

## **STUDIES OF THE ENDOTHELIAL PROTEIN C RECEPTOR**

**STUDIES OF THE ENDOTHELIAL PROTEIN C RECEPTOR**

**By**

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**A Thesis**

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### **Lay Abstract**

Under normal conditions, blood is maintained in a fluid state. Upon injury or infection, the blood begins to form a clot to prevent bleeding. Once bleeding has stopped the clot is dissolved and blood regains its fluid state. The formation of a blood clot is a serious and potentially life threatening disease. A blood clot formed inside a blood vessel can block the flow of blood through the circulation, leading to organ damage. Approximately 50% of blood clots are caused by known genetic or environmental factors, leaving 50% of blood clots caused by unknown factors. In this thesis we investigate the unknown factors that contribute to blood clotting. In patients who have experienced blood clots with no known cause, we have identified genetic mutations in a blood vessel wall protein, known as the endothelial protein C receptor (EPCR) that renders it non-functional. We demonstrate both *in vitro* and *in vivo* that non-functional EPCR not only leads to the formation of a blood clot but also delays the removal of the blood clot. Our *in vivo* studies have also revealed a previously unknown role for EPCR in the bone marrow, likely through its effects on blood coagulation. Taken together, loss of EPCR function contributes to the development of clot formation and likely impacts other organ systems.

## Abstract

The endothelial protein C receptor (EPCR) binds to protein C (PC) and increases the rate of activated protein C (APC) generation by the thrombin-thrombomodulin (TM) complex. APC exerts anticoagulant, anti-inflammatory, and cytoprotective effects, which are EPCR-dependent. The thrombin-TM complex is also a potent activator of thrombin activable fibrinolysis inhibitor (TAFI), leading to impaired clot lysis. Mutations and polymorphisms identified in the EPCR gene, which can affect the efficiency of PC activation, have been associated with an increased risk of thrombosis. In this thesis we investigate the impact of impaired PC binding to EPCR on coagulation, inflammation, and fibrinolysis using novel *in vitro* and *in vivo* models. Using a murine model that harbours a variant of EPCR that does not bind PC (R84A), we demonstrate that upon thrombotic challenge, there is an increase in thrombin generation and fibrin deposition in the lungs. Upon inflammatory challenge, impaired PC/EPCR interactions also result in increased thrombin generation and increased neutrophil infiltration into the lungs. Using cells that express TM and a human variant of EPCR that does not bind PC (R96C), we demonstrate that clot lysis is delayed in normal plasma independent of TAFI activation, suggesting PC and TAFI do not compete for activation by the thrombin-TM complex. In contrast, delayed clot lysis in plasma deficient of PC is a result of greater TAFI activation by the thrombin-TM complex. Taken together, impairment of the PC pathway contributes to thrombosis through pro-coagulant, pro-inflammatory and anti-fibrinolytic mechanisms. Interestingly, mice with EPCR variant R84A, develop bone marrow failure and splenomegaly, revealing a novel role for EPCR in the bone marrow. Taken together,

PC/EPCR interactions regulate the coagulation, inflammation, and fibrinolytic pathways, which may have a significant impact on maintaining hematopoietic homeostasis.

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## Table of Contents

<b>Lay Abstract.....</b>	<b>iii</b>
<b>Abstract.....</b>	<b>iv</b>
<b>Acknowledgments .....</b>	<b>vi</b>
<b>List of Figures.....</b>	<b>xi</b>
<b>List of Tables .....</b>	<b>xiii</b>
<b>List of Abbreviations .....</b>	<b>xiv</b>
<b>CHAPTER 1: GENERAL INTRODUCTION.....</b>	<b>1</b>
<b>1.1 Introduction to hemostasis.....</b>	<b>1</b>
1.1.1 The vascular endothelium .....	1
1.1.2 Platelets .....	2
1.1.3 Coagulation Cascade.....	4
1.1.3.1 The Extrinsic Pathway.....	4
1.1.3.2 The Contact Pathway.....	7
1.1.3.3 The Common Pathway .....	8
<b>1.2 Inhibitors of coagulation .....</b>	<b>9</b>
1.2.1 Tissue factor pathway Inhibitor .....	9
1.2.2 Antithrombin.....	12
1.2.3 The protein C pathway .....	13
1.2.3.1 Activated Protein C .....	16
1.2.3.2 Protein S .....	17
<b>1.3 Fibrinolysis .....</b>	<b>18</b>
<b>1.4 Overview of thrombosis .....</b>	<b>20</b>
1.4.1 Venous thrombus formation .....	22
1.4.2 Congenital risk factors for thrombosis .....	25
1.4.3 Acquired risk factors for thrombosis .....	26
<b>1.5 Evaluating thrombotic potential .....</b>	<b>27</b>
1.5.1 Clotting Assays.....	27
1.5.2 Thrombin generation assays .....	28
1.5.3 Cell-based thrombin generation assays .....	29
<b>1.6 The endothelial protein C receptor .....</b>	<b>30</b>
1.6.1 EPCR structure .....	30
1.6.2 EPCR distribution.....	31
1.6.3 EPCR ligand interactions.....	34
1.6.4 EPCR cell signaling.....	39
<b>1.7 The role of EPCR in disease .....</b>	<b>46</b>
1.7.1 Thrombosis .....	46
1.7.2 Inflammation .....	50
1.7.3 Cancer .....	51

1.7.4 Malaria.....	52
<b>1.8 EPCR mouse models .....</b>	<b>52</b>
<b>CHAPTER 2: THESIS OVERVIEW, RATIONALE, HYPOTHESIS,</b>	
<b>OBJECTIVES AND AIMS .....</b>	<b>54</b>
2.1 Thesis overview, rationale, and hypothesis .....	54
2.2 Objectives and Aims.....	55
<b>CHAPTER 3: CHARACTERIZATION OF MICE HARBORING A VARIANT OF</b>	
<b>EPCR WITH IMPAIRED ABILITY TO BIND PROTEIN C: NOVEL ROLE OF</b>	
<b>EPCR IN HEMATOPOIESIS .....</b>	<b>56</b>
3.1 Foreword .....	56
3.2 Objective: To characterize a murine model that harbours a variant of EPCR that lacks PC/APC binding.....	56
3.3 Key Points: .....	58
3.4 Abstract .....	58
3.5 Introduction .....	60
3.6 Materials and Methods .....	62
3.6.1 Generation of WT and R84A mEPCR stable cell lines.....	62
3.6.2 Determination of the affinity of F1-APC for WT mEPCR and R84A mEPCR.....	62
3.6.3 Generation of homozygous R84A EPCR knock-in mice .....	63
3.6.4 Hematological analysis of murine blood .....	63
3.6.5 Bone marrow transplantation.....	63
3.6.6 Flow cytometric analysis of spleen and bone marrow cells .....	64
3.6.7 Colony-forming cell assays .....	65
3.6.8 Immunoblotting of tissue lysates.....	65
3.6.9 Immunohistochemistry and immunofluorescence.....	66
3.6.10 Thrombotic challenge .....	67
3.6.11 LPS Challenge .....	67
3.6.12 Analysis of murine PC, TAT and IL-6 levels.....	68
3.6.13 Statistics.....	68
<b>3.7 Results.....</b>	<b>69</b>
3.7.1 Interaction of murine F1-APC with WT EPCR and R84A EPCR .....	69
3.7.2 Generation of EPCR <sup>R84A/R84A</sup> mice .....	74
3.7.3 EPCR expression in organs of EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup> mice .....	74
3.7.4 Baseline levels of PC, TAT and IL-6 in EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup> mice .....	74
3.7.5 Thrombotic challenge in EPCR <sup>R84A/R84A</sup> mice .....	81
3.7.6 LPS challenge in EPCR <sup>R84A/R84A</sup> mice .....	84
3.7.7 EPCR <sup>R84A/R84A</sup> mice exhibit splenomegaly.....	84
3.7.8 Hematological analysis of spleen, bone marrow and peripheral blood in EPCR <sup>R84A/R84A</sup> mice .....	89
3.7.9 Bone Marrow Transplantation.....	89

<b>3.8 Discussion .....</b>	<b>98</b>
<b>CHAPTER 4: THE IMPACT OF THE ENDOTHELIAL PROTEIN C RECEPTOR ON THROMBIN GENERATION AND CLOT LYSIS.....</b>	<b>103</b>
<b>4.1 Foreword .....</b>	<b>103</b>
<b>4.2 Objective: To determine if diminished EPCR function can modulate thrombin     generation and fibrinolysis. ....</b>	<b>103</b>
<b>4.3 Summary .....</b>	<b>106</b>
<b>4.4 Introduction .....</b>	<b>108</b>
<b>4.5 Materials and Methods .....</b>	<b>111</b>
4.5.1 Generation of stable cell lines expressing TM and the EPCR variant R96C .....	111
4.5.2 Flow Cytometry .....	111
4.5.3 Thrombin generation assay on cells .....	112
4.5.4 Plasma clot lysis in the presence of cells.....	113
4.5.5 Protein C activation on HEK293 cells expressing TM and EPCR.....	113
4.5.6 TAFI activation on HEK293 cells expressing TM and EPCR .....	114
4.5.7 Statistical Analyses.....	115
<b>4.6 Results.....</b>	<b>116</b>
4.6.1 TM and EPCR expression on HEK293 cells.....	116
4.6.2 Protein C activation by HEK293 cells expressing TM and EPCR.....	116
4.6.3 TAFI activation by HEK293 cells expressing TM and EPCR .....	119
4.6.4 Thrombin generation in the presence of cells expressing TM and EPCR.....	124
4.6.5 Clot lysis in the presence of cells .....	127
<b>4.7 Discussion .....</b>	<b>131</b>
<b>CHAPTER 5.0 GENERAL DISCUSSION AND FUTURE DIRECTIONS .....</b>	<b>136</b>
<b>5.1 EPCR and coagulation .....</b>	<b>138</b>
<b>5.2 EPCR and Inflammation .....</b>	<b>151</b>
<b>5.3 EPCR and Fibrinolysis.....</b>	<b>154</b>
<b>5.4 EPCR and hematopoiesis.....</b>	<b>159</b>
<b>5.5 Conclusions.....</b>	<b>164</b>

## List of Figures

Figure 1.1	The coagulation and fibrinolytic systems	6
Figure 1.2	Fibrin formation	11
Figure 1.3	The protein C pathway	15
Figure 1.4	Proposed mechanism for venous thrombus formation	24
Figure 1.5	Structure of soluble EPCR	33
Figure 1.6	EPCR-mediated anti-inflammatory effects	43
Figure 1.7	EPCR-mediated anti-apoptotic effects	45
Figure 1.8	EPCR-mediated barrier protective effects	48
Figure 3.1	Flow cytometric analysis of HEK293 cells expressing murine WT or R84A EPCR	71
Figure 3.2	Flow cytometric analysis of human and murine F1-FVIIa and F1-FXa binding to mEPCR	73
Figure 3.3	Detection of mice homozygous for the EPCR R84A mutation by PCR	76
Figure 3.4	EPCR expression in EPCR <sup>R84A/R84A</sup> mice	78
Figure 3.5	Thrombotic challenge of EPCR <sup>WT/WT</sup> , EPCR <sup>WT/R84A</sup> , and EPCR <sup>R84A/R84A</sup> mice	83
Figure 3.6	Inflammatory challenge of EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup> mice	86
Figure 3.7	Morphological and histochemical abnormalities in the spleens of EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup>	88
Figure 3.8	Colony forming cell assays with bone marrow, spleen, and peripheral blood cells	94
Figure 3.9	Hematological analysis of the bone marrow and spleen in bone marrow transplanted EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup>	97

	mice	
Figure 4.1	Flow cytometric analysis of cells expressing TM and EPCR	118
Figure 4.2	APC generation in the presence of cells expressing TM and EPCR	121
Figure 4.3	TAFI generation in the presence of cells expressing TM and EPCR	123
Figure 4.4	Thrombin generation in the presence of cells expressing TM and EPCR	126
Figure 4.5	Clot lysis in the presence of cells expressing TM and EPCR	129
Figure 5.1	Impact of impaired PC/EPCR interactions on coagulation, inflammation, and fibrinolysis	140
Figure 5.2	APC generation by TM and EPCR expressing cells in the presence of TM and EPCR blocking antibodies	148
Figure 5.3	APC and thrombin generation in plasma from patient with suspected antibody towards PC pathway in the presence of TM and EPCR expressing cells	150
Figure 5.4	A proposed mechanism for bone marrow failure in EPCR <sup>R84A/R84A</sup> mice	163

### List of Tables

Table 1.1	Alignment of the regions of the Gla domain of hemostatic proteins involved in EPCR binding	37
Table 3.1	Comparison of baseline PC, TAT, and IL-6 antigen levels in EPCR <sup>WT/WT</sup> and EPCR <sup>R84A/R84A</sup> mice	80
Table 3.2	Hematological analysis of the spleen and bone marrow from EPCR <sup>R84A/R84A</sup> mice	90
Table 3.3	Hematological analysis of peripheral blood EPCR <sup>R84A/R84A</sup> mice	92

### **List of Abbreviations**

<b>5IAf-Pg</b>	5-idoacetamidofluorescién-plasminogen
<b>Ang</b>	angiopoietin
<b>ANOVA</b>	1-way analysis of variance
<b>APC</b>	activated protein C
<b>AT</b>	antithrombin
<b>BM</b>	bone marrow
<b>BSA</b>	bovine serum albumin
<b>CD</b>	cluster of differentiation antigen
<b>cDNA</b>	complementary DNA
<b>CLP</b>	cecal ligation and puncture
<b>DAPI</b>	4,6 diamidino-2-phenylindole
<b>DC</b>	domian cassettes
<b>DMEM</b>	Dulbecco modified Eagle medium
<b>Dvl-2</b>	disheveled-2
<b>DVT</b>	deep vein thrombosis
<b>E</b>	embryonic day
<b>EGF</b>	epidermal growth factor
<b>ELISA</b>	enzyme-linked immunosorbent assay

<b>EPCR</b>	endothelial protein C receptor
<b>ETP</b>	endogenous thrombin potential
<b>F</b>	factor
<b>F1.2</b>	Fragment 1+2
<b>FBS</b>	fetal bovine serum
<b>FDP</b>	fibrin degradation products
<b>FITC</b>	fluorescein isothiocyanate
<b>Fl</b>	fluorescein
<b>FPRck</b>	Phe-Pro-Arg-chloromethylketone
<b>Gla</b>	$\gamma$ -carboxyglutamic acid
<b>GP</b>	glycoprotein
<b>h</b>	human
<b>H&amp;E</b>	hematoxylin and eosin
<b>HBS</b>	HEPES buffered saline
<b>HEK293</b>	human embryonic kidney cell
<b>HIT</b>	heparin-induced thrombocytopenia
<b>HK</b>	high molecular weight kininogen
<b>HRP</b>	horseradish peroxidase
<b>HSC</b>	hematopoietic stem cell



<b>HSPC</b>	hematopoietic stem and progenitor cells
<b>IL</b>	interleukin
<b>IVC</b>	inferior vena cava
<b>LPS</b>	lipopolysaccharide
<b>LT-HSC</b>	long-term hematopoietic stem cell
<b>m</b>	murine
<b>MFI</b>	mean fluorescence intensity
<b>MP</b>	microparticle
<b>MPO</b>	myeloperoxidase
<b>NET</b>	neutrophil extracellular trap
<b>NO</b>	nitric oxide
<b>nfK<math>\beta</math></b>	nuclear transcription factor
<b>PAI-1</b>	plasminogen activator inhibitor-1
<b>PAR</b>	protease activated receptors
<b>PBS</b>	phosphate buffered saline
<b>PC</b>	protein C
<b>PCPS</b>	phospholipid vesicles
<b>PCR</b>	polymerase chain reaction
<b>PCTDP</b>	plasma deficient in both PC and TAFI

<b>PE</b>	phycoerythrin
<b>PfEMP1</b>	Plasmodium falciparum erythrocyte membrane protein 1
<b>PK</b>	prekallikrein
<b>PR3</b>	proteinase-3
<b><i>PROCR</i></b>	endothelial protein C receptor gene
<b>ProcrLox</b>	conditional EPCR knockout mouse
<b>PS</b>	phosphatidylserine
<b>PTCI</b>	potatoe tuber carboxypeptidase inhibitor
<b>RCL</b>	reactive center loop
<b>S1P</b>	sphingosine 1 phosphate
<b>S1P1</b>	sphingosine 1-phosphate receptor
<b>SE</b>	standard error
<b>sEPCR</b>	soluble EPCR
<b>serpin</b>	serine protease inhibitor
<b>SNP</b>	single-nucleotide polymorphism
<b>sphk-1</b>	sphingosine kinase 1
<b>sTF</b>	soluble tissue factor
<b>sTM</b>	soluble TM
<b>TAFI</b>	thrombin activable fibrinolysis inhibitor

<b>TAT</b>	thrombin-antithrombin complex
<b>TCR</b>	T cell antigen receptor
<b>TF</b>	tissue factor
<b>TFPI</b>	tissue factor pathway inhibitor
<b>Tie2</b>	tyrosine kinase with immunoglobulin-like EGF-like domains 2
<b>TM</b>	thrombomodulin
<b>TNF<math>\alpha</math></b>	tumor necrosis factor
<b>tPA</b>	tissue plasminogen activator
<b>uPA</b>	urokinase plasminogen activator
<b>UTR</b>	untranslated region
<b>VFKcK</b>	Val-Phe-Lys-chloromethylketone
<b>VKD</b>	vitamin-K dependent
<b>VTE</b>	venous thromboembolism
<b>vWF</b>	von Willebrand factor
<b>WT</b>	wild-type
<b><math>\alpha</math>2-AP</b>	$\alpha$ 2-antiplasmin

## **CHAPTER 1: GENERAL INTRODUCTION**

### **1.1 Introduction to hemostasis**

Hemostasis is a physiological process to maintain blood in a fluid state and to prevent blood loss after vessel injury. The hemostatic process involves both cellular and biochemical events to maintain a delicate balance between the processes of clot formation and clot lysis. In primary hemostasis, platelets aggregate and adhere to components of the subendothelium that is exposed as a result of mechanical, physical or chemical injury to the vessel. The outcome of primary hemostasis is the formation of a mechanical platelet plug to prevent bleeding at the site of an injury. In secondary hemostasis, the coagulation cascade leads to the generation of thrombin, a central event in the hemostatic process. The generation of thrombin converts soluble fibrinogen to insoluble fibrin, forming a fibrin meshwork that stabilizes the platelet plug at the site of injury. The outcome of secondary hemostasis is the formation of a thrombus. A disruption in the hemostatic balance can lead to either hemorrhage or the development of thromboembolic disorders, both of which can be life threatening.

#### **1.1.1 The vascular endothelium**

The vascular endothelium is essential for maintaining the fluidity of the blood by acting as a natural barrier from the prothrombotic underlying tissues. Under physiologic conditions the endothelium provides a thromboresistant surface by preventing platelet adhesion, inhibiting coagulation and releasing fibrinolytic enzymes (Bombeli et al.,

1997). In addition, the endothelium also produces nitric oxide (NO), prostacyclin, and adenosine di-phosphatase to prevent platelet activation (Colman et al., 2001). However, the vascular endothelium is a dynamic system, which under pathophysiological conditions will adapt to the demands of the microenvironment. When the integrity of the vessel wall is disrupted, due to injury or enhanced endothelial cell barrier permeability, collagen located in the basement membrane is exposed, initiating platelet activation. Endothelial cells secrete von Willebrand factor (vWF), allowing for platelet adhesion to the subendothelial collagen (Golebiewska and Poole, 2015). In addition, endothelial cells also express adhesion molecules such as P-selectin and platelet endothelial cell adhesion molecules to facilitate platelet adhesion (Colman et al., 2001). The recruitment and localization of platelets at the endothelial cell surface is important for two reasons: 1) the formation of the hemostatic plug is necessary to prevent bleeding from the injured vessel, and 2) it is important that activated platelets remain localized to the site of tissue injury.

### **1.1.2 Platelets**

Platelets are anucleate cells produced by bone marrow megakaryocytes that circulate in blood in a discoid, non-adherent state (Italiano, Jr. and Shivdasani, 2003). Upon disruption or activation of the endothelial cell wall, platelet adhesion to the subendothelial collagen is mediated by platelet receptor glycoprotein (GP)Ib $\alpha$  and its ligand vWF and other collagen receptors (GPVI, and GPIa) (Ruggeri, 1997). Interaction of GPIb $\alpha$  with vWF also induces platelets to change their shape to become more rounded and form pseudopods (Ruggeri, 1997). Platelet adhesion to the site of injury is further stabilized through the interaction of platelet integrin  $\alpha$ IIb $\beta$ 3 to fibrin(ogen) and

fibronectin,  $\alpha 5\beta 1$  to fibronectin or collagen, and  $\alpha 2\beta 1$  to collagen (Hou et al., 2015). Knockout of vWF or GPIb $\alpha$  in mice results in decreased platelet adhesion and prolonged bleeding times (Denis et al., 1998; Bergmeier et al., 2006). Deficiencies in GPVI or integrins can also result in impaired hemostatic function, though less severe (Nuyttens et al., 2011).

The interaction of GPIb $\alpha$  and integrins with their ligands can lead to platelet activation (Ruggeri, 1997; Wang et al., 2012; Hou et al., 2015). In addition, thrombin is also a potent activator of platelets through binding of GPIb $\alpha$  and cleavage of protease activated receptors (PAR) 1 and 4 on platelets (Andersen et al., 1999; Ramakrishnan et al., 2001; Adam et al., 2003; Kahn et al., 1998). Upon activation, phosphatidylserine (PS) is exposed on the platelet membrane surface promoting thrombin generation and thus further platelet activation (Roberts et al., 2006; Monroe et al., 2002). Activation of platelets by thrombin or other platelet ligands can induce signaling events leading to the release of platelet  $\alpha$ -granules and dense granules (Sambrano et al., 2001). The contents released from platelet granules, which include adenosine di-phosphate and  $\text{Ca}^{2+}$ , promote further platelet activation and granule release (Johnston-Cox et al., 2011; Bergmeier and Stefanini, 2009). In addition, platelets generate thromboxane  $\text{A}_2$  and reactive  $\text{O}_2$  species further amplifying platelet activation (Jin et al., 2002; Arthur et al., 2008). Following platelet activation, platelet aggregation occurs through the interaction of integrin  $\alpha \text{IIb}\beta 3$  and fibrin(ogen), resulting in the formation of a hemostatic plug at the site of injury (Hou et al., 2015).

### **1.1.3 Coagulation Cascade**

The coagulation cascade makes up a critical component of the hemostatic response. A fundamental principle of blood coagulation is the sequential conversion of inactive zymogens to proteolytic enzymes. This process is divided into the extrinsic, contact and common pathways. An overview of the coagulation cascade is provided in Figure 1.1.

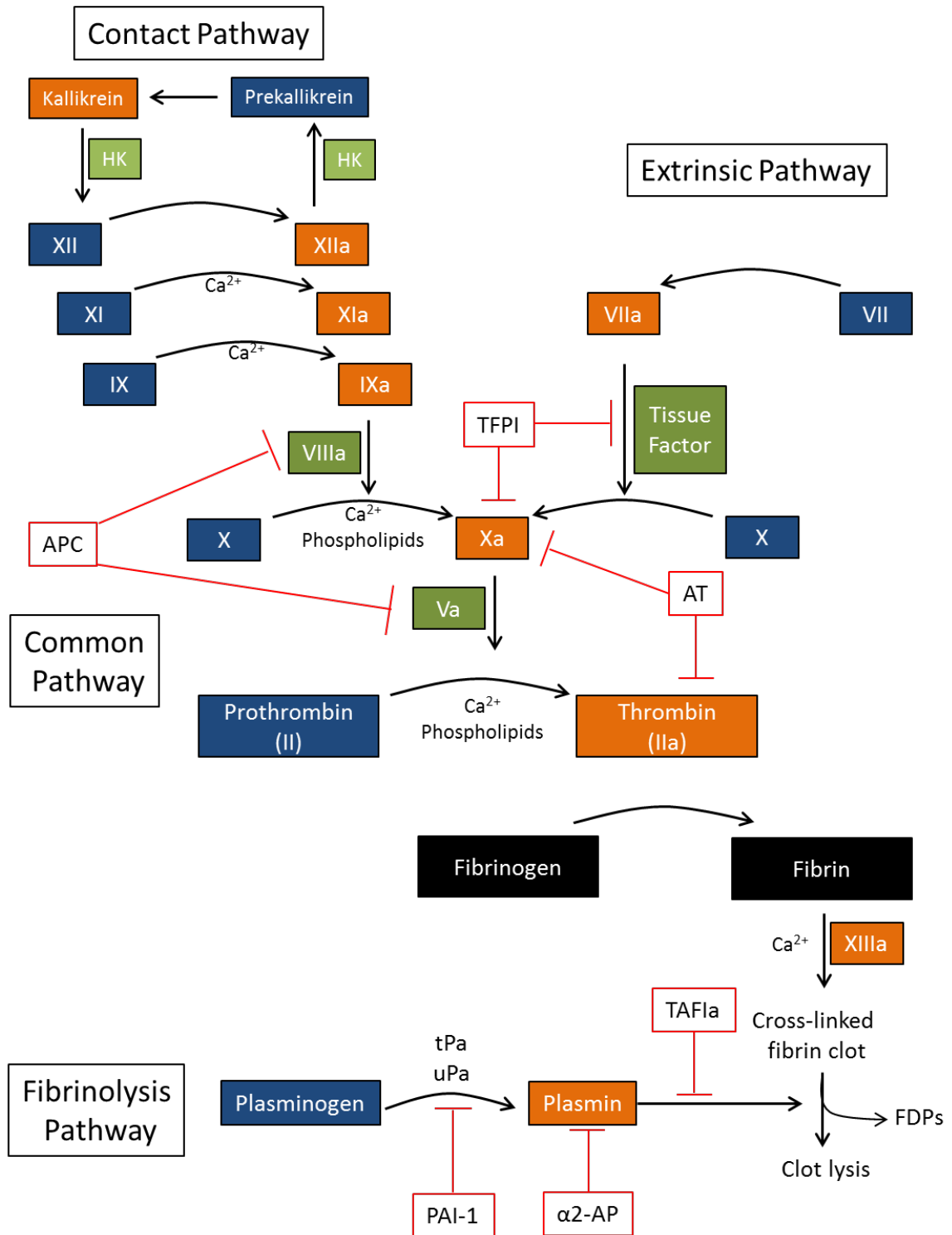
#### **1.1.3.1 The Extrinsic Pathway**

The extrinsic pathway is initiated upon exposure of tissue factor (TF) to the blood. TF is a 47 kDa transmembrane GP that is abundantly expressed by extravascular cells such as fibroblasts and vascular smooth muscle cells (Osterud and Bjorklid, 2006; Drake et al., 1989). Vascular cells that are in contact with blood do not express active TF, however TF procoagulant activity can be induced as a response to injury or inflammatory stimuli in a process known as TF decryption (Mackman et al., 2007; Erlich et al., 1999; Bach, 2006). Once active TF is exposed to the blood, it will bind coagulation factor (F) VII (FVII) and act as a cofactor by promoting proteolysis and activation of FVII to activated FVII (FVIIa). The TF/FVIIa complex proteolytically cleaves FIX to FIXa and FX to FXa on the TF-bearing cell surface (ten Cate et al., 1993; Osterud and Rapaport, 1977). FXa will then bind cofactor FVa to form the prothrombinase complex and together they convert a small amount of prothrombin to thrombin (Monroe et al., 1996). This small amount of thrombin initiates a positive feedback loop on itself through further activation of FV as well as activation of FVIII (Esmon, 1979; Fulcher et al., 1983). The generation of thrombin will also lead to further activation of platelets and the exposure of

**Figure1.1: The coagulation and fibrinolytic systems**

The coagulation and fibrinolytic pathway are made up of the sequential conversion of inactive zymogens (blue) to proteolytic enzymes (orange) in the presence of their cofactors (green). The coagulation process is divided into the extrinsic, contact and common pathways. The ultimate goal of the pathways is to generate thrombin, which can convert soluble fibrinogen to an insoluble fibrin clot. Once hemostasis is restored, the clot is removed by the fibrinolytic pathway. The end product of the fibrinolytic pathway is plasmin which enzymatically digests the fibrin clot. Both the coagulation and fibrinolytic pathways are negatively regulated by inhibitors (red) to prevent uncontrolled clot formation and clot lysis. Activated protein C (APC), Antithrombin (AT), fibrinogen degradation products (FDPs), high molecular weight kininogen (HK), plasminogen activator inhibitor-1 (PAI-1), tissue factor pathway inhibitor (TFPI),  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP). (Figure adapted from Sphan, BT. Br J Anaesth. 2004 93:275-87).





procoagulant phospholipids such as PS on the platelet surface, which provide an anionic membrane for coagulation complexes to assemble (Monroe et al., 1996). The formation of the intrinsic tenase complex (which consists of FVIIIa and FIXa) and the prothrombinase complex (consisting of FVa and FXa) on the surface of activated platelets results in acceleration of FX activation and thrombin generation, respectively (Hoffman and Monroe, III, 2001). Deficiency of TF or FX in humans has not been identified, and therefore likely not compatible with life. In contrast, humans with a severe deficiency of FVII, FVIII (hemophilia A) or FIX (hemophilia B) develop severe bleeding complications, thus underlining the importance of these coagulation factors for normal hemostasis (Tuddenham et al., 1995; Dent et al., 1990).

#### **1.1.3.2 The Contact Pathway**

The contact pathway (also known as the intrinsic pathway) is initiated by exposure of blood to negatively charged molecular surfaces. Non-physiological activators of the contact pathway include kaolin, glass, and silica (Colman and Schmaier, 1997). More recently, physiological activators of the contact pathway have been identified and include collagen (van der Meijden et al., 2009), polyphosphates (Muller et al., 2009), and nucleic acids (von Bruhl et al., 2012). The contact system consists of 4 plasma proteins: FXII, FXI, prekallikrein (PK) and high molecular weight kininogen (HK) (Colman and Schmaier, 1997). Once FXII is bound with a negatively charged surface, a conformational change takes place and FXII is auto-activated. FXIIa catalyzes the activation of PK to kallikrein. In plasma ~ 75% of PK circulates non-covalently bound to HK, which acts as a non-enzymatic cofactor by localizing kallikrein to negatively

charged surfaces (Colman and Schmaier, 1997). Kallikrein feedbacks on FXII, resulting in increased FXII activation. FXIIa in complex with cofactor HK, catalyzes the activation of FXI, resulting in further activation of the downstream coagulation factors FIX, and FX. In contrast to the extrinsic pathway, deficiencies in any of the contact system factors do not result in bleeding complications. As a result the contact pathway was thought not to play an important role in normal hemostasis and only serve as an amplification loop for the extrinsic pathway (Wu, 2015;Pauer et al., 2004). However more recently, a role for contact activation in pathological coagulation has emerged. In support of this, mice deficient in FXII are resistant to thrombotic occlusion (Cheng et al., 2010). These more recent findings suggest a greater role for the contact pathway than was previously understood.

### **1.1.3.3 The Common Pathway**

The converging of the contact and extrinsic pathways forms the final common pathway of the coagulation cascade. FXa generated by both the contact and extrinsic pathway (along with its cofactor FVa), cleaves prothrombin at Arg<sup>320</sup> and Arg<sup>271</sup>, releasing fragment 1 +2 (F1.2), generating active thrombin (Esmon and Jackson, 1974). The loss of F1.2 exposes the functional regions of thrombin (active site and exosite I and II) and allows thrombin to dissociate from phospholipid surfaces to interact with its target substrates. The primary function of thrombin is to convert soluble fibrinogen to an insoluble fibrin clot. The cleavage of fibrinopeptide A from the fibrinogen A $\alpha$  chain by thrombin leads to the formation of fibrin monomers which spontaneously polymerize to form protofibrils (Wolberg and Campbell, 2008). Cleavage of fibrinopeptide B from

fibrinogen B $\beta$  chain by thrombin forms protofibrils that undergo lateral aggregation to form a meshwork of fibrin (Wolberg and Campbell, 2008). Finally, thrombin activation of FXIII results in the formation of covalent crosslinks between adjacent fibrin chains to stabilize and reinforce the platelet plug formed during primary hemostasis (Ariens et al., 2002). This process is illustrated in Figure 1.2.

## **1.2 Inhibitors of coagulation**

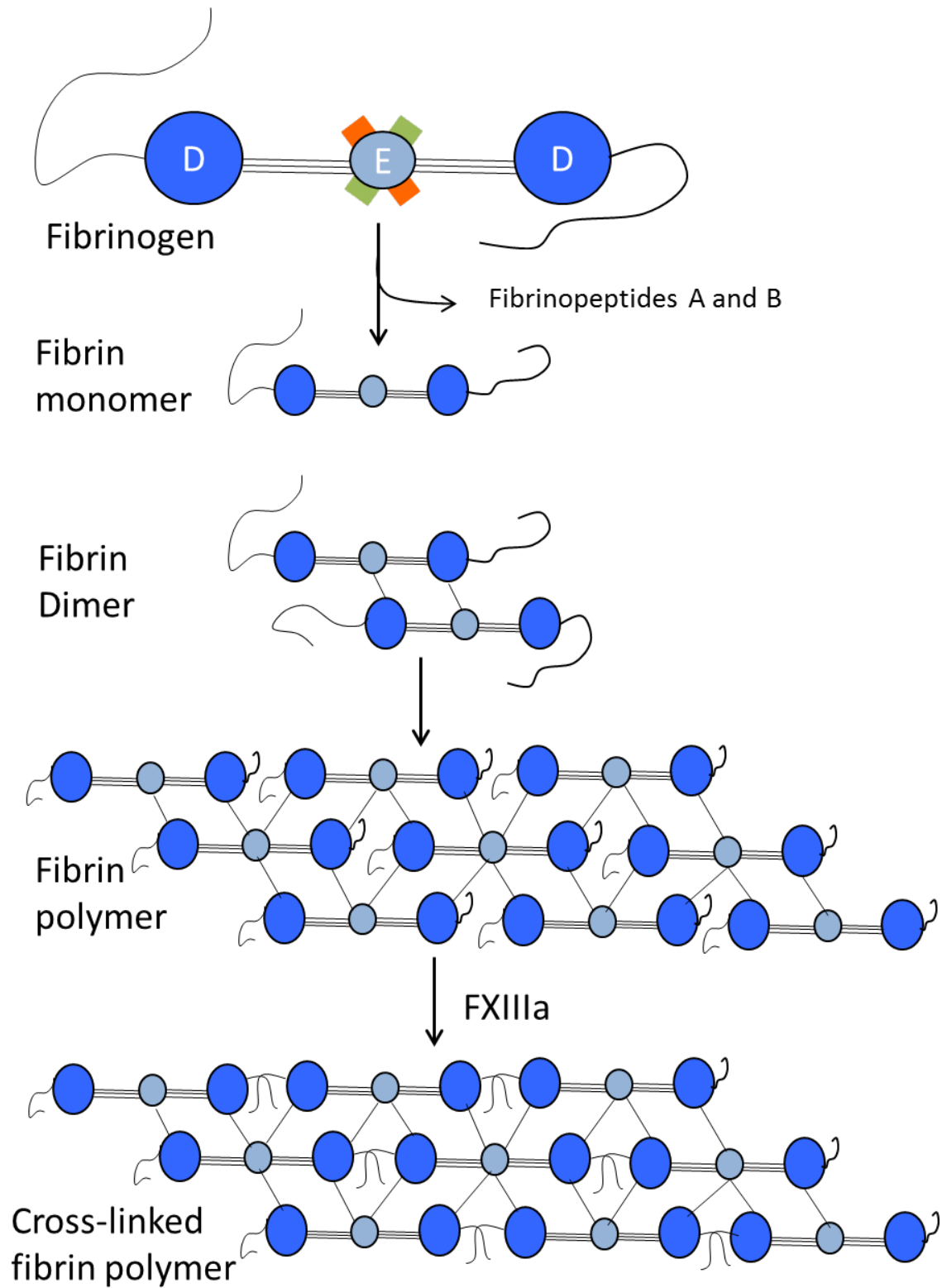
The coagulation cascade is essential for the formation of a thrombus at the site of injury, however the size of a thrombus must be controlled to prevent the occlusion of the blood vessel. As a result of thrombin's ability to amplify its own generation, the negative regulation of coagulation is essential for limiting widespread clot formation. The coagulation cascade is negatively regulated by a number of natural anticoagulant mechanisms, including tissue factor pathway inhibitor (TFPI), antithrombin (AT), and the protein C (PC) pathway (Figure 1.1).

### **1.2.1 Tissue factor pathway Inhibitor**

TFPI is a Kunitz-type protease inhibitor that is present in platelets, on endothelial cells and circulates in plasma at a concentration of  $\sim 200$  pM (Broze, Jr. and Girard, 2012). TFPI inhibits coagulation in two ways: 1) by forming a quaternary complex with TF/FVIIa/FXa and 2) by direct inhibition of FXa (Girard et al., 1989). Kunitz domain 1 in TFPI inhibits the TF/FVIIa complex when FXa is present, and Kunitz domain 2 blocks FXa activity (Girard et al., 1989). More recently it has been demonstrated that protein S acts as a cofactor for TFPI-dependent inhibition of FXa by increasing the affinity of TFPI

### **Figure 1.2: Fibrin Formation**

Fibrinogen consists of 3 pairs of polypeptides ( $A\alpha$ ,  $B\beta$ ,  $\gamma$ ) that are covalently linked near their N-terminals through disulfide bonds, which form the central E domain. The D domain consists of the C-terminal ends of the  $B\beta$  and  $\gamma$  chains. The C-terminal end of the  $A\alpha$  chain protrudes from the D domain and can interact with each other during fibrin clot crosslinking. Thrombin-mediated conversion of fibrinogen to fibrin involves the cleavage of the N-terminal end of the  $A\alpha$  polypeptide, resulting in the formation of fibrin monomers, which spontaneously polymerize to form protofibrils. Cleavage of the N-terminal end of the  $B\beta$  polypeptide by thrombin forms protofibrils that undergo lateral aggregation leading to the formation of the fibrin clot. FXIII further stabilizes the clot by crosslinking fibrin. (Figure adapted from Wolberg AS. Blood. 2007;21:131-142).



for FXa (Hackeng et al., 2006). TFPI contains a third Kunitz domain but its role has yet to be established. Humans with complete TFPI deficiency have not been identified and genetic deletion of TFPI in mice results in embryonic lethality, suggesting that TFPI plays a significant role in maintaining the hemostatic balance (Huang et al., 1997). It has been shown that low levels of TFPI (<10% of normal) in humans are associated with a higher risk of thrombosis (Dahm et al., 2003). In contrast, increased plasma TFPI levels have been associated with bleeding (Vincent et al., 2013). Known as East Texas bleeding disorder, increased plasma TFPI levels are indirectly caused by an alternatively spliced FV that forms a complex with TFPI (Kuang et al., 2001; Vincent et al., 2013). The TFPI/FV complex is retained in the circulation and inhibits thrombin generation by the contact and extrinsic pathway (Vincent et al., 2013).

### **1.2.2 Antithrombin**

AT is a serine protease inhibitor (serpin) that inhibits thrombin generation by inactivating FIXa, FXa, FXIa, FXIIa, and thrombin (Colman et al., 2001). Serpins (including AT) inactivate their target proteases by displaying their reactive center loop (RCL), which is cleaved by the target protease. Following cleavage of the serpins RCL, the protease becomes covalently linked to the serpin and the protease catalytic domain is destroyed, rendering the protease inactive (Lawrence et al., 1995). The ability of AT to inactivate coagulation proteases is further enhanced (~1000 fold) in the presence of glycosaminoglycans, such as heparin and heparan sulfate. Heparins contain a distinct pentasaccharide sequence that binds AT with high affinity inducing a conformational change in AT, accelerating the rate of inactivation of FXa (Olson et al., 1992). For

heparin to accelerate AT-mediated inhibition of thrombin, the heparin chain must be of sufficient length (at least 18 saccharide units) to facilitate bridging of thrombin (Bray et al., 1989).

AT is responsible for approximately 80% of the thrombin inhibition in plasma making it the most important physiological regulator of both thrombin generation and thrombin activity (Fischer et al., 1981). The importance of AT as a regulator of coagulation is demonstrated by the absence of complete AT deficiency in humans, suggesting AT deficiency is not compatible with life. Furthermore, complete deficiency of AT in mice results in embryonic lethality (Ishiguro et al., 2000). In humans, mutations in AT can result in an abnormal reactive domain or an abnormal heparin binding domain, reducing the ability of AT to inhibit its target proteins and increasing the risk of thrombosis (Patnaik and Moll, 2008).

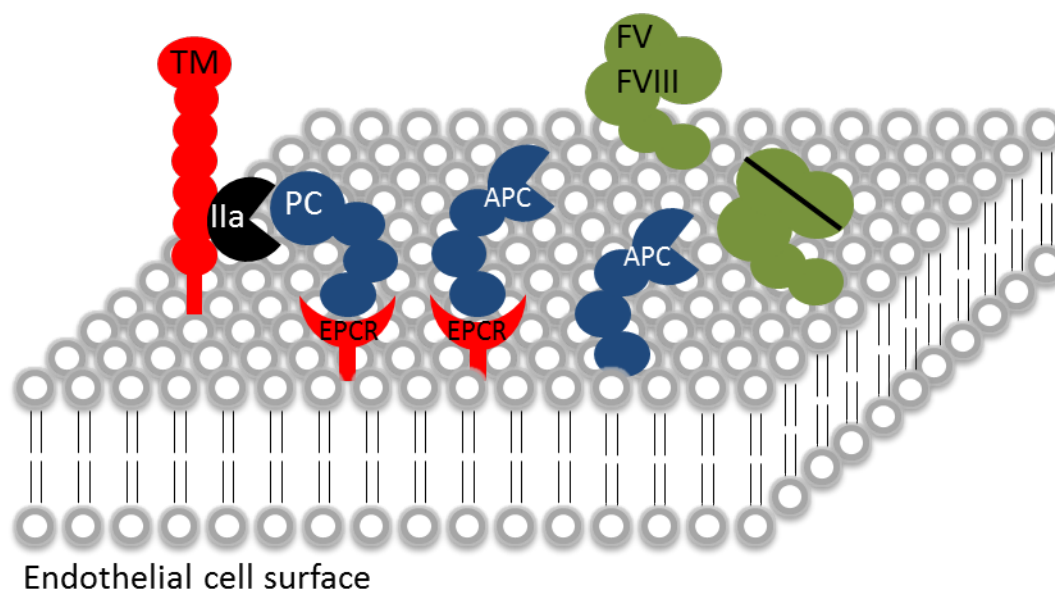
### **1.2.3 The protein C pathway**

The PC pathway limits the amplification and progression of the coagulation cascade through inhibition of the thrombin-mediated positive feedback mechanism (Figure 1.3). PC, a vitamin-K dependent (VKD) GP, is converted by thrombin to its active form, activated PC (APC). However, this reaction is very inefficient and does not produce physiologically relevant concentrations of APC. However, as thrombin levels increase, thrombin will begin to bind thrombomodulin (TM) on the surface of endothelial cells. TM, a 60 kDa transmembrane protein, consists of an N-terminal lectin-like domain, 6 epidermal growth factor (EGF)-like repeats and a serine/threonine-rich domain



**Figure 1.3 The protein C pathway**

Upon thrombin generation, thrombin will bind to thrombomodulin (TM) on the endothelial cell surface. Protein C (PC) bound to its receptor, endothelial protein C receptor (EPCR), presents PC to the thrombin/TM complex, thereby increasing the ability of thrombin to convert PC to activated PC (APC). APC, along with its cofactor protein S (not shown) inactivates FVa and FVIIIa at the endothelial cell surface, down-regulating thrombin generation. (Figure adapted from Rao VM. et al. Blood. 2014;124:1553-62).



(Tsiang et al., 1992). Exosite I of thrombin binds to TM EGF-like repeats 5 and 6 and as a complex will increase the efficiency of thrombin converting PC to APC by approximately 1000-fold (Tsiang et al., 1992; Esmon, 1989). Conversion of PC to APC is further increased by approximately 20-fold *in vivo* when PC, bound to its receptor, endothelial protein C receptor (EPCR), comes into contact with the thrombin-TM complex (Stearns-Kurosawa et al., 1996). Cleavage of PC by thrombin occurs at Arg<sup>169</sup>, resulting in the removal of the activation peptide. APC is then released from EPCR and along with its cofactor protein S, downregulates the thrombin feedback loop by inactivating FVa and FVIIIa (Wildhagen et al., 2011; Hoffman and Monroe, III, 2001).

Complete deficiency of TM, EPCR or PC in mice results in embryonic lethality or neonatal death (Healy et al., 1995; Gu et al., 2002; Jalbert et al., 1998). In humans, homozygous PC deficiency results in neonatal purpura fulminans and venous thrombosis, which can be fatal (Marlar and Neumann, 1990). In addition, acquired PC deficiency can be the result of excessive activation of coagulation, resulting in consumption of PC (Griffin et al., 1982) or an inability to activate PC efficiently due to shedding of EPCR (Faust et al., 2001). These outcomes underline the importance of the PC pathway to normal hemostasis.

#### **1.2.3.1 Activated Protein C**

APC contains an N-terminal  $\gamma$ -carboxyglutamic acid (Gla)-domain, two EGF-like domains, a linking peptide, and a trypsin-like serine protease domain that contains the catalytic triad responsible for proteolysis of FVa and FVIIIa (Griffin et al.,

2007; Wildhagen et al., 2011). The Gla domain of APC has 7  $\text{Ca}^{2+}$  binding sites which when occupied, result in a conformational change allowing APC to interact with the phospholipid membrane and EPCR (Zhang et al., 1992). In addition, the Gla domain also contributes to the interaction of APC with its cofactor protein S (Wildhagen et al., 2011). It has been demonstrated that mutation of key residues within the Gla domain of APC can significantly reduce its anticoagulant activity, underlining the importance of this domain for normal APC function (Zhang et al., 1992).

The catalytic site of APC inactivates FVa by proteolysis at Arg<sup>306</sup>, Arg<sup>506</sup>, Arg<sup>679</sup>, and FVIIIa at Arg<sup>336</sup>, Arg<sup>562</sup>, Arg<sup>740</sup> (Wildhagen et al., 2011). Mutations within the catalytic domain of APC, which affect the interaction and cleavage of FVa and FVIIIa also reduces the anticoagulant activity of APC (Mosnier et al., 2004; Mosnier et al., 2007a). The activity of APC is inhibited by a number of serpins including protein C inhibitor  $\alpha_1$ -antitrypsin and  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP) as well as the protease inhibitor  $\alpha_2$ -macroglobulin (Scully et al., 1993; Heeb et al., 1991). However, these inhibitors of APC are non-specific and also inhibit a large variety of proteases. In addition to its anticoagulant properties, APC also has anti-inflammatory and cytoprotective effects, which are largely dependent on its interaction with EPCR and will be discussed in further detail in section 1.6.4.

### **1.2.3.2 Protein S**

As a non-enzymatic cofactor for APC, protein S shares some homology with APC. Protein S contains an N-terminal Gla-domain, four EGF-like domains and two laminin G-type domains (Castoldi and Hackeng, 2008). Protein S is synthesized by the liver and

endothelial cells and circulates in plasma in a free form (~40%) or in complex with the complement regulatory factor C4b-binding protein (~60%) (Dahlback and Stenflo, 1981). Protein S enhances APC-mediated cleavage of FVa at Arg<sup>306</sup> and FVIIIa at Arg<sup>336</sup> and Arg<sup>562</sup> (Rosing et al., 1995; van de Poel et al., 2001). The APC co-factor activity of protein S requires interaction of the protein S Gla domain and EGF1 domain with APC, and requires protein S to be bound to phospholipid membranes (Saller et al., 2005; Hackeng et al., 2000; Hackeng et al., 1993). Protein S bound to C4b-binding protein has reduced APC co-factor activity (Maurissen et al., 2008). In addition to protein S as a cofactor for APC, protein S is also a cofactor for TFPI inhibition of FXa (Hackeng et al., 2006; Hackeng et al., 2006). Similar to PC-deficiency, protein S deficiency is also associated with a higher risk of thrombosis (Faioni et al., 1997).

### **1.3 Fibrinolysis**

Like the coagulation cascade, fibrinolysis (Figure 1.1) is a highly regulated enzymatic process that serves to control the size of a thrombus and ultimately prevent vessel occlusion. Plasminogen, which circulates in plasma in zymogen form is converted to plasmin by tissue plasminogen activator (tPA) (released from endothelial cells) or urokinase plasminogen activator (uPA) (released from monocytes and macrophages) (Cesarman-Maus and Hajjar, 2005). Once activated, plasmin initiates a positive feedback loop on itself by cleaving single-chain tPA and/or uPA into their more active two-chain form (Cesarman-Maus and Hajjar, 2005; Tate et al., 1987). In the absence of fibrin, tPA is a weak activator of plasminogen and can be rapidly inhibited by plasminogen activator inhibitor-1 (PAI-1) that is released by endothelial cells, and platelets (Sprengers and

Kluft, 1987). In the presence of fibrin, both tPA and plasminogen bind to its surface, and the catalytic efficiency for plasminogen activation by tPA is increased by approximately 500-fold (Hoylaerts et al., 1982). Plasmin that remains bound to fibrin is protected from inhibition by  $\alpha_2$ -AP (Schneider and Nesheim, 2004). Plasmin cleaves fibrin resulting in the generation of FDP and exposure of C-terminal lysine residues (Cesarman-Maus and Hajjar, 2005). Kringle domains in both tPA and plasminogen contain lysine binding sites which further mediate their binding to fibrin leading to further fibrin degradation (Cesarman-Maus and Hajjar, 2005). Binding of tPA and plasminogen can be blocked by thrombin-activable fibrinolysis inhibitor (TAFI) which is activated by thrombin-bound TM (Bajzar et al., 1995). In its active form, TAFIa enzymatically removes lysine residues on fibrin, impairing tPA and plasminogen binding and slowing plasmin generation leading to stabilization of the fibrin clot (Cesarman-Maus and Hajjar, 2005).

Fibrinolytic disorders, including both congenital and acquired, can be a result of a disruption in the balance of fibrinolytic components. This can result in either hyperfibrinolysis resulting in bleeding or hypofibrinolysis resulting in thrombosis. Bleeding as a result of hyperfibrinolysis can be the result of deficiencies in inhibitors including PAI-1 and  $\alpha_2$ -AP or the result of the consumption of fibrinogen during disseminated intravascular coagulation (Mehta and Shapiro, 2008; Carpenter and Mathew, 2008; Hunt, 2014). Hyperfibrinolysis may also be the result of increased release of tPA from endothelial cells combined with the inhibition of PAI-1 release that is often seen in septic shock (Hess et al., 2008). Individuals with hypothyroidism or those who are pregnant may experience thrombotic complications as a result of hypofibrinolysis, which

can be caused by elevated levels of PAI-1,  $\alpha_2$ -AP, TAFI, or fibrinogen (Mazur et al., 2014; Hellgren, 1996). Interestingly, plasminogen deficiency is not associated with thrombosis, but can cause ligneous mucositis (Schuster et al., 2007). Congenital deficiency of tPA and uPA in humans has not been reported, however knockout of tPA or uPA in mice does result in increased fibrin deposition and reduced clot lysis (Carmeliet et al., 1994).

#### **1.4 Overview of thrombosis**

When the regulatory mechanisms of hemostasis become overwhelmed and are unable to control thrombin generation and activity, the result is thrombosis. Thrombosis is a critical event that has the potential to occlude a vessel, preventing blood flow to vital tissues and organs. Arterial thrombosis is a major underlying cause of myocardial infarction and stroke, and venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism, is also associated with significant morbidity and mortality. Based on U.S. rates of incidence, approximately 200,000 people are afflicted with DVT per year in Canada. In approximately 60,000 of these cases DVT progresses to pulmonary embolism, of which approximately 20,000 cases result in death. (Heit, 2002)

The physician Virchow first described the elements that predict the causes of thrombosis in the 19th century. He proposed a triad (known as Virchow's triad), which predicts changes in blood coagulability, changes in the vessel wall, and blood stasis increase the risk of thrombosis [reviewed in (Wolberg et al., 2012)]. A loss of function in

natural anticoagulant pathways, or gain of function in procoagulant pathways can shift the hemostatic balance in favour of a hypercoagulable state. Disruption of normal laminar blood flow (by turbulence or stasis) can contribute to thrombosis by allowing cellular components in the blood, such as platelets to come in contact with the vascular endothelium (Wolberg et al., 2012). Also, a disruption in normal blood flow can inhibit the recirculation and dilution of activated clotting factors as well as entry of anticoagulants, contributing to further activation of coagulation (Wolberg et al., 2012).

The characteristics of thrombosis are vascular-bed specific, with the development of venous thrombosis being different than that of arterial thrombosis (Mackman, 2008). Venous thrombi are a result of extensive fibrin deposition whereas arterial thrombi are a result of extensive platelet activation and recruitment (Wakefield et al., 2008;Undas and Ariens, 2011;Lippi et al., 2011). These differences between arterial and venous clots are partially due to differences that exist in blood flow rates between arterial and venous systems. Arterial clots form under high sheer stress, typically after rupture of an atherosclerotic plaque or damaged endothelium, which likely contributes to the extensive platelet activation (Lippi et al., 2011;Undas and Ariens, 2011). Venous clots form under low sheer stress, thus are more sensitive to the levels of procoagulant and anticoagulant factors in the blood (Wakefield et al., 2008;Undas and Ariens, 2011).

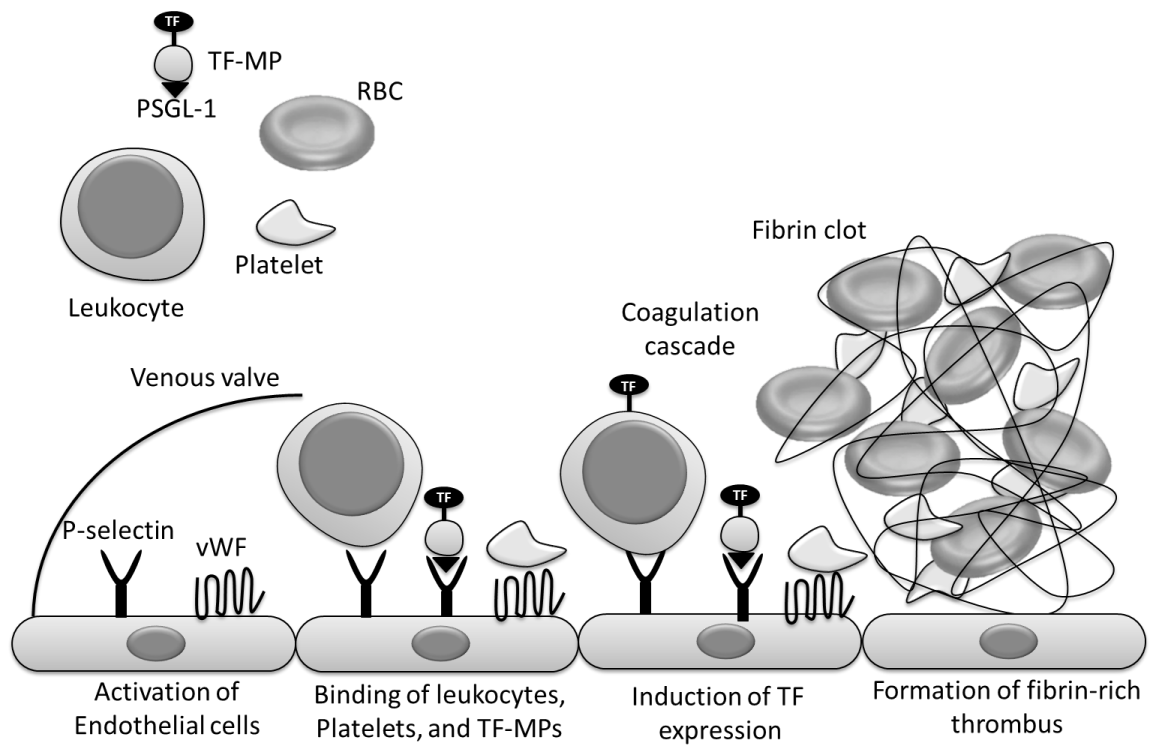


#### **1.4.1 Venous thrombus formation**

With the exception of surgical procedures, venous thrombus formation usually occurs on an intact endothelium (Sevitt, 1974). How is venous thrombosis initiated in the absence of endothelial damage? Evidence from autopsy studies, suggest the venous valvular sinus is a frequent location of thrombus initiation (Sevitt, 1974). This has been attributed to stasis and low oxygen tension in the valvular sinus (Bovill and van, V, 2011). In an experimental model of venous stasis, in which the lumen of the inferior vena cava (IVC) is reduced by 90%, the endothelium becomes activated expressing vWF and P-selectin. The activated endothelium then recruits circulating platelets, leukocytes and leukocyte microparticles (MP) (Brill et al., 2011; von Bruhl et al., 2012; Myers et al., 2003). The captured leukocytes and their MPs are induced to express active TF, triggering thrombus formation. This process is summarized in Figure 1.4. Evidence in support of this is demonstrated by a mouse model of venous stenosis in which there was reduced thrombus formation in mice with genetic deletion of TF in hematopoietic cells (von Bruhl et al., 2012). TF has long been thought to be the only trigger of thrombosis, however there is evidence to suggest other factors that may play a role in initiating thrombus formation. These include polyphosphates released from platelets or bacteria and neutrophil extracellular traps (NET) released by neutrophils during cell death (termed NETosis), both of which can trigger thrombosis through the contact pathway (Brill et al., 2012; Smith et al., 2010; Fuchs et al., 2010).

**Figure 1.4: Proposed mechanism for venous thrombus formation**

The vascular endothelium is activated by hypoxia (created by the venous valve sinus) and/or inflammatory mediators leading to the expression of adhesion molecules P-selectin and von Willebrand factor (vWF). Circulating leukocytes, platelets and tissue factor (TF) positive microparticles (MP) localize to the endothelium by binding to the adhesion proteins. The bound monocytes (and to a lesser extent neutrophils) and/or TF positive MPs are activated to express TF initiating coagulation. Activation of the coagulation cascade overwhelms the natural anticoagulant pathways resulting in the formation of a fibrin-rich clot that also contains platelets and red blood cells. (Figure adapted from Mackman N. J Clin Invest. 2012;122:2331-36).



#### **1.4.2 Congenital risk factors for thrombosis**

Individuals with inherited thrombophilic disorders that result in the loss of function of natural anticoagulants or gain of function of procoagulant factors are at a higher risk of venous thrombosis than the general population [Reviewed in (Anderson and Weitz, 2011)]. Heterozygous AT deficiency is inherited in autosomal dominant fashion and the mutations in the AT gene can result in decreased synthesis of AT (Type I) or reduced functional activity (Type II) (Perry and Carrell, 1996; Demers et al., 1992; van Boven and Lane, 1997). Congenital PC deficiency is also inherited in autosomal dominant fashion and can occur as type I or type II (Griffin et al., 1982). Type I PC deficiency is characterized by reduced PC synthesis, whereas type II is characterized by normal levels of PC antigen with reduced functional activity (Anderson and Weitz, 2011). Protein S deficiency can also be a result of decreased synthesis or reduced functional activity (Anderson and Weitz, 2011). Elevated levels of procoagulant proteins, including FVIII, FIX and FXI as a result of poorly understood genetic mechanisms are also a risk factor for thrombosis (Kraaijenhagen et al., 2000; Meijers et al., 2000; Van, V et al., 2000). One of the most common genetic mutations that increase the risk of thrombosis in Caucasians is known as the FV Leiden mutation. The FV Leiden mutation is a result of a single base substitution producing a R506Q mutation at one of the APC cleavage sites on FV, impairing the APC-mediated inactivation of FVa (Bertina et al., 1994; Bertina et al., 1995; Rees, 1996). Another common genetic mutation that increases the risk of thrombosis is the prothrombin mutation G20210A. This mutation

results in elevated levels of prothrombin, which can enhance thrombin generation (Poort et al., 1996; Wolberg et al., 2003)

#### **1.4.3 Acquired risk factors for thrombosis**

Acquired thrombophilias are a heterogeneous group of disorders, which include antiphospholipid antibody syndrome, heparin-induced thrombocytopenia (HIT), cancer, and pregnancy. Individuals with antiphospholipid antibody syndrome, produce antibodies (known as lupus anticoagulants or anti-cardiolipin antibodies) that target proteins that bind phospholipid surfaces (de Groot and Derksen, 2005). The mechanisms by which antiphospholipid antibodies increase thrombotic risk are not well understood, but it is hypothesized that the antibodies may inhibit the PC pathway, promote endothelial and platelet activation, and may also lead to complement activation (Atsumi et al., 1998; Smirnov et al., 1995; de Groot and Derksen, 2005; Holers et al., 2002). In HIT, heparin use can lead to a serious immune-mediated platelet count drop, which paradoxically causes thrombosis not bleeding (Jang and Hursting, 2005). Although rare, individuals with HIT develop autoantibodies that trigger platelet activation, thereby promoting thrombin generation and the development of thrombosis (Jang and Hursting, 2005; Warkentin, 2003). Cancer is also a major risk factor for thrombosis and accounts for ~ 20-25% of new cases of venous thrombosis (Lee and Levine, 2003). The pathogenesis of thrombosis in individuals with cancer is likely a combination of procoagulant TF expression by tumour cells, reduction of natural anticoagulant proteins by chemotherapy, surgical procedures, and/or immobility leading to stasis (Ruf, 2001; Gale and Gordon, 2001; Swystun et al., 2009; Swystun et al., 2011). Pregnancy and the use of oral

contraceptives can also induce a hypercoagulable state by increasing procoagulant proteins and suppressing anticoagulant proteins (Lim et al., 2007; Sidney et al., 2004; Clark, 2003; Clark et al., 1998).

In approximately 50% of individuals presenting with their first VTE a known acquired and/or congenital thrombophilic factor can be identified leaving 50% with idiopathic VTE (White, 2003; Schafer et al., 2003). In cases of idiopathic VTE, novel thrombophilic factors may exist but are not tested for by current thrombophilia testing. Furthermore, it is difficult to assess the risk of thrombosis for rare thrombophilic factors with low prevalence. More studies are needed to identify and assess novel thrombophilic factors.

## **1.5 Evaluating thrombotic potential**

### **1.5.1 Clotting Assays**

The integrity of the coagulation system is commonly assessed by assays that measure time to clot formation. The activated partial thromboplastin time measures the time it takes plasma to clot when initiated through the contact pathway. The prothrombin time measures the time it takes plasma to clot when initiated through the extrinsic pathway. Although these tests are useful to screen patients who may be at risk for bleeding, they are not sensitive to identifying patients who have a prothrombotic phenotype because the endpoint for these assays is clot formation, when only 5% of the total amount of thrombin that can be generated is formed (Hemker and Beguin,

1995;Mann et al., 2003b;Mann et al., 2003a). Also these tests do not assess the rate at which thrombin is inactivated.

### **1.5.2 Thrombin generation assays**

Thrombin generation assays are functional ways to assess the hemostatic system as the measurements take into account both procoagulant and anticoagulant pathways. The level of thrombin that is measured in particular system is plotted against time resulting in a curve where parameters such as lag-time, time to maximal thrombin, peak thrombin, and the endogenous thrombin potential (ETP) can be derived to assess the ability of a system to regulate thrombin (Hemker and Beguin, 1995;Hemker, 2008). The lag-phase of thrombin generation is characterized by the minute amounts of thrombin generated before the large burst of thrombin that occurs due to FV activation (Baglin, 2005). The lag-phase accounts for less than 5% of the total thrombin generated but does provide enough thrombin to form a fibrin clot (Mann et al., 2003b;Rand et al., 1996). The time to maximal thrombin generation and peak thrombin are measurements of the propagation phase, also considered large-scale thrombin generation. The propagation phase accounts for more than 95% of the thrombin generated in the system (Mann et al., 2003b). Inactivation of thrombin does not have its own distinctive phase, because as thrombin is produced it is quickly inactivated by plasma inhibitors, thus overlapping the propagation phase of thrombin generation (Mann et al., 2003b). The ETP is a measurement of the area under the curve and represents the total amount of thrombin generated (Hemker and Beguin, 1995). The ETP that is obtained from the thrombin

generation curve is a better measurement of global coagulation than clotting assays, as it takes into account both procoagulant and anticoagulant pathways.

### **1.5.3 Cell-based thrombin generation assays**

Thrombin generation is commonly measured in systems that represent the “cascade” model of coagulation, which emphasizes that coagulation is regulated by interactions of plasma proteins (Butenas et al., 1999; Dielis et al., 2008; Duchemin et al., 2008). Although this model provides crucial information to help delineate the determinants of thrombin formation, they fail to address the role that vascular endothelial cells play in the regulation of coagulation. Cell-based models of thrombin generation have developed to better reflect the *in vivo* process of hemostasis which takes place on, and is influenced by different cell surfaces (Hoffman and Monroe, III, 2001). Current cell-based models of thrombin generation include the use of whole blood, platelets, monocytes, neutrophils, and endothelial cells (Ninivaggi et al., 2012; Roberts et al., 2006; Allen et al., 2004; Gould et al., 2014; Coll et al., 2013). Platelets, once activated express PS on their membrane outer leaflet and allow formation of tenase and prothrombinase complexes, resulting in large-scale thrombin generation (Monroe et al., 1996). Monocytes can be induced to express TF by inflammatory stimuli such as C-reactive protein and lipopolysaccharide (LPS), allowing monocytes to act as the TF-bearing cell to initiate coagulation (Steffel et al., 2006). Neutrophils stimulated to release NETs have also been used in thrombin generation assays to demonstrate an increase in thrombin generation initiated through the contact pathway (Gould et al., 2014). Despite expression of TM and EPCR by monocytes and neutrophils, endothelial cells primarily



support PC activation (Zheng et al., 2007b). In support of this, TM expressing neutrophils do not promote thrombin-mediated activation of PC, and monocytes protect FVa from inactivation of APC (Conway et al., 1992; Colucci et al., 2001). Finally, an endothelial cell line EA.hy926 that expresses TM and EPCR has been used to demonstrate the effects of the PC pathway on the parameters of thrombin generation (Coll et al., 2013). The impact of PC activation on thrombin generation is often left unaccounted for during thrombin generation assays, as endothelial cells are not commonly included in thrombin generation assays. In addition The inclusion of soluble TM (sTM) during thrombin generation assays can be used in the absence of endothelial cells to efficiently activate PC, however these conditions fail to account for the role of EPCR.

## **1.6 The endothelial protein C receptor**

### **1.6.1 EPCR structure**

The human EPCR gene (*PROCR*) is located on chromosome 20 at position q11.2, spanning 8kb and consists of 4 exons and 3 introns (Simmonds and Lane, 1999; Fukudome and Esmon, 1994). Exon 1 encodes the 5' untranslated region (UTR) and the signal peptide, exon 2 and 3 encode most of the extracellular domain and exon 4 encodes the transmembrane domain, the cytoplasmic tail, and the 3' UTR (Simmonds and Lane, 1999). The EPCR protein is comprised of 238 amino acids, with a molecular weight of 46 kDa (Fukudome and Esmon, 1994) (Simmonds and Lane, 1999). EPCR shares a similar structural homology to the major histocompatibility class 1 family of proteins, consisting of an eight-strand  $\beta$ -sheet platform for two anti-parallel  $\alpha$ -helices,  $\alpha_1$

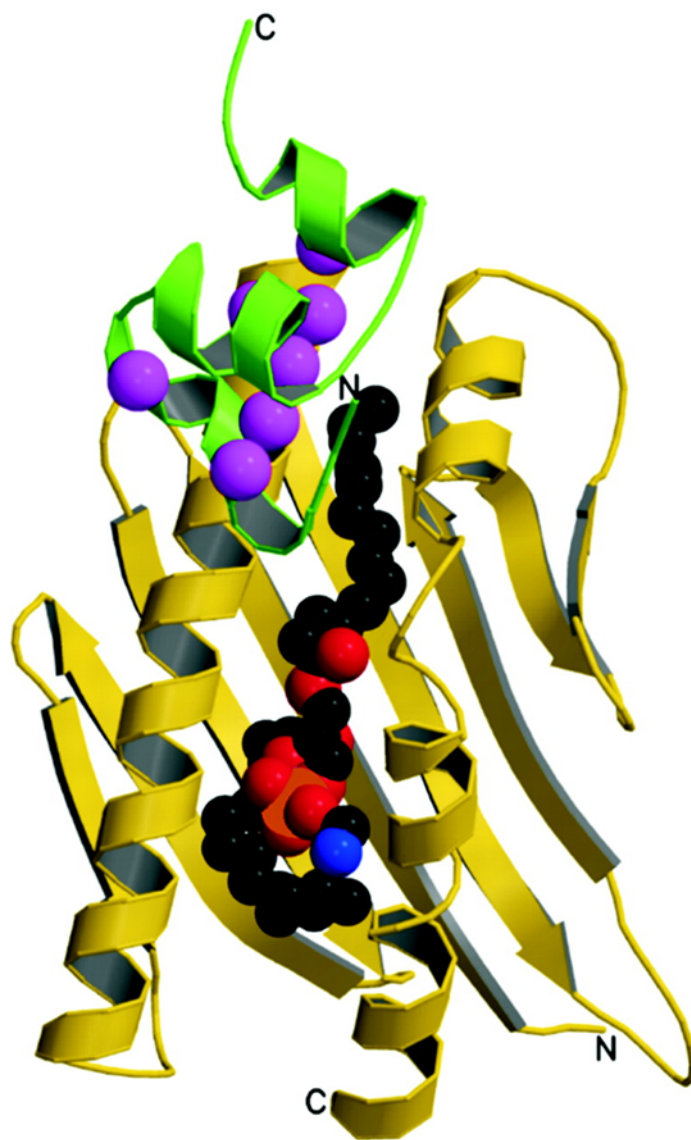
and  $\alpha_2$  (Fukudome and Esmon, 1994). EPCR  $\alpha_1$  and  $\alpha_2$  helical domains provide a surface for VKD ligand binding, which is highly conserved across bovine and murine species (Fukudome and Esmon, 1994) (Fukudome and Esmon, 1995). A hydrophobic groove is formed between the two EPCR  $\alpha$ -helices, where phospholipid is bound and required for protein interaction (Oganesyan et al., 2002) (Figure 1.5). EPCR contains 4 glycosylation sites (Asn-30, Asn-47, Asn-119, Asn-155), which undergo post-translational modifications prior to expression at the cell membrane (Fukudome and Esmon, 1994). The cytoplasmic tail of EPCR contains a palmitoylation site (an unpaired cysteine, Cys-221), thought to play a role in EPCR membrane localization and intracellular trafficking (Fukudome and Esmon, 1994).

### **1.6.2 EPCR distribution**

EPCR was first identified and characterized by the Esmon group in 1994 (Fukudome and Esmon, 1994). Initially it was thought that vascular endothelial cells primarily expressed EPCR, however it has been found to be expressed on variety of different cell types including placental trophoblasts (Gu et al., 2002), vascular smooth muscle cells (Bretschneider et al., 2007), monocytes (Galligan et al., 2001), neutrophils (Sturn et al., 2003), eosinophils (Feistritzer et al., 2003), lymphocytes (Feistritzer et al., 2006), epidermal keratinocytes (Xue et al., 2007), gastric epithelial cells (Nakamura et al., 2005), cardiomyocytes (Wang et al., 2011), osteoblasts (Kurata et al., 2010), chondrocytes (Jackson et al., 2009) and hematopoietic stem cells (HSC) (Balazs et al., 2006). EPCR, unlike TM, is not uniformly distributed throughout the vasculature. EPCR

**Figure 1.5: Structure of soluble EPCR**

In EPCR, the yellow ribbon illustrates two antiparallel  $\alpha$ -helices that sit upon an 8-stranded  $\beta$ -sheet platform to form a groove that is occupied by phospholipid (balls in center). The  $\alpha$ -helices form the VKD-binding domain that interacts with the Gla domain of APC (green ribbon) and its associated  $\text{Ca}^{2+}$  ions (purple balls). The transmembrane domain and cytoplasmic tail are not illustrated. (Figure reproduced from Oganesyan et al. J Biol Chem. 2002; 277: 24851-24854)



is preferentially expressed on larger vessels, and found at much lower concentration in the microcirculation (Laszik et al., 1997). In the larger vessels, the high concentration of EPCR counterbalances the low effective TM concentration due to the high blood volume to surface area ratio, ensuring efficacious PC activation. In the microcirculation, the blood volume to surface ratio is much lower, resulting in a higher effective TM concentration, and efficient PC activation.

### **1.6.3 EPCR ligand interactions**

Studies have identified a number of residues within exon 2 and exon 3 of EPCR that are involved in PC and APC binding. Site-directed mutagenesis of individual residues in EPCR lead to the identification of 10 residues, that when mutated to alanine, resulted in loss of PC/APC binding but retained cell surface expression (Liaw et al., 2001). The identified PC/APC binding region of EPCR is located at the distal end of the  $\alpha_1$  and  $\alpha_2$  helical domains and interacts with the Gla domain of PC and APC (residues 1-45) (Oganesyan et al., 2002;Regan et al., 1997). PC binding to EPCR is dependent on the presence of  $\text{Ca}^{2+}$ , which interacts with the side chains of the Gla domain residues, inducing a conformational change in the Gla domain (Oganesyan et al., 2002). PC binds to EPCR through hydrogen bonds at Gla residues 7, 25, 29, and through hydrophobic interactions at Phe-4 and Leu-8 (Oganesyan et al., 2002). It is predicted that Phe-4 and Leu-8 are the residues most required for EPCR binding (Oganesyan et al., 2002). These key residues involved in PC binding to EPCR are conserved in the Gla domain of FVII, which has also been shown to bind EPCR (Lopez-Sagaseta et al., 2007;Preston et al., 2006;Ghosh et al., 2007). FX contains 4 of the 5 of key residues in its Gla domain with

the substitution of Leu-8 by Met, however there are contradictory results regarding the interaction of FX with EPCR (Sen et al., 2011b). The Gla domains of PC, FVII and FX are summarized in Table 1.1.

Human (h) EPCR (hEPCR) and murine (m) EPCR (mEPCR) have a high degree of homology, as reflected by the ability of both hPC and hFVII to bind to mEPCR with a similar affinity as hEPCR (Liaw et al., 2001;Oganesyan et al., 2002;Lopez-Sagaseta et al., 2009). mPC, and mFVII do not exhibit the critical residues (Phe-4 or Leu-8) required for hPC/hFVII interaction with EPCR (Oganesyan et al., 2002). However, mPC, which has Met-8 substituted for Leu, binds mEPCR and demonstrates similar EPCR-dependent enhancement of PC activation and cell-signaling *in vivo* (Zheng et al., 2007a;Schouten et al., 2010). In contrast, mFVII does not readily bind mEPCR, with an affinity reported to be in the  $\mu$ M range (Puy et al., 2011). The residue Phe-4 that is present in the Gla domain of hFVII, hPC, and mPC is substituted by Leu in mFVII, perhaps accounting for the impaired binding with mEPCR. There has been some evidence to suggest there is an increase in affinity of mFVII for mEPCR in the presence of soluble TF (sTF), however the biological significance of this finding is not known (Disse et al., 2011). Currently, the interaction of FX with EPCR is a controversial topic. One report has shown that hFX binds to endothelial cells in a hEPCR-dependent manner, and could prevent endothelial hPC activation (Schuepbach and Riewald, 2010). A conflicting report has shown that high concentrations of hFX could not prevent EPCR-dependent binding of fluorescently labeled hAPC on endothelial cells, and the affinity of hFXa for EPCR was reported to be in the  $\mu$ M range (Sen et al., 2011b). The Gla domain of hFX has a Met instead of Leu at

**Table 1.1: Alignment of the regions of the Gla domain of hemostatic proteins involved in EPCR binding.**

The key residues involved in PC binding to EPCR that are conserved in the other hemostatic proteins are bolded. (Table adapted from Montes R., et al. Throm. Haem. 2012;107.)

hPC	<sup>1</sup> AN <b>S</b> FLE <b>E</b> L <b>R</b>	<sup>25</sup> EEA <b>K</b> E <b>I</b> F
hFVII	AN <b>A</b> FLE <b>E</b> L <b>R</b>	EEA <b>R</b> E <b>I</b> F
hFX	AN <b>S</b> FLE <b>E</b> M <b>K</b>	EEA <b>R</b> E <b>V</b> F
mPC	AN <b>S</b> FLE <b>E</b> M <b>R</b>	EEA <b>Q</b> E <b>I</b> F
mFVII	AN <b>S</b> LLE <b>E</b> L <b>W</b>	EEA <b>R</b> E <b>I</b> F
mFX	AN <b>S</b> FF <b>E</b> E <b>F</b> K	EE <b>V</b> R <b>E</b> I <b>F</b>



position 8 (similar to mPC), which could account for its weak affinity for hEPCR however, hFX much like mPC does bind to mEPCR with high affinity (Puy et al., 2011). It is unlikely that mFX interacts with mEPCR as it lacks both Phe-4 and Leu-8, however no  $K_d$  calculations have been reported. The differences between human and murine EPCR-dependent interactions are significant and must be taken into account when using murine models to study EPCR function.

More recently, additional novel EPCR ligands have been identified. It has been demonstrated that soluble EPCR (sEPCR) can bind activated neutrophils through interaction with proteinase-3 (PR3), an elastase-like protein that forms a heteromeric complex with the  $\beta 2$  integrin Mac-1 on neutrophils (Kurosawa et al., 2000). sEPCR bound to APC did not prevent sEPCR binding to PR3/Mac-1, suggesting that the EPCR binding site for PR3/Mac-1 is distinct from that of APC (Kurosawa et al., 2000). Once sEPCR is bound, PR3 cleaves EPCR at multiple sites, resulting in its degradation (Villegas-Mendez et al., 2007). Further studies have revealed that EPCR can also interact directly with Mac-1 on monocytes (Fink et al., 2013). Currently it is not known if the interaction of EPCR with PR3/Mac-1 on neutrophils and Mac-1 on monocytes occurs *in vivo*. In addition to neutrophils and monocytes, EPCR can also bind T cells expressing  $\gamma\delta$  T-cell antigen receptor (TCR) (Willcox et al., 2012). T-cells expressing  $\gamma\delta$  TCR play a role in immune surveillance, and may interact with EPCR on the endothelium to target and kill infected or malignant cells (Witherden and Havran, 2012; Willcox et al., 2012). Finally, EPCR can bind plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) subtype domain cassettes (DC) 8 and 13 that are expressed by Plasmodium

falciparum-infected erythrocytes found in patients with severe malaria (Turner et al., 2013). PfEMP1 binding to EPCR, results in the sequestration of infected erythrocytes on the microvascular endothelium, further exacerbating the pathogenesis of malaria (Turner et al., 2013).

#### **1.6.4 EPCR cell signaling**

In addition to the anticoagulant role of EPCR in PC activation, EPCR also plays a significant role in enabling APC-dependent cell signaling. APC bound to EPCR cleaves and activates PAR1, a G-protein coupled receptor, leading to anti-inflammatory activities, anti-apoptotic activity and protection of the endothelial barrier integrity (Riewald et al., 2002; Mosnier et al., 2007b). Thrombin can also cleave PAR1 with ~ 4-fold higher catalytic efficiency than APC (Ludeman et al., 2005). However, in contrast to APC, thrombin-mediated activation of PAR1 elicits cell signaling events leading to pro-inflammatory, pro-apoptotic, and barrier disrupting effects (Riewald and Ruf, 2005). It is not well understood how APC can activate PAR1 and elicit a different cellular response than thrombin.

More recent studies have suggested different mechanisms to explain biased signaling of PAR1 by APC and thrombin. One study has suggested that ligand occupancy of EPCR determines the type of PAR1 signaling (Bae et al., 2007a). It has been demonstrated that when EPCR is occupied by APC, thrombin cleavage of PAR1 elicits a protective signaling response in endothelial cells (Bae et al., 2007a). It has been proposed that occupation of EPCR by APC induces EPCR to dissociate from caveolin-1,

recruiting PAR1 to a protective pathway by coupling PAR1 to  $G_{i/o}$  protein instead of  $G_{12/13}$  and  $G_q$  (Rezaie, 2011; Bae et al., 2007a). Interestingly, occupation of EPCR by PC can also induce EPCR disassociation from caveolin-1 and alter the G-protein coupling specificity of PAR1 (Bae et al., 2007a). In addition, FVIIa bound to EPCR was also able to cleave and activate PAR1, eliciting barrier protective effects comparable to APC (Sen et al., 2011a). A more recent study has suggested the APC mediated PAR1 cytoprotective signaling is mediated by  $\beta$ -arrestin 2 recruitment and activation of the disheveled-2 (Dvl-2) scaffold and not by coupling PAR1 to  $G_{i/o}$  protein (Soh and Trejo, 2011). It has long been thought that APC and thrombin cleave PAR1 at the same site, but more recent studies have demonstrated that APC-bound EPCR cleaves PAR1 predominantly at Arg<sup>46</sup> whereas thrombin cleaves PAR1 at Arg<sup>41</sup> (Mosnier et al., 2012; Schuepbach et al., 2012). Cleavage of PAR1 at different sites generates tethered-ligand sequences that are different resulting in differential activation of PAR1 signaling pathways (Mosnier et al., 2012). Taken together, thrombin cleavage of PAR1 at Arg<sup>41</sup> results in a PAR1 conformation that preferentially binds  $G_{12/13}$  and  $G_q$  proteins leading to signaling events that are pro-inflammatory. APC cleavage of PAR1 at Arg<sup>46</sup> results in a PAR1 conformation that preferentially recruits  $\beta$ -arrestin 2/Dvl-2, activating cytoprotective-signaling pathways.

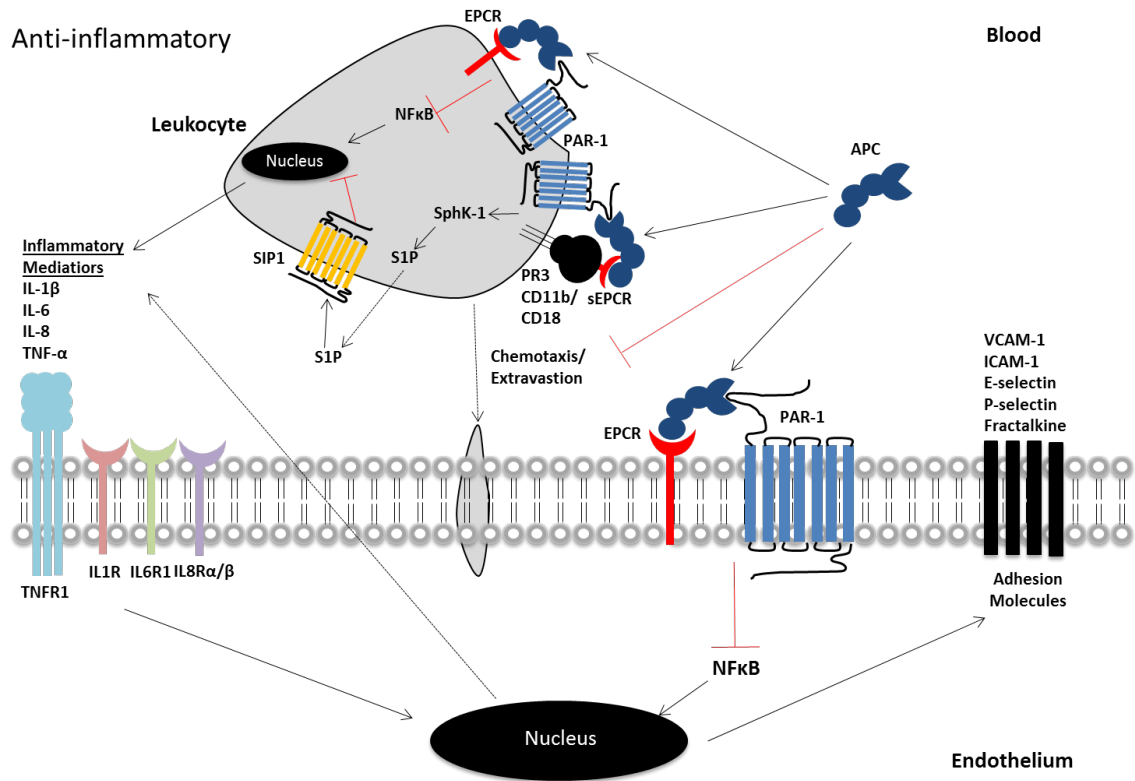
The APC-EPCR-PAR1 induced cytoprotection can be attributed to a combination of cellular responses that include anti-inflammatory, anti-apoptotic and barrier protective effects. In both endothelial cells and leukocytes, activation of PAR1 by APC-EPCR reduces pro-inflammatory gene expression levels and increases anti-inflammatory gene

expression levels through modulation of the p50/p52 subunits of the nuclear transcription factor  $\kappa\beta$  (NF $\kappa\beta$ ) complex (White et al., 2000;Joyce et al., 2001). This includes a decrease in pro-inflammatory cytokines, downregulation of vascular adhesion molecules, and the upregulation of anti-inflammatory cytokine interleukin (IL)-10 (Mosnier et al., 2007b;Joyce et al., 2001). In leukocytes, APC via PAR1 activation or interaction with sEPCR-PR3-Mac1 leads to the suppression of pro-inflammatory activity (Mosnier et al., 2007b;Cao et al., 2010). Taken together, the down-regulation of inflammatory mediators and vascular adhesion molecules reduces leukocyte adhesion and infiltration, resulting in less tissue damage to the underlying tissue (Figure 1.6) (Mosnier et al., 2007b). In support of this, studies have shown that EPCR-dependent APC signaling is protective in animal models of LPS-induced septic shock and acute lung injury (Nold et al., 2007;Schouten et al., 2011;Xu et al., 2009;Frommhold et al., 2011).

APC-EPCR-PAR1 signaling has been shown to reduce endothelial cell apoptosis by decreasing caspase-3 and caspase-8 activation, decreased translocation of PS to the outer cell membrane, inhibiting tumour suppressor protein p53 expression and normalizing the pro-apoptotic Bax/Bcl-2 ratio (Figure 1.7) (Joyce et al., 2001;Mosnier and Griffin, 2003;Cheng et al., 2003;Guo et al., 2004). Induction of APC-EPCR-PAR1 signaling has been shown to reduce tPA induced apoptosis in human brain endothelium, which may contribute to improving outcomes in ischemic stroke patients who receive tPA-dependent thrombolytic therapy (Liu et al., 2004;Cheng et al., 2006). In addition, the anti-apoptotic effects of APC-EPCR-PAR1 signaling have also been shown to be protective in a model of diabetic nephropathy (Isermann et al., 2007).

**Figure 1.6: EPCR-mediated anti-inflammatory effects**

APC/EPCR-mediated activation of PAR-1 in endothelial cells and leukocytes inhibits NF $\kappa$ B signaling, thereby suppressing pro-inflammatory mediator release and down-regulating the expression of adhesion molecules. Binding of APC to sEPCR/PR3/Mac1 on leukocytes activates PAR-1, resulting in the up regulation of sphingosine kinase 1 (sphk-1) and sphingosine 1 phosphate (S1P). Transactivation of the sphingosine 1 phosphate receptor (S1P1) by S1P decreases the pro-inflammatory activity of leukocytes. Together, a decrease in pro-inflammatory mediators and adhesion molecules inhibits leukocyte infiltration into the underlying tissue. (Figure adapted from Wildhagen KC. et al. *Thromb Haemost.* 2011;106:1-12).



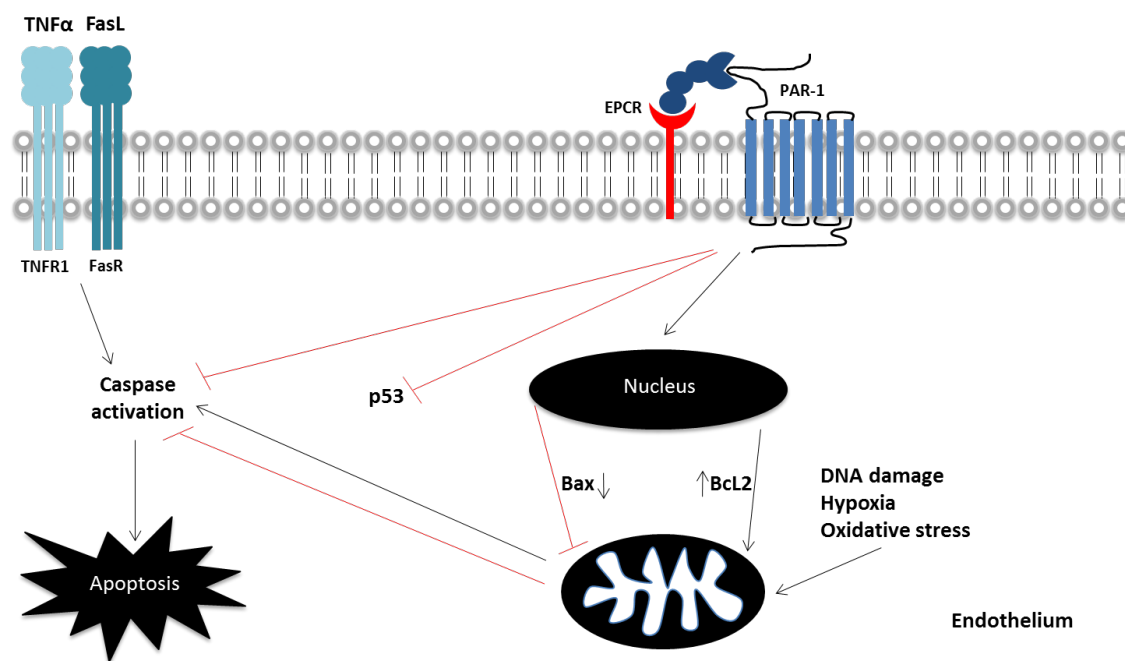
A

**Figure 1.7: EPCR-mediated anti-apoptotic effects**

Apoptosis can be induced by binding of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) or FasL to their receptors on the cell membrane or by DNA damage, hypoxia or oxidative stress. Activation of PAR1 by APC-bound EPCR inhibits caspase activation, decreases the expression of pro-apoptotic genes (p53, Bax) and increases the expression of anti-apoptotic genes (Bcl2). Normalization of the Bax/Bcl2 ratio on the mitochondrial membrane leads to the inhibition of apoptosis. (Figure adapted from Wildhagen KC. et al. *Thromb Haemost.* 2011;106:1-12).

Anti-apoptosis

Blood



B



Inflammatory conditions, such as sepsis, can lead to endothelial cell activation and dysfunction resulting in physical changes to the endothelium that increases vascular permeability. APC decreases vascular permeability through EPCR dependent activation of PAR1, leading to increased concentrations of sphk-1 and S1P and upregulation of tyrosine kinase with immunoglobulin-like EGF-like domains 2 (Tie2) (Van Sluis et al., 2009;Minhas et al., 2010). Binding of S1P to S1P1 receptor leads to activation the PI 3-kinase/Akt pathway, upregulation of RAC-1 GTPases and upregulation of the angiopoietin-1/angiopoietin-2 ratio (Ang1/Ang2) leading to stabilization of the endothelial cell cytoskeleton (Feistritz and Riewald, 2005;Bezuhly et al., 2009;Van Sluis et al., 2009). Ang1 transactivation of Tie2 leads to further activation of the PI 3-kinase/Akt pathway and enhancement of endothelial barrier integrity (Minhas et al., 2010). The improved barrier function results in reduced leukocyte trafficking to the extravascular space (Figure 1.8) (Bartolome et al., 2008).

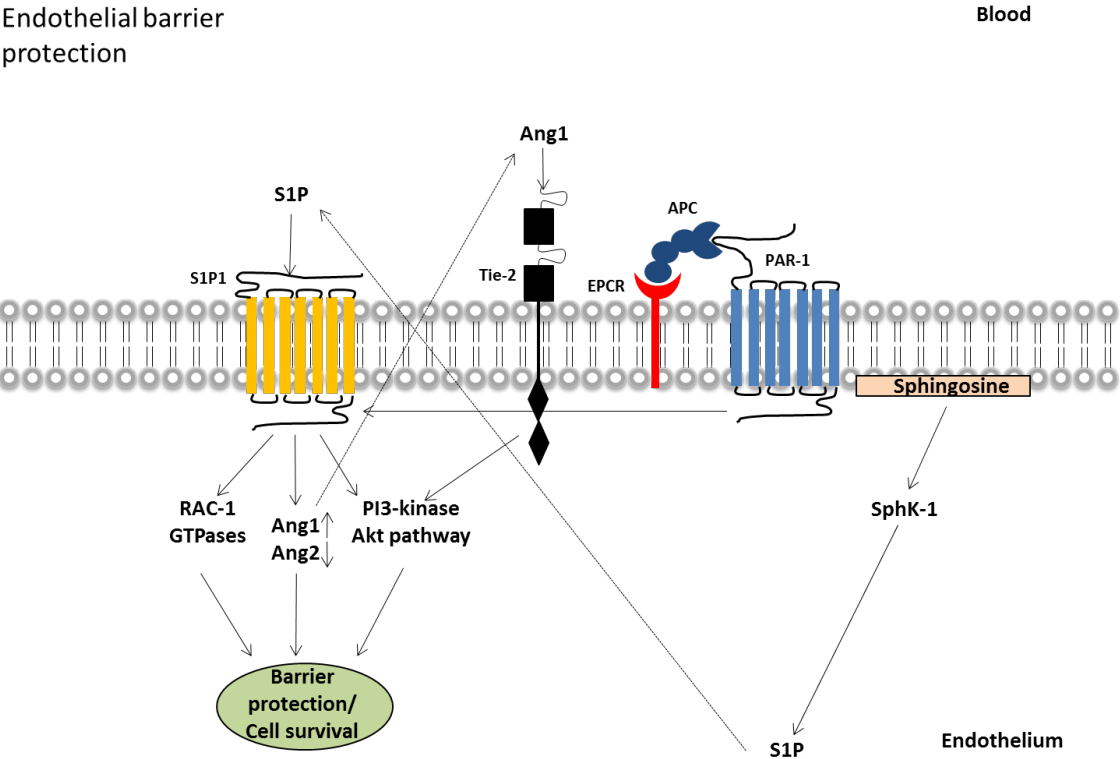
## **1.7 The role of EPCR in disease**

### **1.7.1 Thrombosis**

Deficiencies in EPCR function, much like deficiencies of other components of the PC pathway, are associated with an increased risk of thrombosis (Estelles et al., 1984;Schwarz et al., 1984;Espana et al., 2002;Bertina, 1999). The anticoagulant role of EPCR has been demonstrated in murine models of thrombosis, in which blockade of EPCR with anti-EPCR antibodies accelerates thrombus development (Centelles et al., 2010). Clinically, the presence of high titers of anti-EPCR autoantibodies has been

**Figure 1.8: EPCR-mediated barrier protective effects**

Activation of PAR1 by APC-bound EPCR up-regulates sphk-1 and S1P. Transactivation of S1P1 receptor by S1P leads to increased barrier integrity via activation of the PI 3-kinase/Akt pathway, upregulation of RAC-1 GTPases and Ang1 and downregulation of Ang2. Ang1 binding to Tie2 leads to further activation of the PI 3-kinase/Akt pathway, further enhancing barrier integrity. (Figure adapted from Wildhagen KC. et al. *Thromb Haemost.* 2011;106:1-12).



C

described in patients with DVT (Van, V et al., 2007). The high levels of anti-EPCR antibodies in these patients are associated with elevated D-dimer levels, which are an indication of high coagulant activity, increasing their risk of thrombosis (Van, V et al., 2007). Elevations in circulating levels of sEPCR, may also be associated with an increased risk of thrombosis (Kurosawa et al., 1997; Saposnik et al., 2004). sEPCR is generated through proteolytic cleavage of cell-surface EPCR by the metalloprotease ADAM17 (Kurosawa et al., 1997; Qu et al., 2007). sEPCR binds PC and APC with the same affinity as membrane-bound EPCR, however sEPCR inhibits activation of PC by the thrombin-TM complex, and inhibits the anticoagulant activities of APC (Liaw et al., 2000). Increased sEPCR levels in human plasma could be an indication of endothelial dysfunction and is often seen in patients with sepsis and lupus erythematosus, both characterized by inflammation and a procoagulant phenotype (Liaw et al., 2004; Kurosawa et al., 1998).

A number of mutations and polymorphisms have been identified in *PROCR* gene, which can affect the efficiency of protein C activation. A 23-base pair insertion in exon 3 of the *PROCR* gene, causes a stop codon downstream of its insertion point resulting in a truncated EPCR protein (Biguzzi et al., 2001). The truncated EPCR does not localize to the cell surface, is not secreted, and cannot bind PC, likely conferring an increased risk for thrombosis (Biguzzi et al., 2001). There are currently 4 haplotypes of EPCR identified: The H1 haplotype contains 10 single-nucleotide polymorphisms (SNP), and is associated with increased circulating APC levels, and is protective against VTE (Medina et al., 2004). The H2 haplotype contains the common allele of each SNP. The H3

haplotype contains 4 SNPs and is associated with increased levels of sEPCR (Saposnik et al., 2004). Carriers of the H3 haplotype may be at an increased risk for VTE, although there is contradicting data (Saposnik et al., 2004; Medina et al., 2004; Uitte et al., 2004; Yamagishi et al., 2009). However, the presence of the H3 haplotype in combination with other thrombophilias (i.e. prothrombin G20210A mutation) is associated with an increased risk of thrombosis (Navarro et al., 2008). The H4 haplotype contains 2 SNPs, and is reported to be associated with a slight increase in risk of VTE (Uitte et al., 2004). In addition to EPCR haplotypes, rare SNPs in *PROCR* have been identified in patients with unprovoked VTE that result in variants of EPCR that lack PC binding (Wu et al., 2013). Due to the low prevalence (< 1%) of these SNPs it is difficult to determine if they are associated with a higher risk of thrombosis.

### **1.7.2 Inflammation**

There is increasing evidence that indicates diminished EPCR expression or function can exacerbate inflammatory conditions. Soon after EPCR was discovered, it was first shown that EPCR plays an important role in the host defense against infections. In a baboon model of sepsis, inhibition of PC binding to EPCR by a monoclonal antibody converts the response of a sublethal concentration of *Escherichia coli* to a lethal response (Taylor, Jr. et al., 2000). The baboons that received the EPCR antibody had elevated levels of IL-6 and IL-8, and increased influx of neutrophils in the renal and hepatic microvasculature (Taylor, Jr. et al., 2000). In addition, mice with low EPCR expression exhibited reduced survival following administration of LPS in an endotoxemia model of sepsis (Kerschen et al., 2007). Although endothelial EPCR, as opposed to hematopoietic-

derived EPCR has been implicated in modulating the immune response during endotoxemia, there is evidence to support that EPCR on cluster of differentiation antigen (CD)8<sup>+</sup> dendritic cells is required to reduce the mortality of endotoxemia in mice (Zheng et al., 2007b;Kerschen et al., 2010). The exogenous administration of APC initially succeeded in reducing severe-sepsis associated mortality in patients enrolled in the PROWESS study, however a subsequent study failed to show a survival benefit and APC was withdrawn from the market (Bernard et al., 2001;Ranieri et al., 2012). APC administration has been effective in ameliorating chronic inflammatory conditions including experimental colitis and systemic lupus erythematosus in mice in an EPCR-dependent manner (Scaldaferri et al., 2007;Lichtnekert et al., 2011).

### **1.7.3 Cancer**

The role of EPCR in cancer is complex, as EPCR expressed by both endothelial cells and tumour cells seems to have opposing effects. Expression of EPCR by endothelial cells may limit cancer cell extravasation and metastasis by EPCR-mediated barrier protection. In support of this, overexpression of EPCR in a murine melanoma metastasis model resulted in reduced liver and lung metastasis (Van Sluis et al., 2009;Bezuhly et al., 2009). In contrast, in a model of lung adenocarcinoma EPCR expression resulted in increased metastatic activity, suggesting that the EPCR may have different effects between cancer types (Anton et al., 2012). EPCR expressing breast cancer stem cells have been shown to have increased tumour cell-initiating activities including tumour cell survival, migration and invasion when compared to cells with low EPCR expression (Schaffner et al., 2013). However, in malignant pleural mesothelioma

cells, EPCR expression promotes tumour cell apoptosis, attenuating their tumourgenicity through a EPCR-mediated cytotoxic mechanism that is currently unknown (Keshava et al., 2013). Taken together, EPCR has both anti-cancer and pro-cancer effects that differ between both cancer and cell type, and may be influenced by specific microenvironments.

#### **1.7.4 Malaria**

Severe malaria is associated with expression of subtypes PfEMP1 DCs 8 and 13 by *P. falciparum*-infected erythrocytes. Binding of PfEMP1 to EPCR in the cerebral microvasculature not only leads to sequestration of infected erythrocytes but also impairs PC binding and activation (Turner et al., 2013). Furthermore, blockade of PC and APC binding, impairs EPCR-mediated cytoprotective signaling (Turner et al., 2013). In a post-mortem analysis of children who died of cerebral malaria, fibrin clots and reduced EPCR and TM expression was found in the cerebral microvasculature (Moxon et al., 2013). The loss of TM and EPCR from the microvascular endothelium following the sequestration of infected erythrocytes only further exacerbates the localized procoagulant and inflammatory response (Moxon et al., 2013). Interestingly, high levels of sEPCR are protective in severe malaria, likely through inhibition of PfEMP1 binding to membrane-bound EPCR (Turner et al., 2013; Naka et al., 2014).

#### **1.8 EPCR mouse models**

In the mouse, EPCR is strongly expressed during embryogenesis, primarily on trophoblast giant cells involved in placental development (Crawley et al., 2002). The

physiological importance of EPCR during development is demonstrated in constitutive EPCR knockout mice, which show fibrin-deposition and placental thrombosis around trophoblast giant cells, leading to embryonic lethality before day 10 (E10) (Gu et al., 2002). A conditional EPCR knockout mouse line (ProcrLox) with specific deletion of EPCR in the developing embryo resulted in low EPCR expression in the placenta, however the embryos developed normally (Li et al., 2005b). The viable ProcrLox mice appeared healthy, with no evidence of hemorrhage or spontaneous thrombosis (Li et al., 2005b). It has also been demonstrated, that mice with very low EPCR expression levels were healthy and did not present with a thrombotic phenotype (Castellino et al., 2002). Furthermore, the generation of a transgenic mouse line overexpressing EPCR conferred protection from fibrin deposition in mice challenged with FXa, and rescued mice from a lethal dose of LPS (Li et al., 2005a). Currently, there are no animal models to study the role of EPCR *in vivo* in the absence of PC/APC interaction.



## **CHAPTER 2: THESIS OVERVIEW, RATIONALE, HYPOTHESIS, OBJECTIVES AND AIMS**

### **2.1 Thesis overview, rationale, and hypothesis**

EPCR plays a major role in regulating coagulation and inflammation. Previous studies have identified a number mutations and polymorphisms in the *PROCR* gene, which can affect the efficiency of protein C activation. At this time it is not well established if these functional mutations and polymorphisms in EPCR contribute to the risk of disease, in part due to low prevalence and conflicting results. In addition to the role of EPCR in coagulation and inflammation, the role of EPCR in fibrinolysis has yet to be established. The thrombin-TM complex activates both PC and TAFI. It is not clear if impaired PC binding to EPCR can affect TAFI activation and delay fibrinolysis, further contributing to development of thrombosis.

For the research carried out in this thesis, we hypothesized that impaired binding of PC/APC to EPCR will result in procoagulant, pro-inflammatory, and anti-fibrinolytic effects. To test these hypotheses we have generated a mouse model that contains an EPCR point mutation knock-in that impairs PC/APC binding but retains EPCR extracellular expression. To impair PC/APC binding in a murine model we introduced of a point mutation in exon 2 resulting in a switch from arginine (R) to an alanine (A) at residue 84 (R84A). This mutation was chosen based on previous work that identified the important residues in the *PROCR* gene that are required for PC/APC binding. In addition we also generated a cell line that expressed TM and an EPCR variant (R96C) identified in

a patient with VTE. These cell lines were used to determine the effect of impaired PC/APC binding to EPCR on thrombin generation and fibrinolysis.

## **2.2 Objectives and Aims**

The overall objective of this study is to better understand the mechanism by which EPCR and its ligand(s) regulate coagulation, inflammation and fibrinolysis. Our aims are as follows:

1. To characterize a murine model that harbours a variant of EPCR that lacks PC/APC binding and its response to thrombotic and inflammatory challenges.
2. To investigate the role of the PC pathway in fibrinolysis and whether impaired PC binding to EPCR can attenuate fibrinolysis.

## **CHAPTER 3: CHARACTERIZATION OF MICE HARBORING A VARIANT OF EPCR WITH IMPAIRED ABILITY TO BIND PROTEIN C: NOVEL ROLE OF EPCR IN HEMATOPOIESIS**

### **3.1 Foreword**

The effects of impaired PC/APC binding to EPCR *in vivo* are described in this manuscript. We demonstrate that mice harboring a variant of EPCR that does not bind PC exhibit a procoagulant and pro-inflammatory phenotype when challenged. In addition we have identified a previously unknown role for EPCR in hematopoiesis.

### **3.2 Objective: To characterize a murine model that harbours a variant of EPCR that lacks PC/APC binding.**

The physiologic importance of EPCR is demonstrated in EPCR knockout mice, which show placental thrombosis and early embryonic lethality (Gu et al., 2002). Furthermore, overexpression of EPCR on endothelial cells protects animals against thrombotic and septic challenge (Li et al., 2005a). To study the role of EPCR in the absence of PC/APC binding, we generated a knock-in mouse model harboring a variant of EPCR (R84A) with impaired ability to bind to PC. We hypothesized that impaired PC binding to EPCR would result in a procoagulant and pro-inflammatory phenotype. To test this hypothesis, mice were given a thrombotic or inflammatory challenge and we assessed (A) PC activation, (B) thrombin generation, (C) and organ histology. These objectives were used to demonstrate the importance of PC binding to EPCR in regulating coagulation and inflammation.

**Characterization of mice harboring a variant of EPCR with impaired ability to bind protein C: Novel role of EPCR in hematopoiesis**

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**Running Head:** Role of EPCR in coagulation and hematopoiesis

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**Authorship Contributions:** L. Pepler coordinated the study, performed experiments, interpreted the data and wrote the manuscript. P. Yu performed the bone marrow transplant experiments. DJ Dwivedi assisted with histology experiments. BL. Trigatti contributed to experimental design of bone marrow transplant experiments. PC. Liaw contributed to experimental design, interpretation of data and editing of the manuscript for publication.

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**References:** References for this manuscript have been incorporated into the bibliography at the end of this thesis.

### 3.3 Key Points:

- 1) Mutation of the PC binding domain of EPCR results in viable mice (EPCR<sup>R84A/R84A</sup>) that exhibit a procoagulant and pro-inflammatory phenotype when challenged.
- 2) EPCR<sup>R84A/R84A</sup> mice develop splenomegaly as a result of bone marrow failure, suggesting that EPCR plays an important role in hematopoiesis.

### 3.4 Abstract

The interaction of protein C (PC) with the endothelial protein C receptor (EPCR) enhances activated PC (APC) generation. The physiological importance of EPCR has been demonstrated in EPCR knockout mice, which show early embryonic lethality due to placental thrombosis. In order to study the role of EPCR independent of PC interaction, we generated an EPCR point mutation knock-in mouse (EPCR<sup>R84A/R84A</sup>) which lacks the ability to bind PC/APC. EPCR<sup>R84A/R84A</sup> mice are viable and reproduce normally. In response to thrombotic challenge with factor Xa/phospholipids, EPCR<sup>R84A/R84A</sup> mice generate more thrombin, less APC, and show increased fibrin deposition in lungs and heart compared to wild type mice. EPCR<sup>R84A/R84A</sup> mice challenged with lipopolysaccharide generate less APC, more IL-6 and show increased neutrophil infiltration in the lungs compared to WT controls. Interestingly, EPCR<sup>R84A/R84A</sup> mice develop splenomegaly as a result of bone marrow failure. Bone marrow transplant experiments suggest a role for EPCR on hematopoietic stem cells and bone marrow stromal cells in modulating hematopoiesis. Taken together, our

studies suggest that impaired EPCR/PC binding interactions not only result in procoagulant and pro-inflammatory effects, but also impacts hematopoiesis.

### 3.5 Introduction

The protein C (PC) pathway plays a major role in inhibiting blood coagulation (Esmon, 2003). The pathway is initiated upon binding of thrombin to thrombomodulin (TM) on the surface of vascular endothelial cells. The thrombin-TM complex rapidly converts zymogen PC to its active form activated protein C (APC). APC generation is augmented by ~20-fold *in vivo* by the endothelial protein C receptor (EPCR), a receptor which binds circulating PC and presents it to the thrombin-TM complex (Taylor, Jr. et al., 2001). APC, in conjunction with its cofactor protein S, degrades coagulation cofactors Va and VIIIa, thereby attenuating thrombin generation. Congenital or acquired defects in components of the PC pathway are associated with an increased risk of venous thrombosis (Esmon, 2009; Anderson and Weitz, 2011; Espana et al., 2002)

In mice, EPCR gene disruption results in early embryonic lethality due to fibrin deposition and placental thrombosis (Gu et al., 2002). During embryogenesis, EPCR is expressed primarily on trophoblast giant cells at the feto-maternal boundary (Crawley et al., 2002). Conditional EPCR knockout with specific deletion of EPCR in the developing embryo, but not on trophoblast giant cells, results in viable EPCR deficient mice (*ProcrLox*) with no evidence of spontaneous thrombosis (Li et al., 2005b). EPCR has also been found to be highly expressed on hematopoietic stem cells (HSC) (Balazs et al., 2006), however the biological role for EPCR on HSCs is unclear since selective deletion of EPCR on hematopoietic cells has minimal effects on systemic PC activation (Zheng et al., 2007b).

In addition to augmenting PC activation, EPCR also mediates the anti-inflammatory, anti-apoptotic, and endothelial barrier protective effects of APC (Esmon, 2012;Rezaie, 2010). On endothelial cells, the receptors required for both PC activation (TM and EPCR) and APC-mediated cellular signaling (EPCR and protease activated receptor-1(PAR1)) pathways are colocalized in lipid rafts (Bae et al., 2007b). EPCR serves as a co-receptor for APC-mediated cleavage of PAR1 (Riewald et al., 2002). Occupancy of EPCR by PC/APC switches the specificity of PAR1-dependent signaling from a permeability-enhancing to a barrier-protective response (Bae et al., 2007a). APC-mediated PAR1 cytoprotective signaling requires  $\beta$ -arrestin recruitment and activation of the disheveled-2 scaffold (Soh and Trejo, 2011). *In vivo*, overexpression of EPCR on endothelial cells protects mice against thrombotic or septic challenge (Li et al., 2005a) and inhibition of PC binding to EPCR in baboons converts the response of sublethal concentrations of *Escherichia coli* into a lethal response (Li et al., 2005a;Taylor, Jr. et al., 2000).

Currently, there are no animal models to study the biological role of EPCR independent of its interaction with PC/APC. In this study, we generated a point mutation knock-in mouse model harboring a variant of EPCR (R84A), which lacks ability to bind PC/APC. Our findings suggest that EPCR not only regulates coagulation and inflammation, but also plays an important role in hematopoiesis.



### **3.6 Materials and Methods**

#### **3.6.1 Generation of WT and R84A mEPCR stable cell lines**

Construction and mutagenesis of murine EPCR (mEPCR) was done as previously described (Liaw et al., 2001). Human embryonic kidney cells (HEK293) were transfected with pcDNA3.1( - ) vectors containing WT mEPCR or R84A mEPCR complementary DNAs (cDNAs) using Effectene transfection reagent (Qiagen, Valencia, CA). After 2 weeks of drug selection (400 µg/mL of G418) drug-resistant colonies were isolated, and expression of cell surface WT or R84A mEPCR was assessed using a FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

#### **3.6.2 Determination of the affinity of Fl-APC for WT mEPCR and R84A mEPCR**

To assess the binding of murine APC to WT or R84A mEPCR, murine APC (kindly provided by Dr. Charles Esmon, Oklahoma Medical Research Foundation, Oklahoma City, OK) was labeled at the active site with fluorescein (Fl) (Haematological Technologies, Essex Junction, VT) as previously described (Liaw et al., 2001). HEK293 cells stably transfected with WT or R84A mEPCR were incubated with increasing concentrations of Fl-APC at 4 °C for 15 min in the dark. Bound Fl-APC was detected on the fluorescence-1 channel on a FACSCalibur flow cytometer. Values for  $K_d$  were determined by fitting binding isotherms with a hyperbolic equation using the TableCurve program (Jandel Scientific, San Rafael, CA).

### **3.6.3 Generation of homozygous R84A EPCR knock-in mice**

All animal care and experimental procedures were approved by the McMaster University Research Ethics Board. In collaboration with GenOway (France), the EPCR<sup>R84A/R84A</sup> knock-in mouse was generated using the following strategy: the gene targeting vector consisted of exons 1-3, where exon 2 contained the desired R84A point mutation. The targeting vector was introduced into mouse embryonic stem cells of the 129Sv/Pas background and homologous recombinants were selected by polymerase chain reaction (PCR) screening. The EPCR recombined embryonic stem cells were used in blastocyst injections to generate male chimeras. The male chimeras were then bred with female Flp deleter mice to excise the neomycin selection cassette to generate heterozygous mice carrying the R84A knock-in mutation (EPCR<sup>WT/R84A</sup>). Heterozygous offspring were intercrossed to generate homozygous mice carrying the R84A mutation (EPCR<sup>R84A/R84A</sup>), and backcrossed 3 generations with C57BL/6 mice.

### **3.6.4 Hematological analysis of murine blood**

Blood was collected via cardiac puncture into 3.2% sodium citrate and a complete blood count was done using the Hemavet 950FS hematology cell analyzer (Drew Scientific, Waterbury CT).

### **3.6.5 Bone marrow transplantation**

Recipient mice (10-12 weeks old) were exposed to 14Gy of  $\gamma$ -irradiation from a <sup>137</sup>Cs source using a Gammacel 3000 small animal irradiator. Bone marrow (BM) prepared from the tibias and femurs of donor mice was injected via the retro-orbital sinus.

Mice were allowed to recover for four weeks before blood was collected and DNA isolated using QIAamp DNA Blood Minikit (Qiagen Inc., Valencia, CA) from blood cells for detection of donor-derived genes by PCR. EPCR<sup>R84A/R84A</sup>BM/EPCR<sup>WT/WT</sup> chimeric mice were generated by transplanting BM from EPCR<sup>R84A/R84A</sup> mice into recipient EPCR<sup>WT/WT</sup> mice. EPCR<sup>WT/WT</sup>BM/EPCR<sup>R84A/R84A</sup> chimeric mice were generated by transplanting BM from EPCR<sup>WT/WT</sup> mice into recipient EPCR<sup>R84A/R84A</sup> mice. Control mice were recipient EPCR<sup>R84A/R84A</sup> mice transplanted with EPCR<sup>R84A/R84A</sup> BM and recipient EPCR<sup>WT/WT</sup> mice transplanted with EPCR<sup>WT/WT</sup> BM. Six months post-transplantation, mice were sacrificed and spleen and bone marrow cells were harvested and total cell counts were performed using a hemocytometer.

### 3.6.6 Flow cytometric analysis of spleen and bone marrow cells

Spleen tissue from mice was harvested and made into a single cell suspension by homogenization using the plunger of a 3 mL syringe. Single cell suspension of the bone marrow was prepared by flushing the femurs and tibias of WT and EPCR<sup>R84A/R84A</sup> mice with Dulbecco modified Eagle medium (DMEM). Total cell counts were performed using a hemocytometer. Single-cell suspensions were incubated with FcγRIII/II to block non-specific binding prior to incubation with the following antibodies: fluorescein isothiocyanate (FITC)-anti-Gr-1 (granulocytes), phycoerythrin (PE)-anti-CD41 (megakaryocytes), PE-Cy7-anti-CD45 (leukocytes), PE-Cy7-anti-B220 (B-cells), and allophycocyanin-anti-CD11b (monocyte/macrophage) (BD Biosciences, Franklin Lakes, NJ). To identify hematopoietic stem and progenitors cells (HSPC), spleen and BM single cell suspensions were incubated with the following antibodies: allophycocyanin -(anti-

CD3, -anti-CD45R, -anti-Gr-1, -anti-CD11b, -anti-TER-119), PE-anti-c-Kit, PE-Cy7-anti-Sca-1, (BD Biosciences, Franklin Lakes, NJ). Cells were incubated with antibodies for 30 min at 4°C. The stained cells were washed twice and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and analyzed using a flow cytometer.

### **3.6.7 Colony-forming cell assays**

Isolated spleen, BM, and peripheral blood cells from mice were added to methylcellulose complete media and plated on 35mm dishes as described by the manufacturer (R&D Systems, Minneapolis, MN). Plated cells were incubated for 8 days in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Colonies were identified and enumerated using gridded scoring dishes on an inverted light microscope.

### **3.6.8 Immunoblotting of tissue lysates**

Mouse organs (heart, kidney, liver, lung and spleen) were collected and snap frozen in liquid N<sub>2</sub>. Tissue lysates were prepared by incubating tissues in lysis buffer (Millipore, Billerica, MA), followed by homogenization of tissues. Homogenized tissue was spun down at 5500 x g for 20 minutes at room temperature and the supernatant was collected. Sodium dodecyl sulfate-polyacrylamide gel of the tissue lysates was performed and protein was transferred onto a nitrocellulose membrane. Immunoblotting was performed using a polyclonal rabbit anti-EPCR antibody (Clone G-20) (Santa Cruz Biotechnology, Dallas, TX) and horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Bio-Rad, Mississauga, ON).

### 3.6.9 Immunohistochemistry and immunofluorescence

Mouse tissues were collected and snap frozen in dry ice with optimal cutting temperature embedding medium and stored at -70°C. Tissues were sectioned (8 µm thick) and fixed in ice cold acetone. Sections were blocked for 1 hour with 5% goat serum. Frozen sections were incubated with a rabbit anti-EPCR polyclonal antibody (Clone G-20) overnight at 4°C. Sections were washed with PBS and incubated with a goat anti-rabbit secondary antibody conjugated to Alexa 488 (Life Technologies, Burlington, ON). To stain nuclear DNA, sections were washed in PBS and incubated with 4,6 diamidino-2-phenylindole (DAPI) (Life Technologies, Burlington, ON).

To collect tissues for fibrin staining of factor (FXa)/phospholipid vesicles (PCPS)-challenged mice, WT and EPCR<sup>R84A/R84A</sup> mice were exsanguinated by cardiac puncture, and slowly perfused with 20 mL of 0.9% NaCl containing 200 U/mL of heparin, followed by fixation of tissues in 10% formalin. Tissues were dissected, paraffin embedded and processed for sectioning. Tissue sections (5 µm thick) were stained with a polyclonal rabbit anti-human fibrin antibody (Dako, Glostrup Denmark). The anti-fibrin antibody was detected by incubation with an alkaline phosphatase goat anti-rabbit secondary antibody, followed by incubation with Vector Red, a substrate that generates a bright red product (Vector laboratories, Burlingame, CA). Samples were examined by an Olympus BX41 fluorescent microscope and images collected using an Olympus DP72 camera (Olympus Corporation, Tokyo, Japan). For quantification of fibrin, the mean fluorescence intensity (MFI) of 10 images per experimental sample was measured using Slidebook software and corrected for the autofluorescence of the tissue by subtracting the

MFI of the negative control. To quantitate the amount of red pulp/white pulp in the spleen of WT and R84A mice, the total spleen area and white pulp area of hematoxylin and eosin (H&E) stained splenic sections was calculated using ImageJ software. The area of the white pulp and red pulp was then expressed as a percent of total spleen area.

### 3.6.10 Thrombotic challenge

To determine the effect of the EPCR R84A mutation on PC activation *in vivo*, anesthetized EPCR<sup>WT/WT</sup>, EPCR<sup>WT/R84A</sup>, and EPCR<sup>R84A/R84A</sup> mice were given an intravenous injection (via tail vein) of bovine thrombin (1 nmol/kg) (Sigma, St. Louis, MO) or 0.9% NaCl as a control (Li et al., 2005a). At 8 minutes post injection, blood samples were collected in 3.2% sodium citrate and 0.01 M benzamidine (Sigma, St. Louis, MO). To determine the effect of EPCR R84A mutation on thrombin generation EPCR<sup>WT/WT</sup>, EPCR<sup>WT/R84A</sup>, and EPCR<sup>R84A/R84A</sup> mice were given an intravenous injection (via tail vein) of FXa (Haematological Technologies, Essex Junction, VT) (50 pmol/kg) and PCPS (150 nmol/kg) (Diapharma, West Chester, OH) in 0.9% NaCl (supplemented with 0.1% BSA and 2 mM CaCl<sub>2</sub>) or 0.9% NaCl(supplemented with 0.1% BSA and 2 mM CaCl<sub>2</sub>) as a control. At 10 minutes post injection, blood samples were collected. Whole blood was centrifuged at 1500 g for 10 minutes at room temperature, plasma collected and stored at -70°C.

### 3.6.11 LPS Challenge

Lipopolysaccharide (LPS) (1 mg/kg, Sigma, St. Louis, MO) was injected intraperitoneally into EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice. Whole blood was collected at

0, 2, 4, 6, and 8 hours post LPS injection into 3.2% sodium citrate and 0.01 M benzamidine. Plasma was collected for coagulation and cytokine assays. To measure myeloperoxidase (MPO) activity (a measure of neutrophil infiltration) lungs from LPS-challenged mice were collected 24 hours post LPS challenge and snap frozen in liquid N<sub>2</sub>. MPO activity was measured as previously described (Khan et al., 2014)

### **3.6.12 Analysis of murine PC, TAT and IL-6 levels**

Mouse PC and APC was assayed as described previously (Li et al., 2005a). Mouse thrombin-antithrombin (TAT) complexes were measured using the Enzygnost TAT kit as described by the manufacturer (Siemens, Munich, Germany). Mouse IL-6 antigen was measured using the Quantikine IL-6 enzyme-linked immunosorbent assay (ELISA) kit as described by the manufacturer (R&D Systems, Minneapolis, MN).

### **3.6.13 Statistics**

Results are shown as the mean  $\pm$  the standard error (SE). The student's t-test was used to compare values between EPCR<sup>WT/WT</sup> mice and EPCR<sup>R84A/R84A</sup> mice. A 1-way analysis of variance (ANOVA) was used to compare values between treatment groups of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup>. A P-value < 0.05 was considered to be statistically significant.

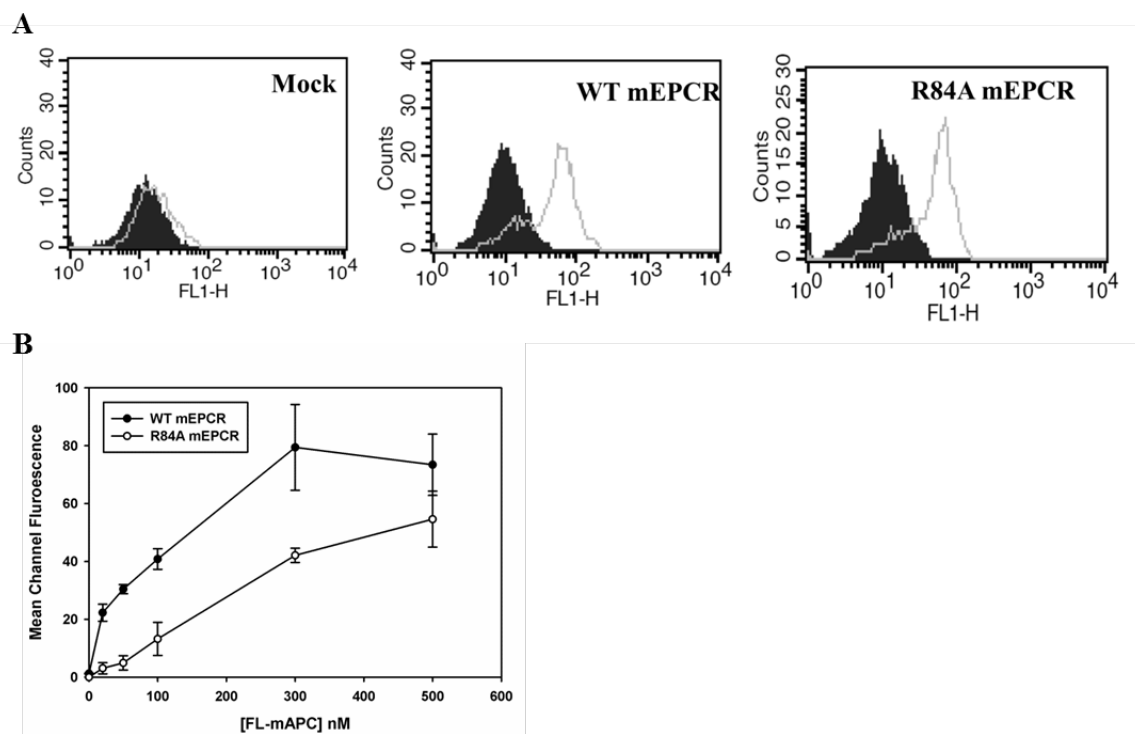
### **3.7 Results**

#### **3.7.1 Interaction of murine FI-APC with WT EPCR and R84A EPCR**

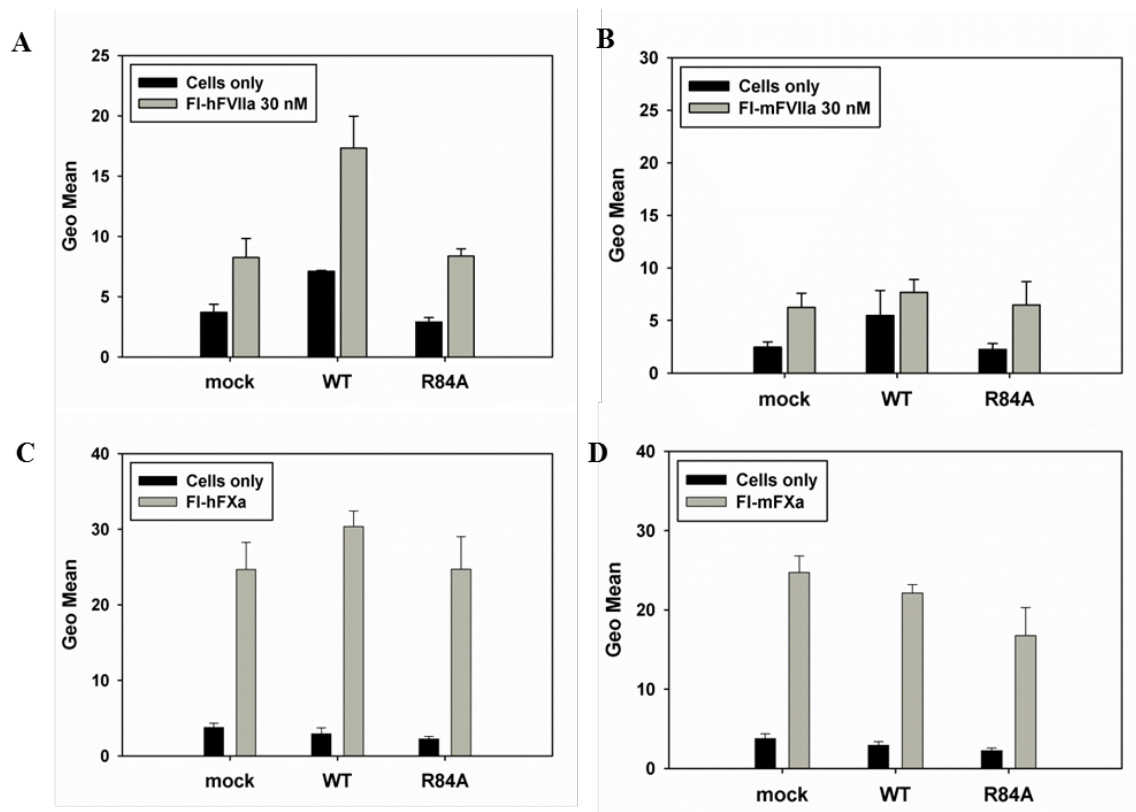
EPCR consists of two antiparallel  $\alpha$ -helices that sit upon an 8-stranded  $\beta$ -sheet platform (Oganesyan et al., 2002). Mutagenesis studies of EPCR and the EPCR crystal structure have shown that the PC-binding domain is located at the distal end of the 2  $\alpha$ -helical segments (Oganesyan et al., 2002; Liaw et al., 2001). To generate a mEPCR variant, which lacks the ability to bind to PC/APC, we mutated Arg<sup>84</sup> (a critical residue required for the binding of EPCR to PC/APC) to Alanine. The cDNAs of WT mEPCR and R84A mEPCR were stably transfected into HEK293 cells, and cell surface EPCR expression was confirmed by the binding of FITC-labelled anti-EPCR polyclonal antibody by flow cytometry (Figure 3.1A). As expected, FI-mAPC saturably bound WT mEPCR but not R84A mEPCR (Figure 3.1B). The binding affinity of FI-mAPC for WT mEPCR is  $43 \pm 12$  nM, consistent with previous reports (Liaw et al., 2001; Fukudome and Esmon, 1994). In contrast, R84A mEPCR binding to FI-mAPC did not saturate even at FI-mAPC concentrations of 500 nM, which is much higher than the circulating concentration of PC [ $\sim 60$  nM] (Figure 3.1B). These studies confirm that the R84A mEPCR variant is expressed on the cell surface but lacks the ability to bind to PC/APC. We are also able to confirm that FI-mFVIIa and FI-mFXa does not bind WT or R84A mEPCR (Figure 3.2)



**Figure 3.1: Flow cytometric analysis of HEK293 cells expressing murine WT or R84A EPCR:** (A) Human embryonic kidney cells (HEK293) were stably transfected with pcDNA3.1(-) vector only (left), or vectors containing the cDNA encoding murine WT (middle) or R84A (right) EPCR. EPCR expression was measured by incubating transfected HEK293 cells with FITC-IgG isotype control (filled histogram) or FITC-labelled goat polyclonal EPCR antibody (grey line). (B) Stably transfected HEK293 cells expressing WT or R84A mEPCR were incubated with murine FI-APC (0-500 nM) in the presence of 3 mM  $\text{CaCl}_2$  and 0.6 mM  $\text{MgCl}_2$  for 15 minutes at room temperature and binding of FI-labelled protein to cells expressing mEPCR was analyzed by flow cytometry.



**Figure 3.2: Flow cytometric analysis of human and murine Fl-FVIIa, and Fl-FXa binding to mEPCR:** Human embryonic kidney cells (HEK293) were stably transfected with cDNAs encoding WT or R84A EPCR. Stably transfected HEK293 cells expressing WT or R84A mEPCR were incubated with 100nM of human or murine Fl-FVIIa (**A and B**) and human or murine Fl-FXa (**C and D**) in the presence of 3 mM  $\text{CaCl}_2$  and 0.6 mM  $\text{MgCl}_2$  for 15 minutes at room temperature. Binding of Fl-labelled protein to cells expressing mEPCR was analyzed by flow cytometry. Human Fl-FVIIa but not murine Fl-FVIIa bound WT mEPCR and the introduction of the R84A mutation in mEPCR also results in impaired Fl-hFVIIa binding. Both Fl-hFXa and Fl-mFXa bound non-specifically to the cell surface of transfected HEK293 cells independent of EPCR.



### 3.7.2 Generation of EPCR<sup>R84A/R84A</sup> mice

To study the biological role of EPCR in the absence of PC/APC binding *in vivo* we generated mice carrying a point mutation in EPCR (R84A). A targeting vector was designed to introduce an R84A point mutation in exon 2 of EPCR, floxed by two loxP sites and a neomycin cassette (Figure 3.3A). Mice homozygous for the EPCR R84A mutation were confirmed by PCR analysis (Figure 3.3B). Both EPCR<sup>R84A/R84A</sup> and EPCR<sup>WT/R84A</sup> mice had normal fertility and lifespans without apparent gross abnormalities.

### 3.7.3 EPCR expression in organs of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice

To confirm that the EPCR R84A mutation does not impair the expression of EPCR, we measured EPCR protein from EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> tissue lysates by western blot analysis. EPCR protein levels were similar in the heart, kidney, liver, lung, and spleen of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice (Figure 3.4A). Expression of EPCR by aortic endothelial cells was also unaffected by the R84A mutation (Figure 3.4B).

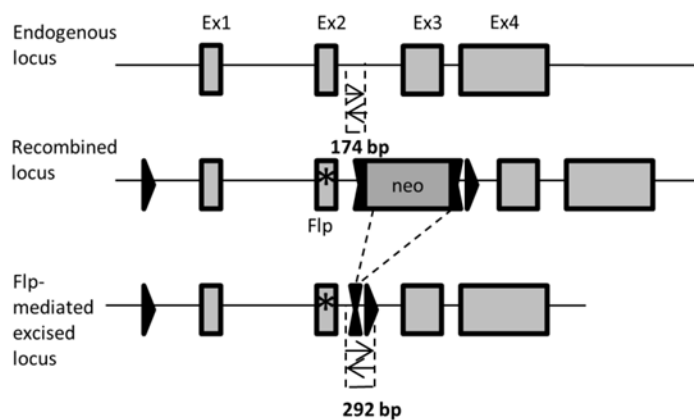
### 3.7.4 Baseline levels of PC, TAT and IL-6 in EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice

Next, we determined the baseline levels of PC, TAT and IL-6 levels in healthy (ie. unchallenged) EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice (Table 3.1). The plasma PC level was ~40% higher in EPCR<sup>R84A/R84A</sup> mice compared to EPCR<sup>WT/WT</sup> mice, consistent with previous studies which showed that EPCR expression on the endothelium binds PC and decreases the circulating PC level (Li et al., 2005b; Li et al., 2005a). Circulating levels of TAT complexes were similar in healthy EPCR<sup>R84A/R84A</sup> mice compared to EPCR<sup>WT/WT</sup> (Table 3.1). Plasma levels of IL-6 (a pro-inflammatory cytokine) are higher in

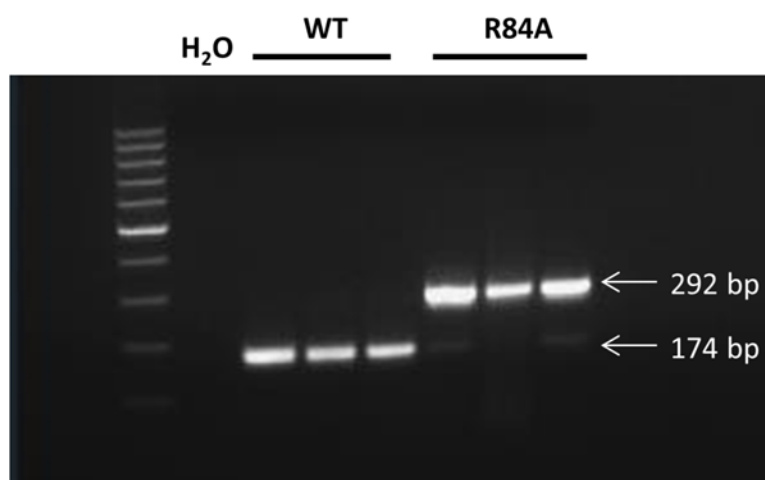
**Figure 3.3: Detection of mice homozygous for the EPCR R84A mutation by PCR:**

**(A)** Schematic diagram of PCR strategy to detect WT or R84A DNA. LoxP sites are represented by single triangles and FRT sites by double triangles. The R84A point mutation in exon 2 is represented by an asterisk. PCR amplification of WT genomic DNA results in the detection of a 174 bp fragment, while flp-excised genomic DNA yields a 292 bp fragment including an FRT and loxP site. **(B)** Mouse genomic DNA containing the EPCR Flp-excised allele was tested by PCR. PCR without DNA template (H<sub>2</sub>O) was used as a negative control.

A



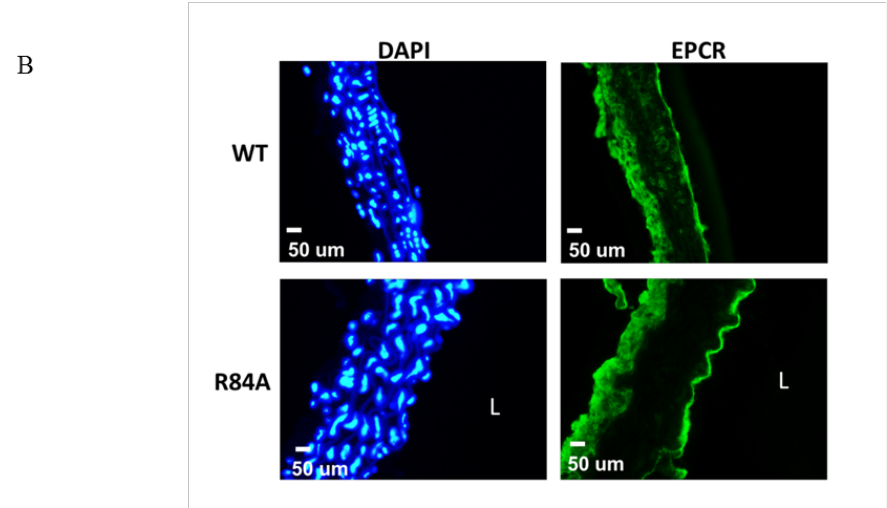
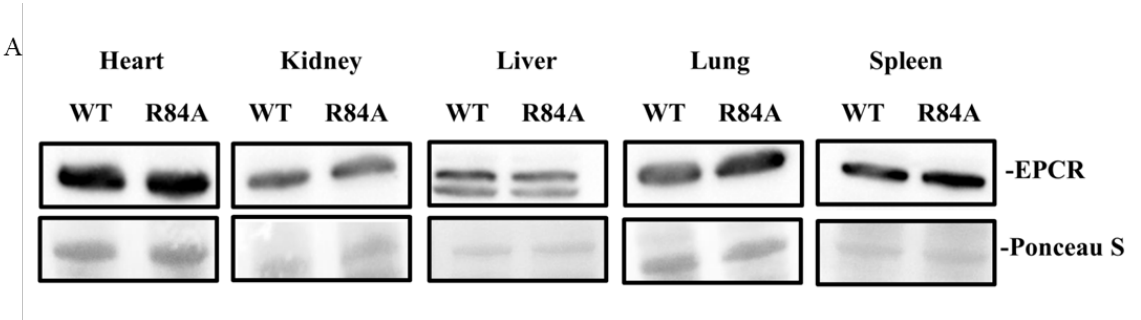
B



**Figure 3.4: EPCR expression in EPCR<sup>R84A/R84A</sup> mice.**

**(A)** Western blot analysis of tissue lysates from heart, kidney, liver, lung and spleen of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice. The nitrocellulose membrane was stained with Ponceau S dye to confirm that similar amounts of  $\beta$ -actin protein were loaded in each lane. **(B)** Frozen sections of aorta from WT and EPCR<sup>R84A/R84A</sup> mice were fixed with 4% paraformaldehyde and stained with a FITC-labeled rabbit polyclonal antibody specific for EPCR, and DAPI, a nuclear stain used to identify cells. The lumen is indicated by L. Blots and images are representative of 3 mice per group.





**Table 3.1: Comparison of baseline PC, TAT and IL-6 antigen levels in EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice.** PC, TAT, and IL-6 antigen levels in healthy EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice were measured by ELISA. Data represents the mean  $\pm$  SE, n > 6 for each group, \* P < 0.05.

<b>Parameter</b>	<b>EPCR<sup>WT/WT</sup></b>	<b>EPCR<sup>R84A/R84A</sup></b>
<b>PC(μg/mL)</b>	0.9 ± 0.1	1.3 ± 0.1 *
<b>TAT (ng/mL)</b>	5.6 ± 1.0	8.4 ± 1.6
<b>IL-6 (pg/mL)</b>	15.6 ± 1.5	22.0 ± 3.3 *

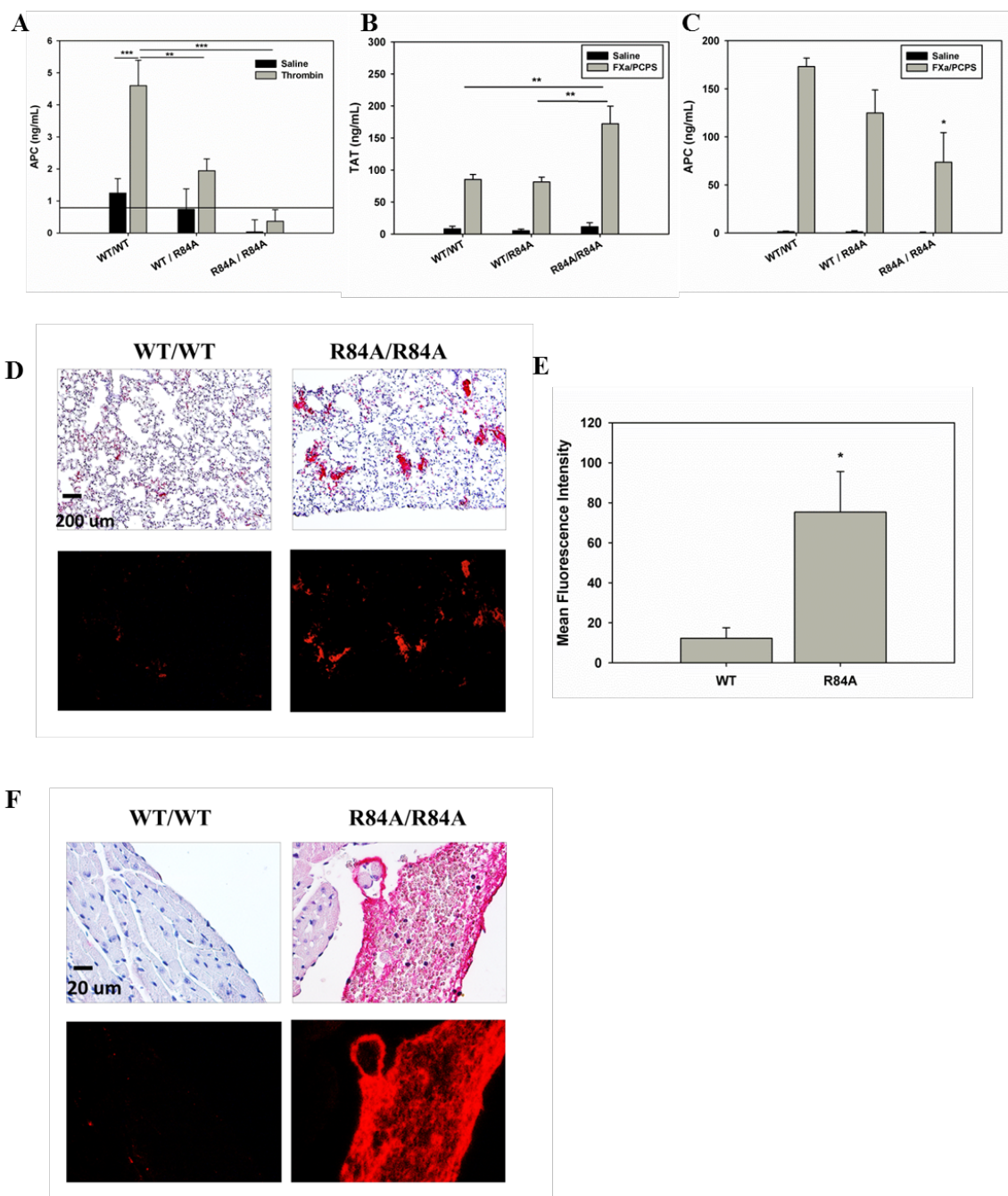
EPCR<sup>R84A/R84A</sup> mice when compared to EPCR<sup>WT/WT</sup> mice. APC was not detectable in the EPCR<sup>WT/WT</sup> or the EPCR<sup>R84A/R84A</sup> mice.

### 3.7.5 Thrombotic challenge in EPCR<sup>R84A/R84A</sup> mice

To determine if mice harboring the EPCR R84A mutation exhibit impaired ability to activate PC, we infused thrombin (1 nmol/kg) into WT, heterozygous, or homozygous mice and measured plasma levels of APC. As shown in Figure 3.5A, in response to thrombin infusion, plasma levels of APC in EPCR<sup>WT/R84A</sup> and EPCR<sup>R84A/R84A</sup> mice were 42% and 8% of those of EPCR<sup>WT/WT</sup> mice, respectively. These studies confirm that introduction of the R84A mutation in EPCR reduces PC activation *in vivo*.

To test if mice harboring the R84A EPCR mutation exhibit a more severe procoagulant phenotype when challenged, we infused EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice with FXa/PCPS, a procoagulant stimulant. Upon infusion of FXa/PCPS, EPCR<sup>R84A/R84A</sup> mice had a ~2-fold increase in TAT levels compared to EPCR<sup>WT/WT</sup> mice whereas the TAT levels were similar between EPCR<sup>WT/WT</sup> and EPCR<sup>WT/R84A</sup> mice (Figure 3.5B). Plasma APC levels were lower in EPCR<sup>R84A/R84A</sup> compared to EPCR<sup>WT/WT</sup> or EPCR<sup>WT/R84A</sup> mice (Figure 3.5C). Histological studies show that there is increased fibrin deposition in the lungs of EPCR<sup>R84A/R84A</sup> mice compared to EPCR<sup>WT/WT</sup> mice (Figure 3.5D and E). EPCR<sup>R84A/R84A</sup> mice also showed evidence of large intraventricular fibrin clots in the heart, which were absent in EPCR<sup>WT/WT</sup> mice (Figure 3.5F).

**Figure 3.5: Thrombotic challenge of EPCR<sup>WT/WT</sup>, EPCR<sup>WT/R84A</sup>, and EPCR<sup>R84A/R84A</sup> mice.** (A) Anesthetized mice were given an intravenous injection (via tail vein) of bovine thrombin (1 nmol/kg) or 0.9% NaCl. At 8 minutes post injection, blood samples were collected via the IVC in the presence of 0.01 M benzamidine. APC levels were measured by enzyme-capture assay. The solid line indicates the lower limit detection for the assay. (B and C) Anesthetized mice were given an intravenous injection (via tail vein) of 50 pmol/kg of FXa and 75 nmol/kg of PCPS in the presence of 0.1% BSA and 2 mM CaCl<sub>2</sub>. At 10 minutes post injection, blood samples were collected via the IVC. TAT (B) and APC (C) levels were measured by ELISA. (D) Immunohistochemical detection of fibrin deposition in the lungs after FXa/PCPS injection. Red alkaline phosphatase reaction product identifies fibrin under bright field (top) and fluorescent (bottom) microscopy. (E) Fluorescence quantification shows that the mean fluorescence intensity of fibrin staining is greater in the lungs of EPCR<sup>R84A/R84A</sup> mice when compared to WT mice. (F) Immunohistochemical detection of fibrin deposition in the ventricles of the heart after FXa/PCPS injection. Images are representative of 3 mice per group. Data represents the mean ± SE, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. n > 3 per group.



### **3.7.6 LPS challenge in EPCR<sup>R84A/R84A</sup> mice**

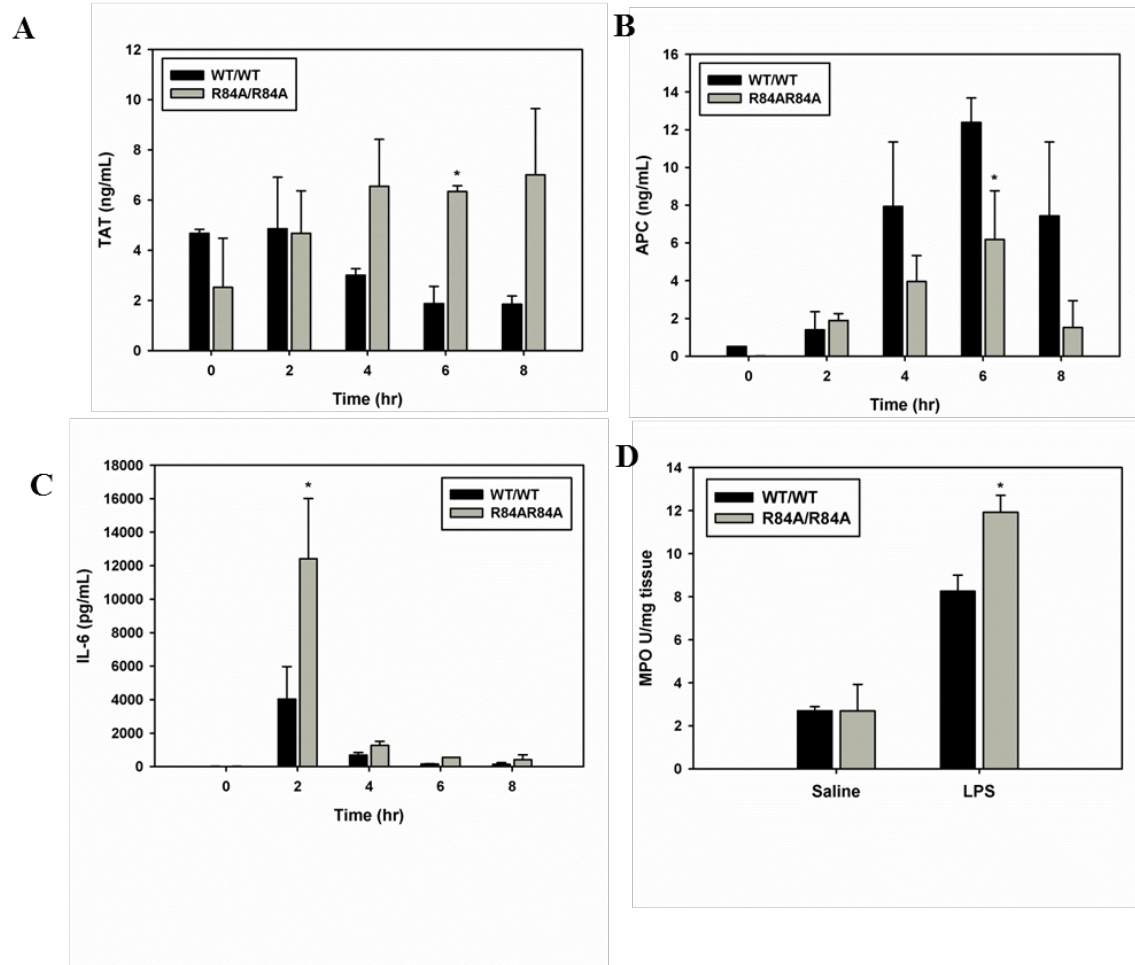
Next, we determined if mice harboring the R84A EPCR mutation exhibit a more pro-inflammatory phenotype when challenged with endotoxin. At 6 hours post-LPS injection, EPCR<sup>R84A/R84A</sup> mice had a ~3-fold increase in TAT levels (Figure 3.6A), and a ~2 fold decrease in circulating APC (Figure 3.6B) when compared to EPCR<sup>WT/WT</sup> mice. In LPS-challenged EPCR<sup>R84A/R84A</sup> mice, circulating IL-6 was significantly increased at 2 hours-post LPS injection when compared to EPCR<sup>WT/WT</sup> mice (Figure 3.6C). Since the lungs are a major site of neutrophil sequestration after LPS challenge, we also measured MPO activity (as a marker of neutrophil infiltration) in the lungs of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice. MPO activity in lung homogenates of EPCR<sup>R84A/R84A</sup> mice was increased compared to EPCR<sup>WT/WT</sup> mice 24 hours after LPS injection (Figure 3.6D).

### **3.7.7 EPCR<sup>R84A/R84A</sup> mice exhibit splenomegaly**

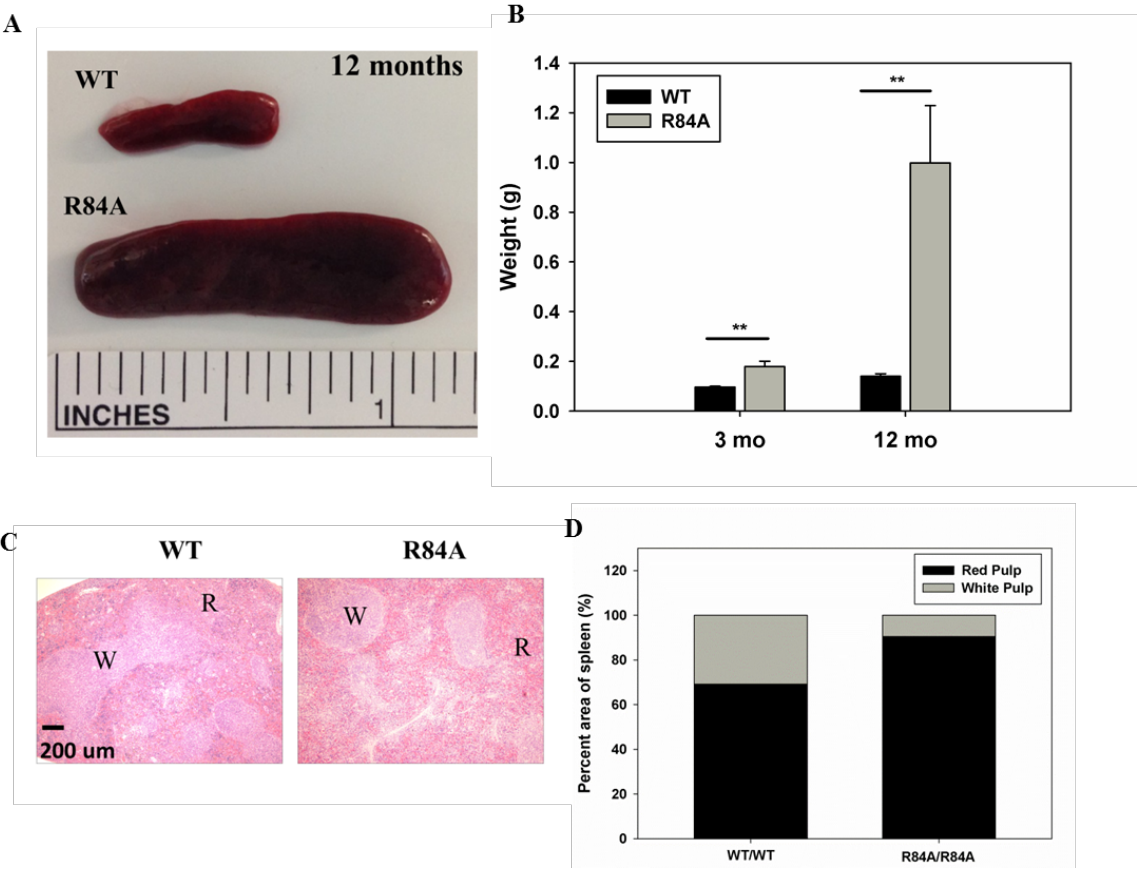
EPCR<sup>R84A/R84A</sup> mice developed a splenic disorder characterized by splenomegaly (Figure 3.7A). There was a significant increase in the splenic weight of EPCR<sup>R84A/R84A</sup> mice compared to WT mice at 3 months of age and further increases in the splenic weight of EPCR<sup>R84A/R84A</sup> mice at 1 year (Figure 3.7B). Histological analysis of the spleen from EPCR<sup>R84A/R84A</sup> mice revealed an increased amount of red pulp ( $90.4 \pm 0.5\%$ ) compared to EPCR<sup>WT/WT</sup> mice ( $69.2 \pm 3.03\%$ ) (Figure 3.7C and D).

**Figure 3.6: Inflammatory challenge of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice.** Mice were given an intraperitoneal injection of LPS (1 mg/kg). At 0, 2, 4, 6, and 8 hours post LPS injection, blood samples were collected via the IVC. **(A)** TAT, **(B)** APC, and **(C)** IL-6 levels were measured by ELISA. **(D)** Lung tissue was collected from mice 24 hours post LPS injection and snap frozen in liquid N<sub>2</sub> and lung MPO activity was measured as described in methods.





**Figure 3.7: Morphological and histochemical abnormalities in the spleens of EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice:** Gross anatomy (**A**) and weight (in grams) (**B**) of spleens from EPCR<sup>R84A/R84A</sup> mice revealed severe splenomegaly compared to EPCR<sup>WT/WT</sup> mice. Data represents the mean  $\pm$  SE,  $n > 5$  per group. Splenic sections from EPCR<sup>R84A/R84A</sup> mice stained with H&E at low magnification (10X) (**C**) reveal and increase in the amount of red pulp (R) but not white pulp (W) compared to splenic sections of age-matched EPCR<sup>WT/WT</sup> mice (**D**) Images representative of 3 mice per group. \*\* $p < 0.01$ .



### **3.7.8 Hematological analysis of spleen, bone marrow and peripheral blood in EPCR<sup>R84A/R84A</sup> mice**

Flow cytometric analysis of the spleen and BM was performed to further characterize the cell populations in EPCR<sup>R84A/R84A</sup> mice (Table 3.2). In the spleen of EPCR<sup>R84A/R84A</sup> mice, there was a significant increase in the percentage monocytes/macrophages (CD11b + cells). The total cell numbers in the bone marrow were decreased in EPCR<sup>R84A/R84A</sup> mice. Hematological analysis of peripheral blood revealed circulating platelets were significantly decreased in EPCR<sup>R84A/R84A</sup> mice compared to EPCR<sup>WT/WT</sup> mice (Table 3.3). These studies suggest that EPCR<sup>R84A/R84A</sup> mice exhibit increased cell numbers in the spleen due to increased extramedullary hematopoiesis as a result of BM failure.

To characterize the progenitor activity of the bone marrow and the spleen in EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice, cell colony forming assays were performed on isolated BM and spleen cells of these mice. There were no observed differences in the number of multi-potential progenitors and lineage-restricted progenitors of erythroid, granulocyte, monocyte/macrophage pathways from cultured BM and spleen cell suspensions from EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice suggesting HSCs are functional in EPCR<sup>R84A/R84A</sup> mice (Figure 3.8).

### **3.7.9 Bone Marrow Transplantation**

To determine if BM failure in EPCR<sup>R84A/R84A</sup> mice is a result of impairment of PC/EPCR binding on hematopoietic cells we performed BM transplants to generate EPCR<sup>R84A/R84A</sup>BM/EPCR<sup>WT/WT</sup> and EPCR<sup>WT/WT</sup>BM/EPCR<sup>R84A/R84A</sup> chimeras. Total BM

Table 3.2: Hematological analysis of the spleen and bone marrow of EPCR <sup>R84A/R84A</sup> mice n > 4 per group											
		Monocyte/Macrophage CD11b <sup>+</sup>		B cells B220 <sup>+</sup>		Granulocytes Gr-1 <sup>+</sup>		Megakaryocytes CD41 <sup>+</sup>		HSPC LSK <sup>+</sup>	
Spleen	Cells/BW (g) x10 <sup>5</sup>	Cells/BW (g) x10 <sup>5</sup>	%	Cells/BW (g) x10 <sup>5</sup>	%	Cells/BW (g) x10 <sup>5</sup>	%	Cells/BW (g) x10 <sup>5</sup>	%	Cells/BW (g) x10 <sup>5</sup>	%
WT	28.0± 3.46	1.61± 0.32	6.26 ± 1.16	18.2 ± 2.19	65.2 ± 1.07	2.04 ± 0.43	7.14 ± 0.75	2.83 ± 1.12	8.76 ± 2.66	0.70 ± 0.33	0.85± 0.32
R84A	72.4 ± 14.1	9.81± 2.45	12.5 ± 2.24	41.7 ± 9.73	55.0 ± 6.78	4.96 ± 1.04	7.29 ± 1.34	8.91 ± 1.26	12.5 ± 1.79	1.5 ± 1.01	1.5 ± 1.12
P value	0.03	0.02	0.04	0.06	0.2	0.03	0.9	0.02	0.2	0.4	0.5
BM											
WT	23.2 ± 1.02	11.0± 1.40	48.3 ± 7.46	5.44 ± 0.46	22.8 ± 2.32	11.4± 1.46	50.0 ± 7.65	4.08± 1.05	17.1 ± 3.57	0.14 ± 0.04	0.37± 0.11
R84A	13.2± 3.16	5.82± 1.45	53.2 ± 3.33	2.19 ± 0.66	19.1 ± 0.99	5.87± 1.67	53.1 ± 2.69	2.89± 0.34	22.5 ± 3.8	0.15 ± 0.05	0.71 ± 0.27
P value	0.03	0.02	0.54	0.01	0.10	0.04	0.72	0.18	0.36	0.8	0.3

**Table 3.3: Hematological analysis of peripheral blood from EPCR<sup>R84A/R84A</sup> mice:**

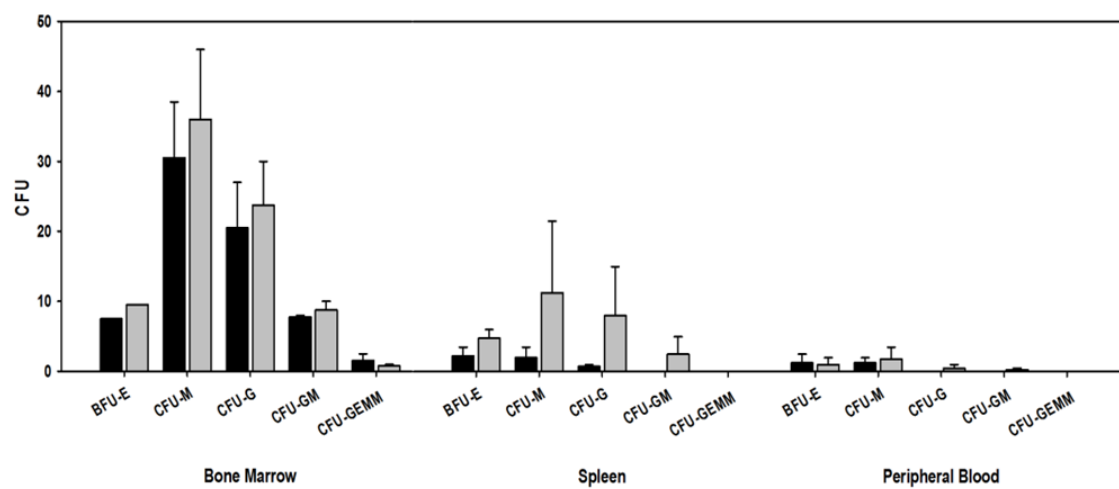
Complete blood count of EPCR<sup>WT/WT</sup> (n= 7) and EPCR<sup>R84A/R84A</sup> mice (n=8), \* p < 0.05.

<b>Parameter</b>	<b>WT</b>	<b>R84A</b>	<b>Normal Range</b>
<b>WBC (K/<math>\mu</math>L)</b>	$5.1 \pm 0.77$	$3.1 \pm 0.36$	1.8 – 10.7
<b>RBC (M/<math>\mu</math>L)</b>	$7.0 \pm 0.27$	$6.0 \pm 0.35$	6.36 – 9.42
<b>Hematocrit (%)</b>	$31.5 \pm 0.98$	$32.4 \pm 2.0$	35.1- 45.4
<b>Platelets (K/<math>\mu</math>L)</b>	$636 \pm 51.9$	$460.5 \pm 36.7^*$	592 - 2972
<b>Lymphocytes (K/<math>\mu</math>L)</b>	$4.2 \pm 0.64$	$2.4 \pm 0.35$	0.9 – 9.3
<b>Monocytes (K/<math>\mu</math>L)</b>	$0.08 \pm 0.02$	$0.12 \pm 0.02$	0.0 – 0.4
<b>Neutrophils (K/<math>\mu</math>L)</b>	$0.86 \pm 0.15$	$0.59 \pm 0.07$	0.1 – 2.4

**Figure 3.8: Colony forming cell assays with BM, spleen and peripheral blood cells:**

Isolated spleen, BM, and peripheral blood cells from EPCR<sup>WT/WT</sup> (black bars) and EPCR<sup>R84A/R84A</sup> (gray bars) mice were added to methylcellulose complete media and plated on 35mm dishes. Plated cells were incubated for 8 days in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Colonies were identified and enumerated using gridded scoring dishes.

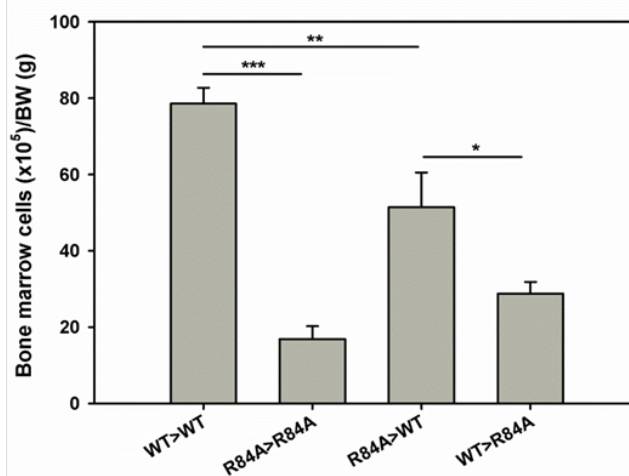




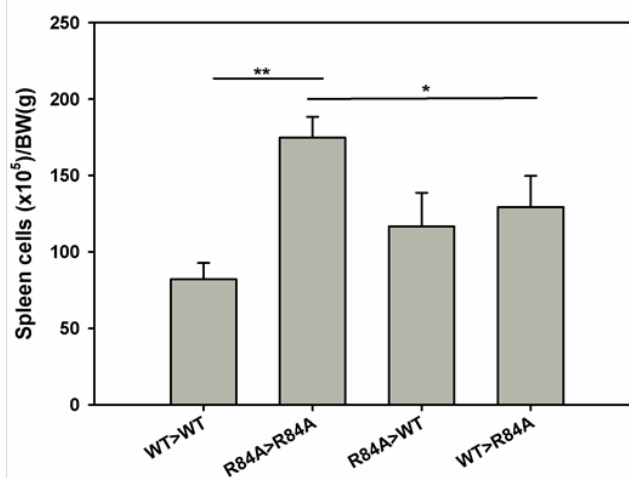
cell counts in  $\text{EPCR}^{\text{R84A/R84A}}\text{BM}/\text{EPCR}^{\text{WT/WT}}$  chimeric mice were significantly decreased compared to control  $\text{EPCR}^{\text{WT/WT}}\text{BM}/\text{EPCR}^{\text{WT/WT}}$  mice (Figure 3.9A). However,  $\text{EPCR}^{\text{WT/WT}}\text{BM}$  was not able to increase the cell count in the BM transplanted  $\text{EPCR}^{\text{R84A/R84A}}$  recipient mice (Figure 3.9A). As a result, the cellularity of  $\text{EPCR}^{\text{WT/WT}}\text{BM}/\text{EPCR}^{\text{R84A/R84A}}$  chimeric mice was significantly decreased when compared to  $\text{EPCR}^{\text{R84A/R84A}}\text{BM}/\text{EPCR}^{\text{WT/WT}}$  chimeric mice. Despite no increase in total cells in the BM of  $\text{EPCR}^{\text{WT/WT}}\text{BM}/\text{EPCR}^{\text{R84A/R84A}}$  chimeric mice, spleen cell numbers and spleen weight significantly decreased compared to control  $\text{EPCR}^{\text{R84A/R84A}}\text{BM}/\text{EPCR}^{\text{R84A/R84A}}$  mice (Figure 3.9B and C).

**Figure 3.9: Hematological analysis of the BM and spleen in BM Transplanted EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice:** EPCR<sup>R84A/R84A</sup> BM/EPCR<sup>WT/WT</sup> chimeric mice (R84A>WT) were generated by transplanting BM from EPCR<sup>R84A/R84A</sup> mice into irradiated recipient EPCR<sup>WT/WT</sup> mice. EPCR<sup>WT/WT</sup> BM/EPCR<sup>R84A/R84A</sup> chimeric mice (WT>R84A) were generated by transplanting BM from EPCR<sup>WT/WT</sup> mice into recipient EPCR<sup>R84A/R84A</sup> mice. Control mice were recipient EPCR<sup>R84A/R84A</sup> mice transplanted with EPCR<sup>R84A/R84A</sup> BM (R84A>R84A) and recipient EPCR<sup>WT/WT</sup> mice transplanted with EPCR<sup>WT/WT</sup> BM (WT>WT). Six months post-BMT mice were sacrificed and total cell counts of BM (**A**), and spleen (**B**) were performed. Splenic weight was assessed (**C**). Data represents the mean  $\pm$  SE, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .  $n > 5$  per group.

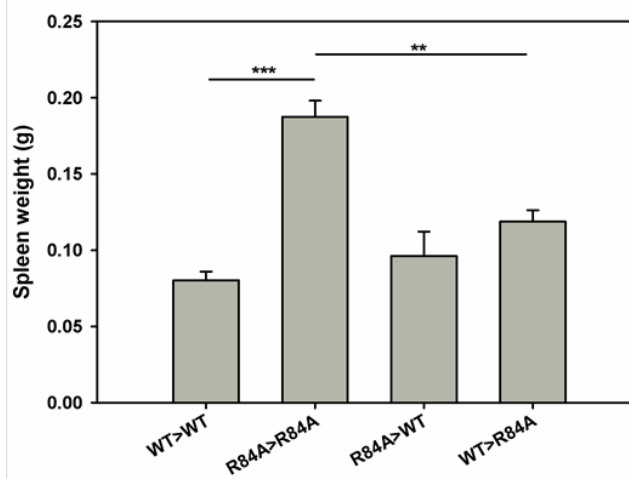
**A**



**B**



**C**



### 3.8 Discussion

The hemostatic balance is tightly regulated during embryonic development, especially at the feto-maternal interface. During development, maternal fibrinogen serves as a hemostatic agent (to assure that normal bleeding during development of vessels is controlled) and also as an anchor for placental-maternal attachment during embryonic development (Suh et al., 1995; Iwaki et al., 2002). The PC system has been shown to be essential for the maintenance of pregnancy. For example, TM-deficient mice do not survive beyond embryonic day E8.5 (E8.5), and death is caused by tissue factor-initiated coagulation at the feto-maternal interface (Isermann et al., 2003). Deletion of the EPCR gene in mice leads to an increase in fibrin deposition around the giant trophoblast cells at day E9.5 and embryonic lethality before day E10 (Gu et al., 2002). Using conditional knock-out strategies, it was shown that EPCR-deficient embryos with EPCR expression on placental giant trophoblast cells can develop normally (Li et al., 2005b). These studies suggest that impaired PC activation at the feto-maternal interface leads to fibrin deposition and abortion of the embryo. However, we have now demonstrated that mice harboring a variant of EPCR that is deficient in PC binding (Figure 3.1B) and PC activation *in vivo* (Figure 3.5A) can reproduce normally and produce viable offspring.

If impairment in PC activation in the EPCR<sup>R84A/R84A</sup> mice does not lead to placental thrombosis and embryonic abortion, how is thrombin generation regulated at the feto-maternal interface? It has been demonstrated that EPCR deficiency is not embryonic lethal in genetically modified mice that express low tissue factor (TF) (approximately 1% of normal), suggesting that decreased thrombin generation rescues

EPCR-deficient embryos (Li et al., 2005b). In this study, we found that the plasma level of PC was ~40% higher in EPCR<sup>R84A/R84A</sup> mice compared to WT mice, suggesting that introduction of the R84A mutation into EPCR results in a redistribution of intravascular PC (Li et al., 2005b; Li et al., 2005a). We postulate that an increase in plasma PC levels may increase the PC activation rate by the thrombin-TM complex. Although we did not detect measureable differences in plasma APC levels between WT and EPCR<sup>R84A/R84A</sup> mice, it is possible that APC levels in the microcirculation (where the effective concentration of TM on vascular endothelial cells is the highest) are sufficiently high in the EPCR<sup>R84A/R84A</sup> mice to support embryo development. Alternatively, a role for EPCR, independent of APC may contribute to the viability of EPCR<sup>R84A/R84A</sup> mice. It has been demonstrated that soluble EPCR (sEPCR), which is present in normal plasma, dose-dependently inhibits FX activation by the sTF/FVIIa complex in the absence of PC/APC (Disse et al., 2011). During embryonic development, EPCR<sup>R84A/R84A</sup> may shed enough sEPCR that is free to interact, and inhibit FX activation by TF/FVIIa complex, thereby downregulating thrombin generation and preventing placental thrombosis and embryonic lethality in an APC-independent manner.

EPCR<sup>R84A/R84A</sup> mice have a normal lifespan and showed no evidence of overt pathological thrombosis. However, upon thrombotic or inflammatory challenge EPCR<sup>R84A/R84A</sup> mice have significant physiological consequences as a result of impaired PC binding to EPCR (Figure 3.5 and Figure 3.6). Similar to EPCR conditional knockout mice (*ProcrLox*) (Li et al., 2005b), EPCR<sup>R84A/R84A</sup> mice also have decreased platelet counts (Table 3.3) (Li et al., 2005b). A decrease in platelet counts and increased

extramedullary hematopoiesis may reflect a sub-acute coagulopathy resulting in hematopoietic compensation and enlargement of the spleen. However, enlargement of the spleen accompanied a by a decrease in total cell counts in the bone marrow of EPCR<sup>R84A/R84A</sup> mice, a phenotype that was not reported in EPCR *ProcrLox* mice, suggests that EPCR, independent of APC interaction may play an important role in maintaining hematopoietic homeostasis within the bone marrow. Despite decreased cell numbers in the BM, and increased cell numbers in the spleen, percentages of B lymphocytes (B220+ cells), granulocyte progenitors (Gr-1+ cells) megakaryocytes (CD41+), and HSPCs (LSK+) in the BM and spleen were similar between EPCR<sup>WT/WT</sup> and EPCR<sup>R84A/R84A</sup> mice (Table 3.2), and peripheral red and white blood cell counts remained normal in EPCR<sup>R84A/R84A</sup> mice (Table 3.3) suggesting that the spleen is able to support normal hematopoiesis.

EPCR is highly expressed on HSCs, and has been used to specifically identify HSCs in murine BM (Balazs et al., 2006). In mice, EPCR expression appears to be limited to HSCs, and is absent on circulating leukocytes (Balazs et al., 2006;Kerschen et al., 2010). In support of this, deficiency of EPCR in hematopoietic cells does not impair the anticoagulant and anti-inflammatory responses in a mouse model of endotoxemia (Zheng et al., 2007b). This is in contrast to humans, in which EPCR expression has been reported in neutrophils, monocytes, eosinophils, and natural killer cells (Sturn et al., 2003;Galligan et al., 2001;Feistritz et al., 2003;Joyce et al., 2004). EPCR is also present within the BM stroma on endothelial cells, chondrocytes (Jackson et al., 2009), and osteoblasts (Kurata et al., 2010). The results of our BM transplant experiments

suggest that EPCR expressed in the stroma may play a greater role in modulating hematopoiesis. In support of this, EPCR<sup>WT/WT</sup>BM/EPCR<sup>R84A/R84A</sup> chimeras have a lower BM cellularity than EPCR<sup>R84A/R84A</sup>BM/EPCR<sup>WT/WT</sup> chimeras. We postulate that stromal derived EPCR plays a greater role in modulating hematopoiesis than EPCR expressed by HSCs, since the latter makes up only a small percentage of the cell population in the BM. However, our results do not exclude a role for EPCR expressed by HSCs as EPCR<sup>R84A/R84A</sup>BM/EPCR<sup>WT/WT</sup> chimeras also had reduced cellularity in the BM although not as severe as EPCR<sup>WT/WT</sup>BM/EPCR<sup>R84A/R84A</sup> chimeras (Figure 3.9).

Coagulation regulators including tissue factor, thrombin, TM, and APC have been shown to be present in the small blood vessels of the bone marrow (Gur Cohen et al., 2013) and studies have revealed a role for the coagulation system in regulating hematopoiesis. Injection of thrombin into mice can induce rapid mobilization of HSPCs into the peripheral circulation, a process which is dependent on thrombin activation of PAR1 and secretion of stromal derived factor-1 (Gur Cohen et al., 2013; Gur-Cohen S et al., 2011). However, it is unlikely that increased thrombin activation of PAR-1 in EPCR<sup>R84A/R84A</sup> as a result of EPCR being unoccupied by PC/APC would result in bone marrow failure, as EPCR *ProcrLox* mice do not demonstrate a similar phenotype. More recently, a study has demonstrated a role for TM and APC in acceleration of HSPC recovery after irradiation, however these effects seemed to be independent of APC/EPCR signaling (Geiger et al., 2012). Our results suggest that EPCR in the absence of PC/APC binding may interact with other ligands, which may affect hematopoiesis, which would account for the EPCR<sup>R84A/R84A</sup> phenotype being absent from mice expressing low levels



of EPCR. *In vitro*, EPCR binds Mac-1 directly on monocytes and indirectly on neutrophils via proteinase-3 (Fink et al., 2013)(Kurosawa et al., 2000). EPCR also binds to the  $\gamma\delta$  T-cell antigen receptor on T-cells (Willcox et al., 2012). At this time it is unclear if these cellular interactions with EPCR *in vivo* are relevant to hematopoiesis.

In summary, we have shown that mice harboring a variant of EPCR that lacks the ability to bind to PC/APC exhibit impaired PC activation and increased thrombin generation in response to thrombotic challenge and inflammatory challenge. In addition, we have identified a possible biological role for EPCR in the regulation of hematopoiesis.

## **CHAPTER 4: THE IMPACT OF THE ENDOTHELIAL PROTEIN C RECEPTOR ON THROMBIN GENERATION AND CLOT LYSIS**

### **4.1 Foreword**

The effects of impaired PC/APC binding to EPCR on thrombin generation and fibrinolysis are described in this manuscript. We demonstrate that a variant of EPCR identified in a patient with VTE that does not bind PC, enhances thrombin generation and impairs fibrinolysis independent of TAFI activation in a cell-based assay. In addition we demonstrate that plasma deficient in PC, inhibits fibrinolysis through increased TAFI activation in a cell-based clot lysis assay.

### **4.2 Objective: To determine if diminished EPCR function can modulate thrombin generation and fibrinolysis.**

Diminished EPCR function is associated with an increased risk of thrombosis. To study the impact of an EPCR variant that does not bind PC/APC on thrombin generation and fibrinolysis we generated cells that expressed TM and a variant of EPCR (R96C) identified in a patient with VTE. We hypothesized that impaired PC binding to EPCR would result in increased thrombin generation as a result of impaired PC activation. We also hypothesized that increased thrombin generation would impair fibrinolysis through an increase in TAFI activation. To test this hypothesis, thrombin generation was initiated in plasma in the presence of cells expressing TM and EPCR and we measured (A) PC activation, (B) TAFI activation, (C) thrombin generation and (D) clot lysis time. These

objectives were used to demonstrate the importance of PC binding to EPCR in regulating coagulation and fibrinolysis.

## **The impact of the endothelial protein C receptor on thrombin generation and clot lysis**

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**Running Head:** EPCR in coagulation and fibrinolysis

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**Authorship Contributions:** L. Pepler coordinated the study, performed experiments and wrote the manuscript. C. Wu assisted with TAFIa assay. DJ Dwivedi and C. Wu assisted with the creation of the cell line expressing EPCR R96C. PY Kim contributed to experimental design of the TAFIa assay and interpretation of data. PC Liaw contributed to experimental design, interpretation of data, and editing the manuscript for publication.

**\*This manuscript has been submitted to the Journal of Thrombosis and Hemostasis:** submission ID JTH-2016-00421

**References:** References for this manuscript have been incorporated into the bibliography at the end of this thesis.

**Essentials:**

- Thrombin activates thrombin-activable fibrinolysis inhibitor (TAFI) and protein C (PC)
- It is not well understood how PC and the endothelial protein C receptor (EPCR) impact clot lysis
- Impaired PC/EPCR binding increases thrombin generation without affecting TAFI activation
- Impairment of EPCR delays clot lysis independent of TAFI activation

**4.3 Summary**

**Background:** When thrombin is bound to thrombomodulin (TM), it becomes a potent activator of protein C (PC) and thrombin-activable fibrinolysis inhibitor (TAFI). Activation of PC is enhanced when PC is bound to the endothelial protein C receptor (EPCR) and presented to the thrombin-TM complex. Activated protein C (APC) inhibits thrombin generation via degradation of factor Va and VIIIa, while activated TAFI (TAFIa) attenuates fibrinolysis.

**Objective:** To determine the impact of EPCR on the generation of APC and TAFIa and how this affects fibrinolysis.

**Methods:** We performed thrombin generation and clot lysis assays in the presence of cells expressing wildtype TM and EPCR (WT cells) or wildtype TM a variant of EPCR (R96C cells) that does not bind PC/APC.

**Results:** In the presence of R96C cells, thrombin generation in normal plasma is increased, as a result of impaired PC activation when compared to WT cells. In addition, clot lysis is delayed in normal plasma in the presence of R96C cells, independent of TAFI activation. In PC deficient plasma, clot lysis is delayed in the presence WT and R96C cells as a result of increased TAFI activation.

**Conclusions:** We demonstrate that impaired EPCR function can be detected by thrombin generation and clot lysis assays on cells expressing TM and EPCR. We also demonstrated that EPCR has both anticoagulant and pro-fibrinolytic effects that are independent of TAFIa.

#### 4.4 Introduction

The protein C (PC) pathway is an important natural anticoagulant pathway that inhibits thrombin generation. When thrombin binds thrombomodulin (TM) on the endothelial cell surface, the substrate specificity of thrombin switches from fibrinogen to PC (Van de et al., 2004). The thrombin-TM complex converts zymogen PC to its activated form, activated PC (APC) (Esmon et al., 1982). The endothelial protein C receptor (EPCR), which binds PC and presents it to the thrombin-TM complex, enhances PC activation by 8-fold *in vitro* and 20-fold *in vivo* (Stearns-Kurosawa et al., 1996; Taylor, Jr. et al., 2001). Activated protein C (APC), along with its cofactor protein S inactivates coagulation factors (F) Va and VIIIa, thereby attenuating thrombin generation.

In addition to activation of PC, the thrombin-TM complex also activates thrombin-activable fibrinolysis inhibitor (TAFI) to activated TAFI (TAFIa) (Bajzar et al., 1996). TAFIa attenuates fibrinolysis by removing the exposed C-terminal lysine residues from fibrin, thereby attenuating plasmin generation and thus, clot lysis (Wang et al., 1998). Although the kinetics of PC and TAFI activation by the thrombin-TM complex are similar (Esmon et al., 1982; Bajzar et al., 1996), it is not well understood if PC-bound EPCR can affect TAFI activation by the thrombin-TM complex. Simultaneous activation of PC and TAFI on human endothelial cells (both umbilical vein and microvascular) that express both TM and EPCR, demonstrate that activation of PC and TAFI by the thrombin-TM complex are likely independent processes, and that the presence of EPCR

does not affect TAFI activation(Wu et al., 2016). How EPCR can affect fibrinolysis, however, remains unanswered.

Clinically, impairment of the anticoagulant PC pathway has been associated with an increased risk of venous thromboembolism (Esmon, 2009;Anderson and Weitz, 2011;Espana et al., 2002). PC deficiency, in which individuals have a congenital or acquired reduction in PC antigen and/or activity, is an independent risk factor for thrombosis and can be identified by *ex vivo* activation of PC by protac, an activator found in snake venom (Bovill et al., 1989;Martinoli and Stocker, 1986). *In vivo*, however, activation of PC is accelerated by the binding of PC to EPCR on the vascular endothelium (Taylor, Jr. et al., 2001). Thus, mutations that impair EPCR/PC binding interactions are predicted to increase the risk the risk of thrombosis (Li et al., 2005b;Pepler et al., 2015;Centelles et al., 2010). In support of this, targeted gene sequencing studies have shown that mutations that impair EPCR-interactions are present in patients with unprovoked VTE but not in controls (Simmonds and Lane, 1999;Biguzzi et al., 2001;Wu et al., 2013;Saposnik et al., 2004;Uitte et al., 2004;Medina et al., 2004).

*In vitro*, thrombin generation and clot lysis assays are commonly performed in the absence of cell surfaces, and therefore not sensitive to deficiencies in EPCR function. We hypothesize that impaired EPCR/PC binding will result in increased thrombin generation due to decreased levels of APC generated. We also hypothesize that increased amount of thrombin generated will lead to inhibition of fibrinolysis due to increased TAFI activation. To test these hypotheses we performed thrombin generation and clot lysis



assays in plasma in the presence of cells that express TM and a variant of EPCR that does not bind PC.

## **4.5 Materials and Methods**

### **4.5.1 Generation of stable cell lines expressing TM and the EPCR variant R96C**

Human embryonic kidney cells (HEK293) expressing both human wild-type TM and EPCR were established as previously described (known as WT cells) (Liaw et al., 2001). HEK293 cells expressing both wild-type TM and R96C EPCR (Known as R96C cells) were established as follows. Generation of the EPCR variant R96C was performed as previously described (Liaw et al., 2001; Wu et al., 2013). HEK293 cells were stably transfected using 2 different pcDNA3.1(-) vectors containing TM and R96C EPCR cDNAs using Effectene transfection reagent (Qiagen, Valenica, CA) as described by the manufacturer. Transfection of HEK293 cells was performed in 6-well dishes in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Burlington, ON) containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). 48 hours post-transfection, the media was changed to DMEM with 10% FBS, 400 µg/mL of G418 and 200 µg/mL of hygromycin (Life Technologies, Burlington, ON). After 2 weeks of drug selection, drug-resistant colonies were isolated, and expression of cell surface TM and EPCR was assessed using a FASCCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

### **4.5.2 Flow Cytometry**

WT and R96C cells were grown to confluency in 6-well dishes, detached with gentle pipetting, and suspended in 1 mL of phosphate buffered saline (PBS), pH 7.4 containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). The cells were

washed twice and incubated with 5 µg/mL of fluorescein isothiocyanate (FITC)-labeled CTM1009, an anti-TM mouse monoclonal antibody or Alexa488- labeled JRK1535, an anti-EPCR mouse monoclonal antibody (Liaw et al., 2001; Stearns-Kurosawa et al., 1996) for 30 min at 4°C. Bound antibody was detected on the fluorescence-1 channel on a FASCCalibur flow cytometer. The fluorescence intensity of each sample was analyzed twice.

#### **4.5.3 Thrombin generation assay on cells**

WT cells, R96C cells, and HEK293 cells (no TM or EPCR) were cultured to 80% confluence in 100 mm dishes in DMEM containing 10% FBS and the appropriate drug selection reagent. At 80% confluency, the cells were washed and harvested in HEPES buffered saline (HBS) and total cell counts were performed using a hemocytometer. Cells were added ( $8 \times 10^4$ ) into the wells of a 96-well black microtiter plate containing HBS, recombiplastin (recombinant human tissue factor (TF) and phospholipids) and citrated normal human pooled plasma or plasma deficient in PC or TAFI (Affinity Biologicals, Ancaster, ON) to a final volume of 75 µL. For normal human plasma, blood was obtained via venipuncture from 10 healthy adult volunteers into 3.2% sodium citrate. Whole blood was centrifuged at  $1500 \times g$  for 10 min at room temperature. Plasma was collected, pooled and stored at -80°C. To determine the effect of TM and EPCR on thrombin generation, cells were pre-incubated with 500 nM of anti-TM antibody (CTM1009) or anti-EPCR antibody (JRK1535) (Stearns-Kurosawa et al., 1996; Liaw et al., 2001) for 30 min at RT. Thrombin generation was initiated by 25 µL of a solution containing 30 mM  $\text{CaCl}_2$  and 2 mM fluorogenic thrombin substrate (Z-Gly-Arg-AMC)(Bachem, Bubendorf,

Switzerland). Thrombin generation was monitored in a SpectraMax M5e plate reader (Molecular devices, Sunnyvale, CA) at 37°C using the Technothrombin thrombin generation assay protocol (Technoclone, Vienna, Austria). Thrombin generation profiles were analyzed using Technothrombin thrombin generation assay software (Technoclone).

#### **4.5.4 Plasma clot lysis in the presence of cells**

PC-deficient, TAFI-deficient or normal plasma was added to flat-bottomed 96-well plates containing  $8 \times 10^4$  cells (HEK293, WT, or R96C). To initiate clot formation and clot lysis, 25  $\mu$ L of a solution containing 2 nM tissue plasminogen activator (tPA, Aviva Systems Bio, San Diego, CA) 30 mM  $\text{CaCl}_2$  and re-lipidated TF in 20 mM Tris, 0.15M NaCl, pH 7.0 was added. To examine the effect of TM and EPCR on clot lysis, TM and EPCR expressing cells were pre-incubated with TM and EPCR blocking antibodies CTM1009 and JRK1535, respectively. To determine the effect of TAFIa on clot lysis, an inhibitor of TAFIa, potato tuber carboxypeptidase inhibitor (PTCI) (Sigma, St. Louis MO) was added to the assay reaction mixture prior to initiation of clot formation. Absorbance was monitored at 450 nm for 2 hours at 37°C in a SpectraMax plate reader. Clot lysis times were determined as the time from half maximal increase to half maximal decrease in absorbance as determined using SoftMax Pro software.

#### **4.5.5 Protein C activation on HEK293 cells expressing TM and EPCR**

To measure PC activation by cells expressing TM and EPCR, a modified thrombin generation assay was used. Briefly, HEK293, WT and R96C cells were added to 96-well plates containing defibrinated plasma. Thrombin generation was initiated as

described previously in the methods. At 0, 5, 10, 20 and 30 min intervals, plasma was removed from the well and added to a solution containing citrate and 20 mM benzamidine for measurement of APC. To quantify APC, an APC enzyme capture assay was performed as previously described (Liaw et al., 2003).

#### **4.5.6 TAFI activation on HEK293 cells expressing TM and EPCR**

As a result of the short half-life and thermal instability of TAFIa, it is difficult to measure TAFI activation in plasma at 37°C. To enhance TAFIa stability in our experiment we used the TAFI variant with Ile at position 325 instead of Thr, which has a longer half-life without changes to its activation kinetics or activity (Schneider et al., 2002). We then prepared plasma that is deficient in both PC and TAFI (PCTDP). TAFI was immunodepleted from the PC-deficient plasma using anti-TAFI monoclonal antibody (mAb16) linked to Sepharose beads as described previously (Wu et al., 2009). PC and/or TAFI were added back at physiological concentrations (70 nM) into PCTDP to produce PCTDP+PC+TAFI, PCTDP+TAFI or PCTDP+PC. To measure TAFI activation by cells expressing TM and EPCR in plasma, a modified thrombin generation assay was used. Briefly, HEK293, WT and R96C cells were added to 96-well plates containing defibrinated PCTDP+PC+TAFI, PCTDP+TAFI or PCTDP+PC. Thrombin generation was initiated as described previously in the methods. At 0, 5, 10, 20 and 30 min intervals, plasma was removed from the well and added to a solution containing 100 µM Phe-Pro-Arg-chloromethylketone (FPRck) and 150 µM Val-Phe-Lys-chloromethylketone (VFKck) (plasmin inhibitor) for measurement of TAFIa. To measure TAFIa, a fluorescent-based assay was used (Kim et al., 2008). Briefly, recombinant plasminogen

possessing S741C mutation labeled with 5-iodoacetamidofluorescein (5IAF-Pg) is incubated with QSY-labeled fibrin degradation products (QSY-FDPs). Upon binding, the QSY moiety quenches the fluorescence of 5IAF-Pg. Removal of the C-terminal lysine residues by TAFIa results in release of 5IAF-Pg, which ultimately results in increased fluorescence. The rate of fluorescence change is directly proportional to TAFIa levels present. Therefore, TAFIa levels were quantified using known amounts of TAFIa to generate a standard curve. The reactions were monitored using SpectraMax M3 plate reader (Molecular devices, Sunnyvale, CA) at 25°C, with the excitation and emission wavelengths set at 480 nm and 520 nm, respectively, with a 495 nm emission cutoff filter.

#### **4.5.7 Statistical Analyses**

Results are expressed as the mean  $\pm$  the standard error (SE). The Student's t-test or a 1-way analysis of variance was used to determine significant difference between experimental groups. A P value  $< 0.05$  was considered to be statistically significant.

## **4.6 Results**

### **4.6.1 TM and EPCR expression on HEK293 cells**

#### **TM and EPCR expression on HEK293 cells**

For this study, the EPCR variant R96C, identified in a patient with unprovoked VTE, which lacks PC/APC binding, was used to study the effect of EPCR on coagulation and fibrinolysis (Wu et al., 2013). WT TM and either WT EPCR or a variant form of EPCR (R96C), were stably transfected into HEK293 cells, and cell surface expression of TM and EPCR was confirmed by flow cytometry (Fig. 4.1). The expression levels of TM and EPCR antigen were similar between WT cells (expressing wild-type TM and EPCR) and R96C cells (expressing wild-type TM and R96C EPCR) (Fig. 4.1). The ratios of TM:EPCR on WT and R96C cells were 1:9 and 1:8, respectively, which is consistent with the TM:EPCR ratio of 1:7 found on human umbilical vein endothelial cells (HUVEC) (Fig. 4.1). Using a modified thrombin generation assay to measure APC, known amounts of soluble TM (sTM) were added to recalcified plasma to measure the conversion of PC to APC. We determined that TM expressed by WT and R96C cells (using  $8 \times 10^4$  cells in 100  $\mu$ L volume) had an activity equivalent to approximately 4 nM of sTM as measured by APC chromogenic activity (not shown).

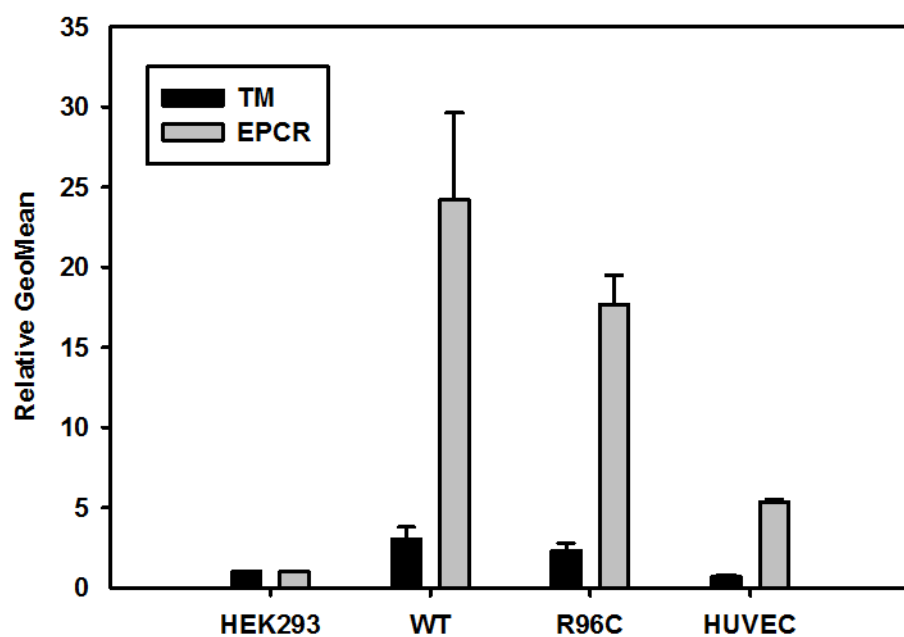
#### **4.6.2 Protein C activation by HEK293 cells expressing TM and EPCR**

To determine the ability of HEK293 cells, WT cells and R96C cells to convert plasma PC to APC, thrombin generation was initiated in defibrinated plasma in the presence of cells and the conversion of plasma PC to APC was measured at different time

**Figure 4.1: Flow cytometric analysis of cells expressing TM and EPCR.**

HEK293 cells were stably transfected with pcDNA3.1(-) vector only or vectors containing the cDNA encoding WT TM and WT EPCR or R96C EPCR. TM and EPCR surface antigen was measured by incubating transfected cells with a FITC-labeled anti-TM mouse monoclonal antibody or an Alexa488-labeled anti-EPCR mouse monoclonal antibody. The binding of fluorescently labeled antibodies to cells was analyzed by flow cytometry on fluorescence channel-1 and geometric mean calculated. Data represents the mean  $\pm$  SE of 3 independent experiments.





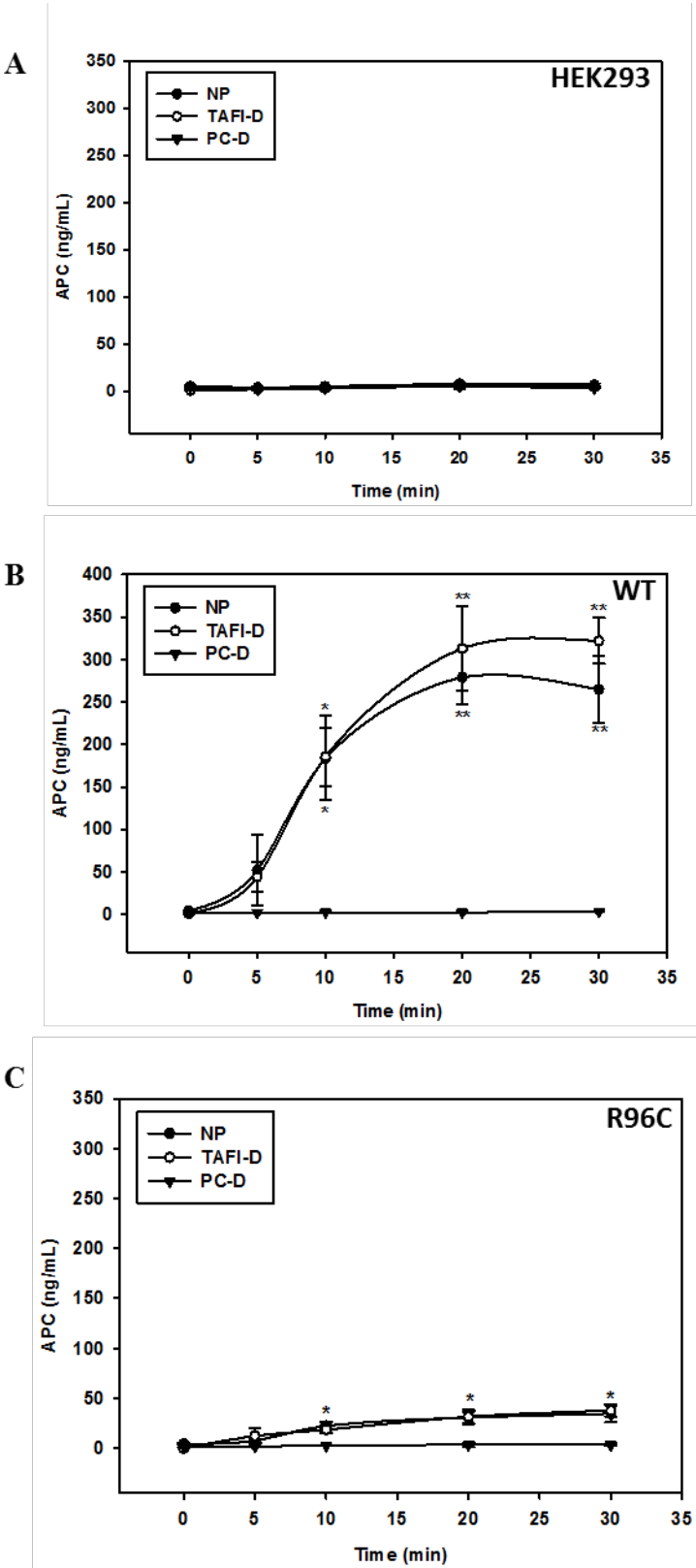
points by an APC-enzyme capture assay. To determine if the absence of TAFI can affect APC generation by thrombin-bound TM, we measured APC generation in normal and TAFI-deficient defibrinated plasma. Initiation of coagulation with TF/CaCl<sub>2</sub> in the presence of HEK293 cells results in very little APC generation after thrombin generation is initiated (Fig. 4.2A). In contrast, WT cells generate a significant amount of APC as early as 10 min post initiation of thrombin generation in both normal and TAFI-deficient plasma (Fig. 4.2B). WT cells in both normal and TAFI-deficient plasma convert similar amounts of plasma PC to APC, suggesting that the absence of TAFI in plasma does not increase PC activation (Fig. 4.2B). The conversion of PC to APC by R96C cells is reduced when compared with WT cells, suggesting that impaired PC binding to the EPCR variant R96C impairs PC activation (Fig. 4.2C). The total amount of PC activation by R96C cells was also similar in normal and TAFI-deficient plasma, further suggesting that TAFI does not affect APC generation in our system (Fig. 4.2C).

#### **4.6.3 TAFI activation by HEK293 cells expressing TM and EPCR**

To determine if PC can affect TAFI activation by the thrombin-TM complex on cells expressing TM and EPCR, we initiated thrombin generation in plasma that is deficient in both PC and TAFI (PCTDP) with PC and/or Ile325-TAFI variant added back and measured TAFI activation. In the absence of cells expressing TM and EPCR, TAFIa generation was similar between PCTDP+PC+TAFI and PCTDP+TAFI (Fig. 4.3A). The amount of TAFIa generated by WT cells and R96C cells is similar in PCTDP+PC+TAFI suggesting impaired PC binding to EPCR does not affect TAFIa generation (Fig. 4.3B and C). However, in the presence of TM, with or without functional EPCR (R96C cells),

**Figure 4.2: APC generation in the presence of cells expressing TM and EPCR.**

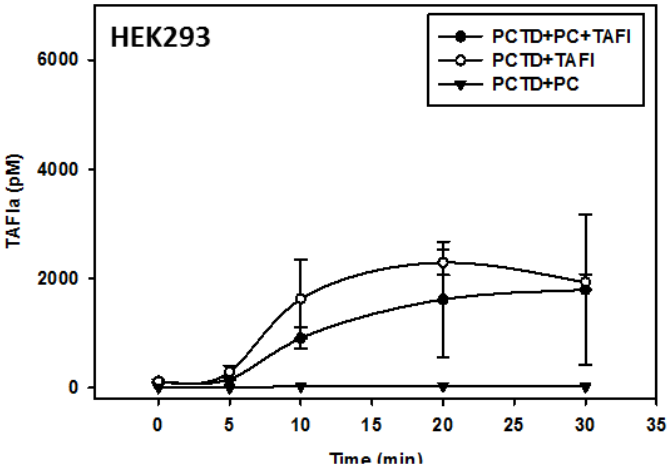
HEK293 (A), WT (B) and R96C (C) cells ( $8 \times 10^4$  cells) were added to 96-well microtiter plates containing defibrinated normal, PC-deficient, or TAFI-deficient plasma. Thrombin generation was initiated with TF and  $\text{CaCl}_2$ . At 0, 5, 10, 20, and 30 min post initiation of thrombin generation, plasma was collected and added to citrate/benzamidine. APC was measured by an APC enzyme-capture assay. Data represents the mean  $\pm$  SE of 3 independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



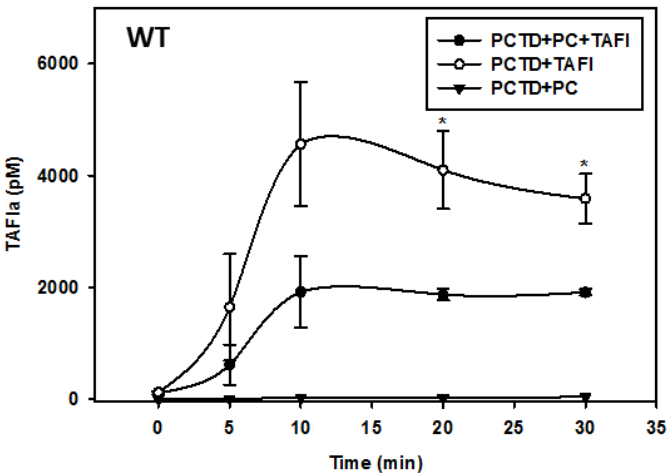
**Figure 4.3: TAFI generation in presence of cells expressing TM and EPCR.**

HEK293 (A), WT (B) and R96C (C) cells ( $8 \times 10^4$  cells) were added to 96-well microtiter plates containing defibrinated PCTD+PC+TAFI, PCTD+TAFI, or PCTD+PC. Thrombin generation was initiated with TF and  $\text{CaCl}_2$ . At 0, 5, 10, 20, and 30 min post initiation of thrombin generation, plasma was collected and added to citrate/FPRck/VFKck. TAFIa was measured by a fluorescent-based assay. Data represents the mean  $\pm$  SE of 2 independent experiments. \*  $P < 0.05$ .

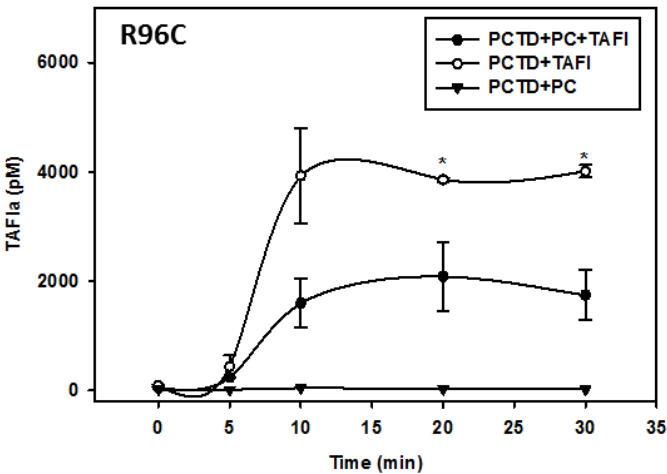
A



B



C



more TAFIa is generated in PCTDP+TAFI when compared with PCTDP+PC+TAFI (Fig. 4.3B and C). This suggests the absence of PC in plasma does increase TAFI activation by the thrombin-TM complex in our system.

#### **4.6.4 Thrombin generation in the presence of cells expressing TM and EPCR**

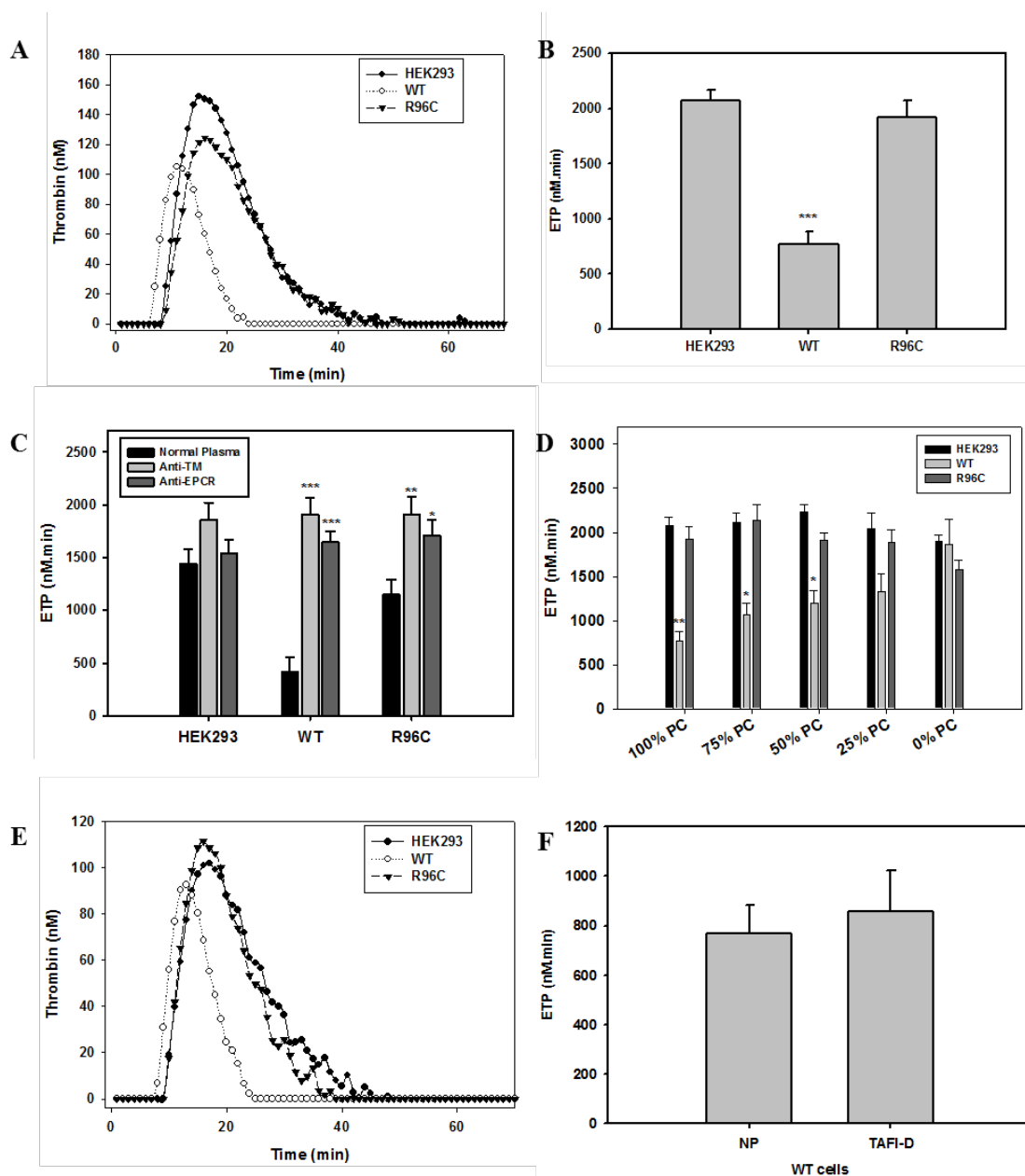
To determine if cells expressing the endothelial components of the PC pathway can modulate coagulation, we performed thrombin generation assays in the presence of cells expressing TM and EPCR. In the presence of WT cells, the endogenous thrombin potential (ETP) (a measure of total thrombin generated) was reduced by approximately 60% when compared with HEK293 cells and R96C cells (Fig. 4.4A and B). Blocking TM or EPCR on WT cells increased thrombin generation, resulting in thrombin levels similar to that observed in the presence of HEK293 cells (Fig. 4.4C). Furthermore, decreasing plasma PC levels (through mixing of normal plasma and PC-deficient plasma) increased thrombin generation in the presence of WT cells. This is demonstrated by normalization of the ETP across all cell types when thrombin generation is initiated in PC-deficient plasma (Fig. 4.4D). Taken together, these results suggest that both TM and EPCR are required for sufficient generation of APC to inhibit thrombin generation in our system. However, in the absence of PC/EPCR interactions, APC-mediated inhibition of thrombin generation is lost.

To confirm that the absence of TAFI does not affect thrombin generation by increasing PC activation by the thrombin-TM complex, we performed thrombin generation assays in TAFI-deficient plasma. Similar to normal plasma, thrombin

**Figure 4.4 : Thrombin generation in the presence of cells expressing TM and EPCR.**

HEK293, WT and R96C cells ( $8 \times 10^4$  cells) were added to 96-well microtiter plates containing normal pooled plasma (**A-C**) or plasma with varying levels of PC (**D**). Thrombin generation was initiated with TF and  $\text{CaCl}_2$  and thrombin generation was measured over time by cleavage of a thrombin specific fluorogenic substrate. A thrombogram was generated (**A**) and ETP calculated for thrombin generation in normal plasma (**B**), thrombin generation in the presence of cells pre-incubated with 500 nM of anti-TM or anti-EPCR antibodies (**C**) and thrombin generation in plasma with varying levels of PC (**D**). Data represents the mean  $\pm$  SE of 5 independent experiments; \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .





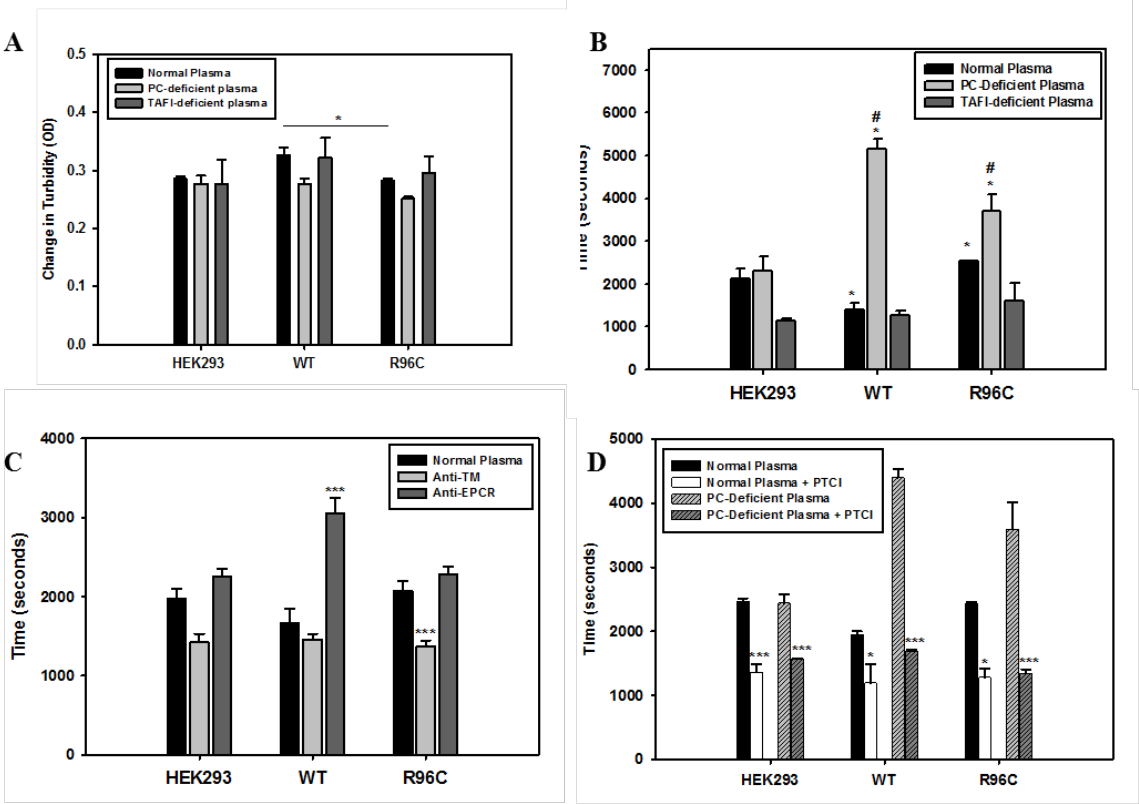
generation in TAFI-deficient plasma is decreased in the presence of WT cells when compared to HEK293 and R96C cells (Fig. 4.4E). However, there are no significant differences between the inhibition of thrombin by WT cells between normal plasma and TAFI-deficient plasma (Fig. 4.4F), confirming that the generation of APC by thrombin is unaffected by TAFI levels.

#### **4.6.5 Clot lysis in the presence of cells**

To determine if cells expressing TM and EPCR can modulate fibrinolysis, we performed clot lysis assays in the presence of HEK293 cells, WT cells and R96C cells. One parameter of fibrin clot structure can be assessed by the change in turbidity over time (measured by the optical density). In the absence of TM and EPCR expressing cells, the change in clot turbidity between normal plasma and PC and TAFI deficient plasma were similar, suggesting no differences in clot structure (Fig. 4.5A). However, in the presence of WT cells, a more turbid clot is formed in normal plasma when compared with clots formed in the presence of R96C cells (Fig. 4.5A). This results in the acceleration of clot lysis, which is 45% faster than the clot lysis time in the presence of R96C cells (Fig. 4.5B). In contrast, plasma deficient in PC show delayed clot lysis in the presence of WT cells and R96C cells when compared with HEK293 cells (Fig. 4.5B). Blocking TM but not EPCR on WT and R96C cells reduced the clot lysis time, suggesting that blocking the thrombin-TM complex inhibits TAFI activation (Fig. 4.5C). Taken together, the absence of PC in the plasma or the presence of R96C EPCR results in delayed clot lysis.

**Figure 4.5: Clot lysis in the presence of cells expressing TM and EPCR.**

PC-deficient, TAFI-deficient or normal plasma was added to 96-well plates containing  $8 \times 10^4$  cells (HEK293, WT, or R96C). To initiate clot formation and clot lysis, TF,  $\text{CaCl}_2$  and tPA was added and absorbance at 450 nm was measured over time and clot turbidity **(A)** and time to clot lysis was calculated **(B)**. Time to clot lysis was also calculated in normal plasma in the presence of TM or EPCR inhibitory antibodies **(C)**, or in the presence of PTCI **(D)**. Data represents the mean  $\pm$  SE of 4 independent experiments; \*  $P < 0.05$  comparing the effect of different cells within a plasma type. #  $P < 0.05$  comparing the effect of different plasmas on a cell type.



To determine if the delay in clot lysis in the absence of PC activation is a result of greater thrombin-mediated TAFI activation, we measured the clot lysis time in the presence of PTCl, an inhibitor of TAFIa. In normal plasma, the clot lysis time was decreased by ~ 40% independent of cell type, confirming that small amounts of thrombin-mediated TAFI activation do occur in our system, independent of thrombin-bound to TM (Fig. 4.5D). In the presence of TM and EPCR, the clot lysis time for PC-deficient plasma was further reduced by ~68% when PTCl was present suggesting that delayed clot lysis in PC-deficient plasma is mediated by increased TAFI activation (Fig. 4.5D).

#### **4.7 Discussion**

In this study, we showed that impaired EPCR function alters the dynamics of thrombin generation and clot lysis. When thrombin generation is performed in normal plasma, more total thrombin is generated when EPCR does not bind PC as a result of decreased APC generation. However the increase in thrombin that is generated does not result in a significant increase in TAFI activation by the thrombin-TM complex but still results in a slight delay in fibrinolysis. This confirms previous findings that there is no difference in TAFI activation on endothelial cells in the presence or absence of EPCR (Wu et al., 2016). The delay in clot lysis observed in the presence of cells expressing the EPCR R96C variant is likely a result of the formation of a less turbid clot. High thrombin concentrations, like those generated when PC binding to EPCR is impaired, produce clots that are less turbid than those produced at low thrombin concentrations (Wolberg, 2007). The clots that are formed at high thrombin concentrations are composed of a dense network of thin fibrin strands that are less permeable making them more resistant to fibrinolysis (Wolberg, 2007).

Although the delay in clot lysis time when there is impaired PC binding to EPCR may be a result of differences in thrombin generation and clot turbidity, there is also prolongation of the clot lysis time when thrombin generation and clot turbidity remain the same. In support of this, we show that in PC-deficient plasma, there is no difference in the total thrombin that is generated between HEK293 cells, WT cells, and R96C cells. However, there is a prolongation of the clot lysis time in the presence of WT cells and R96C cells when compared to HEK293 cells in PC-deficient plasma. This effect of

delayed clot lysis in PC-deficient plasma is lost in the presence of the TAFIa inhibitor PTCI confirming that impaired fibrinolysis is directly related to increased TAFIa generation by the thrombin-TM complex. Increased TAFI activation by the thrombin-TM complex in the absence of PC in plasma is in contrast to Wu *et al.*, who show that TAFI activation on endothelial cells is not affected by the presence of PC, albeit in the buffer system (Wu et al., 2016). This discrepancy may be accounted for by the differences in the amount of thrombin in each system, and the expression level of TM resulting in greater TAFI activation. In our plasma system, peak thrombin levels reached ~160 nM in plasma compared to 10 nM added in the buffer system, and TM expression levels are higher on WT and R96C when compared to HUVEC. Despite no difference in the total amount of thrombin generated by HEK293, WT and R96C cells in PC-deficient plasma, it is likely that more thrombin is available to activate TAFI when PC is absent from the plasma. As a result of increased TAFI activation in PC-deficient plasma, there is attenuation of fibrinolysis via the removal of lysine residues and the down-regulation of plasminogen activation. Taken together, the unregulated thrombin generation as a result of impairment in the PC pathway may contribute to thrombosis by impairing clot lysis in a TAFIa-dependent and independent manner.

Differences in the way impairment of the PC pathway affect thrombin generation and clot lysis may explain why in some clinical conditions thrombus formation occurs in the large vessels while others result in the development of microvascular thrombosis. The distribution of endothelial TM throughout the vasculature is uniform, whereas EPCR is expressed at higher levels in the larger vessels than the microvasculature (Laszik et al.,

1997). However, the effective TM concentration in the vasculature varies greatly from ~0.15 nM in large vessels to ~500 nM in the microcirculation due to the increase in cell surface to blood volume ratio (Esmon, 1989; Esmon, 2000). In our system, the effective TM concentration is ~4 nM, thus mimicking the TM concentration in the larger vessels. At these TM levels, we have shown that impairment of EPCR results in a significant decrease in the amount of APC generated leading to increased thrombin generation and impaired clot lysis, which may contribute to thrombosis in the larger vessels. Clinically, mutations in *PROCR* have been associated with a higher risk of thrombosis. One of the first reported mutations in the *PROCR* was a 23-base-pair insertion in exon 3 that results in a truncated EPCR protein that is not expressed on the endothelial cell surface and thus does not bind PC/APC (Simmonds and Lane, 1999; Biguzzi et al., 2001). This mutation has been identified in individuals with VTE (prevalence of 0.48% vs 0.38% in controls) and myocardial infarction (prevalence of 1.20% vs 0.27% in controls) (Medina et al., 2007). Another mutation in *PROCR* that could contribute to thrombotic risk is the A4600G substitution (H3 haplotype) leading to a Ser219Gly amino acid substitution change in the transmembrane domain of EPCR (Saposnik et al., 2004). This Ser219 change to Gly results in increased EPCR shedding by metalloprotease ADAM17 and carriers of the H3 haplotype have elevated levels of soluble EPCR (sEPCR) (Qu et al., 2007; Qu et al., 2006). Not only is there a loss of membrane bound EPCR, sEPCR binds PC with similar affinity and prevents its activation by the thrombin-TM complex (Liaw et al., 2000). However, the thrombotic risk associated with the H3 haplotype is controversial, as one study found that carriers of the H3 haplotype have an increased risk



of VTE (Saposnik et al., 2004), whereas others have found no association with increased VTE risk (Medina et al., 2004;Uitte et al., 2004). Finally, anti-EPCR auto-antibodies present in ~20% of patients with antiphospholipid syndrome are associated with multiple episodes of venous thrombosis (Hurtado et al., 2004).

Impairment or loss of EPCR function in the microvasculature is less likely to lead to the development of microvasculature thrombosis, as the effective TM concentration is much higher and is likely able to support APC generation independent of EPCR to regulate thrombin generation. However, we have shown that deficiency of PC in the plasma will lead to impaired PC activation and increased thrombin generation resulting in increased TAFI activation and impaired fibrinolysis. There are several clinical situations in which congenital or acquired PC-deficiency may contribute to microvascular thrombosis. Newborns with hereditary homozygous PC-deficiency often present with purpura fulminans, a thrombotic disorder that is characterized by microvascular thrombosis in the dermis (Dreyfus et al., 1991;Gladson et al., 1987). A decrease in PC synthesis caused by complications associated with the administration of the vitamin K antagonist warfarin can lead to microvascular thrombosis and skin necrosis (Gladson et al., 1987). Finally, acquired PC-deficiency during sepsis as a result of disseminated intravascular coagulation leads to widespread microvascular thrombosis causing organ dysfunction (Fisher, Jr. and Yan, 2000;Esmon, 2001). Taken together, these conditions result in the absence of PC interacting with the thrombin-TM complex within the microvasculature. As a result, there is increased thrombin generation and TAFI activation leading to the development of clots that are more resistant to lysis.

We have developed a cell-based system that can measure the impact of the PC pathway on both thrombin generation and clot lysis. We have demonstrated that in the presence of a cell surface, plasma deficiency of PC or impairment of EPCR leads to the formation of clots that are more resistant to lysis. The influence of EPCR function on thrombotic risk has been difficult to determine because it is a membrane-bound protein, and therefore its function cannot be measured in plasma-based assays. We have demonstrated that EPCR has both anticoagulant and pro-fibrinolytic effects. Although the clinical utility of a cell-based assay to determine EPCR function may be limited, our studies suggest that impaired EPCR function may contribute to thrombosis.

## **CHAPTER 5.0 GENERAL DISCUSSION AND FUTURE DIRECTIONS**

There is extensive crosstalk that occurs between the coagulation, inflammation, and fibrinolytic pathways. One of the main interfaces that link these systems together is the PC pathway. The PC pathway is an important modulator of numerous pathological processes through its anticoagulant, anti-inflammatory, and pro-fibrinolytic activities. Under normal conditions or in response to injury, the vascular endothelium acts as a protective barrier between the blood and underlying tissues, partially through the actions of TM and EPCR. TM sequesters thrombin and EPCR sequesters PC at the endothelial cell surface in order to generate adequate amounts of APC to protect the vascular endothelium from procoagulant and pro-inflammatory mediators. Generation of APC by the PC pathway can prevent the progression of thrombosis through its anticoagulant and pro-fibrinolytic activities, and dampen the inflammatory response during infection through its cytoprotective activities. Most of these functions depend on PC/APC binding to its receptor EPCR to efficiently activate PC and to support APC cell signaling.

Clinically, impairment of EPCR function may be associated with a higher risk of thrombosis and exacerbate inflammatory responses. In previous work we have identified SNPs in EPCR that impair PC binding to EPCR in individuals with VTE (Wu et al., 2013). Furthermore, in inflammation-induced diseases like sepsis, loss of cell surface EPCR through shedding impairs PC activation and decreases in circulating PC are associated with organ dysfunction and higher mortality (Zheng et al., 2007a; Macias and Nelson, 2004). Taken together, impairment of the interaction between PC and EPCR

likely contributes significantly to disease progression. Since EPCR is a major mediator of the interplay between the pathways of coagulation, inflammation, and fibrinolysis, we further explored the effects of impaired PC binding to EPCR on these systems using *in vivo* and *in vitro* methods.

The studies described in this thesis were undertaken to further define the role of EPCR in the absence of PC binding. For this research we hypothesized that impaired binding of PC to EPCR would result in procoagulant, pro-inflammatory, and anti-fibrinolytic effects. To test this hypothesis we generated and characterized a novel mouse model that harbours a murine variant of EPCR (R84A) that does not bind PC. We also generated cell lines expressing TM and a human variant of EPCR (R96C) that does not bind PC and developed cell-based assays to determine the effects of impaired EPCR function on thrombin generation and clot lysis. Through these studies we made 4 major findings. First, we demonstrated that impaired PC binding to EPCR, results in increased thrombin generation and fibrin deposition *in vivo*. Second, the inflammatory response to endotoxin was exacerbated in mice with impaired PC binding to EPCR. Third, impairment of PC binding to EPCR delays clot lysis. Finally, we identified an unexpected role for EPCR in regulating hematopoiesis, suggesting the intricate relationship between coagulation, inflammation and fibrinolysis that is mediated by EPCR may be relevant in other previously unknown biological processes. This discussion will focus on the present understanding of the relationship between coagulation, inflammation, and fibrinolysis and how EPCR and PC interactions can impact the interplay between these systems. A schematic to summarize the impact of impaired PC/EPCR interactions on the cross-talk

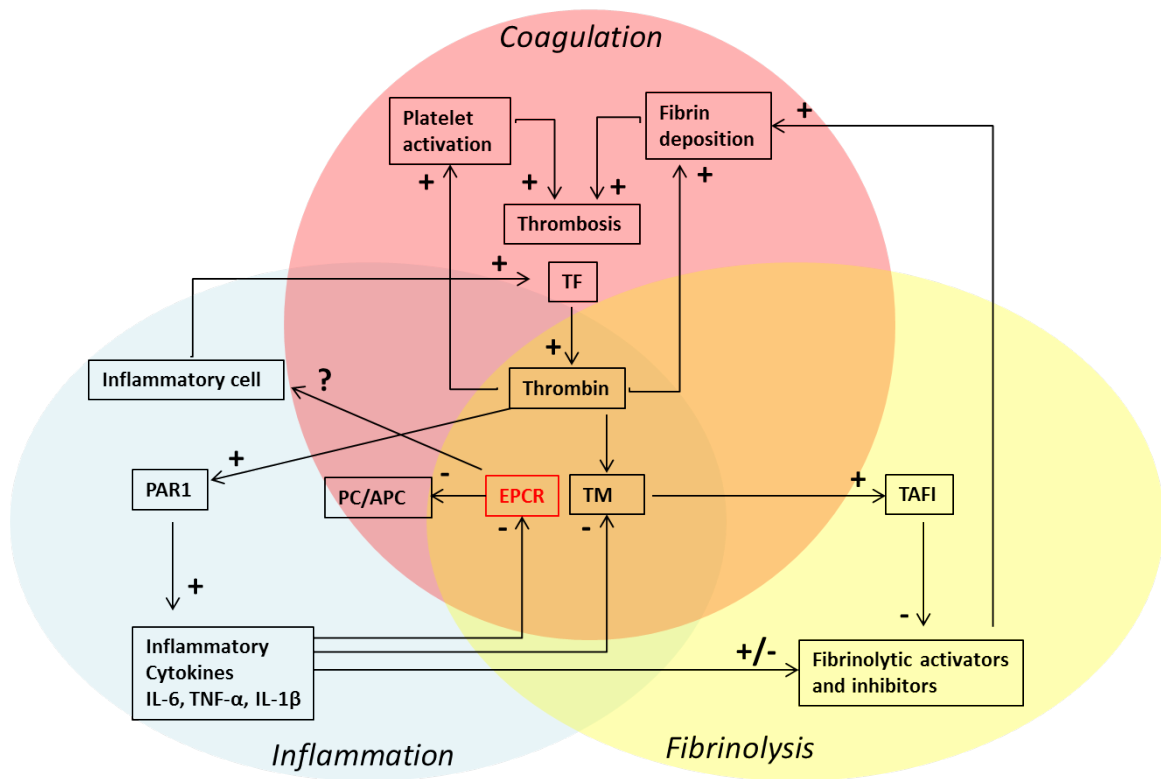
between coagulation, inflammation and fibrinolysis is illustrated in Figure 5.1. This chapter also suggests future studies to extend the studies outlined in this thesis.

### **5.1 EPCR and coagulation**

The PC pathway is a key regulator of procoagulant activity. It had been thought that the PC pathway served only to shut down coagulation through APC-mediated inactivation of FVa and FVIIIa. More recently, the role of the PC pathway has shifted from that of a means to terminate coagulation to one in which the PC pathway acts to prevent clotting on healthy endothelium. In support of this are two findings: First, APC circulates in human plasma at a concentration of ~ 40 pM and a half-life of ~20 minutes (Griffin et al., 2007). This suggests there is continuous formation of small amounts of APC under healthy conditions. Second, APC inactivates FVa at the endothelial cell surface but not on the surface of platelets (Oliver et al., 2002). It has been demonstrated that FVa is protected from APC-mediated cleavage when thrombin generation takes place on platelets (Camire et al., 1998). Taken together, endothelial TM and EPCR convert PC to APC in order to prevent clotting reactions on the uninjured vessels. Any disruption or impairment in the PC pathway has the potential to leave the endothelium unprotected, resulting in the development of thrombosis.

In Chapter 3, we demonstrate that healthy mice that harbor the EPCR variant R84A that does not bind PC, do not develop spontaneous thrombosis. However, upon intravenous injection of FXa, EPCR<sup>R84A/R84A</sup> mice have lower circulating APC,

**Figure 5.1: Impact of impaired PC/EPCR interactions on coagulation, inflammation, and fibrinolysis.** A schematic representation of the major pathways affected by the impairment of PC binding to EPCR. Diminished EPCR function is indicated in red. Arrows with a plus sign indicate a stimulatory effect and arrows with a minus sign indicate an inhibitory effect. Pathways in the red circle represent coagulation pathways; in the blue circle, inflammatory pathways; and yellow circle, fibrinolytic pathways. Figure adapted from Levi M. and van der Poll T. Trends Cardiovasc Med 2005;15:254-259.



resulting in increased TAT levels and fibrin deposition. Furthermore, in Chapter 4 we demonstrate that initiating coagulation with TF and  $\text{CaCl}_2$  in the presence of cells expressing TM and EPCR variant R96C results in increased thrombin generation and impaired fibrinolysis. It is likely that impairment of PC/EPCR interactions on its own is not enough to initiate thrombus development. In support of this is the “multiple-hit hypothesis” which acknowledges that although changes in coagulation and anticoagulation factor levels influences the risk of thrombosis, it is clear that other factors also contribute to thrombotic risk (Esmon, 2009). This is evident in some families with PC deficiency, in which some families have a low incidence of thrombosis, whereas other families have a high incidence of thrombosis (Esmon, 2009). Even in the same family with PC-deficiency, different members can have either a high or low frequency of thrombosis (Bovill et al., 1989). Taken together, the multiple-hit hypothesis suggests there is a threshold at which the activity of procoagulant factors overcomes the activity of anticoagulant factors. The impairment of PC binding to EPCR would lower the threshold at which the generation of thrombin is not adequately regulated by APC at the endothelium. Upon challenge, the lowered threshold is reached as the thrombin that is generated quickly overwhelms the natural anticoagulant pathways and thrombosis occurs.

One of the limitations of the study in Chapter 3 is the use of FXa/phospholipids, to initiate thrombin generation *in vivo*. FXa/phospholipids is a strong thrombotic stimulus that is not physiologically relevant. Current understanding is that the initiation of thrombosis *in vivo* occurs on an intact endothelium as a result of inflammatory mediators or hypoxia (due to disturbed blood flow in the venous valvular sinus)



(Mackman, 2012). When EPCR<sup>R84A/R84A</sup> mice were injected with LPS as a more physiologically relevant inflammatory challenge, TAT levels were increased at 6 hours post-injection (Chapter 3) suggesting that our murine model with impaired PC/EPCR interactions is more sensitive to vascular diseases.

Future studies should be performed with hypoxia as the thrombotic challenge as this would better mimic the initiation of venous thrombosis in humans. In healthy individuals, a reduction in oxygen-hemoglobin saturation, like that which occurs during commercial flights is linked to an increased risk of DVT (Silverman and Gendreau, 2009; Ferrari et al., 1999; Kuipers et al., 2007). Furthermore, mice that have been subjected to hypoxic conditions exhibit increased thrombus formation, partially mediated by changes in the vessel wall and through the recruitment of leukocytes (Brill et al., 2013; Lawson et al., 1997). For future studies, EPCR<sup>R84A/R84A</sup> and EPCR<sup>WT/WT</sup> mice would be placed in a hypoxic chamber with 6% oxygen for different periods of time (Brill et al., 2013). The hypoxia would induce leukocyte adhesion to the hypoxemic vessel wall due to translocation of P-selectin (Pinsky et al., 1996). Subsequent leukocyte activation would induce TF expression, triggering procoagulant pathways (Yan et al., 1998). TF expression, platelet accumulation, and fibrin deposition could be measured in the lungs, kidney and brain of EPCR<sup>R84A/R84A</sup> and EPCR<sup>WT/WT</sup> mice, tissues in which EPCR is known to have a protective effect (von Drygalski et al., 2013). We would expect that as a result of impaired PC activation in EPCR<sup>R84A/R84A</sup> mice, thrombin generation, platelet activation and fibrin deposition would be increased.

An alternative to using a hypoxic chamber as a thrombotic challenge is an IVC stenosis model. The IVC stenosis model has been developed to study the mechanisms of early acute thrombosis (Brill et al., 2011). These studies would include ligating the IVC in EPCR<sup>R84A/R84A</sup> and EPCR<sup>WT/WT</sup> mice to produce a 90% reduction in the IVC lumen size without damaging the endothelium (Brill et al., 2011). Thrombosis in the IVC stenosis model is initiated by a combination of endothelial activation, reduced blood flow, and disturbed blood flow upstream of the stenosis site, producing non-occlusive thrombi that closely mimic clinical scenarios (Brill et al., 2011; von Bruhl et al., 2012). Impaired PC/EPCR interactions would not only result in increased thrombin generation (as our own results in Chapter 3 and 4 suggests), but may also contribute to increased recruitment of leukocytes via the upregulation of adhesion molecules (Joyce et al., 2001). We expect the impairment of the natural anticoagulant PC pathway at the vessel wall in EPCR<sup>R84A/R84A</sup> mice will accelerate thrombus formation and lead to greater thrombus size. These studies would further support a role for EPCR as a risk factor for thrombosis.

In addition to being a cellular receptor for PC/APC, EPCR also serves as a receptor for FVII and FVIIa on the endothelium in humans (Ghosh et al., 2007). The physiological importance of FVIIa binding to EPCR has yet to be established but it may play a role in FVIIa clearance (Ghosh et al., 2007). It has been demonstrated that upon binding of FVIIa to EPCR, the FVIIa-EPCR complex is rapidly internalized through endocytosis (Nayak et al., 2009). However, FVIIa and EPCR are recycled back to the endothelial cell surface suggesting EPCR does not account fully for FVIIa clearance (Nayak et al., 2009). Furthermore, FVII circulates in plasma at 10 nM, (7-fold lower than

PC) and binds EPCR with a similar affinity as PC, suggesting that under normal physiological conditions only a small percentage of FVIIa would bind EPCR (Fukudome and Esmon, 1994; Ghosh et al., 2007). However, hemophilia patients receive large doses of recombinant FVIIa, which can approach plasma PC levels (Hedner, 2004). Under these conditions, EPCR may contribute to FVIIa clearance.

In addition to the role EPCR may play in FVIIa clearance, sEPCR has also been shown to decrease the ability of FVIIa to activate FX (Lopez-Sagaseta et al., 2007). Furthermore, EPCR interaction with FVII reduces FVII activation by FXa by preventing FVII interaction with phospholipid vesicles at the endothelial cell surface (Puy et al., 2010). The ability of (s)EPCR to reduce the procoagulant activity of FVII(a) may have physiological significance. In patients with PC-deficiency, FVII binding to (s)EPCR may confer protection by reducing FVIIa-dependent procoagulant activity. In addition, conditions associated with high levels of sEPCR (i.e. H3 haplotype gene encoding EPCR) may also prevent FVIIa-dependent procoagulant activity. Impairment of PC binding to EPCR would not only reduce the ability of the endothelium to activate PC but may also reduce the EPCR-mediated protection of FVII from activation, conferring even greater thrombotic risk. The impact of FVII(a) interactions with EPCR on coagulation *in vivo* has not been examined.

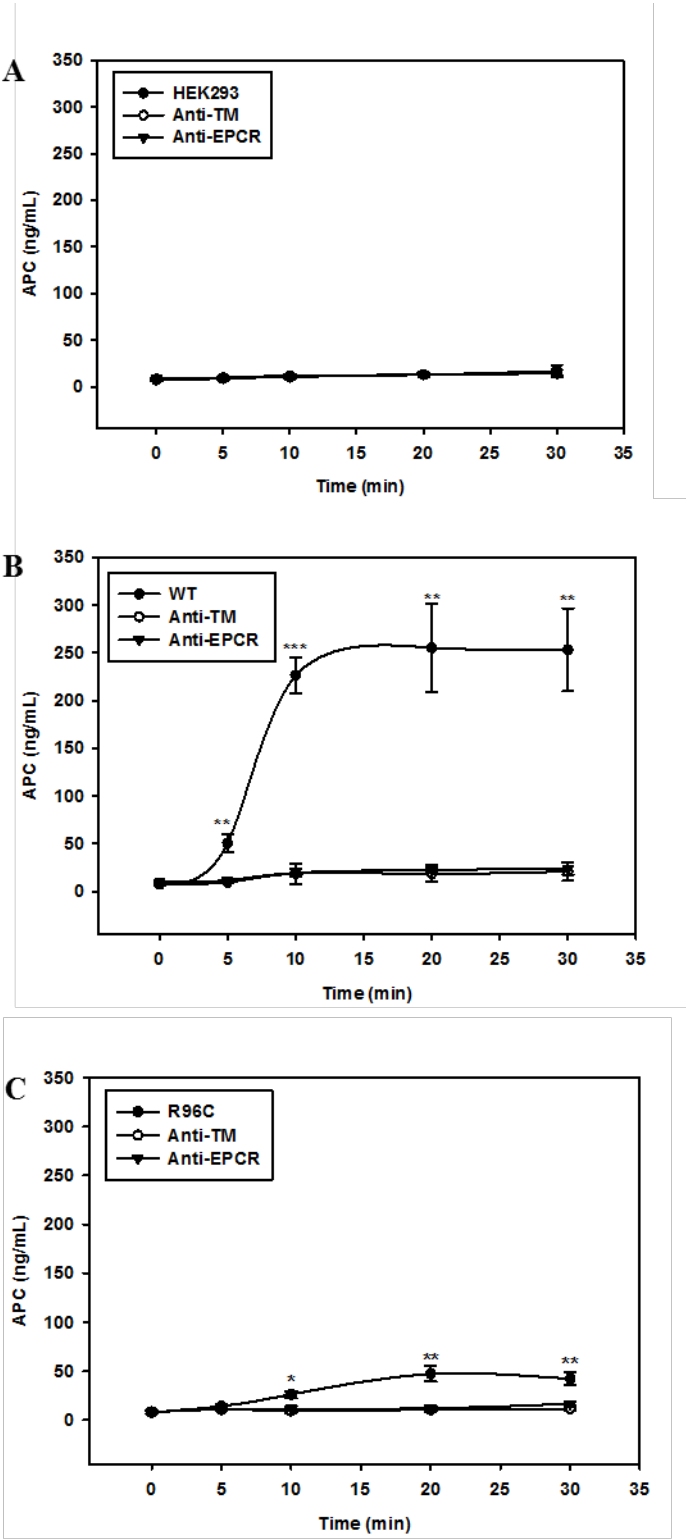
A limitation of our study is the inability to study FVII/EPCR interactions in our EPCR<sup>R84A/R84A</sup> mouse model. Our group and others have demonstrated that while hFVIIa can bind mEPCR, mFVIIa does not (Sen et al., 2011b; Sen et al., 2012). Groups that have

studied FVII/EPCR interactions *in vivo* have used hFVIIa or used mFVIIa with a modified Gla domain that does bind mEPCR (Nayak et al., 2009; Pavani et al., 2014). However, as we have demonstrated in Chapter 3, hFVIIa and mFVIIa do not bind the EPCR R84A variant that is used in our mouse model. Future studies could assess the impact of FVII/EPCR interaction on coagulation *in vitro* using our cell-based thrombin generation assay established in Chapter 4. These experiments would be carried out in PC-deficient plasma to allow FVII to bind to EPCR expressed by HEK293 cells. We would expect that FVII binding to EPCR would reduce thrombin generation. To study FVII/EPCR interactions *in vivo* in the absence of PC/EPCR interactions, a mouse expressing a PC mutation that impairs its binding to EPCR could be generated. Using this mouse model, EPCR interaction with hFVII(a) or modified mFVII(a) could be studied to measure the impact these interactions have on coagulation *in vivo*. These studies would provide mechanistic insights into how EPCR and FVII(a) regulate coagulation.

In Chapter 4 we established a cell-based assay that can measure the kinetics of thrombin generation in plasma that is sensitive to the PC pathway. By including cells expressing TM and EPCR, our thrombin generation assay is sensitive to the inactivation of FVa and FVIIIa by APC in a physiologically relevant manner. In addition, we demonstrate that cells expressing TM and a variant of EPCR (R96C) that does not bind EPCR generate less APC and more thrombin than cells expressing WT TM and EPCR. The EPCR R96C variant was identified in a patient with unprovoked VTE (Wu et al., 2013) and we have demonstrated that impaired PC binding to EPCR in our cell-based system, generates more thrombin and impairs fibrinolysis, supporting the hypothesis that

impaired EPCR function will increase the risk of thrombosis. Clinically, it would not be feasible to generate different cell lines expressing novel EPCR variants to determine the functional impact mutations have on coagulation. However, what we have demonstrated is proof-of-principle that diminished EPCR function is procoagulant and anti-fibrinolytic. There are other clinical scenarios where our cell-based assay may prove to be useful, such as determining the cause of thrombosis in cases where there is a suspected antibody towards the PC pathway. In Figure 5.2, we demonstrate that incubating WT cells with blocking antibodies towards TM or EPCR significantly impairs PC activation. Using plasma from a patient with a suspected antibody towards PC, we show that while APC generation is normal, thrombin generation is increased (Figure 5.3). This may suggest the antibody is inhibiting APC once it is activated, and interfering with inactivation of FVa and FVIIIa. Further studies will be needed to isolate the antibody from the plasma to identify if APC is the target. One of the limitations of using patient plasma in this assay, particularly patients who have experienced thrombotic events, is that they are usually on anti-thrombotic therapy. Plasma that contains, heparin, warfarin or direct thrombin and FXa inhibitors do not generate thrombin under our assay conditions. To address this, plasma would need to be collected when the patient is off anticoagulant therapy (i.e. before surgical procedures) or an antidote will need to be added to the plasma before thrombin generation is initiated. Our cell-based assay may have limited clinical utility but does have research applicability by having the ability to measure thrombin generation under normal and pathological conditions that involve the PC pathway.

**Figure 5.2: APC generation by TM and EPCR expressing cells in the presence of TM and EPCR blocking antibodies.** HEK293 (A), WT (B) and R96C (C) cells ( $8 \times 10^4$  cells) were added to 96-well microtiter plates containing defibrinated normal plasma with or without anti-TM (CTM1009) or anti-EPCR (JRK1535). Thrombin generation was initiated with TF and  $\text{CaCl}_2$ . At 0, 5, 10, 20, and 30 min post initiation of thrombin generation, plasma was collected and added to citrate/benzamidine. APC was measured by an APC enzyme-capture assay. Data represents the mean  $\pm$  SE of 3 independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

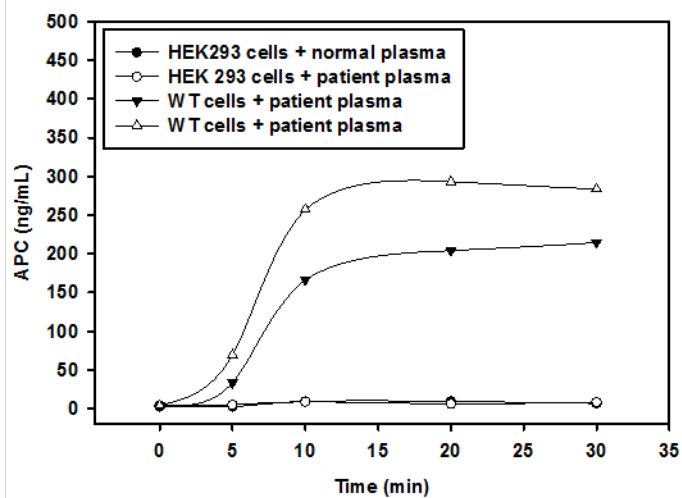


**Figure 5.3: APC and Thrombin generation in plasma from patient with suspected antibody towards PC pathway in the presence of TM and EPCR expressing cells.**

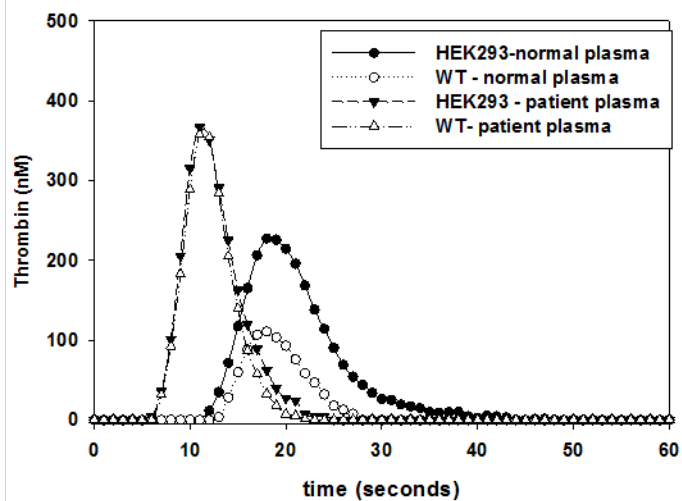
HEK293 and WT cells ( $8 \times 10^4$  cells) were added to 96-well microtiter plates containing normal pooled plasma or patient plasma. Thrombin generation was initiated with TF and  $\text{CaCl}_2$ . **(A)** APC generation was measured over time at 0, 5, 10, 20, and 30 min post initiation of thrombin generation. Plasma was collected and added to citrate/benzamidine. APC was measured by an APC enzyme-capture assay. Thrombin generation was measured over time by cleavage of a thrombin specific fluorogenic substrate. A thrombogram was generated **(B)** and ETP **(C)** calculated for thrombin generation in normal plasma and patient plasma.



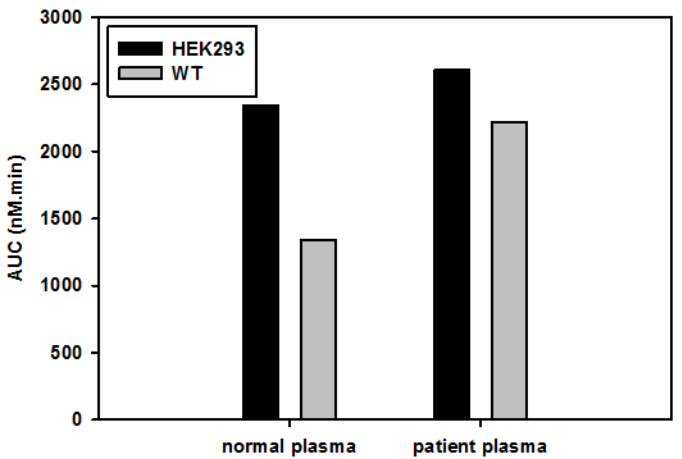
**A**



**B**



**C**



## 5.2 EPCR and Inflammation

There continues to be increasing evidence that diminished EPCR function is a contributory factor in the pathogenesis of many inflammatory conditions. In addition to the role of EPCR as a modulator of coagulation it also plays an important role in modulating inflammation. As we have demonstrated in Chapter 3 and 4, impaired PC binding to EPCR impairs PC activation leading to increased thrombin generation. PAR1 activation by thrombin in the absence of APC-bound EPCR increases the gene expression of the pro-inflammatory cytokine IL-6 (Ludeman et al., 2005; Bae et al., 2007a). In models of experimental endotoxemia, the *in vivo* expression of TF is mostly dependent on IL-6 (van der et al., 1994; Levi et al., 1997). Inhibition of IL-6 in these models completely abrogates the TF-dependent thrombin generation (van der et al., 1994; Levi et al., 1997). In Chapter 3 we demonstrate that LPS challenged EPCR<sup>R84A/R84A</sup> mice have increased plasma levels of IL-6 followed by an increase in TAT levels. This suggests that impaired PC/APC binding to EPCR further exacerbates coagulation resulting in significant cross-talk between coagulation and inflammatory systems.

In experimental endotoxemia models, APC has been shown to inhibit leukocyte adhesion to vascular endothelial cells and reduces the accumulation of neutrophils in the lungs (Grey et al., 1994; Murakami et al., 1996). In Chapter 3, we demonstrate that LPS-challenged EPCR<sup>R84A/R84A</sup> mice have increased neutrophil infiltration as measured by MPO activity. Since PC activation is impaired in EPCR<sup>R84A/R84A</sup> mice it is reasonable to suggest that lower APC levels and higher thrombin levels leads to an upregulation in adhesion molecules on the endothelial cell surface to facilitate leukocyte adhesion.

However, it has been demonstrated *in vitro* that EPCR itself binds neutrophils (via PR3) and monocytes (via Mac-1) under static and flow conditions (Fink et al., 2013; Villegas-Mendez et al., 2007; Kurosawa et al., 2000). It is feasible to postulate that in the absence of PC binding to EPCR in EPCR<sup>R84A/R84A</sup> mice, accumulation of leukocytes in the lungs may in part be mediated by EPCR binding. In support of this is a murine model of pneumococcal pneumonia that demonstrated reduced neutrophil recruitment in the lungs of EPCR knock out mice (ProcrLox) (Schouten et al., 2014). In contrast sEPCR, which is elevated in inflammatory conditions, also binds leukocytes, which may block the attachment and accumulation of leukocytes to the endothelial cell surface.

Future studies would be needed to determine if 1) EPCR variant R84A binds neutrophils via PR3 and monocytes via Mac-1, 2) if these interactions are relevant *in vivo* and 3) if the net effect of the interactions is pro-inflammatory (EPCR R84A facilitates leukocyte recruitment to the endothelial cell surface) or anti-inflammatory (sEPCR R84A blocks accumulation of leukocytes to the endothelial cell surface). To assess binding of EPCR R84A to monocytes and neutrophils, HEK293 cells expressing EPCR variant R84A would be subjected to a flow chamber. LPS stimulated monocytes or neutrophils would be applied with flow to the HEK293 cells and adherent cells would be quantified. To determine if these interactions occur *in vivo*, intra-vital microscopy could be used on EPCR<sup>R84A/R84A</sup> mice to visualize fluorescently labeled monocytes and neutrophils adhering to EPCR on the vessel wall. Using mice that do not express EPCR (ProcrLox) but have a similar phenotype under inflammatory challenge would be a good comparison

to EPCR<sup>R84A/R84A</sup> mice to determine if leukocyte interactions with the vessel can be mediated by EPCR.

APC binding to EPCR has been established as a crucial step in the host response to sepsis as blocking EPCR with anti-EPCR antibodies in baboons challenged with a sub-lethal dose of LPS die more rapidly (Taylor, Jr. et al., 2000). In mice, EPCR expressed by endothelial cells is the major contributor to maintaining an immune response to LPS (Zheng et al., 2007b). Unlike humans, mice do not express EPCR on leukocytes so it is more difficult to determine the role of leukocyte-derived EPCR in the host response to sepsis *in vivo* (Kerschen et al., 2010). However, Kerschen *et al* demonstrated that CD8<sup>+</sup> dendritic cells (the only mature immune cell in mice to express EPCR outside of the BM) do play a significant role in reducing the mortality of endotoxemia in mice. The results of this study indicate that the mortality-reducing effects of APC in mice require engagement of EPCR on both dendritic cells and the endothelium (Kerschen et al., 2010). Unfortunately, the subset of murine CD8<sup>+</sup> dendritic cells does not correlate with specific human dendritic subsets making it difficult to gain insights into the mechanisms of APC therapy in septic patients (Kerschen et al., 2010). Species-specific differences in APC targets as a result of differences in EPCR expression (i.e. leukocytes in humans, dendritic cells in mice) may limit the transferability of mechanistic concepts gained from sepsis pathology in mouse models to clinical scenarios.

Currently, the gold standard for polymicrobial sepsis models in mice is the cecal ligation and puncture (CLP) model as it closely mimics human sepsis progression with

similar hemodynamic changes (Dejager et al., 2011). Future studies could include using a CLP model on EPCR<sup>R84A/R84A</sup> mice to induce a prolonged inflammatory response that is more physiologically relevant compared to using LPS as an acute inflammatory challenge. A previous study of murine pneumococcal sepsis demonstrated that overexpression of EPCR leads to higher bacterial outgrowth in the lungs, blood, spleen, and liver, whereas in the absence of EPCR, bacterial outgrowth was significantly reduced (Schouten et al., 2014). This is in contrast with previous infection models in which overexpression of EPCR is protective, and EPCR knockout increased mortality in mice (Li et al., 2005a; Zheng et al., 2007b). Our EPCR<sup>R84A/R84A</sup> mouse model may be helpful in establishing how EPCR influences bacterial colony growth. One mechanism may be through enhanced activation of coagulation. It has been demonstrated that increased thrombin generation is associated with an enhanced antimicrobial defense as thrombosis and fibrin deposition limit dissemination of microbial pathogens (Sun et al., 2009). Future studies will be needed to determine if impairment of PC binding to EPCR can attenuate bacterial growth in EPCR<sup>R84A/R84A</sup> mice and by what mechanism.

### **5.3 EPCR and Fibrinolysis**

Activation of inflammatory and coagulation pathways also leads to cross-talk with the fibrinolytic pathway. During inflammation, thrombin activation of PAR1 leads to increases in pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$  (Grey et al., 1994). Both TNF- $\alpha$  and IL-1 $\beta$  are central regulators of plasminogen activators and inhibitors involved in the fibrinolytic pathway. Both cytokines are involved in stimulating the release of tPA and uPA from vascular endothelial cells and monocytes to facilitate

fibrinolysis and removal of the fibrin clot (van der et al., 1991). However, there is a delayed but sustained increase in PAI-1 leading to inhibition of fibrinolysis. In addition, the coagulation system attenuates fibrinolysis through thrombin-mediated TAFI activation.

Activation of TAFI and PC by thrombin is enhanced ~1000 fold when thrombin is in complex with TM at the endothelial surface. The protein C pathway has pro-fibrinolytic effects through the downregulation of thrombin and decreased TAFI activation resulting in clots that are less resistant to lysis (Gresele et al., 1998). It has long been speculated that PC-bound EPCR may provide additional pro-fibrinolytic effects by competing with TAFI for activation by thrombin-bound TM. One study demonstrated that in solution PC and TAFI do act as competitive inhibitors in the presence of sTM (Kokame et al., 1998). However in a separate study, Wu *et al* demonstrated that activation of TAFI and PC on HUVEC are independent processes and that TAFI and PC do not compete for activation by thrombin-bound TM (Wu et al., 2016). In Chapter 4, we confirm the findings that impaired PC binding to EPCR does not affect TAFI activation and the absence of TAFI in plasma does not affect PC activation. However, we do demonstrate using our cell-based assay that the absence of PC in the plasma, does increase TAFI activation, which is in contrast to the findings in Wu *et al*. This discrepancy may be accounted for by the differences in the amount of thrombin in each system, and the expression level of TM resulting in greater TAFI activation. In our plasma system, peak thrombin levels reached ~160 nM compared to 10 nM in solution and expression of TM on WT and R96C cells was 2-fold greater than on HUVEC.

Although we failed to demonstrate increased TAFI activation when PC binding to EPCR is impaired there is still a delay in clot lysis likely as a result of increased thrombin generation. It has been well documented that higher concentrations of thrombin produce fibrin clots that are composed of thin, tightly packed fibrin strands that are more resistant to lysis (Wolberg, 2007). In addition, higher thrombin levels may also lead to greater activation of FXIII. Activated FXIII forms bonds between fibrin monomers to strengthen the clot and cross-links  $\alpha_2$ -AP to fibrin which impairs clot lysis by inhibiting plasmin (Sakata and Aoki, 1982). Furthermore, APC can form a 1:1 covalent complex with the serpin PAI-1, thereby reducing the amount of PAI-1 that can inhibit tPA (van Hinsbergh et al., 1985; Sakata et al., 1985). Impaired PC binding to EPCR, which reduces APC generation, may further impair fibrinolysis by reduced APC/PAI-1 complex formation. In contrast, TAFI activation by the thrombin-TM complex was increased in PC-deficient plasma (Chapter 4). As a result of increased TAFI activation, clot lysis was delayed, suggesting that increased TAFI activation may contribute to thrombosis in individuals with PC-deficiency. In support of this, plasma from individuals with congenital type II PC deficiency also demonstrated an increase in clot lysis time that was partially dependent on TAFIa and increased FXIII consumption in comparison to healthy controls (Foley et al., 2012).

The observed increase in thrombin generation as a result of impairment of the PC pathway will have an influence on clot structure and fibrinolysis, and ultimately clot stability. As previously discussed in Chapter 4, high concentrations of thrombin form dense, highly stable clots that are associated with thrombosis, whereas low concentrations

of thrombin form loosely woven, unstable clots that are associated with bleeding disorders (Wolberg and Campbell, 2008). In addition to PC-deficiency, and functional mutations in EPCR, individuals with the prothrombin G20210A mutation, which results in elevated prothrombin levels, also generate higher thrombin levels in *in vitro* thrombin generation assays (Butenas et al., 1999; Wolberg et al., 2003). Increased thrombin generation in these plasmas triggers the formation of densely packed fibrin clots composed of thin fibers and impairs fibrinolysis through increased TAFI activation (Wolberg et al., 2003; Colucci et al., 2004). It has been demonstrated that hypofibrinolysis as measured by the clot lysis assay in plasma from patients with recurrent VTE can accurately predict a 2-fold increased risk of thrombosis (Lisman et al., 2005). In our cell-based clot lysis assay, impairment of PC binding to EPCR and PC-deficiency results in hypofibrinolysis, which may contribute to thrombotic risk in individuals with impairments in the PC pathway.

A limitation of both our cell-based thrombin and clot lysis assay is the absence of whole blood components in the assays. It has been well demonstrated that platelets, leukocytes, and red blood cells contribute to thrombin generation and thrombus formation. In the future whole blood thrombin generation and fibrinolysis assays could be conducted in the presence of these cellular elements, including endothelial cells that express TM and EPCR. Our cell-based thrombin generation and clot lysis assays are also done in the absence of flow, which leads to consumption of coagulation and fibrinolytic factors. Future studies could be conducted *in vivo*, using electrolytic induced thrombosis model in our EPCR<sup>R84A/R84A</sup> mice. This model would allow us to determine the



contribution of impaired PC/EPCR interactions on thrombus development and stability, by measuring thrombus size and embolic events.

In addition to its anti-fibrinolytic activity, TAFIa also plays an important role in inflammation and is another example of the cross-talk that exists between the coagulation, fibrinolysis, and inflammatory systems. It was first discovered that TAFIa inactivates bradykinin, an important mediator of inflammation that is responsible for the classic signs of inflammation, including swelling, heat, redness and pain (Campbell and Okada, 1989; Shinohara et al., 1994; Ueno and Oh-ishi, 2003). TAFIa also plays a significant role in down-regulating the complement system, an important component of the innate immune response. Complement factors, C3a and C5a are pro-inflammatory mediators and potent leukocyte chemoattractants. TAFIa inactivates C3a and C5a thereby reducing their pro-inflammatory effects (Campbell et al., 2002; Myles et al., 2003). Future studies should evaluate the role of impaired PC/EPCR interactions on TAFIa activation *in vivo* and its impact on the complement system. From our *in vitro* studies in Chapter 4, we would expect that TAFI activation in EPCR<sup>R84A/R84A</sup> mice would be similar to that in EPCR<sup>WT/WT</sup> mice. However, EPCR<sup>R84A/R84A</sup> mice generate more thrombin, and thrombin itself is a direct activator of complement factor C5 (Rittirsch et al., 2008). We would expect to see increased complement activation in EPCR<sup>R84A/R84A</sup> mice, and as a result an increased pro-inflammatory response.

#### **5.4 EPCR and hematopoiesis**

When EPCR was first cloned and identified it was thought to be primarily expressed by endothelial cells and act as the physiological receptor for the anticoagulant PC/APC (Fukudome and Esmon, 1995). Additional evidence emerged that APC also has anti-inflammatory properties mediated through EPCR/APC/PAR1 signaling, and through both its anticoagulant and anti-inflammatory actions, enhances fibrinolysis (Riewald and Ruf, 2005; Levi and van der, 2005). The cross-talk between coagulation, inflammation, and fibrinolysis mediated in part by PC/APC interactions with EPCR have been studied primarily as a mechanism to counterbalance intravascular thrombosis and prevent endothelial cell activation. More recently, evidence of close interaction between coagulation, inflammation, fibrinolysis, and complement systems is involved in the regulation and mobilization of HSPCs from the BM (Borkowska et al., 2014; Gur-Cohen et al., 2015). Recently, it has been identified that EPCR is expressed by long-term repopulating hematopoietic stem cells (LT-HSC) in the BM (Balazs et al., 2006; Iwasaki et al., 2010). However, the function of EPCR within the BM has remained unknown until very recently. As a result of the ability of EPCR to regulate the cross-talk between coagulation, inflammation, and fibrinolysis, new functions are emerging for the role of EPCR in the BM.

In Chapter 3, we demonstrated that impairment of PC binding to EPCR in EPCR<sup>R84A/R84A</sup> mice leads to the loss of BM function, and the shifting of hematopoiesis from the BM to the spleen. Through BM transplant experiments we identified that expression of functional EPCR by both HSCs and stromal-derived cells (which include

endothelial cells, chondrocytes, and osteoblasts) play a role in maintaining hematopoietic homeostasis. The BM microenvironment is highly vascularized and there is evidence to support the close association of endothelial cells and HSCs. Some LT-HSCs are located in perivascular niches, adjacent to endothelial cells (Kiel et al., 2005;Kunisaki and Frenette, 2012). In support of the relationship between endothelial cells and HSCs, BM endothelial cells are capable of supporting long-term hematopoiesis *in vitro*, and are essential for hematopoietic recovery from lethal irradiation *in vivo* (Kobayashi et al., 2010;Butler et al., 2010). In addition to endothelial cells, there are also non-vascular cells that likely contribute to maintaining BM HSC niches such as monocytes, macrophages, stromal precursor cells and reticular cells (Ludin et al., 2012;Mendez-Ferrer et al., 2010;Sugiyama et al., 2006).

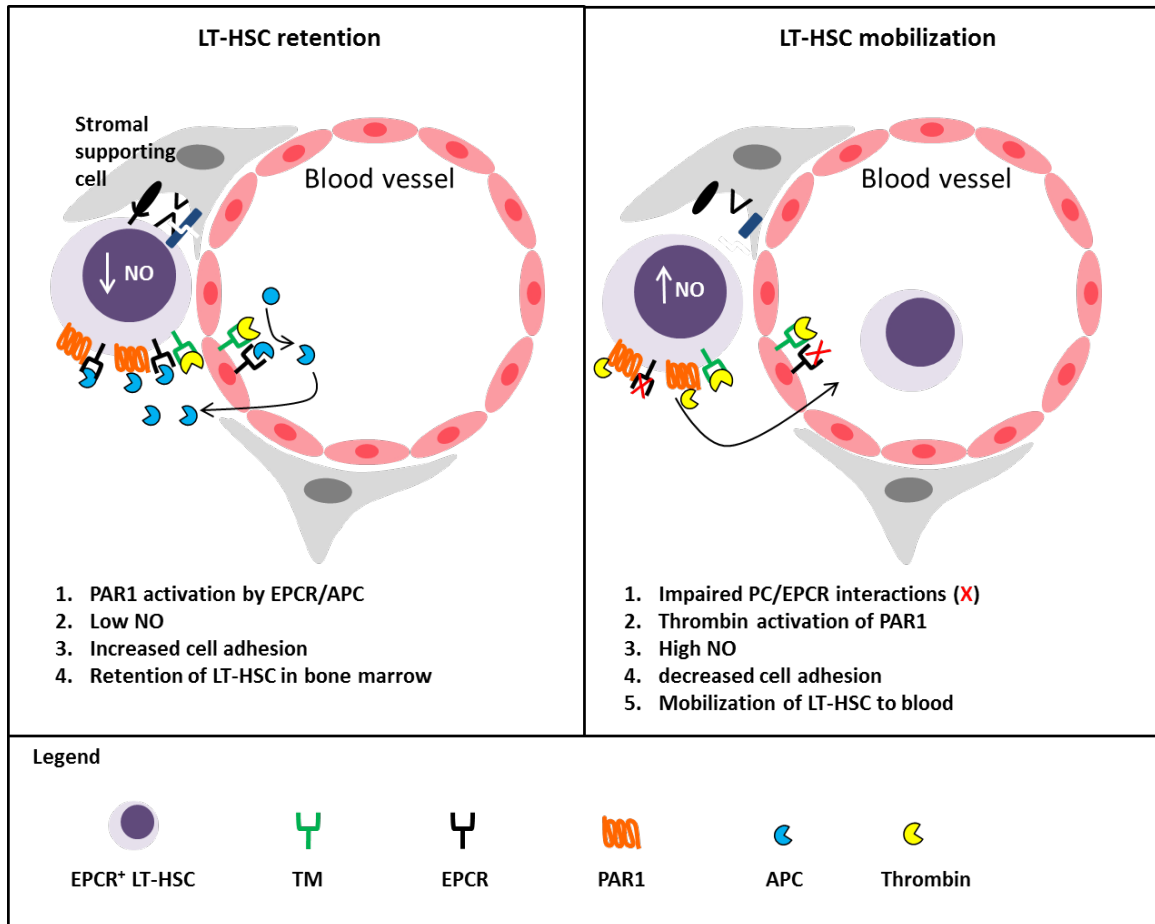
Since our work in Chapter 3 was published, Gur-Cohen *et al* have demonstrated that APC/EPCR/PAR1 signaling regulates the retention of EPCR expressing BM HSCs (Gur-Cohen et al., 2015). Induction of PAR1 signaling by APC/EPCR interactions in BM LT-HSCs leads to a suppression of NO production and promotes cell adhesion. LT-HSCs treated with an EPCR neutralizing antibody, or LT-HSCs expressing low levels of EPCR (in EPCR<sup>Low</sup> mice) failed to compete with normal EPCR<sup>+</sup> LT-HSCs during competitive BM transplant experiments, suggesting that high levels of functional EPCR are needed for proper adhesion and retention within the BM microenvironment. In contrast, mice with impaired thrombin binding to TM, thus reduced APC generation, demonstrated higher levels of HSPCs in the peripheral blood. Furthermore, injection of mice with thrombin resulted in a PAR1 dependent increase in the number LT-HSCs in

the peripheral blood. In agreement with our data that both hematopoietic and stromal EPCR contribute to maintaining the BM microenvironment, Gur-Cohen et al demonstrate that hematopoietic and stromal PAR1 are essential for the thrombin-induced mobilization of LT-HSCs. Thrombin activation of PAR1, enhances NO production and reduces adhesion and retention of LT-HSCs in the BM. In addition to thrombin, LPS has been shown to upregulate TF in the bone marrow microenvironment leading to activation of coagulation and enhanced HSPC recruitment from the BM (Gur-Cohen S et al., 2011). Activation of the complement cascade and plasmin have also been shown to enhance mobilization of HSPCs through alternative mobilization pathways (Borkowska et al., 2014;Tjwa et al., 2008). Taken together, we postulate that impairment of PC binding to EPCR in EPCR<sup>R84A/R84A</sup> mice induces thrombin-mediated PAR1 activation in the BM resulting in the mobilization of EPCR<sup>+</sup> LT-HSCs to the peripheral blood (a schematic of this proposed mechanism is illustrated in Figure 5.4). Loss of LT-HSCs in the BM microenvironment likely contributes to the BM failure that we have observed in our EPCR<sup>R84A/R84A</sup> mice.

In our study in Chapter 3, we identified HSPCs and were limited in our ability to identify LT-HSCs. Future studies with EPCR<sup>R84A/R84A</sup> mice will require the identification of EPCR<sup>+</sup> LT-HSCs by flow cytometry. To determine if PAR1 signaling is responsible for the BM phenotype observed in EPCR<sup>R84A/R84A</sup> mice, we could cross EPCR<sup>R84A/R84A</sup> mice with mice deficient in PAR1. The absence of PAR1 activation by thrombin may restore BM function, and reduce spleen size in EPCR<sup>R84A/R84A</sup> mice. A better

**Figure 5.4: A proposed mechanism for bone marrow failure in EPCR<sup>R84A/R84A</sup> mice.**

It is proposed that PAR1 signaling regulates LT-HSC retention and mobilization in the bone marrow. Under homeostatic conditions PAR1 activation by the APC/EPCR complex decreases NO production, thereby promoting cell adhesion and retention of LT-HSCs. Impairment of PC binding to EPCR results in thrombin activation of PAR1, increasing NO production and downregulation of cell adhesion molecules. LT-HSCs are mobilized to the peripheral blood leading to loss of stem cell function in the BM microenvironment. (Figure adapted from Gur-Cohen S. et al. Ann. N.Y. Acad. Sci. 2016:1-17)



understanding of how APC/EPCR and thrombin manipulate PAR1 signaling in the BM may impact clinical HSPC mobilization protocols for stem cell transplantation.

## 5.5 Conclusions

We have demonstrated that impaired PC/APC binding to EPCR mediates intense cross-talk between coagulation, inflammation, and fibrinolysis pathways (Summarized in Figure 5.1). We demonstrate that EPCR variants that do not bind PC lead to impaired PC activation, increased thrombin generation, increased fibrin deposition and impaired fibrinolysis both *in vitro* and *in vivo*. We also demonstrate that activation of coagulation as a consequence of inflammation is enhanced when ligand binding to EPCR is absent. Finally, we have identified an unexpected role for EPCR in the BM that likely involves regulation of HSC retention and mobilization. Further understanding of how EPCR and its ligands impact the cross-talk between coagulation, inflammation, and fibrinolysis will have relevance for many physiological and pathological processes that involves these systems.

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