electro-Mobility Shift Assay
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
ESR1	Oestrogen Receptor alpha gene
EtOH	Ethanol
FCS	Fetal Calf Serum
GlcCer	Glucosylceramide
HA	Heteroduplex Analysis
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	Histidine

HNF-3	Hepatocyte Nuclear Factor-3
HRE	Hormone Response Element
HRM	High Resolution Melt
HRT	Hormone Replacement Therapy
HSPs	Heat Shock Proteins
I.M.A.G.E.	Integrated Molecular Analysis of Genomes and their Expression
IL-6	Interleukin-6
Kb	Kilobase
Leu	Leucine
LiAc	Lithium Acetate
LNG	Levonorgestrel
mM	Milli Molar
MPA	Medroxyprogesterone
OC	Oral Contraceptive
P ₄	Progesterone
PC	Protein C
PE	Pulmonary Embolism
PEG	Polyethylene glycol
pM	Pico Molar
Poly (dI-dC)	Poly(deoxyinosinic-deoxycytidylic) acid sodium salt
POP	Progesterone Only Pill
PR	Progesterone Receptor
PRE	Progesterone Response Element
Pre-E	Pre-eclampsia
PROS1	Protein S gene
PS	Protein S
PZ	Protein Z
RU486	Mifepristone
Sp1	Stimulating protein 1 (transcriptional factor)
SSCA	Single Stranded Conformational Analysis
STAT-3	Signal Transducer and Activator of Transcription-3
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TF	Tissue Factor
Trp	Tryptophan

TSS	Transcriptional Start Site
Veh	Vehicle
Y-2-H	Yeast-2-Hybrid
Yc media	Yeast complete media
ZPI	Protein Z-dependant Inhibitor
α 1ACT	alpha-1-antichymotrypsin
β -Gal	beta-galactosidase
μ M	Micro Molar

CHAPTER 1

Literature Review

1.1 INTRODUCTION

Maintenance of haemostasis is a delicate balance between pro- and anti-coagulant forces. In the event of vascular injury, pro-coagulant activation generates rapid clot formation, preventing blood loss. Primarily, the anti-coagulant system serves to regulate the extent of the clot development, but is also important for the prevention of coagulant activation in intact, healthy blood vasculature.

1.2 THROMBOSIS

Thrombosis results from excessive clot formation leading to the blockage of arterial or venous vasculature resulting in stroke, pulmonary embolism (PE) or myocardial infarction, all of which can be fatal. Arterial thrombotic events are characteristic of high shear blood flow conditions generated in the artery. They are often associated with atherosclerotic plaque rupture and will not be discussed in this review. Venous thromboembolism (VTE) aetiology is associated with low or static blood flow that can occur for example in the lower extremities (Line, 2001, Furie and Furie, 2005). Unlike arterial thrombi that consist mostly of activated platelets and fibrin compacted tightly together, venous thrombi often contain red blood cells that result in a clot with comparatively less density (Goel and Diamond, 2002). Several outcomes can occur once a thrombus has formed. The clot can remain localised where it will eventually be cleared via the action of fibrinolytic enzymes released from the endothelium and leukocytes that are recruited to the embolus in response to chemotactic factors (Wakefield *et al.*, 1997, Kolev and Machovich, 2003). However, if the embolus becomes mobilized it can become occluded in the veins of the leg or within the pulmonary arteries resulting in a PE.

The incidence of VTE, including deep vein thrombosis (DVT) and PE, affect approximately 1 in 1000 adult persons with mortality occurring in an estimated 30% of cases (Heit *et al.*, 1999b). Furthermore, evidence suggests PE may be the leading undiagnosed cause of death amongst hospitalized patients, many of whom could be saved with standard therapies had the condition been identified (Kroegel and Reissig, 2003). Recent data compiled from six EU member countries estimates that 12% of all deaths per annum are a result of VTE (Cohen *et al.*, 2007). Across the entire EU statistical data shows the number of VTE-related deaths more than doubles the number of deaths attributed to acquired immunodeficiency syndrome (AIDS), breast cancer, prostate cancer and transport accidents combined (Cohen *et al.*, 2007)

Thrombotic risk factors can have both genetic and environmental origins. Environmental risk factors include age, obesity, cancer, immobility (including extended travel times), surgery, trauma and alterations of hormonal status including pregnancy, hormone replacement therapy and contraceptives (Cushman, 2007). Genetic predisposition to a prothrombotic physiology relate to defects in the pro- and anti-coagulants that regulate clot formation via the coagulation cascade (see following section), an example of which is PS deficiency. Inherited PS deficiency occurs in the Caucasian population at a frequency of between 0.03-0.13% (Dykes *et al.*, 2001), but is much higher in Asians occurring at between 0.48-0.63% in the Japanese population (Adachi, 2005) and estimated to be as high as 3.7% in the Thai population (Akkawat and Rojnuckarin, 2005). Although the incidence of PS deficiency overall is low, it is associated with an approximately 10-fold greater risk of developing venous thromboembolism compared to the general population (Adachi, 2005, Martinelli *et al.*, 2001).

1.3 THE COAGULATION CASCADE

Central to the process of coagulopathy is the coagulation cascade, a complex system that is heavily regulated by both positive and negative feedback loops (Figure 1.1). The coagulation cascade was previously divided into the intrinsic and extrinsic pathways that converged into a common pathway, leading to the generation of thrombin. However, it is now widely accepted that contact activation involving FXII does not play a role *in vivo*, leaving the tissue factor (TF) initiated extrinsic pathway model which has been altered to include recent discoveries. The TF-initiated model of activation starts with exposure of TF resulting from vascular injury. Strong expression of TF by cells of the tunica adventitia ensures that in the event of damage to vascular lining, TF will be immediately exposed (Weitz, 2001). TF complexes with activated factor VII (aFVII) which is in constant circulation, equating to 1-2% of the total FVII titre. How FVII is activated and its equilibrium regulated *in vivo* is still heavily debated but it is thought that unbound, circulating aFVII exhibits little or no proteolytic activity until it is complexed with TF (Komiyama *et al.*, 1990). The aFVII/TF complex catalyses the activation of factors IX and X (FIX; FX), with aFX able to generate small, but important, quantities of thrombin from prothrombin (Osterud and Rapaport, 1977, Jesty and Silverberg, 1979, Orfeo *et al.*, 2004). The pico molar quantities of thrombin are enough to activate factors V and VIII (FV; FVIII, serving also to cleave the latter from an association with von Willebrand factor which circulates bound to FVIII (Pittman and Kaufman, 1989, Brummel *et al.*, 2002, Fay, 2004). The formation of the tenase complex (aFVIII/aFIX) on negatively charged phospholipids (such as the surface of platelets) generates large quantities of aFX which bind with aFV to form the prothrombinase complex, also on phospholipid surfaces. The prothrombinase complex dramatically increases prothrombin activation, approximately 300,000-fold greater than that of aFX alone (Nesheim *et al.*, 1979). Positive feedback via thrombin not only

increases further activation of FV and FVIII, but also activates FXI which in turn yields even greater quantities of FIX and FX.

Control of the coagulation cascade occurs at several levels. Once sufficient aFX has been generated, the initial TF/aFVII pathway is shut down via formation of a quaternary complex composed of TF/aFVII and aFX binding to tissue factor pathway inhibitor (TFPI). Thus TFPI removes TF/aFVII and some of the excess aFX from the activation process (Figure 1.1). However, as the total circulatory TFPI concentration is only 2.5nM further downregulatory management is necessary for adequate deactivation. The serine protease inhibitor, antithrombin III (ATIII) further downregulates coagulation, via deactivation of thrombin, but also by deactivating several key procoagulant serine proteases, namely aFVII, aFX, aFIX and aFXI (Figure 1.2). The third major component of the anticoagulant system is the activated Protein C (PC) pathway.

1.4 THE PROTEIN C PATHWAY

PC is a serine protease that circulates in an inactive form that is readily activated by thrombin. The process is increased 20,000 times in the presence of membrane bound thrombomodulin and endothelial cell PC receptor (EPCR) which are both expressed on the surface of endothelial cells (Esmon *et al.*, 1982, Stearns-Kurosawa *et al.*, 1996). Activated PC (aPC) deactivates both the tenase and prothrombinase complexes by proteolytically cleaving aFV and aFVIII, respectively (Esmon, 1987). This mechanism is enhanced 20-fold when aPC is bound to its cofactor protein S (PS) (Rosing *et al.*, 1995, O'Brien *et al.*, 2000). Recent studies have identified the additional requirement of inactivated FV for efficient aPC/PS deactivation of aFVIII (Nicolaes and Dahlback, 2002).

Figure 1.1 Activation of the Coagulation Cascade and Initial Regulation by Tissue Factor Pathway Inhibitor (TFPI) . Exposed TF and aFVII combine to activate FIX and FX. The low levels of aFX generate small quantities of thrombin from prothrombin, enough to activate both FV and FXI and cleave FVIII from vWF leading to the formation of the prothrombinase and tenase complexes. Exponential production of thrombin results leading to cleavage of fibrinogen to fibrin and platelet activation which initiates thrombus formation (blood clot). This process is controlled initially by TFPI which forms a quaternary structure with TF, aFX and aFVII to prevent further activation of FIX and FX.

Figure 1.2 Regulation of the Coagulation Cascade by Antithrombin III (ATIII) and the Protein C (PC) Pathway. ATIII deactivates thrombin, aFVII, aFX, aFVIII and aFXI. The PC pathway is initiated by the activation of PC (APC) by thrombin, a process that is enhanced 20,000 times by the combination of thrombomodulin and endothelial cell protein C receptor (EPCR) which are both expressed on the surface of endothelial cells. Protein S (PS) binds to APC forming the APC/PS complex enhancing APC inactivation of aFV and aFVIII.

1.5 PROTEIN S

PS has several important functional domains (Figure 1.3). The N-terminus requires vitamin K to generate a γ -carboxyglutamic acid rich (Gla)-domain that binds to phospholipids. The Gla-domain (residues 1-45) facilitates binding of the PS molecule to surfaces of activated platelets, platelet microparticles and endothelial cells in the presence of Ca^{+2} ions localising PS to the source of the thrombus formation. Amino acids 46-74, denoted the thrombin sensitive region (TSR), includes arginine residues at positions Arg⁴⁹, Arg⁶⁰ and Arg⁷⁰ (Harris and Esmon, 1985, Dahlback *et al.*, 1992, Furie and Furie, 1992, Hackeng *et al.*, 1993). Proteolytic cleavage of all three by thrombin has been demonstrated with an affinity of Arg⁴⁹>Arg⁷⁰>>Arg⁶⁰; (Lu *et al.*, 1997, Morboeuf *et al.*, 2000, Borgel *et al.*, 2001, Meijer-Huizinga *et al.*, 1994, Heeb and Griffin, 2002). Arg⁶⁰ is only cut if Arg⁴⁹ and Arg⁷⁰ are unavailable. The TSR is reported to also be important for interaction with aPC (Dahlback *et al.*, 1990b, He *et al.*, 1998). Four epidermal growth factor (EGF)-like domains span residues 75-242. EGF1 and possibly EGF2 are required for aPC binding which directs PS cofactor activity. Like the Gla domain, the 3rd and 4th EGF-like domains bind Ca^{+2} ions with high affinity (Hackeng *et al.*, 2000, Mille-Baker *et al.*, 2003, Drakenberg *et al.*, 2005).

Figure 1.3 Schematic of Protein S highlighting key functional domains. Residues 1-45, γ -carboxyglutamic acid (Gla) domain; 46-74, thrombin sensitive region (TSR); 75-242, epidermal growth factor (EGF)-like domains; 243-635, sex hormone binding globulin (SHBG)-like domain. Also shown are the regions of PS that are proposed binding sites for activated Protein C (aPC; blue) and C4b-binding protein (C4b-BP, red). Adapted from Greenberg and Davie (2001).

Unlike the C-terminus of other vitamin K-dependent plasma proteases, which have a serine-protease domain, the large C-terminal of PS (residues 243-635) lacks enzymatic activity (Saposnik *et al.*, 2003). The region is highly homologous with two other plasma proteins, namely another vitamin K dependant protein, growth arrest-specific gene 6 product (GAS6) and the steroid transporter, sex hormone binding globulin (SHBG) (Gershagen *et al.*, 1991, Gershagen *et al.*, 1987, Villoutreix *et al.*, 1997, Manfioletti *et al.*, 1993). Unlike SHBG, the C-terminus of PS does not bind steroid hormones, nor does it undergo dimerisation with either SHBG or other PS molecules (Villoutreix *et al.*, 1997). Like the TSR, the SHBG domain represents another regulatory domain of PS as it has been shown that it is involved in binding to complement C4b-binding protein (C4b-BP) (Saposnik *et al.*, 2003). Approximately 60% of PS circulates in the blood bound to the beta chain of the complement C4b-BP. Only the remaining 40% 'free form' is available for aPC co-factor activity as PS complexed with C4b-BP lacks co-factor activity (Morboeuf *et al.*, 2000).

Recent studies suggest PS to be a multi-functional protein involved in inflammatory and apoptotic processes and, by extrapolation, may have a role in reducing autoimmunity. Anderson *et al.* (2003) showed that PS is required for the efficient phagocytic clearance of cells that have undergone apoptosis, possibly minimising the risk of autoimmunity (Anderson and Shacter, 2004). Recently Hryszko *et al.* (2005) proposed a role in the modulation of inflammation after demonstrating that PS suppressed the binding of plasminogen to the surface of THP-1 cells in addition to downregulating their invasive potential. Lastly, a murine model has demonstrated a significant neuroprotective role for PS post ischemic insult (Liu *et al.*, 2003).

Classification of PS deficiency is based on antigenicity and functionality. The affinity between PS and C4b-BP defines both Type I and III deficiency while Type II is characterised by the strength of PS co-factor activity.

Type I-	Low total and free PS levels
Type III-	Low free with normal total PS levels
Type II-	Low functional activity

The basis for classifying PS deficiency in this way has been criticized as several family studies have identified Type I and Type III deficient individuals within the same kindred, often with the same causative mutation (Zoller *et al.*, 1995, Simmonds *et al.*, 1997, ten Kate *et al.*, 2006). A proposal to restrict classification to either quantitative and qualitative (functional) has yet to be ratified (Gandrille *et al.*, 2000). Several groups have suggested reasons to explain the observed discrepancies between Type I and Type III deficiencies and highlight the many confounding factors that affect PS plasma concentration (Zoller *et al.*, 1995, Simmonds *et al.*, 1997, Rezende *et al.*, 2002, ten Kate *et al.*, 2006). PS levels increase with age and differ markedly between the sexes (Henkens *et al.*, 1995, Dykes *et al.*, 2001). It has also been proposed that variation in the concentration of circulating C4b-BP between individuals could influence free PS availability, although this has been disputed (Ceriello *et al.*, 1990, Zoller *et al.*, 1995, Morboeuf *et al.*, 1998). The existence of anti-PS antibodies in individuals suffering from systemic lupus erythematosus and also end-stage renal failure has been demonstrated, although the extent to which they affect the wider population is poorly documented (Song *et al.*, 2000, Molino *et al.*, 2005). Acquired PS deficiency has been reported in cancer patients, although it is unclear whether this is due to a breakdown in the hepatic system, greater turnover, or a direct result of specific chemotherapy agents

(Battistelli *et al.*, 2005). AIDS patients too experience reduced PS levels that follows disease progression (Dillmon *et al.*, 2005). However, the most widely reported reduction in PS is seen in response to raised oestrogen levels, such as is seen in women who are pregnant or use oral contraceptives (OC).

1.6 THE ORAL CONTRACEPTIVE PILL

The OC pill became widely available in the 1960s, although development began several decades prior. In 1940, Sturgis and Albright reported inhibition of ovulation in women who they administered oestrogens to for the treatment of dysmenorrhea (menstrual cramping), but it was progesterone, not oestrogen, that led to the development of the first oral contraceptive. In 1937, a rabbit model study demonstrated ovulation could be inhibited by the administration of progesterone. However, progress stalled due to the labour intensive process of obtaining progesterone; extraction from over 2000 pregnant sow's ovaries yielded only 1mg of progesterone and it wasn't until 1940 when a chemist named Russell Marker perfected a technique of processing progesterone from the syrup of Mexican yams (Speroff and Darney, 1996).

Further work was required for oral administration of the steroid to be considered, as progesterone is largely inactive when ingested orally (Speroff and Darney, 1996). This issue was overcome in 1951 by Carl Djerassi who engineered and patented the first synthetic progestin from a nortestosterone backbone. Previous studies had shown that removal of the methyl group at C19 of testosterone resulted in a molecule that lacked androgenic properties, but exhibited progestagenic activity. The new progestin, norethisterone, (17 α -ethinyl-19-nortestosterone) orally administered, was 10x more potent than progesterone and was shown to be effective in the treatment of menstrual disorders (Kafriksen and Adashi, 2003, Kovacs, 2003). Within three years, a second

synthetic progestin, norethynodrel, was patented. Almost identical in structure to norethisterone was tested in a small human trial, resulting in 100% cessation of ovulation in all 50 of the female participants after administration of 30mg/day given at days 5 to 25 of their cycle (Rock *et al.*, 1957) (Refer to structural details in Appendices 1-3). Larger clinical trials, carried out in Puerto Rico further supported the initial findings (Pincus, 1958). It was during the Puerto Rican study that the benefits of adding oestrogen were realised when the researchers calculated that the initial supply of norethynodrel contained 1.5% of the oestrogen mestranol (Appendix 1). When follow up studies using a mestranol-reduced stock were initiated, several of the study participants complained of break-through bleeding. This decision formed the basis for the future of the combined OC (COC) pill. Today there are dozens of COCs available that differ primarily by the type of progestin but also in the oestrogen concentration.

A progestin-only form of oral contraceptive, sometimes referred to as the 'minipill', is a viable alternative for women who are intolerant to oestrogen and is particularly useful for breast-feeding mothers concerned with the use of oestrogen, with only negligible amounts of the progestin entering into the milk supply (Shaaban, 1991).

The mestranol component was soon superseded by ethinyl oestradiol (EE) in an attempt to minimise cardiovascular risks and today the oestrogen component of COCs is almost exclusively EE (Woutersz, 1991, Bloemenkamp, 2005). There are two reasons for this. The first is biological. Early COC preparations that incorporated other estrogens, such as β -oestradiol (E_2) or oestradiol valerate (EV), failed due to a high incidence of breakthrough bleeding. This was subsequently shown to be due to a localised, progestin-regulated, increase in the expression of 17 beta-hydroxysteroid-dehydrogenase (17HSD; also known as oestradiol dehydrogenase) within the endometrium (Isomaa *et al.*, 1993, Casey *et al.*, 1994). Oxidation of the -OH group by 17HSD rapidly converts both E_2 and

EV to less potent forms such as estriol (Appendix 1). The ethinyl moiety in EE effectively blocks the 17HSD enzyme, preventing oxidation (Appendix 1) (Wiegratz and Kuhl, 2004). The second reason why EE has dominated as the oestrogen component of both past and present COCs is its lack of patent and therefore there has never been an incentive for the pharmaceutical industry to design newer compounds (Kafrissen and Adashi, 2003).

The use of EE did not substantially reduce the risk of arterial and venous disease. The high concentrations of EE previously used (between 50-100 μ g) were subsequently lowered to below 50 μ g in the 1980's and $\leq$ 35 μ g EE in the 1990's and are termed low dose COCs. Despite the reduction of EE, COC use is still associated with an increased venous thrombotic risk and possibly even an arterial thrombotic risk (Tanis and Rosendaal, 2003, Rosendaal *et al.*, 2003, Van Den Bosch *et al.*, 2003, Wu *et al.*, 2005).

It is known that PS deficiency is associated with an increased thrombotic risk and it is hypothesised that the oestrogen component of COC is responsible for the reduction. However, the COC pill contains a second hormonal component, a progestin, and its affect on PS production is less clear.

In contrast to the largely static development of the oestrogens there has been significant evolution of the progestins. Most of the synthetic progestins have been generated from either a progesterone or testosterone parent molecule.

Progesterone has been manipulated to yield 17 α -hydroxyprogesterone; addition of a hydroxyl group to C17, 19-norprogesterone; removal of the methyl group from C19 and 17 α -hydroxy-19-norprogesterone; addition of a hydroxyl group to C17 of 19-norprogesterone. Progestins generated from 17 α -hydroxyprogesterone are classified as

pregnanes. The hydroxylation of C17 that creates 17 α -hydroxyprogesterone produces a hormonally inactive product (Schindler *et al.*, 2003). Esterification of the C17 generates 17 α -hydroxyprogesterone acetate which is weakly progestogenic but becomes highly active when a methyl group is attached to C6, creating medroxyprogesterone acetate (Appendix 2).

Testosterone formed the basis for the early synthetic progestins when it was discovered that 19-nortestosterone, created by removing the methyl group at C19, lacked androgenicity but gained progestogenic properties. 19-nortestosterone progestins are classified as either gonanes or estranes as defined by the basic molecular skeleton. The two earliest progestins, norethisterone and norethynodrel are examples of estranes while examples of gonanes are levonorgestrel (part of the 2nd generation COCs), desogestrel and gestodene (both progestin components of 3rd generation COCs). Dienogest is a relatively new progestin with unique properties. Although it is a 19-nortestosterone derivative it lacks the C17 ethinyl group common to other 19-nortestosterone derivatives, replaced instead by a cyanomethyl group (Kuhl, 2005) (Appendix 3).

1.7 PREGNANCY

Pregnancy is associated with a shift toward hypercoagulable state due to changes to key components of both the pro- and anti-coagulant pathways, particularly levels of PS which decrease from around 10 weeks. Levels of free PS continue to decline throughout the remainder of gestation, falling to below the normal range for non-pregnant females (not using oral contraceptives) throughout the course of gestation, with the lowest levels evident at delivery (Bremme, 2003, Brenner, 2004). Interestingly, women with a pre-existing PS deficiency have an 8-fold greater chance of developing a venous

thromboembolism as a direct effect of their pregnancy, compared to non-deficient controls (Eldor, 2002).

Levels of both E_2 and P_4 rise significantly over the course of gestation with serum concentrations of both hormones increased 100-fold at term compared to levels observed in non-pregnant women (Figure 1.4) (Dunn *et al.*, 1981). Circulating levels of P_4 rise rapidly in the first 10 weeks, then steadily increase, reaching a maximum at parturition. Other steroids such as testosterone and cortisol are significantly increased during pregnancy (Bammann *et al.*, 1980, Marnach *et al.*, 2003).

Figure 1.4 Oestrogen and Progesterone Levels During Pregnancy. The serum concentrations of both oestrogen [here 17β -oestradiol; (E_2)] and progesterone (P_4)] increase by approximately 100-fold at parturition. Adapted from Dunn *et al.* (1981)

1.8 HORMONE REPLACEMENT THERAPY

Unlike the oestrogen component of COCs, the oestrogen component of hormone replacement therapy (HRT) varies. Conjugated equine oestrogen (a mixture of oestrogens extracted from pregnant mare's urine) and esterified oestrogen are used in many preparations as is 17β -oestradiol (E_2), both in combination with a progestin and stand alone. Natural oestrogens (like E_2) are believed to be associated with a lower risk

of venous thromboembolism than EE, because their effect on haemostasis parameters is weaker (Wiegratz *et al.*, 2004). The progestins included in combined HRT preparations are predominantly medroxyprogesterone or norethisterone. Interestingly, combination HRT is associated with a greater risk of venous thrombosis compared to oestrogen only preparations [Reviewed Wu (2005)].

1.9 OESTROGEN RECEPTOR BIOLOGY

The oestrogen receptor (ER) is the most studied of all the hormone receptors. Its direct involvement in the mediation of oestrogen action was first described by Jensen EV, Jacobsen HI in 1962 (Jensen and Jacobsen, 1962). The last two decades in particular have uncovered several key findings that highlight the complexity of ER biology. Until recently it was thought that there was only one form of ER; ER α , which was cloned and sequenced in 1986 (Greene *et al.*, 1986, Green *et al.*, 1986). A decade later, a second ER; ER β , was cloned from both a rat prostate and ovary and was subsequently identified in humans (Mosselman *et al.*, 1996, Enmark *et al.*, 1997). In this review only ER α will be discussed.

The gene that encodes ER α , ESR1, utilises six different promoters (A-F) with each promoter generating a different mRNA transcript in a tissue specific manner. (Flouriot *et al.*, 1998). Expression of ER α in the liver is governed almost exclusively by the E promoter (Pinzone *et al.*, 2004) which is proposed to be oestrogen inducible, although it has yet to be shown directly. There is circumstantial evidence that supports the theory. For example, it has been shown that levels of E promoter derived ER α mRNAs are higher in the human female liver (Flouriot *et al.*, 1998) who have higher circulating concentrations of oestrogen. A 1999 study by Donaghue *et al.* examined the oestrogen responsiveness of the E promoter *in vitro* using four breast carcinoma cell lines,

demonstrating increases in mRNA transcripts for three out of the four lines (Donaghue *et al.*, 1999). No studies have examined mRNA expression in liver cell lines.

Distributed in both the nucleus and cytoplasm, unliganded ER α is complexed with heat shock proteins (HSPs) that disassociate when the ER α binds to oestrogen, initiating conformational change causing dimerisation with similarly activated ERs. Dimerised ER α translocates to the nucleus and influences gene expression, either increasing or decreasing transcriptional activity (Htun *et al.*, 1999) (Figure 1.5). Commonly referred to as the classic ER α pathway, the dimerised ER α complex binds to oestrogen response elements (EREs) that initiates the recruitment of additional transcriptional factors directly affecting transcription. In addition to the classical mechanism, there are at least three other distinct pathways whereby ER α can exert regulatory control over transcription.

Ligand bound ER α interacts with several key transcriptional factors that in turn bind to their cognate promoter recognition sites. Highly GC-rich promoters are often regulated by stimulating protein 1 (Sp1) transcriptional factors which bind to GC rich motifs. Activated ER α is able to form complexes with Sp1 and subsequently regulate gene transcription. The ER α component itself can either interact indirectly with DNA or interact with ERE or ERE1/2 sequences (Safe, 2001). Upregulation by Sp1/ER α complexes has been widely reported (Duan *et al.*, 1998, Xie *et al.*, 1999, Li *et al.*, 2001, Castro-Rivera *et al.*, 2001, Schultz *et al.*, 2003). Although recent studies have described Sp1/ER α based repression of genes (Kelley *et al.*, 2003, Stossi *et al.*, 2006).

Figure 1.5 The Classic Oestrogen Receptor Pathway. Inactive oestrogen receptor alpha (ER α) bound to heat shock proteins (HSP) circulate in the cytoplasm. Binding to oestrogen (E) initiates a conformational change that dislodges the HSPs and facilitates dimerisation with similarly activated ER α 's. Dimerised ER α 's enter the nuclear membrane and bind to oestrogen response elements (EREs), regulating gene expression.

Like the Sp1 mechanism, ER α forms complexes with Jun/Fos transcriptional factors that bind to AP-1 sites within promoters, regulating transcription. Regulation of gene expression by Jun/Fos/ER α complexes also induces or represses gene expression (Kushner *et al.*, 2000).

ER α regulation of ERE-dependant transcription in the absence of oestrogen has also been shown resulting from phosphorylation of the ER α by protein-kinase cascades initiated by growth factors such as EGF and IGF-1 (Ignar-Trowbridge *et al.*, 1993, Ignar-Trowbridge *et al.*, 1995, Lee *et al.*, 1997).

An accumulation of evidence is mounting that suggests ER α can exert rapid, nongenomic regulatory responses via plasma membrane bound ERs. Ligand binding appears to activate phosphorylation cascades and other signalling pathways. These newly identified ER α mechanisms have only recently been identified.

1.10 PROGESTERONE RECEPTOR BIOLOGY

The so-called superfamily of hormone receptors refers to a division of receptors that include the progesterone, androgen, and mineral- and glucocortico-steriod receptors (PR, AR, MR, GR, respectively) which share structural similarities and recognise consensus sequence response elements that are different from the ERE consensus sequence. Figure 1.6 details the previously accepted HRE motif as it was thought to be in 1995 (Zilliagus *et al.*, 1995).

	-6	-5	-4	-3	-2	-1		1	2	3	4	5	6
Consensus HRE	G	G	T	A	C	A	NNN	T	G	T	T	C	T
	C	T	C	T		C						C	
	T		G										
Consensus ERE		G	G	T	C	A	NNN	T	G	A	C	C	
		A											

Figure 1.6 Hormone Response Element Motifs. Consensus response element sequences for PR, AR, MR and GR (HRE) and for ER (ERE). Subnucleotides denote alternatives for that position. Adapted from Zilliagus *et al.*(1995). It is now accepted that HREs can be even more complex and diverse in comomposition

However, studies since then have identified multiple variations that make prediction of novel HRE binding sites difficult. A recent paper by Stepanova *et al.*(2006) addressed the problem by generating an algorithm compiled from the analysis of >650 experimentally validated HREs and is available online to screen novel sequences; <http://birc.ntu.edu.sg/~pmaria/> (Stepanova *et al.*, 2006). The algorithm generates a value expressed as a similarity coefficient that relates the probability of the sequence entered being a HRE in comparison to the >650 experimentally validated elements.

Although not as extensively studied as the ER, research into the superfamily of hormone receptors has increased markedly over the last decade, especially pertaining to their role as transcriptional regulators of gene expression (Lu *et al.*, 2006). Investigation of PR

biology in particular has dominated largely due to its synergistic role with ER in some breast cancers, but also because of its importance in developmental biology (Fernandez-Valdivia *et al.*, 2005, Cordera and Jordan, 2006).

Like the ERs, the PR has two distinct isoforms, PR-A and PR-B (there is a third PR-C isoform but it is largely undescribed, so for the purposes of this review only the PR-A and PR-B isoforms will be discussed). Unlike the ERs, both forms are transcribed from the same gene utilizing different promoters (Kastner *et al.*, 1990). Interestingly, both promoters are E₂ responsive; via an ER α /Sp1 mechanism for the PR-A expressing promoter and an AP1/Sp1 mechanism for the PR-B promoter (Petz *et al.*, 2004, Flototto *et al.*, 2004).

The PR-A and PR-B isoforms are identical except that PR-B has an additional 164 amino acid N-terminus that accounts for the differences in activation profiles between the two receptors (Takimoto *et al.*, 2003). *In vitro* studies show that ligand bound PR-B is a stronger transactivator than liganded PR-A and studies using malignant breast cells suggest that it is also the more potent form *in vivo* (Richer *et al.*, 2002, Tung *et al.*, 2006).

PRs undergo similar conformational change and dimerisation as the ERs once bound to P₄ or any other of the many progestin isoforms, where upon they then interact with progesterone response elements (PREs) to regulate gene expression (Tsai *et al.*, 1988, Guiochon-Mantel *et al.*, 1989, Stepanova *et al.*, 2006). However, differences in PR binding affinity in addition to distinct pro- and anti- prostogenic, oestrogenic, and androgenic profiles for each of the different progestins, means that no two progestins exhibit the same transactivational effects [see Schindler *et al.*, (2003)] for a thorough review of progestin biology). This is evident physiologically when contrasting the effect

of 2nd and 3rd generation COCs, that contain different progestins, on inflammatory markers, lipid metabolism, haemostatic and fibrinolytic parameters, along with thrombotic and cardiovascular disease risk status (Kemmeren *et al.*, 2001b, Kemmeren *et al.*, 2001a, Kemmeren *et al.*, 2004, Baillargeon *et al.*, 2005, van Rooijen *et al.*, 2006, Van Den Bosch *et al.*, 2003, Tanis and Rosendaal, 2003, Tans *et al.*, 2000)

1.11 THE EFFECT OF HORMONES ON HAEMOSTATIC PARAMETERS

Variation of most of the procoagulants has been observed in response to OC use, pregnancy or HRT to varying degrees. For example, FVII appears to increase in response to raised oestrogen concentrations, such as the latter stages of pregnancy and HRT (particularly oestrogen only preparations) (Brenner, 2004, Cosman *et al.*, 2005). Like PS, variation in FVII is greatest amongst users of 3rd Generation COCs, but unlike PS, elevated FVII is viewed more as a risk factor for arterial rather than venous thrombosis (Koster *et al.*, 1994, Winkler, 2000, Wiegratz *et al.*, 2004). One study identified an ERE in the promoter region of the FVII gene, but surprisingly found that it repressed, rather than enhanced FVII expression (Di Bitondo *et al.*, 2002). Interestingly, some studies suggest that HRT use correlates with reduced FVII levels in post-menopausal women (Borgfeldt *et al.*, 2004, Collins *et al.*, 2006). It may be a result of this identified ERE repression overcoming a pre-menopause upregulatory mechanism.

A more thoroughly investigated hormonally responsive procoagulant is FIX and its response to testosterone. Haemophilia B results from a deficiency of FIX. One form of Haemophilia B, Haemophilia B Leyden, is in most cases corrected with the onset of puberty. It was this property that led to the identification of an androgen response element (ARE) in the promoter region of the FIX gene after researchers studied the FIX

promoter sequences of individuals who did not normalise during puberty. Point mutations that mapped to a specific sequence that subsequent analysis showed was involved in binding to ligand bound AR.

Many of the anticoagulants are influenced by shifts in hormonal balance. The ubiquitous anticoagulant antithrombin (AT) is reported to be regulated by hormones, but it is a contentious issue. Some studies suggest that use of OCs reduces AT levels while other reports indicate little or no change (Norris and Bonnar, 1996, Winkler *et al.*, Tans *et al.*, 2000, Aldrighi *et al.*, 2006). Furthermore, unlike PS, AT levels are not reduced during pregnancy, when both oestrogen and progesterone plasma concentrations are high (Cerneca *et al.*, 1997, Clark *et al.*, 1998). Interestingly though, there has recently been a surge in publications detailing significant AT decreases in postmenopausal women undergoing HRT (Borgfeldt *et al.*, 2004, Al-Farra *et al.*, 2005, Sumino *et al.*, 2005, Sgarabotto *et al.*, 2006).

aPC resistance (aPC-R) is defined as an impaired ability of aPC to deactivate FV. Hereditary aPC-R is linked to the FV-Leiden point mutation which replaces arginine at position 506 with a glutamine (R506Q), destroying the proteolytic cut site. The result is prolonged FV activity and thus greater thrombin activation. In the mid-1990s, acquired aPC-R was reported in pregnant women and women using OCs and, like PS, the use of 3rd generation COCs correlates with a greater impact (Olivieri *et al.*, 1995, Meinardi *et al.*, 1997, Rosing *et al.*, 1997, Rosing *et al.*, 1999, Tans *et al.*, 2000, Alhenc-Gelas *et al.*, 2004, Kemmeren *et al.*, 2004). There is significant debate as to the exact cause of acquired aPC-R; raised FVIII/FVII and reduced PS or phospholipid levels have all been suggested. Recent studies suggest that the expression of a glycosphingolipid, glucosylceramide (GlcCer), may be the causative factor. GlcCer enhances aPC interaction with phospholipid surfaces increasing aPC activity, especially in the

presence of PS (Yegneswaran *et al.*, 2003, Deguchi *et al.*, 2005). *In vitro* studies have shown that GlcCer levels are reduced in response to increased oestrogen, thus providing a potential mechanism to describe acquired aPC-R (Shukla *et al.*, 1992).

1.12 THE EFFECT OF HORMONES ON PROTEIN S

The most consistent hormonally responsive anticoagulant is PS. Reduction in the circulating levels of both free and total PS are significant for users of oestrogen containing COCs, particularly 3rd generation forms (Kemmeren *et al.*, 2004, Wiegratz *et al.*, 2004). During pregnancy, when oestrogen concentration is markedly raised, levels of free and total PS decrease significantly from the 2nd trimester onwards (Brenner, 2004). Currently, there is debate regarding the impact of HRT on PS levels which may reflect the greater variety of oestrogens used in hormone replacement therapies, or results could be complicated by the phenomenon of increasing PS levels as women get older (Goodwin *et al.*, 2002, Oger *et al.*, 2003, Sgarabotto *et al.*, 2006, Basurto *et al.*, 2006). Nevertheless, in younger women the influence of oestrogens on PS levels is significant. Recent publications also suggest that progestins influence PS levels, with some isoforms clearly associated with increased levels of PS. This provides a possible explanation for the greater impact of 3rd generation COCs on free and total PS compared to 2nd generation COCs (Winkler, 1998, Kemmeren *et al.*, 2004).

1.13 PROTEIN S GENETICS

PS is coded for by the PROS1 (or PROS- α) gene that is located near the centromere of chromosome 3 (3p11.1-q11.2) (Ploos van Amstel *et al.*, 1987). A second pseudogene, designated PROS2 (or PROS- β), shares a 97% exonic and 95.4% intronic homology with PROS1 (Gandrille *et al.*, 1997). The PROS1 gene is approximately 101 Kb in length containing 15 exons that produce approximately 3.3 Kb of mRNA transcript (Figure 1.7) (Schmidel *et al.*, 1990). Primarily expressed by the hepatocyte, PS mRNA transcripts have also been identified in megakaryocytes and platelets, endothelial cells, osteoblasts, vascular smooth muscle and nervous system cells and Leydig cells located in the testes (Fair *et al.*, 1986, Dahlback, 1991, Stitt *et al.*, 1995). Reflecting the extensive tissue distribution is the diverse number of transcriptional start sites (TSS) that have been identified from the liver and various cell lines (Lundwall *et al.*, 1986, Ploos van Amstel *et al.*, 1990, Malm *et al.*, 1994, Benzakour and Kanthou, 2000, de Wolf *et al.*, 2005). A limited number of studies have investigated the promoter region of PROS1, identifying several transcriptional regulatory motifs including GC-rich motifs that bind the zinc-finger proteins Sp1 and Sp3 and a possible Hepatic Nuclear Factor 3 (HNF3) binding site (de Wolf *et al.*, 2006a, Tatewaki *et al.*, 2003) (Figure 1.7). Recently, a binding site for phosphorylated Signal Transducer and Activator of Transcription 3 (STAT3) protein was identified (de Wolf *et al.*, 2006b) (Figure 1.7). Phosphorylation of STAT3 was shown to be a consequence of interleukin-6 (IL-6) stimulation, previously identified as an activator of PS expression *in vitro* (Hooper *et al.*, 1995, Hooper *et al.*, 1996, Hooper *et al.*, 1997). To date, identification of any regulatory elements located within the 3' untranslated region of the PROS1 gene have not been described.

Figure 1.7- Schematic of the PROS1 Gene. PS is encoded by the PROS1 gene that maps near to the centromere of chromosome 3 (3p11.1-q11.2). The PS coding region comprises 15 exons (detailed in red) and 14 introns and is flanked by the 5' and 3' untranslated regions (5'UTR and 3'UTR). Previously reported transcriptional binding domains located within the promoter region, Sp1, HNF1, HNF3 and HNF4 are shown (Tatewaki *et al.*, 2003, de Wolf *et al.*, 2006a, Hall *et al.*, 2006). Downward arrows denote dominant initiation start sites for various mRNA transcripts derived from human liver samples and various cell lines (de Wolf *et al.*, 2005)

1.14 AIMS

PS deficiency including the acquired form resulting from pregnancy and COC use. In addition, combination of oral contraceptive use with an underlying hereditary PS deficiency increases the underlying risk of developing a thrombotic complication.

Clinically it is well documented that PS levels are influenced by hormonal status, particularly in users of COCs and in pregnant women, where oestrogen and progesterone (or progestin isoforms) are increased. Screening the PROS1 gene and the PROS1 mRNA transcript for mutations may uncover a region or element(s) that are directly or indirectly responsible for the variation of PS. Alternatively, it is possible that the regulation is not occurring at the transcriptional control but is instead determined by a protein-protein interaction of PS with a hormonally regulated binding partner. A detailed examination of all of these factors will facilitate a better understanding of the mechanism that hormonally regulates PS levels.

Therefore the aims of this thesis are:

- Develop a method to screen the PROS1 gene and the associated mRNA transcript for mutations.
- Investigate the PROS1 gene to identify key hormonally responsive regions that account for the differences in observed levels of circulating PS between users of 2nd and 3rd generation COCs.
- To examine the steroid based mechanisms in the regulation of the identified hormone response elements.
- To identify novel protein binding partners of PS (that may or may not be oestrogen sensitive).

CHAPTER 2

Materials and Methods

2.1 PATIENTS AND CONTROLS

Sample DNA and RNA was derived from two sources. The first group comprised patients selected by a Senior Haematologist (Dr. Ross Baker) at Royal Perth Hospital (Perth, Western Australia) that had a range of conditions including family history of PS deficiency or unexpected fluctuating free PS levels (determined by a monoclonal free PS antigen enzyme-linked immunosorbent assay- Corgenix; USA) (Table 2.1). The second group consisted of 137 pregnant women recruited from King Edward Memorial Hospital (Perth, Western Australia) that were divided into three subgroups; 48 women who experienced normal pregnancy (controls), 41 women who suffered from pre-eclampsia (Pre-E) while pregnant and 48 women who suffered gestational diabetes (GD) (Table 2.2). Pre-E was defined as the development of blood pressure greater than 140/90 mmHg after 20 weeks gestation, with proteinuria of at least 300mg/24h and whose blood pressure returned to normal levels by 3 months post partum without continuing proteinuria (Brown *et al.*, 2000). GD was diagnosed using a 75g oral glucose test and was defined as a fasting blood glucose level greater than 5.5mM and/or a blood glucose level of 8mM or more, 2h post ingestion. Pregnancy blood samples were collected from women at 32 weeks gestation, and 6 weeks and 6 months post-partum. Total, free and functional PS levels were analysed at each time point using the Liatest Protein S, STA-Liatest Free Protein S and STA-Staclot Protein S tests, respectively (Diagnostica Stago, France).

Sample ID [†]	Sex	PS Level (%) [‡]	Patient History [±]
N41	M	77	Control
PS1	F	34-75%	Fluctuating PS levels Thrombosis in Pregnancy
PS2	F	50	SLE, MI cerebral infarction
PS3	F	45	Severe pre-eclampsia
PS4	M	42	PE
PS5	F	52	Pre-eclampsia
PS6	F	114	Fluctuating PS level
PS7	F	81	DVT, Family history PS def.
PS8	F	36	Recurrent DVT during pregnancy
PS9	F	57	Fluctuating PS level
PS10	F	41	Fluctuating PS level
PS11	F	49	Recurrent pregnancy loss
PS12	F	80	Daughter of PS13
PS13	F	54	Post Partum PE
PS14	F	12	PE
PS15	F	89	History of Thrombosis
PS16	F	59	History of thrombosis, DVT
PS17	F	24	Recurrent DVT
PS18	F	75	Family history of DVT
PS19	F	38	PS deficiency, spontaneous miscarriages
PS20	F	82	Persistent DVT in calf
PS21	F	78	Fluctuating PS level
PS22	M	37	PS deficiency
PS23	M	22	Family history of PS deficiency, DVTx2, MI
PS24	F	128	History of Thrombosis

Table 2.1 Details of the PS Deficient Cohort- The cohort was generated from patients selected by a Senior Haematologist (Dr. Ross Baker) at Royal Perth Hospital (Perth, Western Australia) who had a range of conditions including family history of PS deficiency or fluctuating free PS levels; ‡ PS levels mean % of free antigenic PS (normal 50%-150%); SLE= Systemic Lupus Erythematosus; MI= Myocardial Infarction; PE= Pulmonary Embolism; DVT= Deep Vein Thrombosis.

Control (C)				Gestational Diabetes (GD)				Pre-eclampsia (Pre)			
	PS Level (%) [‡]				PS Level (%) [‡]				PS Level (%) [‡]		
N=46	V1	V3	dV3-V1	N=33	V1	V3	dV3-V1	N=38	V1	V3	dV3-V1
C192	49	90	41	GD401	42	87	45	Pre211	49	68	19
C256	58	117	59	GD407	53	111	58	Pre255	49	103	54
C268	42	86	44	GD408	45	99	54	Pre270	59	111	52
C269	47	87	40	GD410	30	82	52	Pre271	49	90	41
C273	54	68	14	GD412	36	107	71	Pre275	61	123	62
C278	59	93	34	GD413	52	102	50	Pre288	34	106	72
C279	47	87	40	GD414	47	51	4	Pre290	45	111	66
C280	51	87	36	GD416	40	84	44	Pre292	59	116	57
C281	56	86	30	GD417	48	82	34	Pre296	76	125	49
C282	44	116	72	GD419	47	80	33	Pre297	68	145	77
C286	51	106	55	GD420	49	91	42	Pre301	40	98	58
C287	47	96	49	GD421	38	86	48	Pre307	58	99	41
C298	70	139	69	GD422	50	125	75	Pre309	77	74	-3
C299	59	97	38	GD423	54	104	50	Pre311	37	51	14
C303	46	115	69	GD424	46	80	34	Pre312	57	118	61
C304	59	100	41	GD431	44	101	57	Pre313	49	88	39
C314	46	87	41	GD433	34	68	34	Pre321	64	99	35
C315	55	106	51	GD436	40	99	59	Pre324	33	62	29
C316	58	103	45	GD437	46	81	35	Pre326	44	89	45
C318	59	92	33	GD438	46	120	74	Pre327	47	86	39
C319	54	93	39	GD439	32	66	34	Pre333	84	136	52
C320	46	77	31	GD440	51	91	40	Pre341	51	133	82
C322	49	106	57	GD441	46	85	39	Pre342	55	104	49
C323	51	67	16	GD442	46	81	35	Pre344	61	116	55
C325	51	97	46	GD443	39	73	34	Pre347	62	88	26
C330	43	76	33	GD444	39	120	81	Pre349	76	141	65
C334	50	65	15	GD446	43	84	41	Pre354	61	126	65
C335	49	135	86	GD448	50	126	76	Pre363	52	96	44
C337	61	96	35	GD453	63	99	36	Pre370	53	122	69
C345	68	96	28	GD456	43	88	45	Pre373	48	80	32
C346	57	93	36	GD457	40	87	47	Pre375	62	81	19
C352	68	131	63	GD460	44	78	34	Pre376	46	79	33
C353	62	114	52	GD462	44	101	57	Pre380	57	113	56
C356	49	77	28					Pre381	33	106	73
C357	47	64	17					Pre390	54	106	52
C362	58	119	61					Pre391	47	63	16
C364	50	77	27					Pre392	56	63	7
C366	57	86	29					Pre393	51	89	38
C368	125	135	10								
C371	49	68	19								
C377	43	98	55								
C379	64	89	25								
C385	56	93	37								
C387	49	79	30								
C388	54	91	37								
C389	61	75	14								

Table 2.2 Details of the Pregnancy Cohort- The pregnancy cohort consisted of 137 pregnant women recruited from King Edward Memorial Hospital (Perth, Western Australia) that were divided into three subgroups; 48 women who experienced normal pregnancy (controls=C), 41 women who suffered from pre-eclampsia while pregnant (Pre-E) and 48 women who suffered gestational diabetes (GD); ‡ PS levels mean % of free antigenic PS (normal 50%-150%);

2.2 DNA AND RNA EXTRACTION

DNA and RNA were extracted from leukocytes obtained from citrated whole blood samples. DNA was obtained using a standard salt based extraction method that has been previously described (Wang *et al.*, 1994). Total RNA was obtained using a commercially available reagent, Ultraspec (Fisher Biotec, Australia) following manufacturers instructions.

2.3 COMBINED SSCA AND HETERODUPLEX ANALYSIS (CSHA)

The Combined Single-stranded conformational analysis and Heteroduplex Analysis (CSHA) method was performed using DNA and RNA derived from the 2 patient groups and builds on the method designed by (Espinosa-Parrilla *et al.*, 2000a). DNA samples were used to amplify the coding sequence of the PROS1 gene and part of the 5'UTR using primers detailed in Appendix 4 and *Taq* DNA Polymerase (Invitrogen, Australia). RNA samples were reverse transcribed using Superscript II (Invitrogen, Australia) and random primers (Promega, Australia) according to manufacturers instructions which was then used to generate five PCR fragments using *Taq* DNA Polymerase (Invitrogen, Australia). Primers for amplification of cDNA were designed to span exon/exon boundaries (Appendix 4). The resultant fragments were cut with combinations of restriction enzymes (Appendix 5). Both the DNA and RNA PCR products were diluted 1:4 as follows: 5µL PCR product, 10µL of ddH₂O and 5µL of CSHA loading dye (95% formamide; 100mM NaOH; 20mM EDTA pH 8.0; 0.05% bromophenol blue), overlaid with mineral oil then heat denatured at 96°C for 5 minutes. The samples were immediately snap frozen in crushed dry ice and incubated for 1 minute, followed by thawing at 4°C immediately prior to loading. A 10µL volume of each mixture was loaded into individual wells of a CSHA gel (16cm x 16cm x 0.75mm Biorad Protean

II); matrix composed of 12% 49:1 (acrylamide:bis-acrylamide) (ICN Biomedicals), 5.0% glycerol (BDH) and 0.5xTBE [45mM TRIS (ICN Biomedicals); 44.5mM boric acid (BDH); 10mM EDTA (BDH)] with 1% ammonium persulfate (APS) (Sigma) and 0.1% N,N,N',N'-tetra-methylethylenediamine (Biorad). Samples were electrophoresed at 4°C in 0.5x TBE buffer for 12 to 16 hours at 300V. The system was verified by identifying known mutations derived from DNA samples kindly provided by Prof. Yolanda Espinosa-Parrilla (Centre de Genètica Mèdica i Molecular, Institut de Recerca Oncològica (IRO), Barcelona, Spain).

2.4 SILVER STAINING

A standard silver staining technique was used to visualise the CSHA polyacrylamide gels. Briefly, post electrophoresis gels were immersed in a 10% EtOH solution with shaking (3min), followed by a wash step (H₂O). This process was repeated with 1% nitric acid (3min) then again with 0.1% AgNO₃ (10min). The silver ion was reduced with a 0.05% formaldehyde/3% Na₂CO₃ solution until bands became visible and the reaction terminated with 10% acetic acid.

2.5 ISOLATION OF EXON 5 FRAGMENT

The identification of a SSCA shift in an exon 5/6 product derived from a patient sample presented a problem vis-à-vis was the shifted band a product of exon 5 or exon 6. Therefore, to elucidate which exon to sequence, a total of 54µL *Pst* I cut exon 5/6 PCR product was combined with 12.5µL of 10x loading dye (30% glycerol; 1xTAE buffer; ddH₂O; 0.05% bromophenol blue) and loaded into a superwell of a 3% agarose gel (Scientifix) in the absence of ethidium bromide (initial experiments showed that ethidium bromide interfered with SSCA formation). The products were electrophoresed

in 1xTAE buffer for 6h at 120V. The gel was removed, divided into three and the two peripheral segments stained in a 0.1% ethidium bromide solution for 15 minutes. The ethidium bromide stained gel segments were then viewed using a UV light source to determine the location of the two bands corresponding to the exon 5 and exon 6 fragments. These were marked by notching the gel. Realignment of the stained segments with the non-stained segment identified the location of the corresponding bands on the non-stained segment and these were excised and gel purified (GelClean; MOBIO, Australia) following manufacturers instructions. Both wild and mutated separated products were analysed via CSHA.

2.6 CELL CULTURE

The breast carcinoma cell line MCF-7 (kindly provided by Dr. Jacqueline Bentel, Royal Perth Hospital, Western Australia) and the liver carcinoma cell lines HuH-7 and HepG2 (kindly provided by Dr. Mark Watson, Royal Perth Hospital, Western Australia) were maintained in Phenol Red Free Dulbecco Modified Eagle Medium (DMEM; MultiCel, Australia) supplemented with 10% FCS, 1x nonessential amino acids (MultiCel, Australia), 1mM sodium pyruvate (MultiCel, Australia), 100U/mL penicillin and 0.1mg/mL streptomycin (Gibco/Invitrogen, New Zealand) at 37°C with 5% CO₂.

2.7 PLASMID CONSTRUCTION FOR TRANSIENT TRANSFECTION STUDIES

The backbone of the pPTMR construct was derived from a previously designed vector, pKH2 containing an EGFP reporter gene (a gift from Dr. Mark Watson, Royal Perth Hospital, Australia). The 950bp fragment of the PROS1 promoter was amplified with the high-fidelity polymerase *pfu*-Turbo (Stratagene USA) (see Appendix 6 for primer sequences). The PCR product and pKH2 were digested with *Eco*RI and *Xho*I (Promega,

Australia). pKH2 was dephosphorylated and the 950bp PROS1 promoter sequence introduced via ligation with T4 DNA Ligase (Promega, Australia). A CMV/DsRed2 cassette (Clontech, USA) was introduced in order to produce a constitutively expressed DsRed2 signal to generate a baseline against which the PROS1 promoter controlled EGFP levels could be normalized. Briefly, the CMV/DsRed2 cassette was excised from pDsRed2-NI (Clontech, USA) with *SspI* and *VspI* (Promega, Australia) and the *VspI* 3' overhang blunted with T4 DNA Polymerase (Promega, Australia). The cassette was then isolated from the parent backbone via gel extraction (GelClean; MOBIO, Australia). The PROS1 promoter/EGFP vector was cut with *SspI* (Promega, Australia), dephosphorylated (Antarctic Phosphatase; New England Biolabs, UK) and the CMV/DsRed2 cassette introduced using T4 DNA Ligase (Promega, Australia). The pPMTR(-406) vector was generated via site directed mutagenesis. Briefly, using *pfu-Turbo* (Statagene, USA) the c-406t PCR product and the pPMTR(-ERE) vector were generated using pPMTR as the template, using site directed mutagenesis primers and primers designed to flank the proposed ERE, respectively (see Appendix 6 for primer details). The PCR product lacked the ERE motif and was ligated with T4 DNA Ligase (Promega, Australia) to form pPMTR(-ERE). The pPMTR(AvrII/DraIII-del) plasmid was generated via restriction enzyme digest of the parent pPMTR vector with *AvrII* and *DraIII* (both New England Biolabs, USA). The overhangs were 'flushed' with T4 DNA polymerase (Promega, Australia) and the product isolated via an agarose gel extraction (GelClean; MOBIO, Australia). The isolated product was ligated with T4 DNA Ligase. The pPMTR(BclI/AvrII) plasmid was constructed using the same parameters except that *BclI* (Promega, Australia) was used in place of *DraIII*. Additionally, as *BclI* is *dam* sensitive a *dam*- pPMTR was used, derived from a plasmid preparation using methylation negative *E-coli* K12 strain ER2925 (*dam*- and *dcm*-; New England Biolabs, USA). The pPMTR(-AvrII) plasmid was generated by restriction enzyme digestion of

pPMTR with *AvrII* followed by incubation with 25U of Mung Bean Nuclease. The resultant product was ligated with T4 DNA Ligase creating a ctagg deletion of the cctagg *AvrII* cut site.

2.8 TRANSIENT TRANSFECTION STUDIES

Constructs were transiently transfected using Transfectin (Biorad, Australia) according to the manufacturers instructions in 12-well plates. Cells were seeded at a density of 4.0 and 2.0×10^5 cells/well for MCF-7s and HuH-7s, respectively. Where specified, plasmids were co-transfected with vectors expressing either progesterone receptor isoform A (PR-A; a gift from Prof. Christine Clarke from the Westmead Institute, Australia), isoform B (PR-B; a gift from Prof. Pierre Chambon from the Institut de Génétique et de Biologie Moléculaire et Cellulaire, France), or oestrogen receptor alpha (ER α ; a gift from Dr. Andrew Redfern from the Western Australian Institute for Medical Research, Australia). Where appropriate, media was supplemented with hormone; 1, 5 or 10nM β -oestradiol (E₂; Sigma, Australia) or 20nM progesterone (P₄; Sigma, Australia), medroxyprogesterone acetate (MPA; Sigma, Australia), D(-)-norgestrel (LNG; Sigma, Australia), corticosterone (CORT; Sigma, Australia) and 10 μ M of the progesterone antagonist mifepristone (RU486; Sigma, Australia) delivered using an ethanol vehicle (final concentration 0.5% EtOH) 5h post-transfection, followed by incubation for 24h at 37°C, 5% CO₂. For all transfection procedures, experimental variables were assessed in triplicate. Following incubation, wells were aspirated and the cells washed with 1xPBS prior to resuspension in 1x PBS containing 1% bovine serum albumin and 0.2% NaN₃. Expression levels of EGFP and DsRed2 were measured using an EPICS Coulter flow cytometer at an excitation of 488nm and emissions detected at 505-545nm and 605-635nm for EGFP and DsRed2, respectively. The CMV/DsRed2

cassette was tested in isolation to determine if addition of hormones and/or co-transfection with the plasmids expressing ER α or PR-B affected expression of the internal control.

2.9 NUCLEAR PROTEIN EXTRACTION

Nuclear proteins were extracted from 1×10^8 MCF-7 cells that had been transiently transfected with the pPR-B expression vector after a 24h exposure to 20nM P₄. Briefly, cells were washed twice in 1xPBS then resuspended in 4mL cold Buffer A (10mM HEPES, pH 7.9; 10mM KCl; 0.1mM EDTA; 0.1mM EGTA). Note- All Buffers were supplemented with protease inhibitors, specifically, 0.5mM dithiothreitol, 1mM Na₃VO₄ and 1x Complete Protease Inhibitor (Roche Diagnostics, Australia) and incubated on ice for 20min. Post incubation 250 μ L of 10% NP-40 was added. The mixture was vortexed briefly then centrifuged for 1min at 13000rpm. The supernatant was removed and the pellet was resuspended in 1mL cold Buffer C (20mM HEPES, pH 7.9; 0.42M NaCl; 1mM EDTA; 1mM EGTA; 20% glycerol) and rocked vigorously for 30min at 4°C. Centrifugation at 13000rpm for 5min (4°C) followed and the supernatant was aliquoted into volumes for storage at -80°C.

2.10 ELECTROMOBILITY SHIFT ASSAYS

Oligonucleotides for the PRE investigation were purchased (Geneworks, Australia) and were as follows: CONSENSUS PRE- sense 5' GAT CCT GTA CAG GAT GTT CTA GCT ACA 3' and antisense 5' TGT AGC TAG AAC ATC CTG TAC AGG ATC 3'; MUTATED CONSENSUS PRE- sense 5' GAT CCT CAT CAG GAT CAA CTA GCT ACA 3' and antisense 5' TGT AGC TAG TTG ATC CTG ATG AGG ATC 3'; PROS1 PRE- sense 5' TGA ACC TAG GGA TCC TGT CCT CTT GAA 3' and antisense 5'

TTC AAG AGG ACA GGA TCC CTA GGT TCA 3'; PROS1 -407 MUTANT PRE-sense 5' TGA ACC TAG GGA TTC TGT CCT CTT GAA 3' and antisense 5' TTC AAG AGG ACA GAA TCC CTA GGT TCA 3'; AP1 CONSENSUS- sense 5' CGC TTG ATG AGT CAG CCG GAA 3' and antisense 5' TTC CGG CTG ACT CAT CAA GCG 3' (all Geneworks, Australia). The AP1 probe was included as an Ab control. The 5' termini of the sense oligonucleotides were end labelled with ³²P using a standard kinase reaction (Promega, Australia) following manufacturers instructions utilising [γ -³²P] ATP (Perkin Elmer, Australia). The labelled oligonucleotides were annealed to their cognate sequences to form the radiolabelled probes. Unlabelled competitor probes were also produced. Appropriate probes (50pmol unlabelled competitor probe and/or 5pmol ³²P-labelled probe) were incubated for 20min at 4°C in 1x Dignam Buffer D [20mM HEPES (pH 7.9), 20% glycerol, 0.1M KCl, 0.2mM EDTA supplemented with 0.5mM dithiothreitol, 1mM Na₃VO₄ and 1x Complete Protease Inhibitor (Roche Applied Science, Australia)] with 1µg of PR-B positive nuclear protein extract, with 1µg of Poly dI•dC (Sigma, Australia). Where indicated, 5µg of mouse anti-human PR-B (sc-811 X; Santa Cruz, USA) was added. A 5%, 29:1 acrylamide/*bis*-acrylamide gel matrix was used to electrophorese the samples for 3h at 200V (4°C) in 0.5x TBE. After drying, the gels were exposed to X-ray film for 24h at -80°C and the film developed using standard techniques.

2.11 PLASMID CONSTRUCTION FOR YEAST-2-HYBRID

The coding region of PROS1 was amplified from an I.M.A.G.E clone (MGC:9207 IMAGE:3909023; Geneservice, UK) with *pfu* Turbo (Stratagene, USA) using primers incorporating *Bam*HI and *Pst*I (Promega, Australia) restriction enzyme sequences (See Appendix 6 for primer details). The 'bait' vector pBTM116 (Bartel and Fields, 1995), a

gift from Dr. Evan Ingley from the Western Australian Institute for Medical Research, was also cut with *Bam*HI and *Pst*I, dephosphorylated with Antarctic Phosphatase (NEB, UK) and gel extracted (MoBio, Australia). The PROS1 fragment was ligated (T4 DNA Ligase; Promega, Australia) in downstream from the LexA-BD coding sequence, in-frame, to generate the LexA-BD/PS fusion protein. The PS/pBTM116 vector was used to screen a combined liver and bone marrow cDNA library (see below). A positive interaction was identified that yielded a clone containing a partial sequence of the haemostatic protein Z (PZ). Therefore, to confirm the interaction the coding sequence of PZ (IMAGE:30915254 MGC:103872; Geneservice, UK) was introduced into the 'fish' vector pGADT7 (Clontech, USA) via a method identical to that of the pBTM116/PS procedure, except that *Cla*I and *Bam*HI (Promega, Australia) restriction sequences were incorporated into the primers to match the corresponding cut sites in the pGADT7 vector (see Appendix 6 for primer details). The resultant pGADT7/PZ contained the PZ coding sequence downstream and in-frame, from the Gal4-AD to facilitate expression of a Gal4-AD/PZ fusion protein.

2.12 CONFIRMATION OF VECTOR SEQUENCE

All vectors were purified from cultures using a Qiagen Maxiprep Kit and the sequences confirmed by DNA sequencing using an ABI Prism 3730 48 capillary sequencer (Applied Biosystems). See Appendix 6 for primer details.

2.13 YEAST-TWO-HYBRID SYSTEM

The pBTM116/PS vector was used to screen combined liver and bone marrow cDNA libraries (Clontech, USA). Briefly, 1.5µg of pBTM116/PS was introduced into competent L40 yeast cells, that lack tryptophan (Trp), leucine (Leu) and histidine (His)

expression (a gift from Dr. Evan Ingley) using a previously described method based on the LiAc/PEG protocol (Gietz *et al.*, 1992). The transformation mix was plated out on Yc^{-Trp} agar plates (Yc agar was 0.17% yeast nitrogen base; 37.85mM (NH₄)₂SO₄; 0.11M glucose; with or without 0.1mM tryptophan, 0.1mM histidine or 0.7 leucine; all Sigma, Australia). 100 colonies were assessed for both β -galactose (β -Gal) activity and negative growth on Yc^{-Trp,-His} agar plates. The β -Gal activity was assessed using the following assay. Briefly, selected colonies were grown on Yc^{-Trp} plates until visible to the naked eye and transferred to a nitrocellulose membrane, prior to emersion in liquid N₂ to fix. The membrane was then soaked in Z-buffer (0.06M Na₂HPO₄; 0.04M NaH₂PO₄; 0.01M KCl; 0.001MgSO₄; all Sigma, Australia) supplemented with 0.03M β -mercaptoethanol (Sigma, Australia) and 2.78M X-gal (Promega, Australia) immediately prior to use. The membrane was incubated at RT until the characteristic blue colour indicating positive colonies was detected. A β -Gal positive/Yc^{-His,Trp} negative colony was selected for a large scale transformation of the combined liver and bone marrow cDNA libraries. A total of 250 μ g of each library was used to transform the L40^{pBTM116/PS} cells, based on the LiAc/PEG methodology of (Gietz *et al.*, 1995). Positives were selected for growth on Yc^{-Trp,-His,-Leu} agar plates, β -Gal positivity and for total growth inhibition in the presence of 10mM 3-aminotriazole, a His inhibitor, to account for any 'leaky' His activity. Plasmids were extracted from positive colonies and the cDNA inserts sequenced (see Appendix 6 for primer details). A 'fish' (pACT2) vector containing a partial fragment that corresponded to the PROZ gene that encodes PZ necessitated performing a follow up small scale PZ/PS transformation. Therefore, competent L40s were co-transformed with the pBTM116/PS and pGADT7/PZ to determine whether there was actually an interaction. Controls were; pACT2/Fgr and pBTM116/GPVI-tail, to generate a positive interaction between protein tyrosine kinase Fgr and glycoprotein VI-tail that has been shown previously in our laboratory (gifts

from Julianna Degenaar). They were constructed using similar PCR based techniques (see Sarich 2002) insert free pGADT7/pBTM116 vectors.

2.14 DATA AND STATISTICAL ANALYSIS

Flow cytometer data generated from the transient transfection work (Section 2.8) was analysed using the following calculation. In order to express the PROS1 promoter controlled EGFP signal as a percentage each sample was normalized against the DsRed2 signal.

$$(MFI^{\#} \text{ EGFP} / MFI^{\#} \text{ DsRed2}) \times 100 = \% \text{ expression}$$

[#]MFI= Mean Fluorescence Intensity

An average of the triplicates per variable, derived from three separate experiments, was calculated and expressed as a change in expression compared to the untreated (Vehicle; EtOH) for the respective vectors. Where indicated, a two-tailed Student's T-test was used to calculate *p*-values. Standard error mean (SEM) used to represent error where indicated in graphical interpretation of data.

CHAPTER 3

Mutation Analysis of the PROS1 Gene

3.1 INTRODUCTION

Genetic analysis of the PROS1 gene has been the focus of a number of studies, resulting in the identification of a large number of mutations and probable polymorphisms (Gandrille *et al.*, 1997, Gandrille *et al.*, 2000). A small percentage of mutations have been characterised *in vitro* and found to be causative for PS deficiency. These include the replacement of cysteine by arginine at amino acid position 474 (C474R) (Yamazaki *et al.*, 1996), G441C, Y444C (Espinosa-Parrilla *et al.*, 2000b), V-24E, G295V, H623P (Rezende *et al.*, 2002), R314H, P375Q and D455Y (Okada *et al.*, 2006) which have all been linked with Type I deficiency. Definitive linkage of mutations with Type II deficiency has been demonstrated for T103N (Giri *et al.*, 2000) which is proposed to interfere directly with aPC binding. A deletion of I203 and D204 in the 4th Gla domain (Baroni *et al.*, 2006) has also been proposed to interfere with aPC binding. Other mutations that are likely to alter PS conformation, C80Y and R275C, have also been defined as causative for Type II deficiency, due to the probable disruption of the molecules secondary structure via the removal and introduction of the cysteine residues, respectively, altering sulphur-sulphur bond pairing (Okada *et al.*, 2006). The limitations of the methodologies used to assess the mutant forms *in vitro* makes definitive classification of Type III deficiencies difficult, but cross-matching the patient's data with experimental results allows a measure of certainty.

Polymorphisms within the PROS1 gene have also been described. The K196E (formally known as K155E, or Tokushima variant) has a high prevalence in the Japanese population (frequency= 0.009) where it is a risk factor for DVT (Miyata *et al.*, 2006, Kimura *et al.*, 2006). Another, polymorphism S460P, otherwise known as the Heerlen mutation, has a frequency Caucasian populations in the range of 0.005-0.009 (Heeb *et al.*, 2004, Gandrille *et al.*, 2000). There is ongoing debate as to whether the Heerlen

variant is a risk factor for thrombosis (Denis *et al.*, 2005, Heeb *et al.*, 2004, Koenen *et al.*, 2004).

To date, no mutations have been linked to a *cis*-element that could form the basis for a mechanism to explain how PS is hormonally regulated. In this study, PS deficient patients were selected from two sources; individuals presenting with low or unexplained fluctuating free PS levels and a cohort of pregnant women who had suffered pre-eclampsia or gestational diabetes while pregnant. To identify potential causative mutations of PROS1 DNA, exonic sequences and a large portion of the promoter region were screened using Combined Single-stranded conformational analysis and Heteroduplex Analysis (CSHA). CSHA exploits the change, or changes, in sequence composition arising from the nucleotide substitutions, deletions or insertions. Figure 3.1 details the process involved. Briefly, PCR product is generated from a target sequence with a given size range of 100 to 300bp (larger sequences can be screened by cutting them into smaller fragments with restriction enzymes). The double stranded PCR product is heat denatured and rapidly cooled, producing single-stranded conformers (single-stranded DNA folded back on itself) and heteroduplexes (single-stranded DNA that has reannealed with complimentary strands and also mutated sequence). The secondary structure of the single-stranded conformers is determined by the nucleotide composition of the sequence. A single base change can dramatically alter the tertiary folding, forming a conformer that migrates through a matrix at a different rate to that of a wild type sequence. Heteroduplexes can be formed when a wild type sense strand reanneals with a mutated antisense strand, forming a 'kink' where the mismatched basepairs are. This too can effect the migration of the heteroduplex compared to that of the reannealed wild and mutated double-stranded products.

Figure 3.1 A Schematic of the CSHA Methodology- PCR product is heat denatured at 96°C for 5min to separate the sense and anti-sense strands prior to snap freezing on dry ice (-80°C). Samples are slowly thawed at 4°C. The process generates both single stranded DNA conformers (single stranded DNA that has folded back on itself) and heteroduplexes (DNA that has re-annealed with complimentary strands). Differences in base composition alter conformers and generate mismatched heteroduplexes, both of which can migrate at different rates when electrophoresed on polyacrylamide gel matrices.

The development of CSHA methodology was designed with a particular emphasis on the promoter region of the PROS1 gene, as it is a known 'hotspot' for hormonal regulation in many other genes, particularly via hormone receptor/response element interactions (Beato *et al.*, 1996, DeMayo *et al.*, 2002, Wan and Nordeen, 2003, O'Lone *et al.*, 2004, Schoneveld *et al.*, 2004).

The CSHA method was further modified to facilitate screening of PROS1 RNA to enable the detection of splice site variants and as a cross-check for the sensitivity of the DNA screen. In addition, several studies have identified hormone influenced post-translational mRNA degradation and protection processes that regulate cytoplasmic levels of particular mRNA transcripts, regulating protein translation (Ing, 2005).

This study is first to screen the PROS1 gene and the associated mRNA transcript with the sole aim of identifying potential hormonally responsive elements, by identifying variants that alter hormonal responsiveness. The decision to derive the nucleic acids from two separate sources, the low PS group and the highly hormonal pregnancy cohort, increases the power of the study.

3.1.1 SENSITIVITY OF CSHA COMPARED TO OTHER DETECTION METHODOLOGIES

Empirical determination of the exact sensitivity of CSHA based on published literature is problematic, given the extensive variability in experimental conditions used by different groups. Earlier publications focused exclusively on single-stranded conformational analysis (SSCA) with mutation detection sensitivities ranging from 69% (Choy *et al.*, 1999) to 97% (Sheffield *et al.*, 1993). However, groups that have

combined SSCA with heteroduplex analysis on the same gel matrix have reported sensitivities close to 100% (Espinosa-Parrilla *et al.*, 2000a, Rossetti *et al.*, 1995).

Other detection platforms include denaturing high performance liquid chromatography (dHPLC), high resolution melt curve analysis (HRM) and the 'gold standard' for novel mutation detection, direct DNA sequencing. Several studies report that dHPLC can detect mutations with a 100% accuracy, however this method requires extensive optimisation of column and running conditions and has a high rate of false positives (Ravnik-Glavac *et al.*, 2002, Colosimo *et al.*, 2003, Chou *et al.*, 2005). Preliminary studies suggest that HRM is a highly stringent methodology, but most studies acknowledge the problems associated with detecting A:T or T:A base changes due to the minimal change in thermal stability between wild and mutated alleles (Chou *et al.*, 2005). DNA sequencing represents the most stringent platform for detecting novel mutations, but the cost is unreasonably expensive for screening large cohorts.

3.2 RESULTS

3.2.1 DEVELOPMENT OF THE METHOD

Several parameters were optimised for the PROS1 CSHA including, gel matrix composition, denaturing and quenching temperatures and electrophoretic conditions including, temperature and time.

Figure 3.2 details the comparison of 29:1 and 49:1 gel matrices (both supplemented with 5.0% glycerol) for 3 and 6 hour time periods at 290V (22°C) for a standard 150bp PCR product. Longer running times resulted in greater band separation and were deemed more preferable.

Running temperatures were assessed in combination with sample denaturing times and quenching conditions (Figure 3.3). Two running temperatures were assessed, 4° and

22°C. The denaturing times were 2, 5 and 10 minutes with both dry ice (-70°) and wet ice (4°C) used to quench the samples. The gel matrix used was 12% (49:1) 5% glycerol. Both gels were electrophoresed for 4 hours at 400V. Denaturing times was not observed to affect band migration or resolution. Dry ice improved band resolution and was therefore selected for future procedures. The greatest variability was evident in the temperature used for electrophoresis. The 4°C temperature results showed a similar separation to 22°C for the upper single-stranded conformer banding, but differed in migration distance for the heteroduplex bands. Therefore, 4°C was selected for future studies as it allowed greater running times without the loss of the heteroduplex information.

Increased running times and voltages were also assessed, including 4, 6 and 8h runs and a range of voltages from 150V through to 400V (results not shown). Following assessment of the various parameters tested, the best conditions were considered to be a matrix composed of 12% 49:1 (acrylamide:*bis*-acrylamide), 5.0% glycerol with electrophoresis performed at 300V for between 12-16 hours (exon dependent) at a constant 4°C.

Positive controls for each exon fragment (samples containing known mutations, kindly donated Dr. Espinosa-Parrilla) were tested with the optimised parameters to confirm the ability of our CSHA method to detect mutations. CSHA detection of each of these positive controls is illustrated in Figures 3.4 and 3.5.

Figure 3.2 Comparison of 29:1 and 49:1 Gel Matrixes Over 3 and 6 Hour Run Times- a) Lanes 1 to 5 and 7 to 11: Replicate control samples electrophoresed at 290V (22°C) through 29:1 and 49:1 acrylamide:*bis*-acrylamide gel matrixes, respectively for 3 hours. Lanes 6 and 12: Negative controls. b) Lanes 1 to 5 and 6 to 10: Replicate control samples electrophoresed at 290V (22°C) through 29:1 and 49:1 acrylamide:*bis*-acrylamide gel matrixes, respectively for 6h.

Figure 3.3 Assessment of a) 4°C and b) 22°C Running Temperature Utilising Various Denaturing and Quenching Parameters- Lane 1: Undenatured sample minus loading dye. Lane 2: Undenatured sample with loading dye. Lanes 3, 4 and 5: Control sample denatured at 96°C for 2, 5 and 10 minutes, respectively, and quenched in normal ice. Lanes 6, 7 and 8: Control sample denatured for at 96°C for 2, 5 and 10 minutes, respectively, and quenched in dry ice.

Figure 3.4 CSHA Analysis of Exons 1-10. CSHA analysis of PROS1 exons 1-10 illustrating banding patterns produced by wild-type (Wild) sequences as well as known mutant controls (Gifts from Dr.Espinosa-Parrilla). Note- The large 778bp promoter/exon1 fragment is subjected to two separate restriction enzyme regimes to cut the PCR product into CSHA compatible sizes (BamHI/SacII and BstOI). Using two different regimes minimizes the possibility of missing possible base changes in or around a cut site. No exon 9 variant sequences were available. Bands corresponding to variant sequences are indicated (arrows).

Figure 3.5 CSHA Analysis of Exons 11-15. CSHA analysis of PROS1 exons 11-15 illustrating banding patterns produced by wild-type (Wild) sequences as well as known mutant controls (gifts from Dr Yolanda Espinosa-Parrilla). Bands corresponding to variant sequences are indicated (arrows).

3.2.2 MUTATIONS IDENTIFIED IN THE CODING REGION

Following confirmation of our CSHA system to adequately detect DNA mutations we then tested our patient samples for the presence of any mutations within their *PROS1* gene. Two novel mutations were identified in exonic coding regions (Figure 3.6A). The first was identified in exon 5/6 obtained from a patient with severe PS deficiency. To identify the exon that corresponded to the band shift the two fragments were isolated after restriction enzyme digestion (see section 2.5 Chapter 2) and re-analysed (Figure 3.6B). The mutation mapped to the exon 5 fragment. Sequencing identified an **act**→**gct** point mutation creating a threonine to alanine change at position 101 (T101A). T101A is located within the EGF1 domain, a domain important for the co-factor function of PS as it is part of the region that binds to aPC.

The second point mutation, **ggt**→**tgt**, was identified in exon 10 and changes a glycine to a cysteine at position 340 (G340C; Figure 3.7). The G340C mutation maps to within the SHBG-like domain of the carboxy terminal.

In addition to the two novel findings, other previously described polymorphisms were identified; R192K (Gandrille *et al.*, 1997) [PS Deficient cohort, patient PS3], and S460P (Heerlen mutation) (Bertina *et al.*, 1990); in both cohorts [PS Deficient cohort, patients PS16, PS19, PS21 and PS22 and the Pregnancy cohort, patient GD416]. The K196E polymorphism, commonly found in the Japanese population, was not identified in either cohort. All of the control mutations were detected. A schematic highlighting the location of each of these mutations is seen in Figure 3.8.

Figure 3.6 Identification of a novel mutation affecting the 1st EGF-like domain. A) Silver stained CSHA gel image showing the band shift for a novel mutation identified in the exon 5/6 screen of the patient's DNA. B) A separation method showed that the shift mapped to exon 5 (see text for details). D) Sequencing of exon 5 revealed an **act→gct** point mutation that changes the threonine to an alanine (T101A). C) The T101A mutation was also identified after CSHA was performed on the patient's RNA.

Figure 3.7 Identification of a second novel mutation affecting the SHBG-like domain. A) Silver stained CSHA gel image showing the band shift for a novel mutation identified after screening exon 10 of the patient's DNA. C) Sequencing of exon 10 revealed a **ggt→tgt** point mutation that changes the glycine to a cysteine (G340C) that can be clearly identified in the chromatogram. B) The G340C mutation was also identified after CSHA was performed on the patient's RNA.

Figure 3.8 Mutations identified in the coding region of protein S. A schematic of the PS showing the location of the two novel mutations, T101A in exon 5 and G340C in exon 10 (highlighted in pink) and previously reported polymorphisms K196E, R192K, S460P and P626L that map to exon's 6, 7, 13 and 15, respectively (highlighted in purple). The previously confirmed Type II PS mutation T103N is shown two residues downstream from T101A. Highlighted in grey (and purple) are the Espinosa-Parrilla controls that were used to validate the CSHA method. Adapted from (Greenberg and Davie, 2001).

3.2.3 MUTATIONS IDENTIFIED IN THE 5' UNTRANSLATED REGION

In order to determine whether clinical observations were due to mutations found within the PROS1 promoter region, CSHA was performed on 950bp upstream from the PROS1 start codon. Three single nucleotide changes were identified in the 5' untranslated region (5'UTR), one was a novel finding while the other two have been previously described. The novel variant, 175 bases upstream from the start codon, changes the guanine to an adenine (g-175a) (Figure 3.9A).

The remaining two variants have been previously reported. The nucleotide substitutions c-407t (Espinosa-Parrilla *et al.*, 2000a) and t-397c (Espinosa-Parrilla *et al.*, 1999), are only 10 bases apart from each other (Figure 3.9B and C) and were identified separately in two women from the Pregnancy Cohort who developed greater than normal, acquired PS deficiency while pregnant.

3.2.4 RESULTS OF MRNA ANALYSIS

The CSHA methodology was also used to test cDNA converted patient mRNA samples. Analysis of PS deficient patient mRNA samples identified a heterozygous splice variant. CSHA analysis alone did not identify the mutation. The splice variant was easily identifiable after electrophoresis of the PCR products on an agarose gel (Figure 3.10A). Sequencing of the PCR product resulted in a chromatogram that developed a dual signal at the exon 2-3 junction. Extraction of the wild-type signal enabled extrapolation of the mutated signal that was subsequently identified as exon 1; thus the mutant allele lacks exon 2 (Figure 3.10B).

No additional findings were identified in the remaining patient mRNA samples. However, the two novel mutations T101A and G340C were re-detected using the

mRNA CSHA protocol (Figures 3.6C and 3.7B) suggesting that the technique is sensitive for analysis of patient mRNA.

Figure 3.9 Point changes identified in the promoter region of PROS1 using CSHA. Two restriction enzyme regimes (*Bam*HI in combination with *Sac*II, and *Bst*OI) were used for CSHA of the combined PROS1 promoter and exon 1 region. (A) A novel g→a point mutation was identified 175 bases upstream from the ATG start codon; g-175a. Two previously identified variants t-397c (B) and c-407t (C) were also identified.

Figure 3.10 Detection of a splice variant by analysis of PROS1 mRNA. A splice abnormality was initially identified from the direct analysis of PROS1 mRNA that had been converted to cDNA, PCR amplified and electrophoresed in an agarose gel (A). The smaller product indicates the mutant allele. Sequencing revealed a dual signal initiating at the exon 2/3 boundary (B). Extrapolation of both signals reveals that the mutant form lacks exon 2.

3.3 DISCUSSION

The sensitivity of DNA CSHA technique was confirmed with the successful identification of all the positive controls supplied by Dr. Espinosa-Parrilla (Figures 3.4 and 3.5). The inclusion of RNA CHSA enabled the identification of possible splice variations, such as the splice variant that results in the deletion of exon 2 (Figure 3.10), but also provided a means of 'cross-checking' a patient's DNA, increasing the power of the CSHA method. Screening of DNA using single stranded conformational analysis (SSCA) in isolation is reported to have a sensitivity of around 90% with increasing sensitivity gained from using either two or more gel matrices and/or by varying the electrophoretic conditions (Jaksch *et al.*, 1995, Gross *et al.*, 1999, Hayashi and Yandell, 1993, Espinosa-Parrilla *et al.*, 2000a). The combination of SSCA with heteroduplex analysis (the CSHA method) further increases sensitivity (Espinosa-Parrilla *et al.*, 2000a). Direct evidence of this is seen in this study with the novel T101A finding, identifiable only by a heteroduplex shift in the initial DNA CSHA and by the non-heteroduplex bands in the RNA CSHA. The size of the fragments used for CSHA greatly affects sensitivity (Hayashi and Yandell, 1993). Cutting the large fragments amplified for use in the RNA CSHA with two different restriction enzyme (RE) regimes generated fragments small enough for analysis. This approach was used for the promoter/exon1, the exons 5/6 and exon 15 fragments. Secondly, the RE's were selected to ensure that the cut sites in the first regime differed in proximity to the cut sites of the second regime. This minimised the possibility of missing any mutations localised in or around a particular cut site. Thus, by combining the RNA CSHA with the DNA CSHA one sample effectively undergoes three different analyses, increasing the overall sensitivity of the technique. The exception is the promoter region which is not assessed via RNA CSHA (however, PCR samples used for CSHA of the promoter region were

also treated with two separate RE conditions, which increased the sensitivity for the PROS1 promoter region DNA CSHA). Using the multiple parameter approach set this CSHA study apart from that of the Espinosa-Parrilla group study in which they relied on differential gel matrices to increase the CSHA sensitivity (Espinosa-Parrilla *et al.*, 2000a). The method used in this present study is less labour intensive as only one gel matrix is required for all the DNA and RNA screening.

The CSHA method identified 3 novel mutations located within the promoter, in exon 5 and exon 10 of the PROS1 gene, respectively. The heterozygous T101A mutation is probably a qualitative mutation, the basis of the rare Type II deficiency and is unlikely to interfere with any endocrine based mechanism. Nevertheless, it represents an important finding as only one other Type II mutation has been described in the EGF1 domain, T103N, two residues downstream from T101A. The T103N mutation has been characterised with the mutant form exhibiting approximately 50% less co-factor activity compared to the wild type (Giri *et al.*, 2000). Similar studies will have to be completed with T101A to determine whether or not it is a functional mutation that directly interferes with aPC binding. Interestingly, the T101 residue is highly conserved across species while the T103 is only found in the human and monkey forms of PS (Table 3.1). The patient (PS2), a 34yr old female, had a history of systemic lupus erythematosus and had suffered both a myocardial infarction and a stroke. Analysis of the patients functional PS status was not carried out due to prophylaxis with warfarin.

The second novel mutation identified in the coding region of PROS1, G340C (PS23), was also heterozygous and is located in the C-terminal SHBG-like domain of PS. As with the T101A mutation, it is unlikely to be involved in an endocrine response. Like T101 the G340 residue is highly conserved across several species (Table 3.2). Introduction of an additional cysteine could interfere with disulphide bond formation,

important for the tertiary structure organisation of the protein (Simmonds *et al.*, 1996) [Figure 3.6- the cysteine residue di-sulphide bridging is a hypothetical representation.] The exact structure of PS has proved elusive due to difficulties associated with crystallising the PS molecule (Gandrille *et al.*, 2000)]. Given the even number of cysteines within the structure of PS, it is possible that the introduction of an additional sulphur residue could disrupt the structure of the PS molecule. Similarly, di-sulphide bridges could be formed with other proteins preventing the mutated protein from being exported into the cytoplasm or causing the incorporation of a secondary protein into the structure. This has been previously suggested for the R474C mutation located in exon 13, where the addition of a cysteine creates a protein with a greater molecular weight compared to the wild-type in non-reducing conditions (Yamazaki *et al.*, 1996).

The third novel finding was the heterozygous g-175a mutation (numbering relative to the ATG start codon) identified in the 5' untranslated region of PROS1. The mutation was identified in a patient who experienced persistent low free PS levels which is likely to be a result of a defective transcriptional initiation process. Two studies have reported that either nucleotides -173, -174 or -175 form the starting point for transcription initiation of PROS1 mRNA synthesis in the liver and the human hepatoma cell line, HepG2 (Ploos van Amstel *et al.*, 1990, Hall *et al.*, 2006). Although multiple transcriptional start sites (TSSs) have been described for PROS1, the -173/74/75 transcript appears to be a highly expressed form in the liver, the principle organ for PS expression (Soria and Bertina, 1997, de Wolf *et al.*, 2005, Hall *et al.*, 2006). Therefore, the substitution of a guanine for an adenine may inhibit or even prevent mRNA initiation at the TSS, reducing the numbers of PROS1 mRNA transcripts and consequently, lowering levels of circulating PS.

Species	Amino Acid Sequence																				
	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110
Human	Y	M	S	C	K	D	G	K	A	S	F	T	C	T	C	K	P	G	W	Q	G
Monkey	Y	M	S	C	K	D	G	K	A	S	F	T	C	T	C	K	P	G	W	Q	G
Bovine	F	M	T	C	K	D	G	Q	A	T	F	T	C	I	C	K	S	G	W	Q	G
Porcine	F	M	T	C	K	D	G	Q	A	M	F	T	C	I	C	K	S	G	W	E	G
Rabbit	Y	L	N	C	K	D	G	Q	A	T	F	T	C	I	C	K	P	G	W	Q	G
Mouse	Y	L	A	C	Q	D	G	Q	A	A	F	T	C	F	V	K	P	G	W	Q	G

Table 3.1 Threonine 101 is highly conserved across species.

The threonine residue at position 101 (T101) is conserved across a variety of species, whereas T103, the location of the previously described T103N Type II mutation, is homologous only with the monkey sequence.

Amino acid numbering shown for Human PS.

Species	Amino Acid Sequence																				
	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349
Human	K	I	T	T	G	G	D	V	I	N	N	G	L	W	N	M	V	S	V	E	E
Monkey	K	I	T	T	G	G	D	I	I	N	N	G	L	W	N	M	V	S	V	E	E
Bovine	K	M	T	T	G	G	K	V	I	N	D	G	L	W	H	M	V	S	V	E	E
Porcine	K	I	T	T	G	G	R	V	I	N	D	G	L	W	N	M	V	S	V	E	E
Rabbit	Q	I	T	T	G	G	Q	V	I	N	D	G	L	W	N	M	V	S	V	E	E
Mouse	Q	I	T	T	G	G	N	V	I	N	N	G	I	W	N	M	V	S	V	E	E

Table 3.2 Glycine 340 is highly conserved across species. The glycine residue at position 340 (G340) is conserved across a variety of species. The G340C mutation introduces an additional cysteine that may affect protein structure or cause cross binding with secondary proteins.

Amino acid numbering shown for Human PS.

The region surrounding position -175 is also very GC-rich and has recently been characterised as a strong *cis*-acting element that binds the ubiquitous Sp1 transcription factor (de Wolf *et al.*, 2006a). As detailed in Section 1.9, Sp1 can form the basis of a hormonal response mechanism via interaction with ligand bound ER α and has also been shown to exert similar transcriptional control via interaction with PR (Owen *et al.*, 1998, Safe, 2001, Safe and Kim, 2004).

Analysis of PS deficient patient mRNA not only confirmed the T101A and G340C mutations, it also re-detected the common PROS1 polymorphism S460P (Heerlen mutation). The frequency of the Heerlen mutation in the PS deficient cohort (frequency= 0.167) was similar to previous reports that examined PS deficient patients who had suffered a venous thrombotic event (Espinosa-Parrilla *et al.*, 2000a, Borgel *et al.*, 1996). The low prevalence of the Heerlen mutation in the pregnancy cohort (frequency= 0.007) correlates with previously published data that has examined the frequency of the Heerlen mutation in the general population (Duchemin *et al.*, 1995) and is not surprising given that the pregnancy cohort was not associated with a thrombotic predisposition.

The other advantage of the mRNA analysis was that it facilitated the identification of splice variants such as the apparent exon 2 splice mutation identified in a patient with severe PS deficiency (Figure 3.10). The truncated splice variant needs to be isolated and expressed to confirm that it is the cause of the PS deficiency. Exon 2 encodes the Gla-Domain required for binding of PS to phospholipid surfaces such as the surface of platelets. Therefore, this mutation could be classified as causative for a Type II

deficiency. The patient was receiving warfarin therapy so no functional data was available for PS.

The disadvantage of the DNA/RNA CSHA system is that it fails to detect large heterozygous deletions of the *PROS1* gene, whether partial or whole gene deletions, as amplification of the PCR, from DNA or cDNA only requires one complete allele to generate a product. A 2005 study (Johansson *et al.*, 2005) used single nucleotide polymorphisms (SNPs) and microsatellite markers to screen for large heterozygous deletions of the *PROS1* in a cohort of PS deficient families. They identified large scale deletions as the causal factor in 18% of PS deficient families recruited into their study that would have otherwise been unidentified. At the time of writing this thesis, a third detection tier has been added to the DNA/RNA CSHA methodology, multiplex ligation-dependent probe amplification (MLPA; <http://www.mrc-holland.com/pages/indexpag.html>; accessed October 2007). Briefly, MLPA analysis uses sets of adjacent probes that are ligated together to prime a standard fluorescently labelled PCR product. Each probe set generates a unique sized fragment due to 'stuffer' sequences that are incorporated into the probe design. Fluorescence intensity is compared with known controls to distinguish allele frequency.

PS Deficient Cohort		
Sample ID	Mutation	Patient History
N41 (Control)	None Detected	Control
PS1	None Detected	Fluctuating PS levels Thrombosis in Pregnancy
PS2	T101A [^]	SLE, MI cerebral infarction
PS3	R192K [#]	Severe pre-eclampsia
PS4	None Detected	PE
PS5	None Detected	Pre-eclampsia
PS6	None Detected	Fluctuating PS level
PS7	None Detected	DVT, Family history PS def.
PS8	None Detected	Recurrent DVT during pregnancy
PS9	-68 to -70 del att [^]	Fluctuating PS level
PS10	None Detected	Fluctuating PS level
PS11	None Detected	Recurrent pregnancy loss
PS12	None Detected	Daughter of PS13
PS13	g-175a [^]	Post Partum PE
PS14	None Detected	PE
PS15	None Detected	History of Thrombosis
PS16	S460P [†] (Heerlen)	History of thrombosis, DVT
PS17	None Detected	Recurrent DVT
PS18	None Detected	Family history of DVT
PS19	S460P [†] (Heerlen)	PS deficiency, spontaneous miscarriages
PS20	None Detected	Persistent DVT in calf
PS21	S460P [†] (Heerlen)	Fluctuating PS level
PS22	S460P [†] (Heerlen)	PS deficiency
PS23	G340C [^]	Family history of PS deficiency, DVTx2, MI
PS24	None Detected	History of Thrombosis
Pregnancy Cohort		
Sample ID	Mutation	
Pre326	c-407t [▽]	Greater than normal reduction in PS while pregnant
Pre390	t-397c [▽]	Greater than normal reduction in PS while pregnant
GD416	S460P [†] (Heerlen)	Gestational Diabetes

Table 3.3- Mutations detected within the two cohorts studied- Note- the Pregnancy Cohort has been abridged to show only those patients where mutations were identified. Four novel mutations (denoted [^]) were identified in the PS Deficient Cohort; g-175a a nucleotide substitution located in the 5'UTR; T101A a missense mutation that maps to exon 5; G340C a missense mutation that maps to exon 10; and -68 to -70 del att and 3 base deletion mapping to Intron A. Previously reported mutations R192K (Gandrille *et al.* 1997), PS3; S460P (Heerlen; Bertina *et al.* 1990), PS16, PS19, PS21, PS22 and GD416; and two point mutations identified in the 5'UTR, c-407t (Espinosa-Parrilla *et al.* 2000) and t-397c (Espinosa-Parrilla *et al.* 1999) that were identified in the Pregnancy Cohort.

While regions of the PROS1 coding region and 5' promoter were screened using CSHA, the 3'untranslated (3'-UTR) region of the PROS1 gene was not included in the screening process. Hormonal control of mRNAs via enhancement or protection of nuclease targeted degradation of the 3'UTR has been demonstrated for several genes and may well be occurring in PROS1 (Staton and Leedman, 1998, Staton *et al.*, 2000, Balmer *et al.*, 2001). However, the promoter is often a key region for hormone based regulation and the three variants detected using the CSHA method can be further investigated to ascertain if they form part of some hormone responsive regions. Particularly the t-397c and c-407t variants which are further examined in the following chapter.

CHAPTER 4

Analysis of the PROS1 Promoter

4.1 INTRODUCTION

As detailed in Chapter 1, acquired PS deficiency has been reported in individuals with raised oestrogen levels, arising during pregnancy or in individuals using combined oral contraceptives (COCs), particularly the 3rd generation forms (Comp *et al.*, 1986, Malm *et al.*, 1988, Oruc *et al.*, 2000, Tans *et al.*, 2000, Mackie *et al.*, 2001, Kemmeren *et al.*, 2004, Uchikova and Ledjev, 2005). The fall in PS levels may be one reason why users of 3rd generation COCs have a 6 to 9 times greater risk of developing VTE compared to non-users and approximately double the risk associated with users of 2nd generation COCs (Middeldorp, 2005). The prothrombotic shift results from upregulation of several key components of the procoagulant and fibrinolytic systems in combination with a down-regulation of the anticoagulation system, in particular reduced PS levels and increased aPC resistance. Previous investigations into the molecular mechanisms influencing these changes have focused on promoter regions of genes of the procoagulant and fibrinolytic systems (Farsetti *et al.*, 1995, Brady *et al.*, 1998, Heit *et al.*, 1999a, Farsetti *et al.*, 2001, Smith *et al.*, 2004). The work discussed in this Chapter represents the first attempt to study hormonal regulation of the PROS1 promoter that governs the production of PS.

Regulation of gene expression by transcriptional factors operating via interaction with the promoter regions of genes is well reported [Reviewed by Metivier *et al.* (2006)]. As discussed in Chapter 1, the hormone receptor class of transcriptional factors are highly active in regulating gene expression via direct and indirect interaction with specific motifs located in the promoter regions of a vast array of genes. A haemostatic example is the gene that encodes FIX (F9). Experimental work performed in the 1990s identified an androgen response element (ARE) in the promoter of the F9 gene following investigation of a form of Haemophilia B known as Haemophilia B Leyden (Morgan *et*

al., 1997, Brady *et al.*, 1998, Heit *et al.*, 1999a). FIX levels of patients with the Leyden form of Haemophilia B are low prior to the onset of puberty, but increase to within, or close to, normal ranges by the end of puberty. A subclassification form of Haemophilia B Leyden, Haemophilia B Brandenburg, fails to respond during puberty and was found to be due to a missense mutation within the ARE motif that disrupted the binding of the androgen receptor.

The FIX ARE mechanism is an example of a *cis*-element acting to increase promoter activity. As PS production is decreased in response to E₂ the primary focus in this study is to identify an element that suppresses, rather than increases, promoter activity. An initial screen of the PROS1 promoter sequence using an online software program (Dragon ERE Finder; <http://sdmc.lit.org.sg/ERE-V2/index>; accessed 1st November 2005) identified a potential oestrogen response element (ERE); positions -526 to -538 relative to the ATG start codon, that could act as a repressor. The majority of previously described EREs generate an increase in promoter output in response to E₂, but there are several reports of EREs that have inhibitory functions (Online database-<http://defiant.i2r.a-star.edu.sg/projects/Ergdb-v2/index.htm>; accessed 1st November 2005) particularly when in conjunction with other transcriptional factors such as Sp1 (Stossi *et al.*, 2006).

However, the identification of the previously described variants t-397c and c-407t using the CSHA method detailed in Chapter 3 suggests the possibility of a secondary *cis*-element that acts to increase activity of the PROS1 promoter (Stossi *et al.*, 2006, Espinosa-Parrilla *et al.*, 1999, Espinosa-Parrilla *et al.*, 2000a). This hypothesis will be discussed later in this Chapter.

To assess the potential transcriptional properties of the PROS1 promoter, the pPMTR vector was created that contained the first 950bp of the promoter region fused to an enhanced green fluorescent protein (EGFP) reporter gene. The vector also contains an additional reporter gene DsRed2, constitutively expressed by a cytomegalovirus promoter (CMV). This chimera was included to produce a baseline signal against which the change in expression of the EGFP signal could be normalised to. Subsequent modifications and deletions of the PROS1 sequence within the pPMTR vector were performed to analyse particularly structural domains.

4.2 RESULTS

4.2.1 ER α DOWNREGULATES THE PROS1 PROMOTER BUT NOT VIA THE ERE CONSENSUS SEQUENCE

A potential ERE motif spanning nucleotides -526 to -538 was identified in the PROS1 promoter. The sequence (5' gatcaaaatgacc 3') differs from the 'classical ERE' motif- 5' ggtcannntgacc 3' only at the second position. To determine whether the presence of this motif was required for oestrogen sensitivity, we constructed three plasmids. One containing the wildtype promoter (pPMTR), a second containing the same promoter but lacking the ERE sequence (pPMTR(-ERE)) and a third with a large deletion that lacked the 3' terminal sequence of the ERE plus an additional 111 downstream bases (pPMTR(BclI/AvrII-del). All three were used in transient transfection experiments (See Figure 4.1 for details).

Breast carcinoma MCF-7 cells were cotransfected with 2 μ g pPMTR and 100ng pER α and the activity of the PROS1 promoter measured. In the presence of excess ER α transcriptional activity of the PROS1 promoter was downregulated by 20% ($p=0.002$). This downregulation was extended to 35% with the addition of 10nM E₂ ($p=0.001$) (Figure 4.2A). When the cotransfection experiments were repeated using pPMTR(-ERE) in place of pPMTR, the presence of excess ER α again resulted in a 20% downregulation of PROS1 promoter activity ($p=0.01$), which further diminished to 40% with the addition of 10nM E₂ ($p=0.01$) (Figure 4.3A). Cotransfection experiments using the larger deletion plasmid pPMTR(BclI/AvrII-del) yielded similar results with a 19% ($p=n/s$) downregulation in the presence of excess ER α that decreased to 41% with the addition of 10nM E₂ ($p=0.002$) (Figure 4.3D).

Interestingly, the cotransfection of pPMTR(-407), which contains the c-407t PROS1 variant, with the pER α vector resulted in a 35% decrease in PROS1 activity ($p=0.002$) which further reduced to 50% with the addition of 10nM E₂ ($p=0.004$). Both responses were significantly different compared to the wildtype vector pPMTR ($p=0.01$ and $p=0.01$, respectively) (Figure 4.3B). A similar result was evident for the pPMTR(AvrII/DraIII-del) vector, with the addition of the pER α vector decreasing activity by 36% ($p=0.02$) which further reduced to 47% with the addition of 10nM E₂ ($p=0.004$). However, only the comparison of the wildtype pER α + 10nM E₂ with the deletion pER α + 10nM E₂ was significant ($p=0.01$) (Figure 4.3C).

The E₂ and ER α responses were significantly more pronounced when transfections were performed in the liver carcinoma cell line HuH-7. Cotransfection with 2 μ g pPMTR, 100ng ER α plus 10nM E₂ (as used in the MCF-7 cell line) suppressed PROS1 activity to levels below detectable limits. Maintaining pPMTR at 2 μ g but reducing the ER α vector to 20ng and E₂ to 1nM reduced the activity of the promoter by 46% compared with no treatment ($p=0.002$) (Figure 4.2B), suggesting that the oestrogen response was much greater in the liver cell line.

Figure 4.1 Schematics of the six vectors used for the transient transfection procedures. The blow up details the 950bp sequence from the PROS1 promoter. Two point mutations, c-407t and t-397c, are shown flanking the PRE half site. Also shown is the putative ERE identified using the Dragon ERE Finder online software and Sp1 binding sites postulated by de Wolf *et al.* (2006). (A) pPMTR vector, 950bp wild type PROS1 fragment that includes the putative ERE and proposed PRE motifs; (B) pPMTR-ERE vector, 950bp PROS1 fragment without ERE (underlined); (C) pPMTR(-407) vector, 950bp PROS1 fragment with c-407t mutation introduced; (D) pPMTR(-AvrII), 950bp PROS1 fragment without *AvrII* sequence; (E) pPMTR(del-BclI/AvrII), 950bp PROS1 fragment without intervening 120bp sequence between the *BclI* and *AvrII* cut sites (blue line); (F) pPMTR(del-AvrII/DraIII), 950bp PROS1 fragment without intervening 120bp sequence between the *AvrII* and *DraIII* cut sites (orange line)

ERE, oestrogen response element; PRE, progesterone response element; EGFP, enhanced green fluorescence protein coding sequence; Amp^R, ampicillin resistance coding sequence; DsRed2, coding sequence for red coral fluorescence protein (DsRed2); CMV, cyclomegalovirus promoter; TB, transcriptional blocker.

MCF-7

A

pPMTR MCF-7 Statistical Analysis

pPMTR	pPMTR	<i>p</i> -value
Vehicle	Vehicle + 20ng pER α	0.002
Vehicle	1nM E ₂ + 20ng pER α	0.001
+20ng pER α	1nM E ₂ + 20ng pER α	0.01

Vehicle
 + 100ng pER α
 10nM E₂ + 100ng pER α

HuH-7

B

pPMTR HuH-7 Statistical Analysis

pPMTR	pPMTR	<i>p</i> -value
Vehicle	Vehicle + pER α	0.002
Vehicle	10nM E ₂ + pER α	0.001
+ pER α	10nM E ₂ + pER α	0.01

Vehicle
 + 20ng pER α
 1nM E₂ + 20ng pER α

Figure 4.2 PROS1 promoter is downregulated by oestrogen MCF-7 cells were transfected with (A) pPMTR with either no treatment (Vehicle), 100ng of pER α vector, or 100ng pER α plus 10nM E₂. (B) Huh-7 cells were transfected with pPMTR with either no treatment, 20ng of pER α vector, or 20ng pER α plus 1nM E₂. In all experiments activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Figure 4.3 Manipulation of the PROS1 promoter alters oestrogen response. MCF-7 cells were transfected with (A) pPMTR(-ERE), (B) pPMTR(-407), (C) pPMTR(BclII/AvrII-del) and (D) pPMTR(AvrII/DraIII-del) with either no treatment (Vehicle) ■, 100ng of pER α vector ■, or 100ng pER α plus 10nM E₂ ■. In all experiments activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

4.2.2 PROGESTERONE UPREGULATES PROS1 PROMOTER ACTIVITY

The wildtype pPMTR vector was used to test the effect of the progestin component of the COC pill on PROS1 transcriptional activity. MCF-7 cells were transiently transfected with pPMTR and incubated in the presence of 20nM progesterone (P_4). Expression of the EGFP reporter gene was measured and analysis indicated the presence of P_4 upregulated the PROS1 promoter activity by 25% ($p=0.0003$). When this experiment was repeated in the presence of the progesterone receptor modulator, RU486, the effect was abolished (Figure 4.4A). This finding suggested that the mechanism driving the upregulation involved a progesterone receptor (PR). As there are two PR isoforms, PR-A and PR-B, the aim was to confirm that the pPR-B isoform was the only form involved in the response. MCF-7 cells were cotransfected with 2 μ g pPMTR, 100ng PR isoform plus or minus 20nM P_4 . The PR-B isoform increased transcription of the PROS1 promoter ($p=0.004$) while the PR-A isoform did not exhibit any significant change (Figure 4.4B).

Analysis of the EGFP reporter gene expression using the MCF-7 cell line, indicated PROS1 promoter activity increased by 35% when cotransfected with 100ng of the pPR-B expression vector in the presence of 20nM P_4 ($p=0.01$) (Figure 4.5A). The effect of progesterone and PR-B on PROS1 transcriptional activity were also measured in HuH-7 cells using the same parameters. In the liver cell line upregulation of PROS1 promoter activity was weaker; 17% in the presence of 20nM P_4 ($p=0.008$), increasing to 25% when cotransfected with 100ng of pPR-B ($p=0.04$) (Figure 4.5B).

4.2.3 IDENTIFICATION OF THE PR BINDING DOMAIN WITHIN THE PROS1 PROMOTER

The t-397c and c-407t PS variants were detected using the CSHA method as discussed in Chapter 3. It was hypothesized that these variants may interfere with PR-B binding. To test this hypothesis, MCF-7 cells were transiently transfected with either the wild type pPMTR or pPMTR(-407) vectors. Initial results indicated the c-407t mutant exhibited an impaired ability to respond to increased P₄. These experiments were repeated with the addition of 100ng pPR-B in the presence or absence of 20nM P₄. The promoter activity of pPMTR(-407) did not significantly increase when 100ng pPR-B was added (2% -407 variant vs 17% wild type; $p=0.01$). The addition of 20nM P₄ to the cotransfection recovered some of the promoter responsiveness, but it was still significantly less than that of the wild type (17% -407 variant vs 35% wild type; $p=0.001$) (Figure 4.6). Analysis of the pPMTR(AvrII/DraIII-del) that lacks most of the proposed PRE (including nucleotides -397 and -407), demonstrated a similar response. There was no increased activity with the pPR-B cotransfection (0% AvrII/DraIII-del vs 17% wild type; $p=0.003$) but there was a degree of recovery in the presence of additional P₄ (18% AvrII/DraIII-del vs 35% wild type; $p=n/s$) (Figure 4.7). Results for the pPMTR(-AvrII) plasmid that has the deletion of nucleotides -411 to -415 demonstrated reduced levels of upregulation compared to the wild type, but the differences were not significant; cotransfection with pPR-B (10% -AvrII vs 17% wild type; $p=n/s$) and cotransfection with pPR-B in the presence of 20nM P₄ (26% -AvrII vs vs 35% wild type; $p=n/s$) (Figure 4.8).

Results for the PRE EMSA showed that both the PROS1 PRE probe and the consensus PRE probes bound to PR-B nuclear derived protein. Addition of 5µg of mouse anti-human PR-B Ab disrupted the interaction, but 0.5µg did not, suggesting that the Ab is directed against a region of PR-B that is required for PRE interaction. Therefore, in the

absence of a supershift, the AP1 control was included to demonstrate that the mouse anti-human PR-B was specific for the PR-B complex only. Addition of 5 μ g of mouse anti-human PR-B did not effect binding of the consensus AP1 probe with AP1. The PROS1(-407) PRE probe exhibited a reduced affinity for PR-B compared to the PRE consensus and PROS1 wild-type PRE (Figure 4.9).

4.2.4 DIFFERENTIAL RESPONSES TO DIFFERENT TYPES OF PROGESTINS

It has been clinically observed that the severity of acquired PS deficiency can differ between the different generations of COCs, therefore to determine whether the type of progestin present led to a variation in the extent of hormone regulation of PROS1 promoter transcriptional activity *in vitro*. Once again the response experiments were repeated, this time using the testosterone derived levonorgestrel (LNG) or the progesterone derived medroxyprogesterone acetate (MPA) in place of P₄. MCF-7 cells were cotransfected with 2 μ g pPMTR and the isoforms of progestin added at a concentration of 20nM. Both LNG and MPA exhibited a more potent effect on the PROS1 promoter activity than P₄, with MPA showing the greatest, more than doubling the response generated by P₄ (30% vs 17%; $p=0.006$) (Figure 4.10).

4.2.5 DIFFERENTIAL RESPONSE TO GLUCOCORTICOID

Progesterone receptors are members of a superfamily of receptors (see Chapter 1) that include the glucocorticoid receptors. Therefore the effect of corticosterone (CORT) was assessed to determine any effect on expression. Using the MCF-7 cell line transiently transfected with 2 μ g of pPMTR, 20nM of CORT upregulated PROS1 promoter activity by 20% ($p=0.04$) (Figure 4.11).

Figure 4.4 Progesterone upregulates the PROS1 promoter via a PR-B dependant mechanism. (A) MCF-7 cells transfected with pPMTR and either 10µM of the PR modulator mifepristone (RU486), or 10µM RU486 + 20nM P₄ were added; (B) MCF-7 cells transfected with pPMTR and either no cotransfection, or cotransfection with either 100ng pPR-A, or 100ng pPR-B in normal media supplemented with or without 20nM P₄. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Figure 4.5 Progesterone upregulates the PROS1 promoter via a PR-B dependant mechanism. (A) MCF-7 cells transfected with pPMTR and either no cotransfection, or cotransfection with 100ng pPR-B in normal media supplemented with or without 20nM P₄; (B) HuH-7 cells transfected with pPMTR and either no cotransfection, or cotransfection with 100ng pPR-B in normal media supplemented with or without 20nM P₄. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Statistical Analysis

pPMTR	pPMTR(-407)	<i>p</i> -value
Vehicle + pPR-B	Vehicle + pPR-B	0.01
20nM P ₄ + pPR-B	20nM P ₄ + pPR-B	0.001

Figure 4.6 Introduction of c-407t reduces PR-B induced upregulation. MCF-7 cells transfected with pPMTR or pPMTR(-407) cotransfected with either 100ng of pPR-B, or 100ng pPR-B + 20nM P₄. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Statistical Analysis

pPMTR	pPMTR(AvrII/DraIII-del)	<i>p</i> -value
Vehicle + pPR-B	Vehicle + pPR-B	0.003
20nM P ₄ + pPR-B	20nM P ₄ + pPR-B	(n/s)

Figure 4.7 Deletion of PRE reduces PR-B induced upregulation. MCF-7 cells transfected with pPMTR or pPMTR(AvrII/DraIII-del) cotransfected with either 100ng of pPR-B, or 100ng pPR-B + 20nM P₄. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Statistical Analysis

pPMTR	pPMTR(-AvrII)	<i>p</i> -value
Vehicle + pPR-B	Vehicle + pPR-B	n/s
20nM P ₄ + pPR-B	20nM P ₄ + pPR-B	n/s

Figure 4.8 Deletion of the nucleotides -411 to -415 at the 5' terminal of the PRE reduces PR-B induced upregulation but not significantly. MCF-7 cells transfected with pPMTR or pPMTR(-AvrII) cotransfected with either 100ng of pPR-B, or 100ng pPR-B + 20nM P₄. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

5μg PR-B Ab (pre oligo addition)		✓						✓							✓			
5μg PR-B Ab (post oligo addition)	✓							✓										
³² P-CON PRE	✓	✓	✓	✓														
CON PRE				✓														
³² P-MUT CON PRE					✓	✓												
MUT CON PRE						✓												
³² P-PROS1 PRE								✓	✓	✓	✓							
PROS1 PRE											✓							
³² P-PROS1(-407) PRE													✓	✓				
PROS1(-407) PRE														✓				
³² P-CON AP1																✓	✓	✓
CON AP1																		✓

Figure 4.9 Electromobility Shift Assay (EMSA) confirmation of PR-B based mechanism. The proposed PROS1 PRE was used to probe 1μg of PR-B positive MCF-7 nuclear protein extract. The PROS1 PRE probe formed complexes identical in size to a consensus PRE probe (CON PRE). Introduction of c-407t variant [PROS1 (-407) PRE] diminished complex formation. PR-B binding was disrupted by the addition of 5μg of anti-PR-B prior to the addition of probe, but not after complex formation (post oligo addition). Addition of 5μg of mouse anti-human PR-B did not alter AP1 complex formation when a consensus AP1 (CON AP1) probe was used.

Statistical Analysis

Condition 1	Condition 2	<i>p</i> -value
Vehicle	P ₄	0.001
Vehicle	LNG	0.007
Vehicle	MPA	0.0006
P ₄	LNG	n/s
P ₄	MPA	0.006
LNG	MPA	0.01

Figure 4.10 Progestin isoforms exhibit different levels of transcriptional activation. The effect of 20nM progesterone (P₄); levonorgestrel (LNG); medroxyprogesterone acetate (MPA); on expression profile of pPMTR. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

Figure 4.11 Corticosteroid upregulates the PROS1 promoter. MCF-7 cells transfected with pPMTR with and without 20nM corticosterone. Activity of the PROS1 promoter was measured as a percentage of EGFP expression normalized against constitutive DsRed2 expression over a 24h period.

4.3 DISCUSSION

The primary aim of this chapter was to identify an oestrogen responsive element (ERE) within the PROS1 promoter that governed the oestrogen mediated downregulation. The transient transfection method demonstrated that both ER α alone and in combination with E₂, reduced the activity of the 950bp of the PROS1 promoter contained in the pPMTR vector in both the MCF-7 and HuH-7 cell lines. Initial experiments that assessed the responsiveness of E₂ only (without cotransfection with the ER α expressing vector) using the MCF-7 cell line, also downregulated the PROS1 promoter fragment, but gradually diminished with increasing passage number. The apparent loss of ER α expression has previously been reported, as has the requirement for cotransfection with ER α expression vectors when investigation of other gene promoters using MCF-7s has been examined (Porter *et al.*, 1997, Duan *et al.*, 1998, Sun *et al.*, 1998, Wang *et al.*, 1999, Xie *et al.*, 1999, Qin *et al.*, 1999, Dong *et al.*, 1999, Saville *et al.*, 2000). Similarly, low levels of ER α expression in HuH-7s has been reported (Cheng *et al.*, 2006), explaining the requirement for cotransfection with pER α when performing the transient transfection studies using that cell line.

Nevertheless, the human liver does express high levels of ER α *in vivo* (Pelletier, 2000) and although it has not been conclusively demonstrated, it is postulated that hepatic ER α expression in humans increases in an oestrogen-dependant manner (Pinzone *et al.*, 2004). In humans, the gene that encodes ER α , ESR1, utilises multiple promoters in a tissue specific manner with promoter E the dominant form in the liver (Flouriot *et al.*, 1998, Reid *et al.*, 2002). Greater expression of promoter E ER α mRNA is observed in female liver biopsies compared to male samples (Flouriot *et al.*, 1998). Conversely, levels of both total and free PS are lower in premenopausal women than in men

(Goodwin *et al.*, 2002, Koenen *et al.*, 2005). However, total and free PS levels increase in non-OC using post-menopausal women and may be a result of the corresponding reduction in circulating oestrogen acting to reduce ER α expression in the liver (Goodwin *et al.*, 2002).

The pPMTR transient transfection model demonstrated that the 950bp of the PROS1 promoter is repressed by the ER α and ER α /E₂, but how and where ER α is acting is uncertain. Removing the putative ERE sequence spanning nucleotides -526 to -538 failed to reverse the inhibition, ruling out a 'classical' ER α mechanism. This suggests an alternative ER α pathway is operating via another site within the promoter region. Both the large deletion plasmids, pPMTR(AvrII/DraIII-del) and pPMTR(BclI/AvrII-del) demonstrated inhibition responses similar to the wild type. Therefore the *cis*-acting element is most likely located in the 324bp downstream from the *Dra*III restriction site. This contains the Sp1 elements identified by de Wolf *et al.* (2006). It is possible that ER α is not binding directly to the PROS1 promoter but is instead exerting influence via interaction(s) with Sp1 transcriptional factors. A number of studies have detailed Sp1/ER α regulation of gene promoters; both up- and down-regulation (Safe, 2001). Sp1/ER α transcriptional regulation does not involve direct DNA binding by ER α , but rather ER α bound Sp1 which binds to GC rich Sp1 sequences within the promoter. These GC rich motifs identified in the PROS1 promoter region were confirmed as Sp1 interactive domains that influence transcription to varying degrees (de Wolf *et al.*, 2006a). To confirm that ER α is regulating expression via the Sp1 motifs, further investigations are required. Castro-Rivera *et al.* (2001) described an Sp1/ER α mechanism that mediated E₂-responsiveness of the Cyclin D1 promoter, by performing an EMSA and a Chromatin-Immunoprecipitation (ChIP) assay. The EMSA used ER-positive cell extracts probed with consensus, mutated consensus and Cyclin D1

promoter derived Sp1 ³²P-labelled probes, further demonstrating a supershift when an anti-Sp1 antibody was added. These results were confirmed by a ChIP assay demonstrating that both anti-Sp1 and anti-ER α antibodies were able to immunoprecipitate the proposed Cyclin D promoter Sp1 elements, post E₂-induction.

For the first time, this study demonstrates that the PROS1 gene is upregulated by progesterone via a mechanism that involves binding of ligand activated PR-B to a region surrounding position -407 within the promoter. This represents a counterbalance to the effect of oestrogen and ER α which is important in both pregnancy and COC use settings.

The initial observation that progesterone upregulated expression of the PROS1 promoter initiated the investigation into a progestin based mechanism. The response to progesterone was stronger in the MCF-7 breast cell carcinoma line than it was in the HuH-7 liver carcinoma cell line. This may reflect differences in the PR expression between the two lines.

Neutralizing the progestin response using the progesterone receptor modulator, RU486, suggested that one of the two PR isoforms was involved and was subsequently shown to be PR-B. Identification of the PR-B binding motif originated with the investigation of the functional effects of the c-407t variant, one of the two promoter region variants described in Chapter 3. The previously described variants t-397c and c-407t were detected separately in two patients with pregnancy induced high levels of oestrogen and progesterone who also had greater than normal decreases in their total (tPS), free (frPS) and functional (fuPS) PS levels at 32 weeks gestation compared to levels taken six months post partum (patient 1- 39%, 45% and 62%; patient 2- 64%, 52% and 51%; control pregnancies- 30%, 40% and 41% for tPS, frPS and fuPS, respectively) (data

kindly supplied by Leesa Ivey and Kate Maslen). Therefore, it was hypothesized that the low PS levels were due to the presence of the base change(s), disrupting PR-B binding and reducing the upregulatory effect of progesterone resulting in greater PS reduction in response to the increased oestrogen. Both variants map to a highly probable (87.5%) progesterone responsive motif (Figure 4.1) identified by a recently designed algorithm developed specifically for complex progesterone response elements (Stepanova *et al.*, 2006). The *in vitro* observation that PR-B increased the activity of the pPMTR vector (containing the wildtype PROS1 promoter), but not the pPMTR(-407) vector, strongly suggested that c-407 forms part of a key binding site for the P₄/PR-B complex. The same response was apparent when the experiment was repeated with the pPMTR(AvrII/DraIII-del) plasmid in which most of the PRE is deleted. As with pPMTR(-407), there was increased activity of the PROS1 promoter when cotransfected with the pPR-B plasmid. Interestingly, both plasmids did recover a degree of increased promoter activity when 20nM P₄ was included with the cotransfection, suggesting PROS1 sensitivity to P₄ may be conferred at more than one site. Cotransfection of the pPR-B plasmid with the pPMTR(-AvrII) upregulated expression of the PROS1 promoter marginally suggesting the 5' terminus of the PRE is not required for binding pPR-B.

Surprisingly, the presence of the -407t mutation enhanced the downregulatory effect of E₂ on the PROS1 promoter reflecting the clinical observation of the patient's gestational response. It is well reported that E₂ increases expression of the PR gene in MCF-7 cells and that the PR-B isoform is up-regulated in an ER α dependent manner (Schultz, *et al.*, 2003, Mukherjee *et al.*, 2005, Nardulli *et al.*, 1988, Wei *et al.*, 1988, Read *et al.*, 1988). Thus the introduction of excess ER α could increase PR-B availability in both the pPMTR and pPMTR(-407) transiently transfected cell lines. The promoter containing

the c-407t mutant was unable to respond to the increase of available PR-B as effectively as the wild type and therefore the downregulatory action of ER α and ER α /E₂ was more pronounced.

The EMSA protocol reinforced the initial findings resulting from the expression studies, confirming the proposed PRE as a progesterone-responsive motif, and demonstrating that it was able to bind activated PR-B with a similar affinity to that of the consensus PRE probe. Furthermore, the PROS1(-407) PRE probe that incorporated the t-407c variant exhibited a reduced affinity for PR-B, reflecting the expression study results for the pPMTR(-407) vector.

Identifying the upregulatory effect of progestins on the PROS1 promoter is important for users of oral contraceptives. The demonstration in the present study that the testosterone derived levonorgestrel (LNG), and the progesterone derived medroxyprogesterone acetate (MPA) induce differential upregulation of PROS1 promoter transcriptional activity (MPA significantly greater than P₄) indicates the type of progestin prescribed is important. Clinical data suggests that 3rd generation COCs are associated with significantly lower levels of free PS when compared to data for 2nd generation COCs (Kemmeren *et al.*, 2004, Oral Contraceptive and Hemostasis Study Group, 2003). Both desogestrel (DSG) and gestodene (GSD) are still protected by patents and as such are not available for purchase. When GSD becomes available in the future (DSG is a prodrug and would not be suitable for cell culture) an interesting follow up experiment would be to compare the affect on PROS1 promoter expression with other progestins in isolation (i.e. without the presence of EE).

Progestin	PR	ER	GR	MR
Progesterone	50	0	10	100
Medroxyprogesterone acetate	115	0	29	160
Levonorgestrel	150	0	1	75

Table 4.1 Relative binding affinities of three progestin isoforms for hormone receptors. The relative binding affinities of progesterone, medroxyprogesterone acetate and levonorgestrel for progesterone receptor (PR; promegestone, 100%), oestrogen receptor (ER; 17 β -oestradiol, 100%), glucocorticoid receptor (GR; dexamethasone, 100%) and mineralcorticoid receptor (MR; aldosterone, 100%). Adapted from (Kuhl, 2005)

The PROS1 promoter was also upregulated by the glucocorticoid corticosterone. This is of particular interest and suggests one of several possibilities. As discussed in Chapter 1, the PRs form part of a superfamily of nuclear receptors that includes the glucocorticoid receptor (GR) and mineralcorticoid receptors (MR). Table 4.1 details the binding affinities of P₄, MPA and LNG for PR, GR, MR and ER, highlighting the variability between both different progestins and receptors. The same is also true for glucocorticoid that can cross bind with PR and activate progesterone inducible genes and may explain the corticosterone effect (Leo *et al.*, 2004).

The upregulation could also be operating via a direct GR based mechanism. GR is highly expressed in the human liver where it regulates a wide variety of genes (Rabbitt *et al.*, 2002). A secondary mechanism such as this could explain why both the pPMTR(-407) and pPMTR(AvrII/DraIII-del) vectors demonstrated increased PROS1 promoter activity when additional P₄ was added to the pPR-B cotransfection experiments. Table 4.1 details the binding affinity of P₄ for GR, showing a low but significant affinity.

Therefore the excess P₄ may be driving a upregulatory responsive GRE located in the PROS1 promoter.

A large component of the work presented in this chapter are results derived from *in vitro* expression studies using the pPMTR vector or variations of the pPMTR vector. A criticism that may be levelled at this system is the relatively low changes in expression observed post treatment when compared to changes observed for other hormonally responsive promoter studies for example, the 17-fold change found in the investigations of the F9 gene due to the ARE disrupting point mutation (Brady *et al.*, 1998). However, the PROS1 promoter is known to be a low activity promoter (personal correspondence with Prof. David Lane, Hammersmith Hospital, London), a phenomenon that may actually reflect translational rather than transcriptional output. The PROS1 promoter does not have a consensus Kozak sequence lacks most of the AUG flanking nucleotides required for strong ribosomal initiation (Kozak, 1987). Therefore, the low levels of hormone responsiveness may reflect the overall low level of PS production.

In summary, work detailed in this chapter demonstrates that transcription of the PROS1 gene is influenced by the presence of both oestrogen and progesterone, which downregulate and upregulate transcription, respectively. The oestrogen response is mediated via ER α , while the progesterone response requires the PR-B isoform. In addition, a region within the promoter has been identified that is involved in the binding of ligand bound PR-B receptor. Work will continue towards elucidating the ER α based mechanism. These findings provide an explanation for the development of acquired PS deficiency during pregnancy and in users of COCs, in particular, highlighting the importance of progesterone and synthetic progestins in both pregnancy and COC users, respectively.

Future studies will focus on isolating the region(s) of the PROS1 promoter that direct the ER α mediated down-regulation by continuing with deletion studies and by performing the EMSA and ChIP assays discussed earlier in this chapter, based on the experimentals conducted by Castro-Rivera *et al.* (2001).

CHAPTER 5

Identification of Novel Protein S Binding Partners

5.1 INTRODUCTION

The interaction between the complement protein C4b binding protein (C4b-BP) and PS has been well described (Dahlback and Stenflo, 1981, Walker, 1989, Dahlback *et al.*, 1990a, Dahlback, 1991, Nelson and Long, 1992, Fernandez *et al.*, 1993, Fernandez and Griffin, 1994, Hardig and Dahlback, 1996, Fernandez *et al.*, 1998). The high affinity binding relationship between the two molecules regulates levels of circulating free PS, which is the only form able to act as cofactor to activated protein C. It has been suggested that acquired PS deficiency may be a function of increased circulating levels of C4b-BP although it has not been conclusively demonstrated (Esparza-Gordillo *et al.*, 2003). Nevertheless, it represents a possible mechanism that could limit the availability of free form PS.

Other than PS interaction with aPC, no other protein's have been directly associated with PS. The possibility of a novel protein/protein interaction involving PS may form the basis of potential hormone regulated mechanism. That is, if the concentration of a PS binding partner increased in a hormonal dependant manner, then a reduction in the availability of free PS would result. An example of this form of endocrine regulation can be seen in the relationship between insulin-like growth factor 1 (IGF-1) and its carrier IGF binding protein 1 (IGFBP1), referred to as the IGF-1/IGFBP1 axis. IGFBP1 is part of a larger family of IGF transporter proteins that are expressed by the liver (Rajaram *et al.*, 1997). IGFBP1 inhibits IGF-1 activity by reducing availability for IGF receptor binding (Pierce *et al.*, 2006). Rapid changes in IGFBP1 expression occur in response to glucocorticoid and insulin levels that upregulate and downregulate transcription respectively, via interactions with response elements located in the promoter region of the IGFBP1 gene (Goswami *et al.*, 1994, Suwanichkul *et al.*, 1994). Growth Hormone (GH) also represses IGFBP1 expression in a yet to be determined

mechanism (Lu *et al.*, 1999). Interestingly, levels of IGFBP1 are significantly reduced in postmenopausal women using oestrogen only forms of HRT. The reduction is attenuated by progestins that exhibit strong androgenic properties such as levonorgestrel (Heald *et al.*, 2000, Campagnoli *et al.*, 2003, Heald *et al.*, 2005).

Therefore, the aim of this study is to investigate whether a similar mechanism to that of the IGF-1/IGFBP1 axis exists for PS, by determining if a novel PS binding protein exists that is itself hormonally regulated. To do this, a method successfully employed by previous investigators, the Yeast-2-Hybrid (Y-2-H) System will be used.

5.2 THE YEAST-2-HYBRID SYSTEM

The Y-2-H System was designed in 1980's as a technique to identify novel protein-protein interactors when the unique properties of the yeast GAL4 transcriptional activator were recognised (Fields and Song, 1989). Fields and Song exploited the independent properties of the two key functional domains of the GAL4 protein (a *Saccharomyces cerevisiae* transcriptional activator), the N-terminal DNA binding domain (BD) and the C-terminal activation domain (AD). The BD interacts with a highly specific upstream activating sequence (UAS), while the AD binds to RNA polymerase. When separated, the two domains lack function but are still able to activate transcriptional activity if they physically, but not necessarily covalently, interact (Ma and Ptashne, 1988) (Figure 5.1). Therefore, if two proteins are interactors, forming fusion proteins comprised of the AD with protein X and the BD with protein Z creates a bridge between the AD and BD, facilitating transcription (Figure 5.2B). If the proteins do not interact (proteins X and Y; Figure 5.2A) then the reporter gene will not be transcribed. In the Y-2-H system, specific reporter genes are used to select for positive interactors. The fusion proteins are produced by inserting the cDNA that encodes a

protein of interest into a vector containing the coding sequence for the BD (the 'bait'). Successful expression will yield the required chimeric protein. The 'fish' chimeras are derived from cDNA libraries constructed using vectors containing the coding sequence for the AD.

Figure 5.1 Schematic illustration of the principle underlying the mechanism of the Y-2-H system. Fields and Song (1989) discovered that the *Saccharomyces cerevisiae* GAL4 protein consists of two distinct domains, an N-terminal DNA binding domain (BD) and a C-terminal activation domain (AD). The BD demonstrates a high affinity for an upstream activation sequence (UAS) *cis*-element, while the AD interacts with RNA polymerase (RNA Pol). The two domains retain functionality, initiating transcription when they physically, but not necessarily covalently, interact.

Figure 5.2 Schematic illustrating how the Y-2-H system identifies protein-protein interactors via the creation of fusion proteins. Fusion proteins, comprising of the 'bait' (or known protein) amalgamated with the binding domain (BD) moiety and the 'fish' protein (derived from cDNA libraries for initial large scale screens) fused to the activation domain (AD) factor, will fail to activate transcription if the bait and fish are not interactors (A). However, if they do interact, that will bring the BD and AD together, facilitating activation of RNA polymerase (RNA Pol), leading to transcription of the reporter genes, *HIS3* and *LacZ* (B).

5.2.1 LIMITATIONS OF THE YEAST TWO HYBRID SCREEN

The Y-2-H system is an elegant methodology, however it does have limitations. Generation of the fusion proteins that exploit the AD and BD interaction can result in aberrant folding of the protein of interest that could destroy key binding sites. The fusion proteins may also be toxic, slowing the growth of, or killing the host cell (Van Crielinge and Beyaert). Proteins, that in their native form require post-translational modifications to initiate binding, such as phosphorylation or glycosylation, may not interact with designated binding partners (Fields and Sternglanz, 1994). The major criticism of the Y-2-H system is the high number of false positives that can be identified, such as mitochondrial proteins, heat shock proteins, collagen related proteins and zinc finger proteins. However, comprehensive lists are available online that can be used to reference findings after a screen has been completed to eliminate them from follow up investigation (Van Crielinge and Beyaert).

5.2.2 MATCHMAKER YEAST-2-HYBRID SYSTEM

The MatchMaker™ Y-2-H system used in this study is a dual reporter, LexA-GAL4 based process (Brent and Ptashne, 1985) which utilises a LexA BD (an *E-coli* repressor protein) and GAL4 AD to drive transcription of two reporter genes. The first, *LacZ*, encodes β -galactosidase enabling the identification of positive colonies by using X-galactose (X-gal; 5-bromo-4-chloro-3-indolyl- β -D-galactoside) which is cleaved by β -galactosidase producing a blue by-product. The second, *HIS3*, enables the yeast host to manufacture endogenous histidine required for survival. Using the L40 yeast strain (genotype *his3 Δ 200 trp1-901 leu2-3,112 ade2 LYS2<(4lexAop-HIS3) URA3<(8lexAop-LacZ) GAL4*) increases the overall stringency of the method by using bait and fish plasmids that encode for the *TRP1* and *LEU2* genes, respectively. This allows for triple

knock-out growth selection by using media lacking histidine, tryptophan and leucine (the *TRP1* and *LEU2* gene products allow the yeast to produce endogenous tryptophan and leucine).

To search for potential protein interactors with PS, the Y-2-H screen was performed using expression clones containing cDNA derived from liver and bone marrow sources. As PS is produced in both of these tissues, it was determined that these libraries would offer the best chance for identifying novel PS interactors.

5.3 RESULTS

The initial large scale screen generated over 100 colonies that were able to grow on Yc agar (-His,Trp,Leu). Forty of these were subsequently assessed using the β -gal assay and for growth in the presence of 10 and 20mM of the histidine competitive inhibitor 3-amino-1,2,4-triazole (3AT) which left 6 viable colonies (Figure 5.3). Sequencing of each of these 6 colonies identified the coding regions of a mitochondrial isolate, two defensin isolates, a cytoskeletal talin protein and an α -1-antichymotrypsin isolate that was detected previously in a separate study performed in this laboratory. The remaining sequence matched a previously reported sequence for the anticoagulant Protein Z, PROZ gene (Table 5.1). The PROZ fragment mapped to the 5' terminal of the PROZ gene comprising 102bp of promoter sequence and 25bp of coding sequence that included the ATG start codon. (Figure 5.4).

Colony No.	Finding	Sequence Length	Probable False Positive	Possible Interactor
#2	fragment of PROZ gene	127bp	-	✓
#112	α -1-antichymotrypsin	1569bp	-	✓
#110	defensin	359bp	-	✓
#111	defensin	664bp	-	✓
#12	talin 1	799bp	?	?
#19	mitochondrial isolate	519bp	✓	-

Table 5.1- Sequencing results of positives obtained from large scale Yeast-2-Hybrid Screen. Sequences obtained from plasmid preparations of positive interactors included, a fragment that corresponded to the protein Z (PROZ) and an α -1-antichymotrypsin and two defensin gene isolates. The talin-1 was also identified and could be an interactor, however it is structural membrane protein which is unlikely to be exposed to the extracellular environment. The mitochondrial isolate is likely to be a false positive.

Figure 5.3- Colony screening of positives obtained from large scale Yeast-2-Hybrid Screen. Selected colonies from the large scale screen of the liver and bone marrow cDNA libraries that utilised the pBTM116/PS vector as bait, assessed for; A) growth on 10 and 20mM 3-amino-1,2,4-triazole (3AT) and ; B) β -galactosidase (β -Gal) activity.

The small scale screen was followed up by cloning the entire PROZ cDNA into the pGADT7 vector. The decision to follow up PZ rather than any of the other isolates was based on similarities with PS as both play roles in downregulating the coagulation cascade. This was used in a cotransformation procedure with the PS/pBTM116 vector to ascertain if the full length PZ/AD fusion protein was able to interact with the PS/BD protein to drive transcription of the HIS3 and LacZ reporter genes. In excess of 200 colonies grew on Yc agar (-His,Trp,Leu) plates (Figure 5.5A) and 100 were then isolated by transferring them onto a grid Yc agar (-His,Try,Leu) plate (Figure 5.5B). Of these 100 colonies, 40 of these were able to grow in the presence of 10 and 20nM 3AT supplemented into Yc agar (-His, Trp, Leu) plates and 6 of these were subsequently shown to be β -Gal positive (results not shown).

PROZ Sequence	acacagctgcacaggtcgtgccccgaggctcctgctgccggcacctccccagc
PROZ Sequence	ctggactttggctgtgtttgtagccctgtcccagcactccgggtggga ^{M A} <u>atggca</u>
	^{G C V P L L -}
PROZ Sequence	<u>ggctgcgtcccactgctcc</u>

Figure 5.4- The PROZ sequence identified in the large scale Y-2-H screen. The PROZ sequence was detected using the full length PROS1 cDNA bait (PS/pBTM116) in a large scale screen of the bone marrow and liver cDNA libraries.. Note- Bold denotes mRNA.

A **B**

Figure 5.5 Results of small scale Yeast-2-Hybrid screen. The small scale screen using the PZ/pGADT7 and PS/pBTM116 vectors generated dozens of colonies that were able to grow on Yc agar (-His, Trp, Leu) plates, A); and continued to grow after re-plating, B).

5.4 DISCUSSION

The results of the initial Y-2-H liver and bone marrow cDNA library screen using the PScDNA containing pBTM116 vector identified 6 clones of interest. DNA sequencing identified these clones as; the anticoagulant PZ (or a PZ-related protein), another plasma derived protein, α -1-antichymotrypsin; a cytoskeletal protein, talin 1; inflammatory response defensin proteins (2) and a mitochondrial isolate. All six isolates demonstrated growth in the presence of 20nM 3AT supplemented into Yc agar (-His, Trp, Leu) plates and exhibited β -Gal activity, suggesting that the protein-protein interactions were strong. Alignment of the six sequences identified found no homology between them, including the two defensin isolates (see Appendix 7)

Of the six findings, the mitochondrial isolate stands out as a definite false positive. Mitochondrial proteins are routinely isolated in many Y-2-H screens and listed as known false positives (Van Crielinge and Beyaert). Talin-1 is a structural protein that connects intracellular actin fibres with membrane bound molecules (Senetar and McCann, 2005). Therefore the talin-1 discovery may also represent another false positive, although it is interesting to note that one study did demonstrate talin-1 androgen responsiveness in a prostatic stromal cell line (Betts *et al.*, 1998).

The two defensin isolates are of interest as they are inflammatory mediated peptides that are predominantly produced by neutrophils (De Smet and Contreras, 2005). The role of PS in the inflammatory response was discussed in Chapter 1. In addition, a previous report has demonstrated enhanced binding of C4b-BP bound PS to the surface of neutrophils in the presence of calcium (Furmaniak-Kazmierczak *et al.*, 1993). An

interaction between PS and defensins may exist to anchor the antimicrobial defensins to the neutrophils, localising at site of infection and inflammation. There is no published data detailing regulation of defensin expression by hormones other than glucocorticoids (Chaly *et al.*, 2000).

Work performed previously in this laboratory also identified an α -1-antichymotrypsin (α 1ACT) protein-protein interaction, in that case, with C-reactive protein (Sarich, 2002). Like PS, α 1ACT is involved in acute phase inflammatory responses, with plasma levels increasing 4.5 fold in response to inflammation and, is also synthesized in the liver (Kalsheker *et al.*, 2002). α 1ACT is a member of the large serine protease inhibitor family, that includes antithrombin and plasminogen activator inhibitor-1, which are mediators of complement activation and blood coagulation (van Gent *et al.*, 2003). Interestingly α 1ACT, like the defensins, also relates to neutrophils. The primary role of α 1ACT is as an inhibitor of the proteinase Cathepsin G which is released by stimulated neutrophils (Owen *et al.*, 1995). Cathepsin G is a potent activator of platelets and promotes the generation of thrombin via prothrombinase activation and fibrin monomer release (Goel and Diamond, 2003, Sambrano *et al.*, 2000). Cathepsin G lacks any activity when bound to circulating α 1ACT (Kalsheker *et al.*, 2002). Of particular interest is the finding that α 1ACT mRNA expression was induced by E_2 , when studied using the breast carcinoma cell line, MCF-7 (Yamamura *et al.*, 2004). No data showing increased α 1ACT protein levels in response to E_2 has been published. However, if E_2 up-regulation of α 1ACT protein levels does occur and α 1ACT is a true interactor with PS, then a possible oestrogenic mechanism could be proposed and warrants further investigation.

The PZ isolate was of particular interest. The positive PZ fragment was not a full length PZ cDNA but rather a fragment that corresponded to sequence of the promoter region and the 5' terminal of exon 1. Using the pGADT7 expression vector to generate a full length PZ chimera protein resulted in growth on the selective Yc agar plates (-His,Trp,Leu/+3AT) when the full length PZ was cotransformed with the full length PScDNA/pBTM116 vector suggesting a strong interaction between the two species. Unfortunately, further confirmation experiments could not be carried out due to the inability to generate liquid cultures required for plasmid extraction and subsequent sequence analysis. Several reports discuss the phenomenon of protein toxicity whereby growth of the host yeast strain is inhibited or terminated by the overexpression of the introduced foreign protein (Van Crielinge and Beyaert, 1999). If this was the case with the full length PZ then it may explain why only the N-terminal PZ fragment was identified in the original liver and bone marrow cDNA library screen.

There are many similarities between PS and PZ that further add to the interest of an *in vivo* relationship. Both are anticoagulant proteins that lack proteolytic activity, acting instead as cofactors; PS as cofactor to aPC and PZ a cofactor to protein Z-dependant inhibitor (ZPI). ZPI inhibits aFX activity in a process that is increase 1000-fold via the interaction of PZ (Han *et al.*, 2000). However, unlike the well characterised link between low PS levels and increased thrombotic risk, the effect of low PZ in isolation, does not appear to be a risk factor for venous thrombosis (Santacroce *et al.*, 2006, Martinelli *et al.*, 2005, Al-Shanqeeti *et al.*, 2005). A possible link to stroke has been suggested but is still heavily debated (Sofi *et al.*, 2005, McQuillan *et al.*, 2003, Heeb *et al.*, 2002, Vasse *et al.*, 2001).

The PS/PZ interaction is unlikely to be a hormonally responsive regulatory factor governing changes in the levels of PS seen in COC users and pregnant women. PZ

levels are relatively consistent between the sexes, unlike PS which is significantly higher in males compared to females, and suggests that PZ expression is not influenced by hormonal status (Quack Loetscher *et al.*, 2005). Interestingly, in direct contrast to PS, levels of PZ significantly increase throughout gestation and in users of COCs (Al-Shanqeeti *et al.*, 2005). In both cases, the increased concentration of PZ may be a result of the oestrogen related reduction in PS levels, altering the stoichiometric ratio between the binding partners.

5.4.1 FOLLOW UP STUDIES REQUIRED TO CONFIRM THE PS/PZ INTERACTION

Future work to confirm an interaction between PS and PZ will involve either using a GST Pull-Down assay or a co-immunoprecipitation technique. GST Pull-Down assays involve 'tagging' one of either of the proteins of interest (6xHIS-, GST, biotin) providing a means for teathering the protein to an affinity matrix (eg- glutathione). The suspected binding protein is then added followed by electrophoretic analysis SDS PAGE using silver staining, western blotting or radiographic techniques to visualise the interaction. Co-immunoprecipitation is similar, but instead of immobilising one of the proteins, monoclonal or polyclonal antibodies directed against one of the two proteins are used to pull out the protein of interest and its binding protein, which is subsequently captured using Agarose A or G beads.

These techniques should also be applied to investigate the possible PS/ α 1ACT interaction if a small scale study using full length PS cDNA as a bait and α 1ACT cDNA as the fish is positive.

CHAPTER 6

Concluding Remarks and Future Directions

The central aim of this thesis centred on identifying a mechanism to explain why PS levels decrease in response to raised oestrogen concentrations. Several strategies were applied to determine the exact molecular process involved, including analysis of the PROS1 gene and its promoter in addition to the PS molecule itself. Investigation of the PROS1 promoter region uncovered a possible regulatory link between transcriptional regulation and oestrogen via a process involving ER α . Although the exact mechanism is unclear the preliminary work done in this thesis provides a starting point for future experimental work detailed in Chapter 4.

However, it is the identification of progesterone as an activator of PROS1 transcription that possibly represents the greater finding, providing an important counterbalance to the downregulatory effect of oestrogen, especially in the context of oral contraceptive use. As detailed in the first chapter, combined oral contraceptive formulations comprise both an oestrogen and a progestin component. The oestrogen concentration may vary but in close to 100% of preparations is the synthetic ethinyl oestradiol (a small number contain mestranol, a prodrug that is converted to ethinyl oestradiol). As detailed in Chapter 1, the progestin component can be one of dozens of different progestin isoforms derived from parent molecules such as testosterone, progesterone and more recently spironolactone (drospirenone). It is well reported that these progesterone homologues each have separate and distinct biological profiles. Data included in this thesis reinforces this clearly, finding that the progesterone derived medroxyprogesterone significantly increased PROS1 promoter activity when compared to the parent molecule.

Therefore, it is the transcriptional activation by progestins that stands as the most significant finding of this body of work, providing an explanation for the observation that users of 3rd generation oral contraceptives have lower circulating levels of total and

particularly free PS (Kemmeren *et al.*, 2004). Interestingly, progestin-only formulations (progestin only pill; POP) have been shown to increase the concentration of total and free PS (Kemmeren *et al.*, 2004). Winkler *et al.* (1998) found that users of POPs containing the 3rd Generation progestin, desogestrel had greater PS increases compared to users of levonorgestrel containing POPs. However, the desogestrel concentration assessed was 2.5 stronger than that of levonorgestrel. Kemmeren *et al.* (2004) addressed this variance and assessed both progestins side-by-side in POP formulations and found the levonorgestrel containing form to be the more potent.

Currently, work on a new 'hormone-free' form of OC is well underway. These latest OCs will use progesterone receptor modulators (PRMs) such as RU486 (Chabbert-Buffet *et al.*, 2005, Baird *et al.*, 2003). This thesis demonstrates a direct involvement of PR-B in the up-regulation of the PROS1 promoter, a mechanism that is blocked by RU486. Thus, the long term use of PRMs may predispose some individuals to an increased thrombotic risk, resulting from reduced PS production.

Furthermore, identification of the *cis*-element within the promoter that binds PR-B (nucleotides -397 to -417), initiating the progestin response, could explain other endocrine related PS observations. As previously discussed, unlike the oestrogen receptors, progesterone receptors share homology with other hormone receptors including mineralcorticoid, glucocorticoid and androgen receptors that interact with similar *cis*-elements in the promoter regions of genes. Data presented in Chapter 4 demonstrates the responsiveness of the PROS1 promoter to corticosterone and by extrapolation, could explain the clear differences between the PS levels of males and females. If the *cis*-element is capable of responding to ligand bound androgen receptor then the higher levels of testosterone in males could be generating greater expression of PS.

There are several other interesting observations that can be made in regards to the finding that the PROS1 promoter is responsive to the glucocorticoid corticosterone. For example it is known that PS levels increase with age (Goodwin *et al.*, 2002), particularly in women post-menopause (Henkens *et al.*, 1995), a time when cortisol levels increase (Woods *et al.*, 2006). Although the obvious reduction in oestrogen in postmenopausal women is probably the overriding hormonal change altering PS levels.

Finally, as discussed in Chapter 5, the identification of PZ as a possible PS binding partner may explain the previously described increase in PZ levels observed during pregnancy and although levels of PZ have been reported to increase in users of oral contraceptives, there is no comparative 2nd generation/3rd generation OC data currently available.

Therefore, the work presented in this thesis describes a new interaction between the haematological and endocrine systems. However, it also details several results that will form the basis for some fascinating work to be continued in future studies. The final figure (Figure 6.1) summarises the findings of this body of work and the aspects of PS production that require further investigation.

Figure 6.1 Protein S production. A summary of the work presented in this thesis and its relation to the global picture of PS production. The novel coding region T101A and G340C mutations were detected via CSHA. The CSHA also initiated the identification of the PRE/PR-B based up-regulatory mechanism within the PROS1 promoter region after the detection of the t-397c and c-407t variants. Oestrogenic down-regulation of the PROS1 promoter was also demonstrated but the exact process remains unclear, although it may involve a mechanism incorporating the Sp1 motifs previously identified by de Wolf *et al.* (2006). Both the progestin and oestrogen mechanisms probably require the involvement of additional transcriptional factors (TFs) that still need to be identified. The rate of up-regulation of the PROS1 promoter by progestin's is isoform dependant, explaining the differential effects of 2nd and 3rd generation Combined Oral Contraceptives (COCs) on PS levels. The use of Progestin Only Pill (POPs) formulations, which lack an oestrogen component, are associated with increased PS levels. The identification of protein Z (PZ) as a possible binding partner to PS may explain the relationship in pregnancy between decreased PS and increased PZ levels. Several key questions remained unanswered such as the effects, if any, of oestrogens and progestins on the 3' Untranslated region (3' UTR) of PROS1, mRNA stability, translation and protein stability.

REFERENCES

- Adachi, T. (2005) *Curr Drug Targets*, **6**, 585-92.
- Akkawat, B. and Rojnuckarin, P. (2005) *J Med Assoc Thai*, **88 Suppl 4**, S249-54.
- Aldrighi, J. M., De Campos, L. S., Eluf Gebara, O. C., Petta, C. A. and Bahamondes, L. (2006) *Gynecol Endocrinol*, **22**, 1-4.
- Al-Farra, H. M., Al-Fahoum, S. K. and Tabbaa, M. A. (2005) *Saudi Med J*, **26**, 1930-5.
- Alhenc-Gelas, M., Plu-Bureau, Guillonneau, S., Kirzin, J. M., Aiach, M., Ochat, N. and Scarabin, P. Y. (2004) *J Thromb Haemost.*, **2**, 1594-1600.
- Al-Shanqeeti, A., van Hylckama Vlieg, A., Berntorp, E., Rosendaal, F. R. and Broze, G. J., Jr. (2005) *Thromb Haemost*, **93**, 411-3.
- Anderson, H. A., Maylock, C. A., Williams, J. A., Paweletz, C. P., Shu, H. and Shacter, E. (2003) *Nat.Immunol.*, **4**, 87-91.
- Anderson, H. A. and Shacter, E. (2004) *Curr Pharm Des*, **10**, 929-37.
- Baillargeon, J. P., McClish, D. K., Essah, P. A. and Nestler, J. E. (2005) *J Clin Endocrinol Metab*, **90**, 3863-70.
- Baird, D. T., Brown, A., Cheng, L., Critchley, H. O. D., Lin, S., Narvekar, N. and Williams, A. R. W. (2003) *Steroids*
The 2nd International Symposium on Progestins, Progesterone Receptor Modulators and Progesterone Antagonists, **68**, 1099-1105.
- Balmer, L. A., Beveridge, D. J., Jazayeri, J. A., Thomson, A. M., Walker, C. E. and Leedman, P. J. (2001) *Mol Cell Biol*, **21**, 2070-84.
- Bammann, B. L., Coulam, C. B. and Jiang, N. S. (1980) *Am J Obstet Gynecol*, **137**, 293-8.
- Baroni, M., Mazzola, G., Kaabache, T., Borgel, D., Gandrille, S., Vigano' D'Angelo, S., Marchetti, G., di Iasio, M. G., Pinotti, M., D'Angelo, A. and Bernardi, F. (2006) *J Thromb Haemost*, **4**, 186-91.
- Bartel, P. L. and Fields, S. (1995) *Methods Enzymol*, **254**, 241-63.
- Basurto, L., Saucedo, R., Zarate, A., Martinez, C., Gaminio, E., Reyes, E. and Hernandez, M. (2006) *Gynecol Obstet Invest*, **61**, 61-4.
- Battistelli, S., Vittoria, A., Cappelli, R., Stefanoni, M. and Roviello, F. (2005) *Eur J Surg Oncol*, **31**, 798-802.

- Beato, M., Chavez, S. and Truss, M. (1996) *Steroids*, **61**, 240-51.
- Benzakour, O. and Kanthou, C. (2000) *Blood*, **95**, 2008-14.
- Bertina, R. M., Ploos van Amstel, H. K., van Wijngaarden, A., Coenen, J., Leemhuis, M. P., Deutz-Terlouw, P. P., van, d. L., I and Reitsma, P. H. (1990) *Blood*, **76**, 538-548.
- Betts, A. M., Collett, G. P., Neal, D. E. and Robson, C. N. (1998) *FEBS Lett*, **434**, 66-70.
- Bloemenkamp, K. W. (2005) *Thromb Res*, **115 Suppl 1**, 1-6.
- Borgel, D., Duchemin, J., Alhenc-Gelas, M., Matheron, C., Aiach, M. and Gandrille, S. (1996) *J Lab Clin Med*, **128**, 218-27.
- Borgel, D., Gaussem, P., Garbay, C., Bachelot-Loza, C., Kaabache, T., Liu, W. Q., Brohard-Bohn, B., Le Bonniec, B., Aiach, M. and Gandrille, S. (2001) *Biochem J*, **360**, 499-506.
- Borgfeldt, C., Li, C. and Samsioe, G. (2004) *Climacteric*, **7**, 78-85.
- Brady, J. N., Notley, C., Cameron, C. and Lillicrap, D. (1998) *Br J Haematol*, **101**, 273-9.
- Bremme, K. A. (2003) *Best Pract Res Clin Haematol*, **16**, 153-68.
- Brenner, B. (2004) *Thromb.Res.*, **114**, 409-414.
- Brent, R. and Ptashne, M. (1985) *Cell*, **43**, 729-36.
- Brown, M. A., Hague, W. M., Higgins, J., Lowe, S., McCowan, L., Oats, J., Peek, M. J., Rowan, J. A. and Walters, B. N. (2000) *Aust N Z J Obstet Gynaecol*, **40**, 133-8.
- Brummel, K. E., Paradis, S. G., Butenas, S. and Mann, K. G. (2002) *Blood*, **100**, 148-52.
- Campagnoli, C., Abba, C., Ambroggio, S. and Peris, C. (2003) *Maturitas*, **46 Suppl 1**, S39-44.
- Casey, M. L., MacDonald, P. C. and Andersson, S. (1994) *J Clin Invest*, **94**, 2135-41.
- Castro-Rivera, E., Samudio, I. and Safe, S. (2001) *J Biol Chem*, **276**, 30853-61.
- Ceriello, A., Giugliano, D., Quatraro, A., Marchi, E., Barbanti, M. and Lefebvre, P. (1990) *Diabetes*, **39**, 447-9.

- Cerneca, F., Ricci, G., Simeone, R., Malisano, M., Alberico, S. and Guaschino, S. (1997) *Eur J Obstet Gynecol Reprod Biol*, **73**, 31-6.
- Chabbert-Buffet, N., Meduri, G., Bouchard, P. and Spitz, I. M. (2005) *Hum Reprod Update*, **11**, 293-307.
- Chaly, Y. V., Paleolog, E. M., Kolesnikova, T. S., Tikhonov, II, Petratchenko, E. V. and Voitenok, N. N. (2000) *Eur Cytokine Netw*, **11**, 257-66.
- Cheng, X., Shimizu, I., Yuan, Y., Wei, M., Shen, M., Huang, H., Urata, M., Sannomiya, K., Fukuno, H., Hashimoto-Tamaoki, T. and Ito, S. (2006) *Life Sci*.
- Chou, L. S., Lyon, E. and Wittwer, C. T. (2005) *Am J Clin Pathol*, **124**, 330-8.
- Choy, Y. S., Dabora, S. L., Hall, F., Ramesh, V., Niida, Y., Franz, D., Kasprzyk-Obara, J., Reeve, M. P. and Kwiatkowski, D. J. (1999) *Ann Hum Genet*, **63**, 383-91.
- Clark, P., Brennand, J., Conkie, J. A., McCall, F., Greer, I. A. and Walker, I. D. (1998) *Thromb Haemost*, **79**, 1166-70.
- Cohen, A. T., Agnelli, G., Anderson, F. A., Arcelus, J. I., Bergqvist, D., Brecht, J. G., Greer, I. A., Heit, J. A., Hutchinson, J. L., Kakkar, A. K., Mottier, D., Oger, E., Samama, M. M. and Spannagl, M. (2007) *Thromb Haemost*, **98**, 756-764.
- Collins, P., Flather, M., Lees, B., Mister, R., Proudler, A. J. and Stevenson, J. C. (2006) *Eur Heart J*, **27**, 2046-53.
- Colosimo, A., Guida, V., Scolari, A., De Luca, A., Palka, G., Rigoli, L., Meo, A., Salpietro, D. C. and Dallapiccola, B. (2003) *Genet Test*, **7**, 269-75.
- Comp, P. C., Thurnau, G. R., Welsh, J. and Esmon, C. T. (1986) *Blood*, **68**, 881-885.
- Cordera, F. and Jordan, V. C. (2006) *Semin Oncol*, **33**, 631-41.
- Cosman, F., Baz-Hecht, M., Cushman, M., Vardy, M. D., Cruz, J. D., Nieves, J. W., Zion, M. and Lindsay, R. (2005) *Thromb Res*, **116**, 1-13.
- Cushman, M. (2007) *Semin Hematol*, **44**, 62-9.
- Dahlback, B. (1991) *Thromb Haemost.*, **66**, 49-61.
- Dahlback, B., Frohm, B. and Nelsestuen, G. (1990a) *J Biol Chem*, **265**, 16082-16087.
- Dahlback, B., Hildebrand, B. and Malm, J. (1990b) *J Biol Chem*, **265**, 8127-8135.
- Dahlback, B. and Stenflo, J. (1981) *Proc Natl Acad Sci U S A*, **78**, 2512-6.

- Dahlback, B., Wiedemer, T. and Sims, P. J. (1992) *Biochemistry*, **31**, 12769-12777.
- De Smet, K. and Contreras, R. (2005) *Biotechnol Lett*, **27**, 1337-47.
- de Wolf, C. J., Cupers, R. M., Bertina, R. M. and Vos, H. L. (2005) *J Thromb Haemost.*, **3**, 410-412.
- de Wolf, C. J., Cupers, R. M., Bertina, R. M. and Vos, H. L. (2006a) *J Biol Chem*, **281**, 17635-43.
- de Wolf, C. J., Cupers, R. M., Bertina, R. M. and Vos, H. L. (2006b) *Arterioscler Thromb Vasc Biol*, **26**, 2168-74.
- Deguchi, H., Bouma, B. N., Middeldorp, S., Lee, Y. M. and Griffin, J. H. (2005) *J Thromb Haemost*, **3**, 935-8.
- DeMayo, F. J., Zhao, B., Takamoto, N. and Tsai, S. Y. (2002) *Ann N Y Acad Sci*, **955**, 48-59; discussion 86-8, 396-406.
- Denis, C. V., Roberts, S. J., Hackeng, T. M. and Lenting, P. J. (2005) *Arterioscler Thromb Vasc Biol*, **25**, 2209-15.
- Di Bitondo, R., Hall, A. J., Peake, I. R., Iacoviello, L. and Winship, P. R. (2002) *Hum Mol Genet*, **11**, 723-31.
- Dillmon, M. S., Saag, M. S., Hamza, S. H., Adler, B. K. and Marques, M. B. (2005) *AIDS Res Hum Retroviruses*, **21**, 753-6.
- Donaghue, C., Westley, B. R. and May, F. E. (1999) *Mol Endocrinol*, **13**, 1934-50.
- Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J. C., Harigai, M., Samudio, I., Kladde, M. P., Vyhldal, C. and Safe, S. (1999) *J Biol Chem*, **274**, 32099-107.
- Drakenberg, T., Ghasriani, H., Thulin, E., Thamlitz, A. M., Muranyi, A., Annala, A. and Stenflo, J. (2005) *Biochemistry*, **44**, 8782-9.
- Duan, R., Porter, W. and Safe, S. (1998) *Endocrinology*, **139**, 1981-90.
- Duchemin, J., Gandrille, S., Borgel, D., Feurgard, P., Alhenc-Gelas, M., Matheron, C., Dreyfus, M., Dupuy, E., Juhan-Vague, I. and Aiach, M. (1995) *Blood*, **86**, 3436-3443.
- Dunn, J. F., Nisula, B. C. and Rodbard, D. (1981) *J Clin Endocrinol Metab*, **53**, 58-68.
- Dykes, A. C., Walker, I. D., McMahon, A. D., Islam, S. I. and Tait, R. C. (2001) *Br J Haematol*, **113**, 636-41.

- Eldor, A. (2002) *Eur J Obstet Gynecol Reprod Biol*, **104**, 3-13.
- Enmark, E., Peltö-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M. and Gustafsson, J. A. (1997) *J Clin Endocrinol Metab*, **82**, 4258-65.
- Esmon, C. T. (1987) *Science*, **235**, 1348-52.
- Esmon, N. L., Owen, W. G. and Esmon, C. T. (1982) *J Biol Chem*, **257**, 859-64.
- Esparza-Gordillo, J., Soria, J. M., Buil, A., Souto, J. C., Almasy, L., Blangero, J., Fontcuberta, J. and de Cordoba, S. R. (2003) *Immunogenetics*, **54**, 862-6.
- Espinosa-Parrilla, Y., Morell, M., Borrell, M., Souto, J. C., Fontcuberta, J., Estivill, X. and Sala, N. (2000a) *Hum Mutat*, **15**, 463-73.
- Espinosa-Parrilla, Y., Morell, M., Souto, J. C., Tirado, I., Fontcuberta, J., Estivill, X. and Sala, N. (1999) *Hum Mutat*, **14**, 30-9.
- Espinosa-Parrilla, Y., Yamazaki, T., Sala, N., Dahlback, B. and de Frutos, P. G. (2000b) *Blood*, **95**, 173-9.
- Fair, D. S., Marlar, R. A. and Levin, E. G. (1986) *Blood*, **67**, 1168-1171.
- Farsetti, A., Misiti, S., Citarella, F., Felici, A., Andreoli, M., Fantoni, A., Sacchi, A. and Pontecorvi, A. (1995) *Endocrinology*, **136**, 5076-83.
- Farsetti, A., Narducci, M., Moretti, F., Nanni, S., Mantovani, R., Sacchi, A. and Pontecorvi, A. (2001) *Endocrinology*, **142**, 3380-8.
- Fay, P. J. (2004) *Blood Rev*, **18**, 1-15.
- Fernandez, J. A. and Griffin, J. H. (1994) *J Biol Chem*, **269**, 2535-2540.
- Fernandez, J. A., Griffin, J. H., Chang, G. T., Stam, J., Reitsma, P. H., Bertina, R. M. and Bouma, B. N. (1998) *Blood Cells Mol Dis*, **24**, 101-112.
- Fernandez, J. A., Heeb, M. J. and Griffin, J. H. (1993) *J Biol Chem*, **268**, 16788-16794.
- Fernandez-Valdivia, R., Mukherjee, A., Mulac-Jericevic, B., Conneely, O. M., DeMayo, F. J., Amato, P. and Lydon, J. P. (2005) *Semin Reprod Med*, **23**, 22-37.
- Fields, S. and Song, O. (1989) *Nature*, **340**, 245-6.
- Fields, S. and Sternglanz, R. (1994) *Trends Genet*, **10**, 286-92.

- Flototto, T., Niederacher, D., Hohmann, D., Heimerzheim, T., Dall, P., Djahansouzi, S., Bender, H. G. and Hanstein, B. (2004) *J Steroid Biochem Mol Biol*, **88**, 131-42.
- Flouriou, G., Griffin, C., Kenealy, M., Sonntag-Buck, V. and Gannon, F. (1998) *Mol Endocrinol*, **12**, 1939-54.
- Furie, B. and Furie, B. C. (1992) *N Engl J Med*, **326**, 800-6.
- Furie, B. and Furie, B. C. (2005) *J Clin Invest*, **115**, 3355-62.
- Furmaniak-Kazmierczak, E., Hu, C. Y. and Esmon, C. T. (1993) *Blood*, **81**, 405-11.
- Gandrille, S., Borgel, D., Ireland, H., Lane, D. A., Simmonds, R., Reitsma, P. H., Mannhalter, C., Pabinger, I., Saito, H., Suzuki, K., Formstone, C., Cooper, D. N., Espinosa, Y., Sala, N., Bernardi, F. and Aiach, M. (1997) *Thromb Haemost.*, **77**, 1201-1214.
- Gandrille, S., Borgel, D., Sala, N., Espinosa-Parrilla, Y., Simmonds, R., Rezende, S., Lind, B., Mannhalter, C., Pabinger, I., Reitsma, P. H., Formstone, C., Cooper, D. N., Saito, H., Suzuki, K., Bernardi, F. and Aiach, M. (2000) *Thromb Haemost.*, **84**, 918.
- Gershagen, S., Fernlund, P. and Edenbrandt, C. M. (1991) *J Steroid Biochem Mol Biol*, **40**, 763-9.
- Gershagen, S., Fernlund, P. and Lundwall, A. (1987) *FEBS Lett*, **220**, 129-135.
- Gietz, D., St Jean, A., Woods, R. A. and Schiestl, R. H. (1992) *Nucleic Acids Res*, **20**, 1425.
- Gietz, R. D., Schiestl, R. H., Willems, A. R. and Woods, R. A. (1995) *Yeast*, **11**, 355-60.
- Giri, T. K., Garcia, d. F. and Dahlback, B. (2000) *Thromb Haemost.*, **84**, 413-419.
- Goel, M. S. and Diamond, S. L. (2002) *Blood*, **100**, 3797-803.
- Goel, M. S. and Diamond, S. L. (2003) *J Biol Chem*, **278**, 9458-63.
- Goodwin, A. J., Rosendaal, F. R., Kottke-Marchant, K. and Bovill, E. G. (2002) *Arch Pathol Lab Med*, **126**, 1349-66.
- Goswami, R., Lacson, R., Yang, E., Sam, R. and Unterman, T. (1994) *Endocrinology*, **134**, 736-43.
- Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M. and Chambon, P. (1986) *J Steroid Biochem*, **24**, 77-83.

- Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. and Shine, J. (1986) *Science*, **231**, 1150-4.
- Gross, E., Arnold, N., Goette, J., Schwarz-Boeger, U. and Kiechle, M. (1999) *Hum Genet*, **105**, 72-8.
- Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M. and Milgrom, E. (1989) *Cell*, **57**, 1147-54.
- Hackeng, T. M., Hessing, M., van 't Veer, C., Meijer-Huizinga, F., Meijers, J. C., de Groot, P. G., van Mourik, J. A. and Bouma, B. N. (1993) *J Biol Chem*, **268**, 3993-4000.
- Hackeng, T. M., Yegneswaran, S., Johnson, A. E. and Griffin, J. H. (2000) *Biochem J*, **349 Pt 3**, 757-764.
- Hall, A. J., Peake, I. R. and Winship, P. R. (2006) *Br J Haematol*, **135**, 538-46.
- Han, X., Fiehler, R. and Broze, G. J., Jr. (2000) *Blood*, **96**, 3049-55.
- Hardig, Y. and Dahlback, B. (1996) *J Biol Chem*, **271**, 20861-20867.
- Harris, K. W. and Esmon, C. T. (1985) *J Biol Chem*, **260**, 2007-10.
- Hayashi, K. and Yandell, D. W. (1993) *Hum Mutat*, **2**, 338-46.
- He, X., Shen, L., Villoutreix, B. O. and Dahlback, B. (1998) *J Biol Chem*, **273**, 27449-27458.
- Heald, A., Kaushal, K., Anderson, S., Redpath, M., Durrington, P. N., Selby, P. L. and Gibson, M. J. (2005) *Gynecol Endocrinol*, **20**, 176-82.
- Heald, A., Selby, P. L., White, A. and Gibson, J. M. (2000) *Am J Obstet Gynecol*, **183**, 593-600.
- Heeb, M. J. and Griffin, J. H. (2002) *Blood Cells Mol Dis*, **29**, 190-9.
- Heeb, M. J., Koenen, R. R., Fernandez, J. A. and Hackeng, T. M. (2004) *J Thromb.Haemost.*, **2**, 1766-1773.
- Heeb, M. J., Paganini-Hill, A., Griffin, J. H. and Fisher, M. (2002) *Blood Cells Mol Dis*, **29**, 139-44.
- Heit, J. A., Ketterling, R. P., Zapata, R. E., Ordonez, S. M., Kasper, C. K. and Sommer, S. S. (1999a) *Haemophilia*, **5**, 73-5.

- Heit, J. A., Silverstein, M. D., Mohr, D. N., Petterson, T. M., O'Fallon, W. M. and Melton, L. J., 3rd (1999b) *Arch Intern Med*, **159**, 445-53.
- Henkens, C. M., Bom, V. J., Van der, S. W., Pelsma, P. M., Sibinga, C. T., de Kam, P. J. and van der, M. J. (1995) *Thromb.Haemost.*, **74**, 1271-1275.
- Hooper, W. C., Phillips, D. J. and Evatt, B. L. (1996) *Thromb Res*, **81**, 315-26.
- Hooper, W. C., Phillips, D. J. and Evatt, B. L. (1997) *Thromb Haemost*, **77**, 1014-9.
- Hooper, W. C., Phillips, D. J., Ribeiro, M., Benson, J. and Evatt, B. L. (1995) *Thromb Haemost*, **73**, 819-24.
- Hryszko, T., Suzuki, Y., Mogami, H. and Urano, T. (2005) *FEBS Lett*, **579**, 6023-6.
- Htun, H., Holth, L. T., Walker, D., Davie, J. R. and Hager, G. L. (1999) *Mol Biol Cell*, **10**, 471-86.
- Ignar-Trowbridge, D. M., Pimentel, M., Teng, C. T., Korach, K. S. and McLachlan, J. A. (1995) *Environ Health Perspect*, **103 Suppl 7**, 35-8.
- Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S. and McLachlan, J. A. (1993) *Mol Endocrinol*, **7**, 992-8.
- Ing, N. H. (2005) *Biol Reprod*, **72**, 1290-6.
- Isomaa, V. V., Ghersevich, S. A., Maentausta, O. K., Peltoketo, E. H., Poutanen, M. H. and Vihko, R. K. (1993) *Ann Med*, **25**, 91-7.
- Jaksch, M., Gerbitz, K. D. and Kilger, C. (1995) *Clin Biochem*, **28**, 503-9.
- Jensen, E. V. and Jacobsen, H. I. (1962) *Recent Prog Horm Res*, **18**, 387-414.
- Jesty, J. and Silverberg, S. A. (1979) *J Biol Chem*, **254**, 12337-45.
- Johansson, A. M., Hillarp, A., Sall, T., Zoller, B., Dahlback, B. and Hallden, C. (2005) *Thromb Haemost.*, **94**, 951-957.
- Kafrissen, M. and Adashi, E. (2003) In *Williams Textbook of Endocrinology*(Eds, Larsen, P. R., Kronenberg, H. M., Melmed, S. and Polonsky, K. S.), pp. 665-708.
- Kalsheker, N., Morley, S. and Morgan, K. (2002) *Biochem Soc Trans*, **30**, 93-8.
- Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990) *Embo J*, **9**, 1603-14.

- Kelley, K. M., Rowan, B. G. and Ratnam, M. (2003) *Cancer Res*, **63**, 2820-8.
- Kemmeren, J. M., Algra, A. and Grobbee, D. E. (2001a) *J Intern Med*, **250**, 441-8.
- Kemmeren, J. M., Algra, A. and Grobbee, D. E. (2001b) *BMJ*, **323**, 131-134.
- Kemmeren, J. M., Algra, A., Meijers, J. C., Tans, G., Bouma, B. N., Curvers, J., Rosing, J. and Grobbee, D. E. (2004) *Blood*, **103**, 927-33.
- Kimura, R., Honda, S., Kawasaki, T., Tsuji, H., Madoiwa, S., Sakata, Y., Kojima, T., Murata, M., Nishigami, K., Chiku, M., Hayashi, T., Kokubo, Y., Okayama, A., Tomoike, H., Ikeda, Y. and Miyata, T. (2006) *Blood*, **107**, 1737-8.
- Koenen, R. R., Christella, M., Thomassen, L. G., Tans, G., Rosing, J. and Hackeng, T. M. (2005) *Thromb Haemost*, **93**, 853-9.
- Koenen, R. R., Gomes, L., Tans, G., Rosing, J. and Hackeng, T. M. (2004) *Thromb Haemost*, **91**, 1105-14.
- Kolev, K. and Machovich, R. (2003) *Thromb Haemost*, **89**, 610-21.
- Komiyama, Y., Pedersen, A. H. and Kisiel, W. (1990) *Biochemistry*, **29**, 9418-25.
- Koster, T., Rosendaal, F. R., Reitsma, P. H., van der Velden, P. A., Briet, E. and Vandenbroucke, J. P. (1994) *Thromb Haemost*, **71**, 719-22.
- Kovacs, G. T. (2003) *Aust N Z J Obstet Gynaecol*, **43**, 4-9.
- Kozak, M. (1987) *Nucleic Acids Res*, **15**, 8125-48.
- Kroegel, C. and Reissig, A. (2003) *Respiration*, **70**, 7-30.
- Kuhl, H. (2005) *Climacteric*, **8 Suppl 1**, 3-63.
- Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M. and Webb, P. (2000) *J Steroid Biochem Mol Biol*, **74**, 311-7.
- Lee, A. V., Weng, C. N., Jackson, J. G. and Yee, D. (1997) *J Endocrinol*, **152**, 39-47.
- Leo, J. C., Guo, C., Woon, C. T., Aw, S. E. and Lin, V. C. (2004) *Endocrinology*, **145**, 1314-21.
- Li, C., Briggs, M. R., Ahlborn, T. E., Kraemer, F. B. and Liu, J. (2001) *Endocrinology*, **142**, 1546-53.
- Line, B. R. (2001) *Semin Nucl Med*, **31**, 90-101.

- Liu, D., Guo, H., Griffin, J. H., Fernandez, J. A. and Zlokovic, B. V. (2003) *Circulation*, **107**, 1791-6.
- Lu, D., Xie, R. L., Rydzewski, A. and Long, G. L. (1997) *Thromb Haemost*, **77**, 1156-63.
- Lu, N. Z., Wardell, S. E., Burnstein, K. L., Defranco, D., Fuller, P. J., Giguere, V., Hochberg, R. B., McKay, L., Renoir, J. M., Weigel, N. L., Wilson, E. M., McDonnell, D. P. and Cidlowski, J. A. (2006) *Pharmacol Rev*, **58**, 782-97.
- Lu, X., Shi, Z. and Murphy, L. J. (1999) *Mol Cell Endocrinol*, **149**, 19-28.
- Lundwall, A., Dackowski, W., Cohen, E., Shaffer, M., Mahr, A., Dahlback, B., Stenflo, J. and Wydro, R. (1986) *Proc Natl Acad Sci U S A*, **83**, 6716-20.
- Ma, J. and Ptashne, M. (1988) *Cell*, **55**, 443-6.
- Mackie, I. J., Piegsa, K., Furs, S. A., Johnson, J., Bounds, W., Machin, S. J. and Guillebaud, J. (2001) *Br.J Haematol.*, **113**, 898-904.
- Malm, J., He, X. H., Bjartell, A., Shen, L., Abrahamsson, P. A. and Dahlback, B. (1994) *Biochem.J.*, **302** (Pt 3), 845-850.
- Malm, J., Laurell, M. and Dahlback, B. (1988) *Br J Haematol*, **68**, 437-443.
- Manfioletti, G., Brancolini, C., Avanzi, G. and Schneider, C. (1993) *Mol Cell Biol*, **13**, 4976-85.
- Marnach, M. L., Ramin, K. D., Ramsey, P. S., Song, S. W., Stensland, J. J. and An, K. N. (2003) *Obstet Gynecol*, **101**, 331-5.
- Martinelli, I., Legnani, C., Bucciarelli, P., Grandone, E., De, S., V and Mannucci, P. M. (2001) *Thromb Haemost.*, **86**, 800-803.
- Martinelli, I., Razzari, C., Biguzzi, E., Bucciarelli, P. and Mannucci, P. M. (2005) *J Thromb Haemost*, **3**, 2817-9.
- McQuillan, A. M., Eikelboom, J. W., Hankey, G. J., Baker, R., Thom, J., Staton, J., Yi, Q. and Cole, V. (2003) *Stroke*, **34**, 2415-2419.
- Meijer-Huizinga, F., Mertens, K. and van Mourik, J. A. (1994) *Thromb Haemost*, **72**, 408-14.
- Meinardi, J. R., Henkens, C. M., Heringa, M. P. and van der Meer, J. (1997) *Blood Coagul Fibrinolysis*, **8**, 152-4.
- Metivier, R., Reid, G. and Gannon, F. (2006) *EMBO Rep*, **7**, 161-7.

- Middeldorp, S. (2005) *Gend Med*, **2 Suppl A**, S3-9.
- Mille-Baker, B., Rezende, S. M., Simmonds, R. E., Mason, P. J., Lane, D. A. and Laffan, M. A. (2003) *Blood*, **101**, 1416-1418.
- Miyata, T., Kimura, R., Kokubo, Y. and Sakata, T. (2006) *Int J Hematol*, **83**, 217-23.
- Molino, D., De Lucia, D., Marotta, R., Perna, A., Lombardi, C., Cirillo, M. and De Santo, N. G. (2005) *Kidney Int*, **68**, 1223-9.
- Morboeuf, O., Aiach, M. and Gandrille, S. (1998) *Br J Haematol*, **101**, 10-5.
- Morboeuf, O., Borgel, D., Gaussem, P., Vincenot, A., Pittet, J. L., Aiach, M. and Gandrille, S. (2000) *Thromb Haemost.*, **84**, 604-610.
- Morgan, G. E., Rowley, G., Green, P. M., Chisholm, M., Giannelli, F. and Brownlee, G. G. (1997) *Br J Haematol*, **98**, 79-85.
- Mosselman, S., Polman, J. and Dijkema, R. (1996) *FEBS Lett*, **392**, 49-53.
- Mukherjee, K., Syed, V. and Ho, S. M. (2005) *Oncogene*, **24**, 4388-400.
- Nardulli, A. M., Greene, G. L., O'Malley, B. W. and Katzenellenbogen, B. S. (1988) *Endocrinology*, **122**, 935-44.
- Nelson, R. M. and Long, G. L. (1992) *J Biol Chem*, **267**, 8140-8145.
- Nesheim, M. E., Taswell, J. B. and Mann, K. G. (1979) *J Biol Chem*, **254**, 10952-62.
- Nicolaes, G. A. and Dahlback, B. (2002) *Arterioscler Thromb Vasc Biol*, **22**, 530-8.
- Norris, L. A. and Bonnar, J. (1996) *Br J Obstet Gynaecol*, **103**, 261-7.
- O'Brien, L. M., Matri, M. and Fay, P. J. (2000) *Blood*, **95**, 1714-20.
- Oger, E., Alhenc-Gelas, M., Lacut, K., Blouch, M. T., Roudaut, N., Kerlan, V., Collet, M., Abgrall, J. F., Aiach, M., Scarabin, P. Y. and Mottier, D. (2003) *Arterioscler Thromb Vasc Biol*, **23**, 1671-6.
- Okada, H., Yamazaki, T., Takagi, A., Murate, T., Yamamoto, K., Takamatsu, J., Matsushita, T., Naoe, T., Kunishima, S., Hamaguchi, M., Saito, H. and Kojima, T. (2006) *J Thromb Haemost*, **4**, 2003-9.
- Olivieri, O., Friso, S., Manzato, F., Guella, A., Bernardi, F., Lunghi, B., Girelli, D., Azzini, M., Brocco, G., Russo, C. and et al. (1995) *Br J Haematol*, **91**, 465-70.

O'Lone, R., Frith, M. C., Karlsson, E. K. and Hansen, U. (2004) *Mol Endocrinol*, **18**, 1859-75.

Oral Contraceptive and Hemostasis Study Group. The effects of seven monophasic oral contraceptive regimens on hemostatic variables: conclusions from a large randomized multicenter study (2003) *Contraception*, **67**, 173-85.

Orfeo, T., Brufatto, N., Nesheim, M. E., Xu, H., Butenas, S. and Mann, K. G. (2004) *J Biol Chem*, **279**, 19580-91.

Oruc, S., Saruc, M., Koyuncu, F. M. and Ozdemir, E. (2000) *Aust N Z J Obstet Gynaecol*, **40**, 448-50.

Osterud, B. and Rapaport, S. I. (1977) *Proc Natl Acad Sci U S A*, **74**, 5260-4.

Owen, C. A., Campbell, M. A., Sannes, P. L., Boukedes, S. S. and Campbell, E. J. (1995) *J Cell Biol*, **131**, 775-89.

Owen, G. I., Richer, J. K., Tung, L., Takimoto, G. and Horwitz, K. B. (1998) *J Biol Chem*, **273**, 10696-701.

Pelletier, G. (2000) *Histol Histopathol*, **15**, 1261-70.

Petz, L. N., Ziegler, Y. S., Schultz, J. R., Kim, H., Kemper, J. K. and Nardulli, A. M. (2004) *J Steroid Biochem Mol Biol*, **88**, 113-22.

Pierce, A. L., Shimizu, M., Felli, L., Swanson, P. and Dickhoff, W. W. (2006) *J Endocrinol*, **191**, 379-86.

Pincus, G. (1958) *Postgrad Med*, **24**, 654-60.

Pinzone, J. J., Stevenson, H., Strobl, J. S. and Berg, P. E. (2004) *Mol Cell Biol*, **24**, 4605-12.

Pittman, D. D. and Kaufman, R. J. (1989) *Thromb Haemost*, **61**, 161-5.

Ploos van Amstel, H. K., Reitsma, P. H., van der Logt, C. P. and Bertina, R. M. (1990) *Biochemistry*, **29**, 7853-7861.

Porter, W., Saville, B., Hoivik, D. and Safe, S. (1997) *Mol Endocrinol*, **11**, 1569-80.

Qin, C., Singh, P. and Safe, S. (1999) *Endocrinology*, **140**, 2501-8.

Quack Loetscher, K. C., Stiller, R., Roos, M. and Zimmermann, R. (2005) *Thromb Haemost*, **93**, 706-9.

- Rabbitt, E. H., Lavery, G. G., Walker, E. A., Cooper, M. S., Stewart, P. M. and Hewison, M. (2002) *Faseb J*, **16**, 36-44.
- Rajaram, S., Baylink, D. J. and Mohan, S. (1997) *Endocr Rev*, **18**, 801-31.
- Ravnik-Glavac, M., Atkinson, A., Glavac, D. and Dean, M. (2002) *Hum Mutat*, **19**, 374-83.
- Read, L. D., Snider, C. E., Miller, J. S., Greene, G. L. and Katzenellenbogen, B. S. (1988) *Mol Endocrinol*, **2**, 263-71.
- Reid, G., Denger, S., Kos, M. and Gannon, F. (2002) *Cell Mol Life Sci*, **59**, 821-31.
- Rezende, S. M., Lane, D. A., Zoller, B., Mille-Baker, B., Laffan, M., Dalhback, B. and Simmonds, R. E. (2002) *Thromb Haemost.*, **87**, 258-265.
- Richer, J. K., Jacobsen, B. M., Manning, N. G., Abel, M. G., Wolf, D. M. and Horwitz, K. B. (2002) *J Biol Chem*, **277**, 5209-18.
- Rock, J., Garcia, C. R. and Pincus, G. (1957) *Recent Prog Horm Res*, **13**, 323-39; discussion 339-46.
- Rosendaal, F. R., Van Hylckama Vlieg, A., Tanis, B. C. and Helmerhorst, F. M. (2003) *J Thromb Haemost*, **1**, 1371-80.
- Rosing, J., Hoekema, L., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., Varadi, K., Schwarz, H. P. and Tans, G. (1995) *J Biol Chem*, **270**, 27852-8.
- Rosing, J., Middeldorp, S., Curvers, J., Christella, M., Thomassen, L. G., Nicolaes, G. A., Meijers, J. C., Bouma, B. N., Buller, H. R., Prins, M. H. and Tans, G. (1999) *Lancet*, **354**, 2036-40.
- Rosing, J., Tans, G., Nicolaes, G. A., Thomassen, M. C., van Oerle, R., van der Ploeg, P. M., Heijnen, P., Hamulyak, K. and Hemker, H. C. (1997) *Br J Haematol*, **97**, 233-8.
- Rossetti, S., Corra, S., Biasi, M. O., Turco, A. E. and Pignatti, P. F. (1995) *Mol Cell Probes*, **9**, 195-200.
- Safe, S. (2001) *Vitam Horm*, **62**, 231-52.
- Safe, S. and Kim, K. (2004) *Prog Nucleic Acid Res Mol Biol*, **77**, 1-36.
- Sambrano, G. R., Huang, W., Faruqi, T., Mahrus, S., Craik, C. and Coughlin, S. R. (2000) *J Biol Chem*, **275**, 6819-23.
- Santacroce, R., Sarno, M., Cappucci, F., Sessa, F., Colaizzo, D., Brancaccio, V., Grandone, E. and Margaglione, M. (2006) *J Thromb Haemost*, **4**, 2417-22.

- Saposnik, B., Borgel, D., Aiach, M. and Gandrille, S. (2003) *Eur.J Biochem*, **270**, 545-555.
- Sarich, J. (2002) Murdoch University, Perth.
- Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J. A. and Safe, S. (2000) *J Biol Chem*, **275**, 5379-87.
- Schindler, A. E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J. R., Schweppe, K. W. and Thijssen, J. H. (2003) *Maturitas*, **46 Suppl 1**, S7-S16.
- Schoneveld, O. J., Gaemers, I. C. and Lamers, W. H. (2004) *Biochim Biophys Acta*, **1680**, 114-28.
- Schultz, J. R., Petz, L. N. and Nardulli, A. M. (2003) *Mol Cell Endocrinol*, **201**, 165-75.
- Senetar, M. A. and McCann, R. O. (2005) *Gene*, **362**, 141-52.
- Sgarabotto, M., Baldini, M., Dei Cas, A., Manotti, C., Luciana Barilli, A., Rinaldi, M., Benassi, L. and Bacchi Modena, A. (2006) *Thromb Res*.
- Shaaban, M. M. (1991) *J Steroid Biochem Mol Biol*, **40**, 705-10.
- Sheffield, V. C., Beck, J. S., Kwitek, A. E., Sandstrom, D. W. and Stone, E. M. (1993) *Genomics*, **16**, 325-32.
- Shukla, A., Shukla, G. S. and Radin, N. S. (1992) *Am J Physiol*, **262**, F24-9.
- Simmonds, R. E., Ireland, H., Kunz, G. and Lane, D. A. (1996) *Blood*, **88**, 4195-4204.
- Simmonds, R. E., Zoller, B., Ireland, H., Thompson, E., de Frutos, P. G., Dahlback, B. and Lane, D. A. (1997) *Blood*, **89**, 4364-70.
- Smith, L. H., Coats, S. R., Qin, H., Petrie, M. S., Covington, J. W., Su, M., Eren, M. and Vaughan, D. E. (2004) *Circ Res*, **95**, 269-75.
- Sofi, F., Cesari, F., Gensini, G. F., Abbate, R. and Fedi, S. (2005) *Stroke*, **36**, 1821; author reply 1821-2.
- Song, K. S., Park, Y. S. and Kim, H. K. (2000) *Arthritis Rheum*, **43**, 557-60.
- Soria, J. M. and Bertina, R. M. (1997) *Thromb Haemost*, **77**, 755 (abstract).
- Speroff, L. and Darney, P. (1996) *A Clinical Guide for Contraception*, Williams and Wilkins, Baltimore.

- Staton, J. M. and Leedman, P. J. (1998) *Endocrinology*, **139**, 1093-100.
- Staton, J. M., Thomson, A. M. and Leedman, P. J. (2000) *J Mol Endocrinol*, **25**, 17-34.
- Stearns-Kurosawa, D. J., Kurosawa, S., Mollica, J. S., Ferrell, G. L. and Esmon, C. T. (1996) *Proc Natl Acad Sci U S A*, **93**, 10212-6.
- Stepanova, M., Lin, F. and Lin, V. C. (2006) *Comput Biol Chem*, **30**, 339-47.
- Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D. R., Jones, P. F. and et al. (1995) *Cell*, **80**, 661-70.
- Stossi, F., Likhite, V. S., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (2006) *J Biol Chem*, **281**, 16272-8.
- Sumino, H., Ichikawa, S., Sawada, Y., Sakamoto, H., Kumakura, H., Takayama, Y., Sakamaki, T. and Kurabayashi, M. (2005) *Thromb Res*, **115**, 359-66.
- Sun, G., Porter, W. and Safe, S. (1998) *Mol Endocrinol*, **12**, 882-90.
- Suwanichkul, A., Allander, S. V., Morris, S. L. and Powell, D. R. (1994) *J Biol Chem*, **269**, 30835-41.
- Takimoto, G. S., Tung, L., Abdel-Hafiz, H., Abel, M. G., Sartorius, C. A., Richer, J. K., Jacobsen, B. M., Bain, D. L. and Horwitz, K. B. (2003) *J Steroid Biochem Mol Biol*, **85**, 209-19.
- Tanis, B. C. and Rosendaal, F. R. (2003) *Semin Vasc Med*, **3**, 69-84.
- Tans, G., Curvers, J., Middeldorp, S., Thomassen, M. C., Meijers, J. C., Prins, M. H., Bouma, B. N., Buller, H. R. and Rosing, J. (2000) *Thromb Haemost*, **84**, 15-21.
- Tatewaki, H., Tsuda, H., Kanaji, T., Yokoyama, K. and Hamasaki, N. (2003) *Thromb Haemost.*, **90**, 1029-1039.
- ten Kate, M. K., Mulder, R., Platteel, M., Brouwer, J. L., van der Steege, G. and van der Meer, J. (2006) *Haematologica*, **91**, 1151-2.
- Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J. A., Tsai, M. J. and O'Malley, B. W. (1988) *Cell*, **55**, 361-9.
- Tung, L., Abdel-Hafiz, H., Shen, T., Harvell, D. M., Nitao, L. K., Richer, J. K., Sartorius, C. A., Takimoto, G. S. and Horwitz, K. B. (2006) *Mol Endocrinol*, **20**, 2656-70.
- Uchikova, E. H. and Ledjev, I. I. (2005) *Eur.J Obstet.Gynecol.Reprod.Biol*, **119**, 185-188.

Van Criekinge, W. and Beyaert, R., Vol. 2007, pp. Online Source.

Van Criekinge, W. and Beyaert, R. (1999) *Biol Proced Online*, **2**, 1-38.

Van Den Bosch, M. A., Kemmeren, J. M., Tanis, B. C., Mali, W. P., Helmerhorst, F. M., Rosendaal, F. R., Algra, A. and Van Der Graaf, Y. (2003) *J Thromb Haemost*, **1**, 439-44.

van Gent, D., Sharp, P., Morgan, K. and Kalsheker, N. (2003) *Int J Biochem Cell Biol*, **35**, 1536-47.

van Rooijen, M., Hansson, L. O., Frostegard, J., Silveira, A., Hamsten, A. and Bremme, K. (2006) *J Thromb Haemost*, **4**, 77-82.

Vasse, M., Guegan-Massardier, E., Borg, J. Y., Woimant, F. and Soria, C. (2001) *Lancet*, **357**, 933-934.

Villoutreix, B. O., Garcia, d. F., Lovenklev, M., Linse, S., Fernlund, P. and Dahlback, B. (1997) *Proteins*, **29**, 478-491.

Wakefield, T. W., Strieter, R. M., Prince, M. R., Downing, L. J. and Greenfield, L. J. (1997) *Cardiovasc Surg*, **5**, 6-15.

Walker, F. J. (1989) *J Biol Chem*, **264**, 17645-17648.

Wan, Y. and Nordeen, S. K. (2003) *Recent Prog Horm Res*, **58**, 199-226.

Wang, L., Hirayasu, K., Ishizawa, M. and Kobayashi, Y. (1994) *Nucleic Acids Res*, **22**, 1774-5.

Wang, W., Dong, L., Saville, B. and Safe, S. (1999) *Mol Endocrinol*, **13**, 1373-87.

Wei, L. L., Krett, N. L., Francis, M. D., Gordon, D. F., Wood, W. M., O'Malley, B. W. and Horwitz, K. B. (1988) *Mol Endocrinol*, **2**, 62-72.

Weitz, J. I. (2001) *J Thromb Thrombolysis*, **12**, 7-17.

Wiegatz, I. and Kuhl, H. (2004) *Trends Endocrinol Metab*, **15**, 277-85.

Wiegatz, I., Lee, J. H., Kutschera, E., Winkler, U. H. and Kuhl, H. (2004) *Contraception*, **70**, 97-106.

Winkler, U. H. (1998) *Contraception*, **57**, 203-209.

Winkler, U. H. (2000) *Contraception*, **62**, 11S-20S; discussion 37S-38S.

Winkler, U. H., Holscher, T., Schulte, H., Zierleyn, J. P., Collet, W. and Schindler, A. E. (1996) *Gynecol Endocrinol*, **10**, 265-71.

Woods, N. F., Carr, M. C., Tao, E. Y., Taylor, H. J. and Mitchell, E. S. (2006) *Menopause*, **13**, 212-21.

Woutersz, T. B. (1991) *Int J Fertil*, **36 Suppl 3**, 26-31.

Wu, O. (2005) *Gend Med*, **2 Suppl A**, S18-27.

Wu, O., Robertson, L., Langhorne, P., Twaddle, S., Lowe, G. D., Clark, P., Greaves, M., Walker, I. D., Brenkel, I., Regan, L. and Greer, I. A. (2005) *Thromb Haemost.*, **94**, 17-25.

Xie, W., Duan, R. and Safe, S. (1999) *Endocrinology*, **140**, 219-27.

Yamamura, J., Miyoshi, Y., Tamaki, Y., Taguchi, T., Iwao, K., Monden, M., Kato, K. and Noguchi, S. (2004) *Cancer Sci*, **95**, 887-92.

Yamazaki, T., Katsumi, A., Kagami, K., Okamoto, Y., Sugiura, I., Hamaguchi, M., Kojima, T., Takamatsu, J. and Saito, H. (1996) *Blood*, **87**, 4643-50.

Yegneswaran, S., Deguchi, H. and Griffin, J. H. (2003) *J Biol Chem*, **278**, 14614-21.

Zilliacus, J., Wright, A. P., Carlstedt-Duke, J. and Gustafsson, J. A. (1995) *Mol Endocrinol*, **9**, 389-400.

Zoller, B., Garcia, d. F. and Dahlback, B. (1995) *Blood*, **85**, 3524-3531.

APPENDICES

Appendix 1- Oestrogen Isoforms

17β-Oestradiol (E₂); the predominant isoform *in vivo*.
17α-Ethinyloestradiol (EE); a synthetic oestrogen used in most COC preparations and mestranol, an isoform used in initial COC development. Both EE and mestranol have an ethynyl group attached to C17 that increases their half lives due to a resistance to hepatic metabolism. Oestradiol valerate (EV) is an example of an esterified oestrogen that are included in some HRT preparations.

Appendix 2- Manipulation of the Progesterone Molecule

(PREGNANES)

A schematic illustrating how progesterone is manipulated to form medroxyprogesterone (MPA) and cyproterone acetate. Both progestins are widely used in both COC and HRT preparations. MPA is used in many progestin-only oral contraceptives.

Appendix 3- Manipulation of the Testosterone Molecule

A schematic illustrating how testosterone is manipulated to form; gonanes, including the 2nd generation levonorgestrel and the 3rd generation gestodene and desogestrel; and estranes, norethisterone and dienogest

Appendix 4- Primers used to amplify PROS1 DNA and cDNA for CSHA

Exon/ Frag	Primer Name	# nt	T _m (°C)	Sequence (5'- 3')
5' UTR & 1	PS ERE For	20	50	ACT GAA GTC TTT ATC GGA GC
	PSintronARev	20	50	AGA AAA TCC TAC CAA CCA GG
2	PS-2-5 [#]	20	48	GTC ATA CAA TTC ATA GGC AG
	PS Exon 2 Rev	22	55	AAC AGA AGG AAG TAC AGG CTG G
3	PS Ex3 For#2	21	46	GGT TTG CTA AGA TAT GTT TTC
	PS Ex3 Rev#2	21	46	TCC CAA GGA TAA TGA AAT TAC
4	PS Exon 4 For	20	48	CAA GTA TGT GTG TCT ACT <u>CT</u>
	PS Exon 4 Rev	20	48	TCA ATT GAT GGT AGA AGT GC
5-6	PS-5/6-5 [#]	24	49	<u>G</u> GC TTC AGG ATT TTT ATT <u>ATA</u> <u>GTA</u>
	PS-5/6-3 [#]	22	49	CTA ACT GGG ATT ATT CTC ACA <u>T</u>
7	PS-7-5 [#]	21	50	CAC AAA TCA AGG GTT CTT TGG
	PS Exon 7 Rev	22	53	CAG TAA TGA <u>TAC</u> CAC CAT CAG <u>C</u>
8	PS Exon 8 For	20	43	ATA AGA TTG AAC ATT TAG CG
	PS Exon 8 Rev	18	43	CAG GTG AGA AGT TAA <u>GTA</u>
9	PS Exon 9b For	25	54	CAC ACA AAC ATT AAG CAA TAA <u>CCC G</u>
	PS Exon 9 Rev	21	52	CAT TGG TAT TGG TTC CTC ACC
10	PS Exon 10 For	21	48	AGC <u>TTT</u> CTG TAT TTC <u>TTA CCC</u>
	PS Exon 10 Rev	24	56	TAC AGA CTG CAT CAA AGT GGG A <u>TG</u>
11	PS Exon 11 For	23	48	GTA <u>ATA</u> CTT GGT TAT TTG GTA <u>GT</u>
	PS Exon 11 Rev	20	48	GAA TGA ACA AGC TTG GAT <u>C</u>
12	PS Exon 12 For	18	48	AAT GCA CTC CTT GAC <u>TGG</u>
	PS12Rev#2 [#]	20	52	TGG GCA CAC AGT AGA TAC <u>TC</u>
13	PS Exon 13 For	22	51	GCA TTG AT ATG CTT CTG TTT C
	PS Exon 13 revb	30	53	GTC CTT TGA GGT AAA TAC TGC TAT GTA <u>TGC</u>
14	PS Exon 14 For	19	44	ATA TTG ATT CTT TGC <u>TCC G</u>
	PS Exon 14 Rev	19	44	CTG AAT AAA AAG TAA GCA G
15	PS Exon 15 For	22	49	GGA TTA GAA TTT GGT TGG AAA <u>C</u>
	PS Exon 15 Rev	22	49	TTA <u>TAA</u> GCA GAG AAA AGA <u>TGC C</u>
F1	2ndPSmRNAFor	22	57	TTC GAA ATG AGG GTC CTG GGT G
	PSmRNAR1	24	57	AGG AAC AGT GGT AAC TTC CAG GTG
F2	PSmRNAF2	25	51	GTG TGA ATT TGA CAT AAA TGA ATG C
	PSmRNAR2	25	51	AAA TCA AAT TCT GCT GAA AAT CTG C
F3	PSmRNAF3	26	58	CCA AGA TCA GAA GAG TTG TGA GGT TG
	PSmRNAR3	24	56	ATT CCA GAA GCT CCT TGC TTC ATC
F4	PSmRNAF4	25	54	AAC TCA AAC CGA TTA ACC CTC G
	PSmRNAR4	22	55	TGG CAC TGA ATG GAA CAT CTG G
F5	PSmRNAF5	23	57	CAA TCT GGA GTT GTC GAC ACC AC
	1stPSmRNARev	28	55	ACA CAC AAG GAA AAG GTA TTA TAA GCA G

[#] Denotes primers previously designed by Reitsma *et al.* (1994).

Underlined bases denote mismatches with PROS2

Italicised bases denote penultimate mismatch base to PROS1 and PROS2

F1→F5 denotes cDNA primers.

Appendix 5- Restriction Enzyme Combinations used for CSHA

Enzyme (opt °C)	ERE & Exon 1 818bp	Exons 5-6 363bp	Exon 15 332bp	Frag 1 562bp	Frag 2 528bp	Frag 3 562bp	Frag 4 579bp	Frag 5 393bp
Bam HI & Sac II (37°C)	254bp, 342bp, 222bp							
Bst OI (60°C)	191bp, 83bp, 170bp, 230bp, 126bp, 18bp							177bp, 216bp
Pst I (37°C)		272bp, 233bp						
Bsr I (65°C)			132bp, 127bp			294bp, 268bp		
Msp I (37°C)				232bp, 330bp				
Hsp92 II (37°C)				383bp, 179bp	295bp, 233bp			
Bfm I (37°C)					235bp, 293bp			
Dra III (37°C)						236bp, 326bp		
Dde I (37°C)							199bp, 185bp, 195bp	

Appendix 6- Sequencing, insert amplification and mutagenesis primer sequences

Vector	Insert or Primer Information	# nt	Sequence (5'- 3')
pPTMR	950bp fragment of PROS1 5' UTR	27	ACG CTC GAG CAC GAT CAG GAG TTC AGA
		27	TTG AAT TCA TTC GAA GCG CGC GGA GGC
pPTMR(-407)	c-406t mutagenesis primers	34	CTT CTG AAC CTA GGG ATT CTG TCC TCT TGA ACC C
		34	GGG TTC AAG AGG ACA GAA TCC CTA GGT TCA GAA G
pPTMR(-ERE)	Primers for removal of the proposed ERE	26	ATT TGC TTT TAC TAT CAC CAT AGT TC
		25	CCA CAT GTT TTT TCG GTC TTG TAT G
pPTMR related	Primers used to confirm sequences of pPTMR, pPTMR(-407), pPTMR(-ERE), pPTMR(-AvrII), pPMTR(del-BclII/AvrII) and pPMTR(del-AvrII/DraIII)	20	ACT GAA GTC TTT ATC GGA GC
pPTMR related		19	GCA CGA CTT CTT CAA GTC C
		19	TGA AGT TCA CCT TGA TGC C
pPTMR related		22	ACC ATG GCC TCC TCC GAG AAC G
		22	CTA CAG GAA CAG GTG GTG GCG G
pPTMR related			
pBTM116	2053bp fragment of PROS1 cDNA	27	TTG GAT CCA AAT GAG GGT CCT GGG TGG
		32	ATC TGC AGT TAT AAG CAG AGA AAA GAT GCC TT
pBTM116 related	Primers used to confirm sequence of PROS1 cDNA in pBTM116 vector	27	TTG GAT CCA AAT GAG GGT CCT GGG TGG
pBTM116 related		32	ATC TGC AGA CCC AGT TTG AAA AGA GCG AAG AC
pBTM116 related		26	TTG GAT CCA TGA CCC GGA AAC GGA TT
		26	ATC TGC AGC TTC ATT GCA TGG CAG AG
pBTM116 related		27	TTG GAT CCG CTG TGT CAA TGC CAT TCC
		25	ATC TGC AGG GCA CAC TGA AAC AAC C
pBTM116 related		27	TTG GAT CCA GAA GAG TTG TGA GGT TGT
		28	ATC TGC AGT CCA GCT TCG TAT ACA TCC A
pBTM116 related		28	TTG GAT CCA TGG ATG TAT ACG AAG CTG G
		32	ATC TGC AGT TAT AAG CAG AGA AAA GAT GCC TT
pGADT7	1225bp fragment of PROZ cDNA	25	TTA TCG ATA TAT GGC AGG CTG CGT C
		27	GTG GGA TCC CAG TTA CTT CAT GAT CTG
pACT2	Primers used to sequence pACT2 inserts	20	CCA GAT TAC GCT AGC TTG GG
		20	CAG TTG AAG TGA ACT TGC GG

Appendix 7- Sequence Alignment of Yeast-2-Hybrid Findings

Col1110	-----	
Col1111	-----	
Col119	GNNCCTTNNNNNNNTNTGGGCCGGGGANCGAATTCGCGGCCGCGTCGACGATGAGTTNGCA	60
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	TCACTCTGNACATATTCCCTTTTTTNNGGGAAGTAGGGAGCGTGCTCNTTGNGCNGCGGAT	120
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	GCGATCCNNAANNTACACAGNGCGGANGCCANCGNANTCATTCAGTNCTCCTCNTGAGNAA	180
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	AGATNNGATGACAGGANGTNGCAAGCTGNGCTNTTCCCNATANANCCTGNNGCGGTGGACA	240
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	GACTCTCTGGAGTTCTGAGAGATAGGNGAGCTCTACCTGCCAAANTTTTCCATCTCGAGG	300
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	GACTATNACCTGAACGACNTACTTCTCCAGNTGGGNATTGAGGAAGCCTTCANCNNCNG	360
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	GCTNACCTGNNANGGATCACAGGGGCCAGGAACCTANCNGNCTCCANGNGGTCCATAAC	420
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	NGGTGNGCTTGATGTATNTGANGAGGGCATGAGAATNNTCTGCTGNNACTTTAGTCAAAA	480
Col112	-----	
Col2	-----	
Col1112	-----	

Col1110	-----	
Col1111	-----	
Col119	TCACCCTCCTTTTNTGCATTAGTGGAGACAAGGANCATTGTGCGTTTCAANAGGNCCTTNC	540
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	TGATGATCATTGTCCCTACAGACACCCAGAACATCTTCTTNATGAGCAAATGCNCCANTC	600
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	CCAAGCAAGNCTTAGAGCTTGCCATCAAGCAGTGGGGCTCTCAGTAAGGAACCTTGAATG	660
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	CAAAGCTGGATGCCTGGGTCTCTGGGGCACAGNCTGGCCCCCTGTGCACCGNANTGGCCAT	720
Col112	-----	
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	GGTATGTGTGGCCCTGNCTGCTTTTCCTTGGAAGGNGGACAGCGATTNCCCTGTGTAGCT	780
Col112	-----TNNNNNNNNNNNNNNNNNNNNNNNTNNGGNCGCG	32
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----	
Col119	CTCACATGCACAGGGGTNCCATGGACTCTTNANNTCTGGGAGGGTCCTGGGCCTCCTGAA	840
Col112	TCGACAAGAGCAGGAGAATGAGACAGTGGTGGTGAAAGAGAAGATGGTTGGCGGCATTGC	92
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	-----GGNGGCCGGGGATCGAATTCGCGGCCGC	28
Col119	CAGCAATAAANAATTTTCGTTGGNAGAAAAANAANAANAAAAAATANTGTCNG	900
Col112	CCAGATCATCGCAGCACAGGAAGAAATGCTTCGGAAGGAACGAGAGCTGGAAGAGGCGCG	152
Col2	-----	
Col1112	-----	
Col1110	-----	
Col1111	GTGCACCTGGGACAGAGGACTGCTGTCTGCCCTCTCTGGTCACCCTGCCTAGCTAGAGGA	88
Col119	TANTATTTNTTNNNTNTGNNNGNANNNTTNTTATTANTTNNCANCTNTGTTNTNAANNANA	960
Col112	GAAGAACTGGCCCAGATCCGGCAGC--AGCAGTACAAGTTTCTGCCTTCAGAGCTTCGA	210
Col2	-----	

Col1112	-----	
Col1110	-----	
Col1111	TCTGTGACCCCAGCCATGAGGACCCTCGCCATCCTTGCTGCCATTCTCCTGGTGGCCCTG	148
Col119	NAAAANNNTNTNTNCTNNTNATNCNCNGTNNNTGTNTNNTNNTCNAANNNTNTTNTNNATC	1020
Col112	GATGAGCACTAAAGAAGCCTCTTCTATTTAATGCAGACCCGGCCCAGAGACTGTGCGTGC	270
Col2	-----	
Col1112	-----GNNNNG	6
Col1110	-----	
Col1111	CAGGCCCAGGCTGAGCCACTCCAGGCAAGAGCTGATGAGGTTGCTGCAGCCCCGGAGCAG	208
Col119	NNNTATCNTCCTCNTTNNNTGCGNTGCNTNCGNTTTTTTTTTTNTTTNTTTTCACCGGTANAA	1080
Col112	CACTACCAAAGCCTTCTGGGCTGTCTGGGGCCCAACCTGCCCAACCCCAGCACTCCCCAAA	330
Col2	-----	
Col1112	NGGNCGCGTCGACACCAAACCTACGCCAAAATCCATTTCACTATCATATTCATCGGCGTA	66
Col1110	-----	
Col1111	ATTGCAGCGGACATCCCAGAAGTGGTTGTTTCCCTTGCAATGGGACGAAAGCTTGGCTCCA	268
Col119	NGGGTNNNGAGTTCCCTAGCGNAGCCTNNNCATNCATNTTTTNGTNTNTACNNNNNCNNTT	1140
Col112	GTGCCTGCCAAACCCCAGGGCCTGGCCCCGCCAGTCCCGCAGTACATCCCCCTGTCCCCCT	390
Col2	-----	
Col1112	AATCTAACTTTCTTCCC--ACAACACTTTCTCGGCCTATCCGGAATGCCCCGACGTTACT	124
Col1110	-----GGGNNTCGAATTCGCGGCCGC--GTCG	25
Col1111	AAGCATCCAGGCTCAAGGAAAAACATGGCCTGCTATTGCAGAATACCAGCGTGC--ATTG	326
Col119	CNTNATNACCTTNTGTANNNGTGNNGNATNTNCACNTGCTNNNTNTNNGTNNACTCNNTA	1200
Col112	CCCCAACCCCAAGTGCCCTTCATGCC---CTAGGGCCCCCAAGTGCCCTGCCCT---CCC	444
Col2	-----	
Col1112	CGGACTACCCCGATGCATACACCACATGAAACATCCTATCATCTGTAGGCTCATTCATTT	184
Col1110	ACGGAGAACGTCGCTATGGAACCTGCATCTACCAGGGAAGACTCT-----GG	72
Col1111	CAGGAGAACGTCGCTATGGAACCTGCATCTACCAGGGAAGACTCT-----GG	373
Col119	CNTNNNNCGNTNGCTGGNNNNNNNNNTNNTCNNNTGGNGNGANCNTNCNATCANGCNTAAN	1260
Col112	CAGAGTATTAACGCTCCAAGAGTATTATTAACGCTGCTGTACCTC-----GATCTGAAT	498
Col2	-----	
Col1112	CTCTA-ACAGCAGTAATATTAATAATTTTCATGATTTGAGAAGCC-----T	229
Col1110	GCATTCTGCTGCTGA--GCTTGCAG--AAAAAGAAAAATGAG-----CTCAAATTTG	121
Col1111	GCATTCTGCTGCTGA--GCTTGCAG--AAAAAGAAAAATGAG-----CTCAAATTTG	422
Col119	CCNTTNGCTNTTNTCTNNNTGCANCGNATAANANTNATNGGGTTNAATNTGNANGNTTN	1320
Col112	CTGCCGGGGCCCCAGCCCACTCCACCCTGCCAGCAGCTTCCAGCCAGTCCCCACAGCCTC	558
Col2	-----	
Col1112	TCGCTTCGAAGCGAAAAGTCCTAATAGTAGAAGAACCCTCCA-----TAAACCTG	279
Col1110	CTTTGAGAGCTACAGGGAATTGC-TATTACTCCTGTACCTTCTGCTCAATTTTCCT-TTCC	179
Col1111	CTTTGAGAGCTACAGGGAATTGC-TATTACTCCTGTACCTTCTGCTCAATTTTCCT-TTCC	480
Col119	TCTTNTGTCAAAAAAGNNTANNATNNTTCTCCAANATAAATAGGNNNAGTTTATNANNNN	1380
Col112	ATCAGCTCTCTTACCGTTTTTTT--GATACTATCTTCCCCCACCCTCAGCTACCCATAGG	616
Col2	-----	
Col1112	---GAGTGACTATATGGATGCCCCCACCCTACCACACATTCTGAAGAACCCGTATACATA	336
Col1110	TCATCCCAAATAAAATGCCTTGTTACAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-AAAAAAAAA	238
Col1111	TCATCCCAAATAAAATGCCTTGTTACNAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-AAAAAAAAA	539
Col119	TCNTNCTATANNNTNGNTGACNNTGNANNAGANCNTGTATNNGTCATGGT-CTGNCTNNTC	1439
Col112	GGCTGCAGAGTTATAAGCCCCAAACAGGTCATGCTCCAATAAAAATGATTCTACCTTCNN	676

Col2	-----	
Col112	AAATCTAGACAAAAAAGGAAGGAATCGAACCCCCCAAAGCTGGTTTCAAGCCAACCCCAT	396
Col110	AAAAAAAAAAAAA-----AAAAAAAAAAAAAAAAAAAAAAAAANCCCCNGGGNNNTTTTTTNNN	294
Col111	AAAAAAAAAAAAA-----AAAAAAAAAAAAAAAAAAAAAAAAANNNCCCCNGGGNNNTTTTTTTNN	595
Col119	CNTNTNCTANACTA-----NAGCTANNAATGTAGANATGCTGGANGANATGCTNNNTTTAT	1495
Col112	AAAAAAAAAAAAA-----AAAAAAAAAAAAAAAAAAAAAAAAAACNCNGNAANTTTTTTTTT	732
Col2	---ACACAGCTGCA-----CAGGTCGTGCCCCGAGGCTCCTGCTGCCGGCACCTCCCCCAG	53
Col112	GGCCTCCATGACTTTTTCAAAAAAAAAAAAAAAAAAAAAAAAAAACCNNGGGNNNTTTTTNNNAN	456
	*	
Col110	NNNNNNNNNNNAAAAANCCCCNNGGGTTTTTTTTTN-----N---GGGGGGGGGNNNNNNNNNN	347
Col111	NNNAAAAANNAAAAAACCNCNGGNTTTTTTTTTT-----TTTTNGGGGGGNNNNNNNNNN	651
Col119	TNTTTNATGAGNNNGATCTCATNNNCCTNATCN-----TAGTGNGTNTNNTTTCTGNTGCG	1551
Col112	TTTTNAANTTTNAAAAANCCCCNNGGGTTTTTTTTTT-----TTTTGGGGGGGNNNNNNNNNN	788
Col2	CCTGGACTTTGGCTGTGTTTGTAGCCCTGTCCCAGCACTCCGGGTGGGAATGGCAGGCTG	113
Col112	NNGAAAAANNGGAAAAACCCGGGGGNTNNTTTTT-----TNNNGGGNGNNNNNNNNNNNNNN	512
	*	
Col110	NNNNNNNNNNNN-----	359
Col111	NNNNNNNNNNNN-----	664
Col119	TATCATANTNGTNTGATG	1569
Col112	NNNNNNNNNNNN-----	799
Col2	CGTCCCCTGCTCC-----	127
Col112	NNNNNNNN-----	519