THE ROLE OF VASCULAR GAS6 IN THE PATHOPHYSIOLOGY OF VENOUS THROMBOSIS

By

Richard Robins

Faculty of Medicine, Department of Physiology

McGill University, Montreal, Quebec, Canada

August 2012

A thesis submitted to McGill University, Faculty of Graduate Studies and Research, in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Dedicated to Herbie

~Rest in Peace, old friend~

Abstract

Gas6 is a vitamin K-dependent, secreted protein that amplifies platelet aggregation and secretion in response to platelet agonists. Gas6$^{-/-}$ mice are protected from experimentally induced lethal venous and arterial thromboembolism. This protection has been attributed to defective aggregation in platelets from Gas6$^{-/-}$ mice. However, this platelet phenotype was only observed when platelets were challenged by one agonist, ADP, and only at a concentration of 5.0 μM. This subtle platelet abnormality resulting in a rather dramatic clinical phenotype raises the possibility that Gas6 from a source other than platelets contributes to thrombus formation. We hypothesize that Gas6 derived from the vasculature plays a role in venous thrombus formation. Gas6$^{-/-}$ mice are protected against venous thrombosis induced by 0.37 M $FeCl_3$ in the inferior vena cava (IVC). Bone marrow transplantation experiments generating mice with selective ablations of Gas6 from either the hematopoietic or vascular compartments demonstrate an approximately equal contribution by Gas6 from both compartments to thrombus formation. Platelet depletion in wild type or Gas6$^{-/-}$ mice followed by reconstitution with platelets from either WT or Gas6$^{-/-}$ mice confirm that Gas6 from compartments other than the platelet contribute to thrombosis development. Furthermore, Gas6$^{-/-}$ mice are hyporesponsive to $FeCl_3$ mediated tissue factor induction in venous endothelium, as observed by immunofluorescence micoscopy and further validated by a functional assay. In addition, *in vitro*, Gas6$^{-/-}$ endothelial cells are hyporesponsive to thrombin mediated tissue factor mRNA induction. Taken together, these results suggest that vascular derived Gas6 contributes to thrombus formation *in vivo* and can partially be explained by

the ability of Gas6 to promote endothelial tissue factor induction. We also begin to explore the involvement of the FOX family transcription factor FoxO1 as a downstream mediator of Gas6 induced expression of VCAM-1 during endothelial activation. These findings support the notion that vascular Gas6 may play a pathophysiologic role in venous thromboembolism.

Sommaire

Gas6 est une protéine vitamine-K dépendante, sécrétée qui contribue à l'agrégation des plaquettes. Les souris déficientes en Gas6 ($Gas6^{-/-}$) sont protégées contre les thromboses artérielles et veineuses. La résistance à la thromboses des souris déficientes en Gas6 était attribuée à une réponse plaquettaire défectueuse. Cependant, la phénotype plaquettaire était observée uniquement avec le traitement des plaquettes avec une concentration faible de l'ADP (5.0μM). Cette observation indique la possibilité d'une contribution de Gas6 d'une source distincte des plaquettes. Nous faisons l'hypothèse que Gas6 dérivé d'une source vasculaire contribue à la formation d'une thrombus. Les souris $Gas6^{-/-}$ sont protégées contre la formation des thromboses dans la veine cave inférieure induite par 0.37 M $FeCl_3$. En utilisant des greffes de moelle osseuse, nous avons généré des souris avec les déficits de Gas6 soit dans le compartiment vasculaire ou hématopoïétique. La formation des thromboses dans les souris chimériques était intermédiaire entre les deux groupes contrôles. L'épuisement des plaquettes dans les souris WT, suivi de la reconstitution avec des plaquettes $Gas6^{-/-}$ a confirmé les résultats obtenus par les greffes de moelle osseuse.

En outre, l'induction du facteur tissulaire était atténuée dans les cellules vasculaires des souris $Gas6^{-/-}$. Additionellement, *in vitro*, les cellules endothéliales étaient aussi hypo réactives en ce qui concerne l'induction du facteur tissulaire. Nos résultats suggèrent que Gas6 dérivé d'une source vasculaire contribue à la formation *in vivo* d'une thrombose, en partie dû a l'induction du facteur tissulaire. Nous avons commencé à examiner l'hypothèse à savoir si la protéine FoxO1 est impliquée dans l'induction de VCAM-1 parmi les cascades de signalisation de Gas6. Donc, en guise de conclusion nos résultats soutiennent l'idée que Gas6 vasculaire peut jouer un rôle physiopathologique dans la thrombose veineuse.

Preface

This dissertation was written in accordance with the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research at McGill University. It is written as a manuscript-based thesis. Chapter 1 will serve as a general review of the literature and introduction to the field as a whole. Chapter 2 represents a completed study which has been accepted for publication (Robins *et al.*, Blood 2012). It contains additional experiments that were omitted from publication as well as an expanded discussion with connecting text to Chapter 3, which at the time of writing remains a work in progress that will be submitted for publication in the following year. Chapter 4 includes a general discussion and conclusions. At the end is a list of references for all four chapters.

Acknowledgements

To my supervisor Dr. Mark Blostein, I have many thanks to express. First, thank you for taking a chance on me those 5 years ago and lending me a bench in your lab. Thank you for supporting me over the course of my studies. Thank you for your patience, the limits of which I have probably tested. Thank you for allowing me to stay in the lab to complete my PhD as I've ~~probably~~ definitely, on multiple occasions given you reason not to. Thank you for pushing these projects forward with excellent troubleshooting advice, especially during times where I've lost focus. Thank you for the bagels. Thank you for lending an ear to personal problems, of which we've lost count. Thank you for sending me to conferences, allowing me to see new parts of the world. And finally, thank you for correcting the thesis. I hope having me around was worth it in the end.

To the members of the Blostein lab, both past and present, thank you for all the advice you've given me. I've learned from all of you. In particular, I would like to address Dr. Catherine Lemarié, who gave my work a real shot in the arm when things seemed to have hit a wall. From the moment she joined the lab she has helped me in all aspects of research. She has taught me numerous techniques that I needed to move forward as well as troubleshooting abilities that I'll surely use throughout my career. I'd like to thank her for taking a real interest in the projects I've been assigned by both keeping up to date with my experimental progress and the field in general by suggesting new reading material. Overall, because of her I feel closer to being a well-rounded

scientist. Research is all about reporting new scientific facts. To say that I would not be writing this thesis without all she's done for me is both an understatement of monumental proportion and as factual as any of the science reported here. There's no way I'll ever be able to repay my thanks to her. I'd also like to express a sincere apology to her and everyone in the lab for any times I might have been unpleasant to be around.

Thank you to my committee members, Drs. John White, Sabah Hussain, and Alvin Shrier for providing encouragement and advice at all of our meetings and for whatever reason deeming me worthy of the PhD program. I really appreciated the meetings being somewhat relaxed with much needed humor. To Dr. White especially, thank you for sparing office time and advice every now and then.

To Christine Pamplin at McGill Physiology, thank you for keeping track of my progress over the years. I would never have been a member of this department if it wasn't for your all your help.

To the Animal Health Care technicians at the LDI: Véronique Michaud, Yvhans Chery and Kathy-Ann Forner, thank you for providing exceptional technical assistance all throughout.

I also have to give a special thanks to Mrs. Mary Jane Li-Cheong-man for her help in translating the abstract.

To my incredible family: Mom, Dad and Karen. Thanks for the endless supply of love, support, laughs, encouragement and irritation. I know I'm not man enough to ever say it in person, but I love you all. To my oldest and dearest friends Hakeem and Josh, my brothers from other mothers, and only sources of social interaction, thank you both for sticking by me in good times and bad throughout the years. Hakeem, you've transcended the barrier from friendship to brotherhood so long ago and have been, without a doubt, the biggest influence in my life. And to Josh, thanks for all the laughs and don't ever change.

To Alex and Rob at the comic shop, thank you for being one of the most consistent sources of joy in my life for going on 20 years. No matter how difficult things might have gotten at times over the course of my studies, you guys have always been there to make every Wednesday feel like Christmas morning, giving me something to look forward to. I really appreciate the dedication and care you put into supporting my hobby. As long as there are superhero stories to tell, I'll be there to see you guys every week.

And last but certainly not least, to Eliza, there isn't enough time in the world to properly say what has to be said. So to put it simply, thank you for saving my life and making it worth living.

\(^_^)/ -Bye everybody!

Contribution of Authors

The candidate performed the majority of the research presented in this thesis. In regards to Chapter 2, the protocol for endothelial cell isolation as well as the model of platelet depletion and reconstitution was conceived and established by Dr. Catherine Lemarié. Dr. Jianqiu Wu assisted with blood collection and preparation of PRP. Dr. Catherine Lemarié and Dr. Jianqiu Wu also were responsible for histologic analysis of Tissue factor. Figures 2-3C and 2-4A were prepared by Dr. Catherine Lemarié. Intravenous injections of bone marrow cells and PRP were performed by Veronique Michaud and Yvhans Chery, respectively. Mouse irradiations were handled by the Radiation Oncology department at the Jewish General Hospital. The candidate performed all the research in Chapter 3, with Dr. Catherine Lemarié providing critical guidance and troubleshooting advice for all experiments.

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Chapter 3

List of Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
Akt	Protein Kinase B
APC	Activate Protein C
ApoE	Apolipoprotein E
aPTT	Activate partial thromboplastin time
Ark	Adhesion related kinase
AT	Antithrombin
ATP	Adenosine triphosphate
BMC	Bone marrow cell
BMP2	Bone Morphogenic Protein 2
BMT	Bone marrow transplant
BSA	Bovine serum albumin
cAMP	Ayclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	cyclin guanosine monophosphate
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CT scan	Computerized tomography scan
DAF	Decay accelerating factor
DAF-16	*C. elegans* FOXO homolog

DDB1	DNA damage-binding protein 1
dFoxO	*D. melanogaster* FOXO homolog
DIC	Disseminated intravascular coagulation
DNA	Dexoyribonucleic acid
DVT	Deep vein (venous) thrombosis
EBM	Endothelial Basal Medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGM2	Endothelial growth medium-2
eNOS	Endothelial nitric oxide synthase
EPCR	Endothelial cell protein C receptor
Epo	Erythropoietin
Erk	Extracellular-signal-regulated kinase
ESM-1	Endothelial specific molecule 1
ET-1	Endothelin-1
E1A	Adenovirus early region 1A protein
HSC	Hematopoietic stem cell
f	coagulation factor
FACS	Fluorescence-activated cell sorting
Fas	apoptosis antigen 1 (FasR)
FasL	Fas ligand
FBS	Fetal bovine serum
$FeCl_3$	Ferric chloride

FISH	Fluorescence *in situ* hybridization
FN	Fibronectin
FOX	Forkhead box protein
GADD45	Growth arrest and DNA damage protein 45
Gas6/Gas6	Growth arrest specific 6 (gene/protein)
GhRH	Gonadotropin-releasing hormone
Gla	γ-carboxyglutamic acid
Gy	Gray
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IL-1β	Interleukin 1β
IL-6	Interkeukin 6
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal
i.v	Intravenous
IVC	Inferior vena cava
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
LMWH	Low molecular weight heparin
kDa	Kilodalton
MAPK	Mitogen activated protein kinase
MLEC	Murine lung endothelial cell
MMP	Matrix metalloproteinase

MnSOD	Manganese superoxide dismutase
MP	Microparticle
MRI	Magnetic resonance imaging
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
MxCre	Cre-recombinase
Nck2	Cytoplasmic protein NCK2
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
NTN	Nephrotoxic nephritis
NO	Nitric oxide
OTC	Optimal tissue compound
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PC	Protein C
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PRP	Platelet rich plasma
PS	Protein S

PT	Prothrombin time
PTB	Phosphotyrosine binding
PTS	Post-thrombotic syndrome
qPCR	Quantitative polymerase chain reaction
RanBPM	Ran binding protein M
rGas6	Recombinant murine Gas6
rhGas6	Recombinant human Gas6
RNA	Ribonucleic acid
RTK	Receptor Tyrosine Kinase
SGC	Soluble guanylyl cyclase
SHBP	Sex hormone binding protein
SH2	Src homology 2
Sirt1	Sirtuin 1
SOCS-1	Suppressor of cytokine signalling 1
STAT	Signal transducer and activator of transcription
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAM	Acronym:Tyro3, Axl, Mer
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
t-PA	Tissue plasminogen activator
TXA2	Thromboxane A2
uPA	Urokinase plasminogen activator

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
VTE	Venous thromboembolism
vWF	von Willebrand factor
WT	Wild-type

CHAPTER 1:

GENERAL INTRODUCTION TO VENOUS THROMBOEMBOLISM AND GAS6

1.1 Venous Thromboembolism (VTE)

Venous thromboembolism (VTE) represents an important cause of morbidity and mortality that occurs in approximately 900,000 people annually in North America. VTE comprises deep-vein thrombosis (DVT) and pulmonary embolism (PE), whose complications can lead to the post-thrombotic syndrome (PTS), pulmonary hypertension and death [1]. DVT, as the name implies is the formation of a blood clot (thrombus) within a deep vein, usually in the leg. PE, the second manifestation of VTE occurs when the thrombus formed within the deep vein dislodges and travels via the blood stream to the pulmonary artery, causing blockage. Approximately 30% of those afflicted with VTE will die within 30 days of disease onset [2]. The recurrence rate among VTE survivors is approximately 30%. The incidence of VTE has reportedly changed very little in the last three decades, despite the introduction of improved prophylaxis, the use of effective and safe prevention methods. These include pharmacological and mechanical interventions such as heparin [3] and compression stockings [4], respectively. The apparent consistency in the reported incidence of VTE could be the result of an increase in size of the population at risk (higher average age), exposure of new risk factors or underutilization of available prophylaxis [5].

There are numerous factors that can predispose one to VTE. These include age (it is predominantly a disease of older age), male gender, surgical intervention, trauma, prolonged immobility, malignancy, weakness or paralysis from neurologic disorder and varicose veins. Risk factors exclusive to females include pregnancy, oral contraception and hormone replacement therapy [5]. The predisposing factors to VTE are rooted in the triad originally proposed by German physician Rudolf Virchow. Virchow proposed that a thrombotic event could be initiated as the result of one of the following: changes in amount or activity of the soluble clotting proteins, disruption of the vascular wall or stasis of blood flow (Fig.1-1) [6]. We have since come to understand the molecular and cellular mechanisms that underlie these alterations.

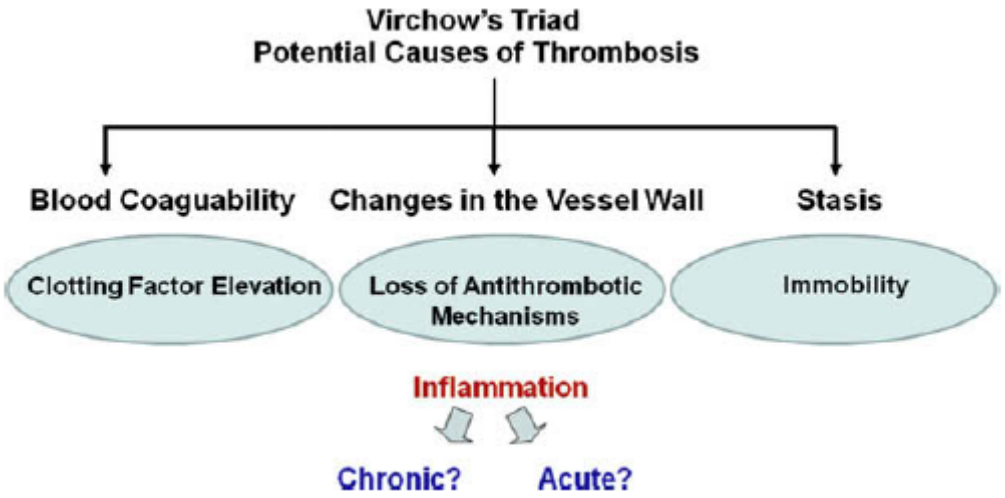


Figure 1-1. Virchow's Triad. The potential factors that predispose one to a thrombotic event. The triad presented here is modified to include the effects of inflammation. Chronic low levels of inflammation is more of a factor for arterial thrombosis, while acute inflammation does in fact increase venous thrombosis [7]**.**

Anatomically, it is believed that venous thrombosis begins at the level of the venous valves, which are important for maintaining blood circulation within the legs. Proper function of venous valves ensures blood flow toward the heart. Autopsy and phlebography studies suggest that the venous valvular sinus is an important location of thrombosis initiation. Less commonly, a thrombus will develop in other sites including retinal veins, intra-abdominal veins, upper limbs and the central nervous system (CNS) [8].

A reduction in the overall incidence of VTE will require improvements in the recognition of those at risk and reduction of risk exposure. Additionally, the development of new prophylaxis as well as its widespread and safe usage should reduce the incidence of VTE [5].

1.2 Hemostasis and Laminar Blood Flow

Hemostasis is the physiological process that halts bleeding. Thrombosis is a disorder of hemostasis that increases coagulation. Hemostasis results in the transformation of blood components from liquid to solid, which in turn is the result of a complex network of enzymatic activators and inhibitors. Over the course of time, blood coagulation has evolved into a highly sophisticated defence mechanism that detects bodily injuries and prevents exsanguination. Considering that the average individual is

injured roughly 4000 times over the course their life [9], it is necessary that hemostatic mechanisms act quickly and are localized specifically to where there is a breach in vascular integrity. Interestingly, the evolution of hemostasis can be traced back to over 350 million years by examining the primitive coagulation system of invertebrates such as *Limulus* (Horseshoe crabs) [9]. Primary hemostasis is the phenomenon of platelets binding to exposed collagen of the subendothelial matrix and is the first line of defence following vascular injury. Primary hemostasis is characterized by the formation of a so-called platelet plug, a process that is conserved among early vertebrates [10]. The second line of defence, or secondary hemostasis, involves tissue factor (TF) and the activity of thrombin and fibrin formation [11]. Data from *in vivo* studies on thrombus formation do however show that the accumulation of platelets and the generation of fibrin occur simultaneously [12].

Proper hemostasis depends on laminar blood flow. The normal condition for blood flow throughout most of the circulatory system is laminar and is characterized by concentric layers flowing in parallel through the vessel (Klabunde, R.E). Laminar flow is required for proper hemostasis in the following ways. Laminar flow produces shear stresses that are maximal at the vessel wall, ensuring proper endothelial cell morphology and function. The expression of endothelial anticoagulant molecules such as nitric oxide (NO), prostacyclin (PGI_2) and tissue-type plasminogen activator (t-PA) are all shear dependent. The prothrombotic and proinflammatory endothelial cell markers such as TF, von Willebrand factor (vWF), endothelin, ICAM-1 and VCAM-1 are also shear dependent. Shear dependent endothelial cell reactivity ensures that platelet adhesion and

fibrin deposition are confined to areas of endothelial injury. Laminar flow also concentrates platelets near the vessel wall, ensuring that upon vessel injury, they are well placed to interact with vWF and molecules of the subendothelium [13].

1.3 Virchow's Triad

1.3.1 Abnormal Flow (Stasis)

To reiterate, the initiation of DVT occurs within the lower extremities of the body. Initiation of DVT is usually at the level of the venous valves, which are abundant in these areas. The function of venous valves is to guide the direction of blood flow and prevent its reflux. Venous valves are most numerous in the lower extremities because it is here that blood return works against oxygen tension. When standing, the venous valves have been shown to open and close roughly 20 times per minute [14]. The flow of blood through these valves creates vortices within the valve pockets that force blood to move in the appropriate direction. When a person is immobilized, there is less venous blood flow in the lower extremities. This onset of blood stasis is maximal within the venous valve sinus pockets, thus serving as common initiation sites for thrombotic events (Figure 1-2) [14]. Leukocytes and platelets have a tendency to become trapped within valve pockets and stasis promotes the interaction between platelets, leukocytes and coagulation enzymes [15, 16]. In the case of DVT, the component of Virchow's triad describing abnormal blood flow usually implies stasis of flow [17].

At a molecular level, the endothelial lining of the venous valves have been shown to express several of the well-established anticoagulant molecules. The expression of these anticoagulant proteins is sensitive to the microenvironment and any changes, such as in the case of hypoxia or inflammation, could potentially lead to their downregulation and the initiation of a thrombotic event [7, 18, 19]. The pockets of the venous vales are prone to hypoxia. Blood within venous valve pockets rapidly become hypoxic under experimental conditions of immobility. The oxygen content of blood within venous valve pockets rises to that of the lumenal blood under experimental conditions mimicking mobility, suggesting that the endothelium covering valve cusps depends on pocket or lumenal blood for oxygen. Endothelial cells of the venous valve pockets will therefore become hypoxic under conditions of immobile blood flow [20]. Additional mechanisms explaining stasis induced venous thrombosis have also been proposed. One possibility has to do with the ratio of endothelial cell surface area to blood volume among the different vascular beds. Blood is exposed to approximately 1000 times more endothelial cell surface area while passing through the microcirculation than it is in larger vessels. This means that as blood travels from the larger vessels into the microcirculation, the efficacy of the natural anticoagulants present on endothelial cells increases dramatically. When stasis of blood occurs, such as from a period of prolonged immobility, the amount of time blood spends in the larger vessel is increased. Consequently, blood is less exposed to the high concentration of anticoagulants in the microcirculation, increasing the risk for development of a thrombotic event [7, 21]. It is generally well accepted that

reducing stasis in the legs reduces the risk of VTE. Regular exercise and the avoidance of immobility serve to reduce the risk of both venous and arterial thrombosis.

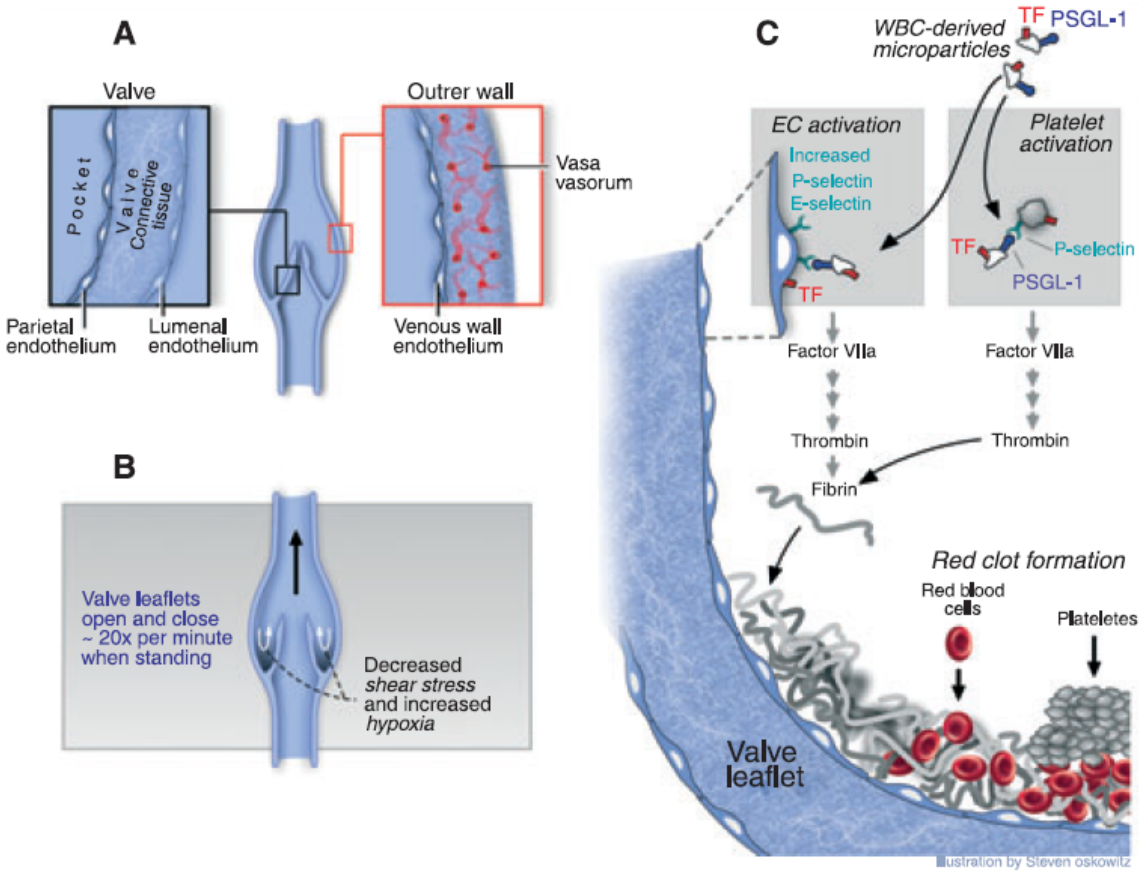


Figure 1-2. Development of DVT. (A) The veins of the lower limbs under normal conditions. Veins of the lower limbs contain high numbers of valves. (B) The valves cycle, and under normal conditions, stasis is reduced within the valve pockets. (C) Stasis and/or loss of vascular integrity can lead to TF-dependent thrombus formation [14].

1.3.2 Coagulability of Blood

The second predisposing mechanism for development of a thrombotic event as proposed by Virchow is a change in the coagulability of blood. The blood coagulation cascade has traditionally been viewed as two processes that both culminate in the generation of fibrin [22]. The extrinsic (contact) and intrinsic pathways merge at the level of blood coagulation factor Xa (common pathway) [9].

1.3.2.1 The Extrinsic Pathway

The extrinsic pathway (TF dependent pathway) plays the major role in initiating thrombus formation following vascular injury. The extrinsic pathway begins with TF, an integral membrane glycoprotein that is expressed by cells of the subendothelium, a critical component of the vessel wall. In the event of vascular injury, TF, which is normally sequestered from blood, becomes exposed to, and serves as the receptor for blood coagulation factor (f) VII (or VIIa), which circulates in low concentration. TF and fVII form a 1:1 complex, in a calcium dependent manner. Once bound to TF, zymogen fVII can be converted to active fVIIa by the cleavage of a single peptide bond (facilitated by various serine proteases), uncovering its intrinsic serine protease activity. The TF:fVIIa complex on the damaged cell membrane can then mediate the conversion of zymogen fX to the active fXa. Factor Xa in turn mediates the cleavage of prothrombin to

thrombin, the key enzyme of the coagulation cascade [23]. The amount of thrombin initially produced by the activity factor of fXa is relatively low (0.1-1.0 nM). Thrombin will then go on to convert fibrinogen to fibrin. This will be discussed in more detail within the context of thrombus resolution. The TF:fVIIa complex can also activate fIX and the intrinsic pathway (Fig. 1-3).

1.3.2.2 The Intrinsic Pathway

The intrinsic pathway of blood coagulation (fVIII dependent) serves to amplify the generation of fXa from the extrinsic pathway. The intrinsic pathway is in fact 50 times more efficient at activating fX. In the intrinsic pathway, fX is activated by a membrane bound complex of activated fVIIIa and fIXa (Figure 1-3). The complexes formed in both the extrinsic and intrinsic blood coagulation cascades are highly dependent on exposure to phosphatidylserine on cell membranes [15].

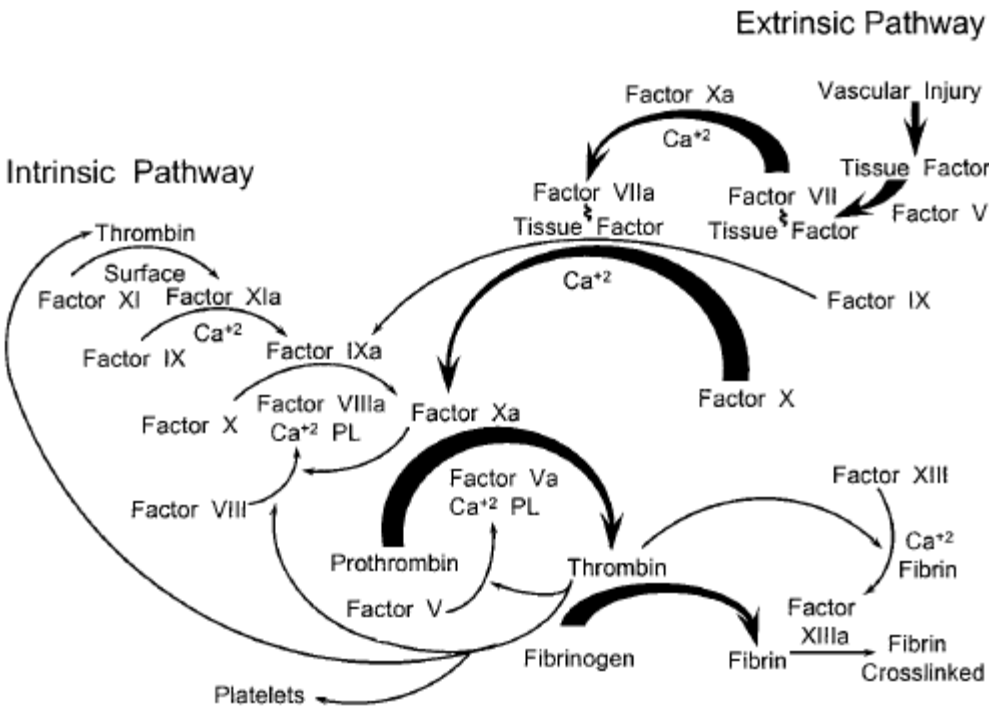


Figure 1-3. The Blood Coagulation Cascade. The intrinsic and extrinsic pathways of blood coagulation merge at the level of fXa (common pathway), resulting in fibrin formation at a site of vascular injury. The term "Waterfall sequence" has been used to described these events [24].

1.3.2.3 Thrombophillia

Changes in the composition of blood, as described by Virchow are usually the result of genetic abnormalities. The genetic risk factors for VTE involve the genes that encode for proteins involved in the blood coagulation cascades or those that regulate it. The earliest reports of families with predispositions to thrombotic events were published during the first part of the twentieth century. Advancements in our understanding of the pathophysiological mechanisms that underlie development of VTE have led to the general consensus that approximately 60% of the risk for development of VTE lies in

genetic factors. The predisposition to development of VTE from genetic factors has been termed 'thrombophilia'. The blood coagulation cascade involves the sequential activity of numerous serine proteases (Fig. 1-3). The activities of these proteases are in turn regulated by a group of proteins that are referred to as the natural anticoagulants. The main natural anticoagulants include antithrombin (AT), protein C (PC) and protein S (PS). A genetic deficiency in any one of these natural inhibitors of coagulation increases one's risk of VTE onset [8].

1.3.2.3.1 Antithrombin (AT)

AT is a member of the serpin (serine protease inhibitor) family of proteins. AT, as the name implies, principally downregulates thrombin activity. At the molecular level, AT exerts its action by forming equimolar complexes with its substrates, thereby masking the substrate's active site [25]. The physiological targets of AT are mainly the proteases involved in the intrinsic pathway of blood coagulation. AT exerts its effects on the activated factors Xa, IXa, XIa, XIIa and to a greater extent fIIa (thrombin) [26]. Interestingly, AT is also capable of causing the dissociation of the factor TF:fVIIa complex and prevents its reformation. In terms of published findings, AT was the first of the natural anticoagulants to be identified as an inheritable risk factor for thrombosis. A heterozygous deficiency of the AT gene is sufficient to increase one's risk for VTE. Women and men are affected equally and a homozygous deficiency in the gene encoding AT is not only exceedingly rare but is presumed to be incompatible with life. The gene

encoding AT is located on chromosome 1q23-25 and has several exons. The first mutation linked to AT deficiency was identified in 1983 and since then the identification of numerous other mutations revealed the heterogeneous nature of AT deficiency. AT deficiency has been subdivided into type I and type II. Type I refers to low plasma levels of AT while type II is the presence of an AT variant in plasma [8].

Central to the activity of AT are the heparins, sulfated polysaccharides that represents the initial choice anticoagulant for treating and preventing thrombotic events. Heparins are the therapeutic derivatives of the naturally occurring heparans. Heparins are injectable polymers that range in molecular weight [27]. *In vivo*, AT circulates in an inactive form until it binds heparan side chains expressed on endothelial cells [28]. The unfractionated form of heparin (i.e. 18 saccharide units) forms a ternary complex with AT and thrombin which results in the inactivation of thrombin. In contrast, the low molecular weight heparins (LMWH) bind AT, changing its substrate specificity to factor Xa [29].

1.3.2.3.2 Protein C (PC) and Protein S (PS)

The anticoagulant role of PC was first identified in 1960 [30]. The protein C anticoagulant pathway represents a significant mechanism for controlling thrombosis. The enzymes comprising this anticoagulant pathway include PC, PS, thrombin, thrombomodulin (TM) and the endothelial cell protein C receptor (EPCR). During the

process of blood clotting, the concentration of thrombin in the affected area increases. A significant amount of this thrombin will bind to TM on the surface of endothelial cells. The thrombin-thrombomodulin complex then serves to activate protein C (In this particular reaction, thrombin activates PC while TM acts as cofactor). Activated protein C (APC) generation is 20-fold higher *in vivo* when PC is bound to EPCR. The enzymatic function of APC is to inactivate the blood coagulation factors Va and VIIIa. PS has no enzymatic activity but rather serves as a cofactor for activated PC [31].

In the 1980's, Griffin *et al.* were the first to recognize genetic deficiencies in PC and PS as inheritable risk factors for VTE. It is now firmly established that loss of function mutations in the PC gene on chromosome 2q13-14 is a cause of VTE. PC deficiency is also subdivided into type I and type II. Type I PC deficiency refers to inadequate amounts of the protein, whereas in type II, protein activity is reduced [32]. The human genome contains two PS genes (*PROS1* and *PROS2*). *PROS1* encodes the active protein while *PROS2* is a pseudogene. Loss of function mutations in *PROS1* is another well established risk factor for VTE [8].

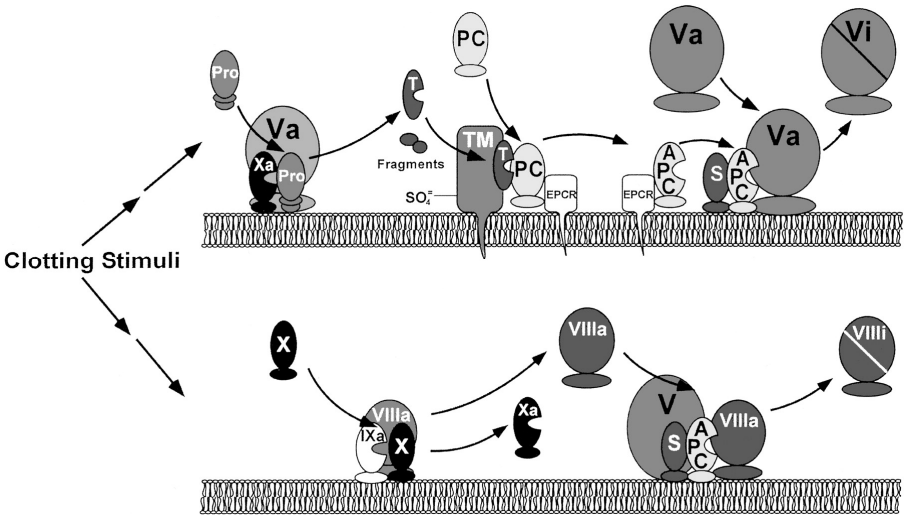


Figure 1-4. Protein C pathway. In addition to causing fibrin formation and activation of endothelial cells and platelets, thrombin can also bind to thrombomodulin (TM). Once bound to TM, thrombin rapidly activates PC, a process that is further enhanced by EPCR. Activated protein C (APC) dissociates from TM and inactivates factors Va and VIIIa [31].

1.3.2.3.3 Factor V Leiden

Thus far, the genetic abnormalities discussed are those involved in anticoagulant pathways. Mutations in the active enzymes of the coagulation cascade proper have also emerged as inheritable risk factors for VTE. A mutation in the factor V gene (G1691A in exon 10) was discovered as a risk factor for venous thrombosis that is present among the general population. This genetic mutation corresponds to a change in the primary amino acid sequence of factor V at position 506, where the normal arginine residue is replaced by glutamine [33]. This particular mutation in fV differs from those related to PC, PS or AT in that it is more common and is caused by a single mutation (i.e. no heterogeneity). This mutation has been named factor V Leiden, after the city in the Netherlands where it

was discovered in 1994. The mutation confers resistance to inactivation by PC, thus causing a hypercoagulable state [34]. Factor V Leiden is a low risk inherited mutation with high prevalence. This became apparent during some initial laboratory studies which showed that resistance to APC was high among patients with venous thrombosis, but also quite frequently observed among healthy control subjects. This means that additional environmental or genetic factors are required for thrombosis initiation when factor V Leiden is present [7, 35] .

The factor V Leiden mutation has been established as the most common inheritable disorder associated with venous thrombosis. In addition, factor V Leiden cosegregates with other inheritable thrombotic risk factors such as AT deficiency [36], PC deficiency [37] and PS deficiency [38]. These observations show that the presence of double or multiple defects in hemostatic genes increases the penetrance of thrombosis.

1.3.2.3.4 Prothrombin G20210A

Prothrombin (factor II) is the precursor form of the active enzyme, thrombin. A common genetic risk factor for DVT is a polymorphism located in the non-coding region of the prothrombin gene. The G20210A mutation results in enhanced mRNA stability of the transcribed gene and elevated levels of plasma prothrombin. This mutation is present almost exclusively in Caucasians [39-41]. Prothrombin mutation G20210A is a common

but mild risk factor for venous thrombosis. Prothrombin G20210A is sometimes coinherited with carriers of factor V Leiden [42].

1.3.2.3.5 Additional Mutations

In addition to the thrombophilia conferred by mutations in PC, PC, AT, fV and prothrombin, numerous other genetic abnormalities have been reported that predispose to VTE. These emerging candidates include fXIII (accelerates fibrin cross-linking), EPCR, TM (although very little evidence exists), and elevated levels of plasma fVIII. There are some genes that are not directly involved in hemostasis that may enhance one's risk for VTE. Such is the case in patients with hyperhomocysteinemia, where blood levels of homocystein increase. This has been observed as a predisposing factor to venous thrombosis, albeit a weak one [7, 35].

1.3.3 Disruptions in the Vascular Bed

Virchow proposed that disruptions in the vascular bed contribute to the onset of a thrombotic event. The endothelial cell has been referred to as the 'Master Regulator' of the hemostatic balance and has already been discussed within the context of Virchow's other predisposing factors. Endothelial cells display a number of properties that can directly or indirectly influence the hemostatic balance in a given vascular bed. Endothelial cells possess the ability to express both anticoagulant and procoagulant

molecules, adhesion molecules and substances that affect vasomotor tone. Endothelial cells are often described as existing in one of two states: quiescent or activated [6]. This is an oversimplification. It must be noted that the so-called states of activation and quiescence among endothelial cells vary greatly among the different vascular beds in the body [43-45]. For the sake of simplicity, quiescent and activated endothelium will henceforth be defined as follows: quiescent endothelium refers to a state in which the cells provide a surface that is anticoagulant, antiadhesive and vasodilatory. Contrarily, activated endothelium provides a surface that is procoagulant, proadhesive and vasoconstricting (Fig. 1-5) [6].

The term endothelial 'activation' was coined by Willms-Kretschmer in the 1960s. Prior to this, the endothelium was viewed as simply the inner lining of the blood vessel that remained essentially inert. The term describes the change in both function and morphology of the cells. Two stages of endothelial activation exist. Type I activation refers to the initial stimulation of the cell and generally does not involve *de novo* protein synthesis or increased gene transcription. Rather, type I activation is characterized by endothelial cell retraction, expression of P-selectin and the release of vWF. Type II activation requires more time to occur following initial stimulation of the cell. Type II activation is characterized by enhanced gene transcription and protein synthesis of proinflammatory and prothrombotic molecules [46].

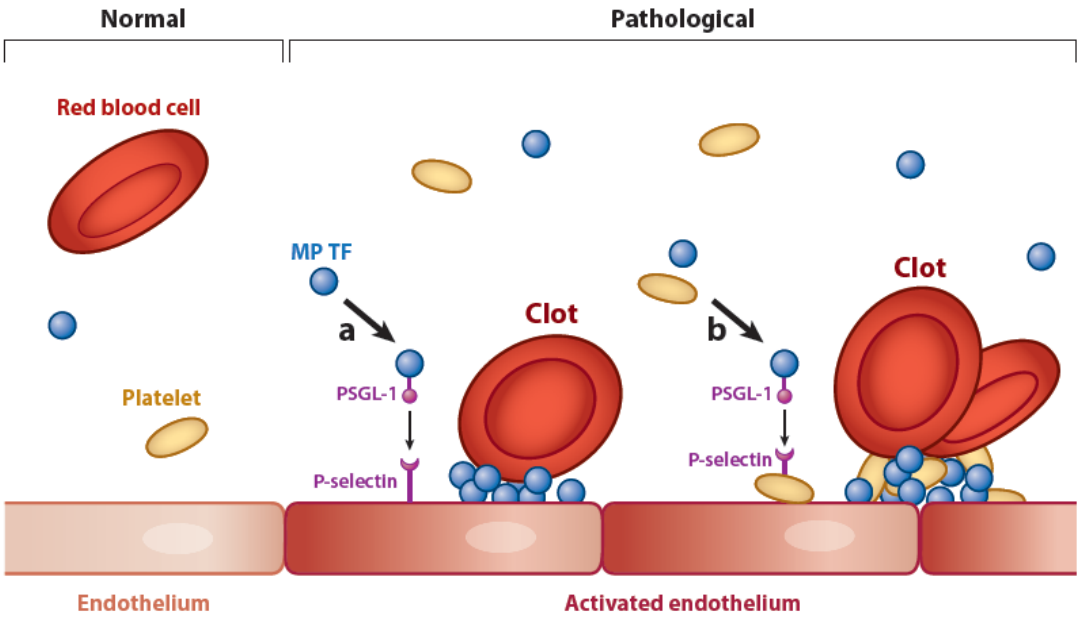


Figure 1-5. Endothelial cell activation promotes formation of a venous clot. Healthy, quiescent endothelium on the left expresses high levels of the anticoagulant molecules and provides an antithrombotic phenotype. Under pathological conditions (on the right), activated endothelial cells express TF and adhesion molecules that promote thrombus formation [47].

Proper spatial and temporal expression of the anti- and procoagulant molecules will ensure that proper hemostasis does not become pathological thrombosis. Endothelial activation induced by hypoxia or inflammation can alter the expression profile of these molecules. Important among the anticoagulant molecules expressed by quiescent endothelial cells include TM, t-PA, tissue factor pathway inhibitor (TFPI) and heparan sulphate, In general, activated endothelial cells will express induced levels of TF, vWF, plasminogen activator inhibitor-1 (PAI-1) [48] and the protease activated receptors (PARs), the latter being the receptor for thrombin [6].

Except in the case of surgery, direct injury to the venous endothelium of the deep vascular beds is rare. Changes in the integrity of the endothelial linings of the vascular bed are much more commonly a consequence of stasis, hypoxia or inflammation. Among the different vascular beds in the body, venous endothelium has a greater capacity to mount an inflammatory response than arterial. This can lead to induction of E- and P-selectin which enhances interactions between endothelial cells and leukocytes. Hypoxia has also been demonstrated to alter the overall transcription activity within cultured endothelium [49].

It is widely accepted that the major contributor regarding the onset of venous thrombosis derived from the vasculature is TF. TF is required as the initiator of the coagulation response. The exact source of TF however, has been a source of some controversy mostly as the result of different experimental model systems used. For example, there exist *in vitro* model systems which show that TF associated with leukocytes or microparticles (i.e. derived from the blood) is involved in initial thrombus formation [50]. In contrast, experiments with mice expressing low (1% of normal) TF in the blood show that following stasis, it is TF derived primarily from the vessel wall that is required for thrombus formation [7, 14, 51]. TF will be discussed in greater detail within the context of *in vivo* thrombus formation.

Virchow's triad states that a thrombotic event can arise as the result of changes in the blood composition, alterations to the vascular wall or by stasis of blood flow. It should be made clear that the initiation of venous thrombosis requires more than a single

abnormality in the triad. The etiology of most VTE is multifactorial. Interactions can occur when two risk factors in combination produce an effect that exceeds the sum of their separate effects. While the individual risk factors (acquired or inherited) for VTE discussed thus far surely fall within Virchow's triad, many authors do agree that these factors work dynamically and that venous thrombosis is a multicausal disease [52].

1.4 Diagnosis of DVT

There are three categories of tests commonly used to assess the probability of DVT. The first is clinical probability which is an assessment based on the patient's history and clinical findings. Second are D-dimer assays. The third category comprises imaging studies which include venous ultrasonography and less frequently, venography, CT scan or MRI. Testing often requires more than one assessment in combination and choosing one strategy over another should ultimately benefit the patient's outcome [53].

DVT refers to thrombus formation within the deep veins of the body (i.e. in the calf or thigh). Local and systemic complications are frequent in DVT patients. A common complication of DVT is the post-thrombotic syndrome (PTS), formerly known as the post-phlebitic syndrome, which is characterized by swelling and chronic pain in the affected limb, and an overall lower quality of life [54]. Diagnosis of DVT has evolved over the years. Prior to 1995, patients with a suspected DVT underwent serial testing via compression ultrasonography. The lack of compressibility by ultrasonography of the

venous segment in question was the diagnostic criterion used. This test was repeated in the patient one week later if results were negative. The results of these serial tests were usually negative and only 10-25% of patients suspected of having DVT actually had the disease. This method had technological limitations in that, depending on the imaging center, sonographic imaging was limited to the proximal veins. Approximately 70-80% of DVTs involve proximal veins, therefore if the suspected DVT involved the distal calf veins which were not scanned, results were negative and serial testing was required [55].

Diagnostic D-dimer testing is based on endogenous fibrinolysis. The D-dimer antigen is a unique degradation product of fibrin and is formed by the sequential actions of three enzymes. The first enzymatic step is thrombin cleavage of the fibrinogen molecule which exposes a polymerization site for homodimerization (or dimerization with a single fibrin molecule). The second step is covalent cross-linkage of fibrin monomers by fXIIIa. The third and final step in D-dimer formation is its release from cross-linked fibrin molecules by the action of plasmin on specific residues (Fig. 1-6) [56]. In healthy subjects, it is uncommon to detect fibrin degradation products (D-dimer, fragment E complex) in circulation, whereas high molecular weight fragments that contain D-dimers are detectable in patients with disseminated intravascular coagulation (DIC) and other thrombotic disorders. D-dimer assay kits are available commercially. D-dimer testing has been accepted clinically when used in conjunction with clinical judgement as a means of selecting patients for ultrasound imaging [57].

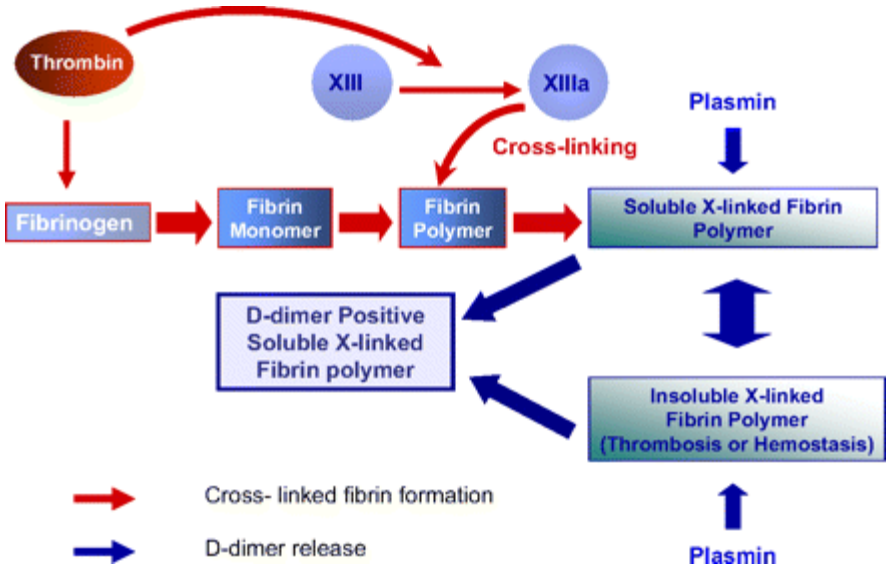


Figure 1-6. D-dimer formation. Thrombin first converts fibrinogen into fibrin monomers. Thrombin remains associated with fibrin and activates factor XIII, which circulates bound to fibrinogen. Formation of the D-dimer antigen is the result of the sequential enzymatic activities of thrombin, factor XIIIa and plasmin and is detectable by commercially available assays [56].

Objective testing for DVT is critical in making a diagnosis. Clinical assessment alone is unreliable and a misdiagnosis can result in fatal PE.

1.5 Survival and Recurrence following Deep Vein Thrombosis and Pulmonary Embolism

Patients must be followed closely after the first incidence of DVT or PE. In one study, the risk of death in patients following PE was reported as being 18 fold higher than

those with DVT alone. The time frame for first recurrence of VTE can range from one week to ten years. The diagnosis of a clinically suspected recurrent venous thrombosis is often more difficult to diagnosis than the initial episode. Patients who survive the initial DVT are prone to chronic swelling and pain in the leg, since the valves in the deep veins can become damaged as a consequence of the thrombotic process, resulting in venous hypertension. Ulcerations of the skin can occur as well as impaired mobility, significantly reducing quality of life. Among early descriptive studies of the post-thrombotic syndrome, the incidence was recorded in approximately 50% of patients with venous thrombosis. This number has shrunk over time, likely as a result of improved initial anticoagulation [58].

1.6 Treatment of DVT

The treatment of DVT must be considered both in the short and long term. In the short term, the goal is to prevent the extension of the thrombus and development of PE. In the long term, therapy will ideally prevent recurring events. The initial therapy involves therapeutic doses of unfractionated heparin or low molecular weight heparin (LMWH). The introduction of LMWH for home treatment has changed DVT from a traditionally in-patient disease to an out-patient disease. Statistical analyses since the clinical introduction of LMWH reveal fewer deaths, hemorrhages and recurrent VTE[59]. For long term treatment, LMWH used in conjunction with vitamin-K antagonists such as Warfarin is proven to be effective in the prevention of recurrent thrombotic events.

Oral anticoagulants were first introduced in the 1940s and to this day, vitamin-K antagonists remain approved for long term use in the prevention and or treatment of most thrombotic disorders. In addition to LMWHs, there are fXa inhibitors such as Apixaban and direct inhibitors of thrombin such as Dabigatran etexilate. Thus, various participating enzymes of the coagulation cascades have been targeted for anticoagulant therapy [59]. The clinical effectiveness of oral anticoagulation therapy is assessed by laboratory tests including the Prothrombin time (PT) and the activated partial thromboplastin time (aPTT). The PT is used most often to monitor the effects of Warfarin-based anti-coagulant therapy on patients with DVT or other thrombotic disorders. The aPTT is commonly used to monitor the effects of unfractionated heparin therapy [60].

The duration of long term therapy will vary depending on the nature of the initial DVT. Some additional interventions exist as well. Usually reserved for cases with a massive DVT is systemic thrombolysis, the use of pharmacological agents designed to dissolve a clot, such as recombinant tissue plasminogen activator [61]. The downside to this intervention is the increased risk for major hemorrhage and other more severe complications. It is also possible to insert an inferior vena cava filter. This method has been shown to prevent PE, but increases the risk of recurrent DVT [55].

1.7 Thrombus Formation *in vivo*

VTE which comprises DVT and PE is a disorder of hemostasis, the process that halts bleeding from the damaged blood vessel. Injury to the blood vessel wall and subsequent extravasation of blood initiates a series of events in the wall itself and in blood that culminate in the cessation of bleeding [11]. Venous thrombi are intravascular deposits that are composed of fibrin and erythrocytes, with variable amounts of platelets and leukocytes [58]. The contributions to *in vivo* thrombus formation from both the hematopoietic and vascular compartments will be discussed.

1.7.1 Role of Platelets

Central to thrombus formation are blood derived platelets. The hemostatic role of platelets was first identified in the 1880s by Bizzozero [62]. Platelets are produced from megakaryocytes in a process called thrombocytopoesis. The megakaryocyte precursors differentiate from hematopoetic stem cells (HSCs) that receive specific growth factor signals. HSCs differentiate and proliferate into progenitor cells which then undergo polyploidization via endomitosis, cytoplasmic maturation, platelet formation and release. During the stage of cytoplasmic maturation, the granules specific to platelets are formed. In 1996, the bone marrow was identified as a major source of thrombopoetin, a growth factor that specifically regulates megakaryocyte differentiation. Thrombopoetin also induces morphological changes of megakaryocytes and begins proplatelet formation (Fig. 1-7) [63, 64].

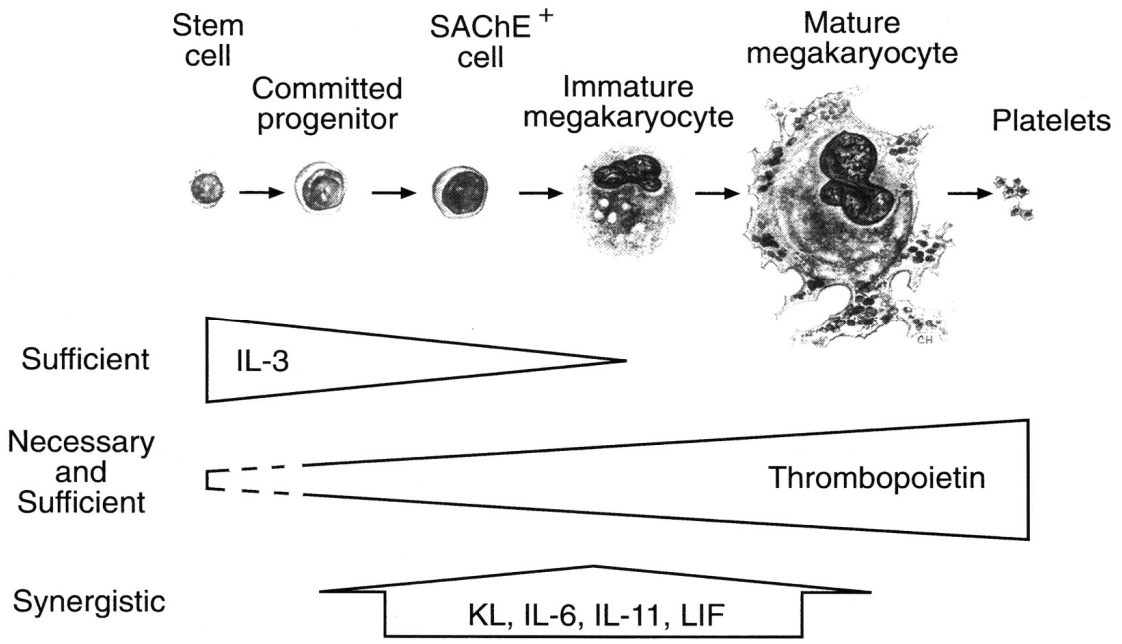


Figure 1-7. The roles of Thrombopoietin in platelet production. Thrombopoietin increases the number and size of megakaryocytes in the bone marrow as well as influencing their maturation [65].

Platelet membranes express a number of mobile transmembrane receptors that are critical for their function. They include a number of integrins, (αIIbβ3, α2β1, α5β1, α6β1 and αVβ3), glycoproteins (Ib, IX and V), Toll-like receptors, thrombin receptors (PAR-1 and PAR-4), ADP receptors, TXA2 receptors and receptor tyrosine kinases (RTKs). The primary function of platelets is in hemostasis and is carried out via proper function of the aforementioned transmembrane receptors. Increasing evidence shows that

platelets are involved in other processes such as inflammation, tumor growth, metastasis and immunologic defence of the host. The intracellular make up of platelets is composed of a cytoskeleton, a tubular system, mitochondria, peroxisomes and the α- and δ-storage granules. Upon platelet activation, the contents of these granules are released promoting further activation and adhesiveness. The contents of the α-granules include vWF, fibrinogen and P-selectin. The contents of the δ-granules include ADP, ATP, histamine and serotonin. The dynamic process of thrombus formation must also be subject to negative regulation to ensure that growth of the thrombus at the site of vascular injury is limited [66].

The formation of a platelet plug at a site of vascular injury occurs in three phases that must be properly coordinated in time and space. Hemostasis begins with the initiation phase which requires the initial binding of platelets to exposed subendothelium. Platelets initially become tethered to the damaged vessel wall via membrane receptors that function in the absence of cellular activation. This allows for rapid interactions that overcome the constraints on bond formation that is imposed by blood flow [67]. Therefore, the initial recruitment is of unstimulated platelets and only a subgroup of these will become activated during thrombus formation [11]. Following initiation is the extension phase that is characterized by the recruitment and activation of addition platelets from the circulation. These additional platelets are recruited to the growing hemostatic plug in a process called aggregation. The αIIβ3 integrin on platelets is critical in mediating aggregation. During the extension phase, a monolayer of activated platelets is created that is firmly attached to the vascular lesion. This occurs within seconds [67].

The third and final stabilization phase prevents premature dissolution of the platelet plug and ensures that wound healing is allowed to occur. The stabilization phase has been described as dynamic in that continuous signalling between platelets is required in order to prevent dissolution [66, 68].

"Inside-out" and "outside-in" are terms used to describe the bidirectional nature of signalling through integrin receptors. αIIbβ3 is the platelet integrin that mediates aggregation and the ability of these cells to form stable thrombi *in vivo* depends on both inside-out and outside-in signalling. The ability of platelets to bind fibrinogen is critically regulated by intracellular signals, the so called "inside-out" signals that induce conformational changes in αIIbβ3 required for extracellular ligand binding of fibrinogen. Once the extracellular domains of αIIbβ3 are bound to fibrinogen, it is believed that the integrin generates additional signals to their short cytoplasmic tails, termed "outside-in", that are important for thrombus stability. The bidirectional signals are thought to be carried out by protein-protein interactions between the cytoplasmic tails of integrins and several adapter proteins [69].

1.7.2 Role of the Vascular Wall

The blood vessel wall is composed of the inner endothelial cell lining which makes contact with blood. Subendothelial layers are comprised of smooth muscle cells and a layer of adventitial cells which express TF [70]. Under normal conditions the endothelial cell layer provides a thromboresistant surface. As previously mentioned, it

does so through the expression of molecules such as NO, PGI_2 and CD39, which inhibit platelet aggregation. When a lesion disrupts the integrity of the vessel wall, TF and other components of the extracellular matrix (ECM) become exposed to blood, initiating thrombus formation. The ECM constituents that platelets come into contact with will depend on the depth of injury. In general, the main components of the basement membrane that become exposed to blood following a superficial disruption of the endothelial lining are proteoglycans, collagen type IV, laminin and fibulin [67].

The collagens make up approximately 40% of the blood vessel wall. Collagen molecules form insoluble scaffolds essential for tissue integrity and provides a surface for the adhesion of vascular cells. At least 25 types of collagen have been identified. Collagen types I, III, IV, V and VI are widely distributed and can be found in the vessel wall. Again, collagens are not typically exposed to blood. Upon vessel injury, blood flows over subendothelial structures including connective tissue which contains a high amount of collagen. Collagen represents the only matrix constituent that mediates both platelet adhesion and activation [71].

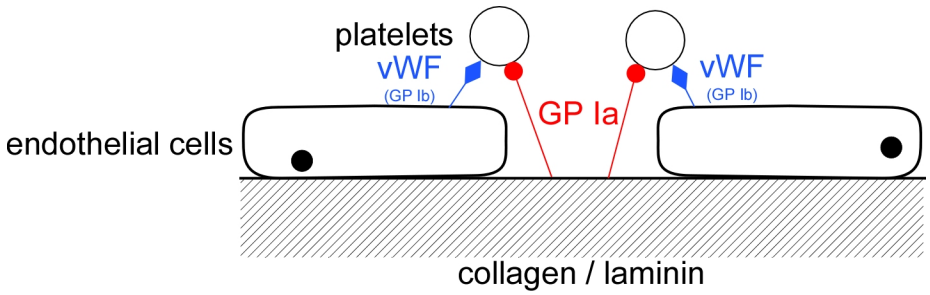


Figure 1-8. Collagen Binding to Platelets. Upon vascular injury, exposed collagens of the ECM come into contact with and activate platelets via platelet glycoprotein (GP) 1b. **(http://www.ric.edu/faculty/jmontvilo/335.htm. Montvilo, JA)**

Collagen initiates platelet adhesion and activation while TF initiates the proteolytic blood coagulation cascade which culminates in thrombin generation and fibrin deposition. TF can also contribute to platelet activation. The dominating pathway (collagen or TF) to platelet activation will depend on the particular injury or disease. Platelet adhesion to collagen occurs via molecular interactions between platelet glycoprotein VI with collagen and the platelet glycoprotein Ib-V-IX with vWF factor (bound to collagen) (Fig. 1-9). In addition to its role in mediating adhesion, glycoprotein VI is also critical for the initial platelet activation and granule release. Thrombin dependent platelet activation occurs independently of glycoprotein VI and vWF. In this instance, thrombin forms a complex with the active fVIIa. This complex in turn activates factor IX which leads to thrombin generation. Thrombin activates platelets by cleavage of PAR1 (PAR4 in mice) on the platelet surface. This in turn causes platelets to release ADP, serotonin and TXA2 activating additional platelets, amplifying thrombus formation [67].

1.7.2.1 Tissue Factor

Tissue Factor has been recognized as a major initiator of blood coagulation for over a century. In 1972, Macfarlane found that TF activates fX in the presence of fVIIa. Two individual groups subsequently purified human TF in the mid 1980s [72, 73]. Following its purification, it was sought to isolate the TF gene. This was accomplished near simultaneously by four individual groups in 1987 who reported their findings on cDNA clones for human TF [74-77]. A few years later, murine TF cDNA clones were isolated [78, 79]. The TF gene is located on human chromosome 1p21-p22 and the complete sequence was published in 1989 by Mackman. The TF gene is approximately 13 kilobases long and is comprised of six exons and five introns. The 5' end of the gene contains CpG islands [80]. The transcribed product is a 47 kDa transmembrane glyprotein composed of 263 amino acids. 219 of these comprise the enzyme's extracellular domain, 23 the transmembrane domain and 21 the cytoplasmic tail. TF belongs to the Class II cytokine receptor superfamily and its extracellular domain shares homology with the interferon receptors. TF is also subject to posttranslational modifications such as N-linked glycosylation on three separate asparagine residues that affect the overall charge of the enzyme but not its capacity to initiate blood coagulation. The extracellular domain has two disulphide bridges between Cys49-Cys57 and Cys186-Cys209, the latter of which is essential for TF activity. The initial view of TF was that it formed an extravascular 'envelope' that surrounded blood vessels and came into contact with the soluble plasma coagulation proteins following disruption of the vessel wall. When this was first visualized histologically, TF positive staining was found predominantly within the adventitia and much less so within the media. Within the

media, TF is inducible on smooth muscle cells by growth factors and TF induction on endothelial cells has been demonstrated both *in vivo* and *in vitro* by various stimuli and disease models, respectively. Among the various organs, TF expression is non-uniform. In mice, high levels of TF are found in the brain, lung, heart, kidney, uterus, testis, skin and placenta. Mouse organs that express lower levels of TF include the liver, spleen, skeletal muscle and thymus. In humans, high levels of TF are found in highly vascularized organs such as the brain and placenta. At the cellular level, the various cells expressing TF include astrocytes, alveolar cells, and cardiomyocytes [81, 82].

Following disruption in integrity of the blood vessel, the coagulation cascade is initiated by the binding of blood coagulation fVII (or fVIIa) to TF. The aberrant expression of TF can trigger intravascular thrombosis, the consequence of disease states such as cancer, sepsis and atherosclerosis. [70]. In 1996, publications from three laboratories demonstrated that knocking out the murine TF gene results in extremely high rates of embryonic lethality [83-85]. These findings revealed the importance of TF in vascular development. The targeted disruption of the TF gene caused the death of embryos as a result of severe embryonic bleeding. The vasculature of the yolk sacs were also disorganized in $TF^{-/-}$ embryos [70].

Having described the roles of vascular TF in hemostasis and thrombosis, it is noteworthy that TF can also be found in the circulation. It was first proposed by Giesen *et al*. that the circulating pool of TF may contribute to thrombosis by sustaining the propagation of a thrombus whose growth was initiated by vascular TF [47, 50].

Circulating TF has been found in peripheral neutrophils and monocytes but the major source are small, negatively charged membrane vesicles called microparticles (MPs) [50, 86]. Leukocytes are the only blood cells known to actually synthesize TF and leukocyte associated TF has been detected in human thrombi [51, 87]. An interesting reaction occurs between leukocytes and platelets whereby TF is transferred from leukocytes to platelets in a reaction that depends on MPs and P-selectin [88]. Platelet specific expression of TF remains an area of debate and various groups have produced conflicting results. Early publications that sought to resolve the issue showed that TF was detectable in platelet α-granules [89] but was later challenged when Butenas *et al.* failed to detect measurable TF activity in platelets challenged with collagen [90].

Membrane coated MPs are shed from almost all cells during apoptosis. MPs have been shown to contribute to intravascular thrombin formation by the exposure of phosphatidylserine and TF [91]. Elevated levels of procoagulant MPs have been detected in patients with the antiphospholipid syndrome and a history of venous thrombosis [92]. Other pathological conditions whose prognoses correlate with plasma levels of TF include angina and acute myocardial infarction. It had been proposed that the pool of TF in the circulation has the capacity to contribute to thrombogenesis *in vivo*, possibly by sustaining propagation of a thrombus via the continued production of thrombin on the its surface. Vascular TF will eventually be sequestered from the blood during the thrombotic process due to rapid deposition of platelets and fibrin on the exposed vessel, thereby physically separating it from circulating blood factors [51]. Blood derived TF is capable of supporting thrombus formation *in vitro* [50] although a series of *in vivo* experiments

designed to explore the functional role of blood derived TF revealed that it is primarily TF from the vascular wall that contributes to thrombus formation [51].

TF also exists as an alternatively spiced or soluble form which is capable of mounting a procoagulant response in the presence of phospholipids. The alternatively spliced form of TF is still a recent discovery and it remains to be seen if it has a role in the pathogenesis of VTE [93]. TF also has the capacity to initiate downstream signalling events. The 21 amino acid cytoplasmic tail of TF contains two phosphorylation sites that are recognized by Protein kinase C and the proline-directed kinase. The processes regulated by TF dependent signalling are broad, including effects on gene transcription, protein translation, apoptosis and reorganization of the cytoskeleton. At the plasma membrane the phospholipid content of microdomains can regulate fVIIa:TF dependent signalling as evidenced by the fact that it is modulated by lipid rafts and caveolae [94]. Some clinical applications for TF include maxillofacial surgery and the induction of gel formation of platelet-rich plasma (PRP) in bone preparation [82].

1.8 Negative Regulation of Thrombus Growth

Endothelial cells form a barrier between tissues and blood and have numerous roles in proper hemostasis. The group of molecules known as the endothelins are released primarily from endothelial cells and participate in hemostatic vasoconstriction [95]. The

growth factors released by endothelial cells such as VEGF are involved in vascular repair by promoting cell proliferation [96]. Another endothelial derived factor of utmost importance in the formation of a hemostatic plug is vWF which is absolutely required for platelet binding through glycoprotein Ib-IX. The endothelial cell provides a thromboresistant surface with expression of TM (Protein C pathway), heparin sulfate and tissue factor pathway inhibitor (TFPI) which as the name implies regulates TF dependent blood coagulation. TFPI regulates the initiation of coagulation by inactivating active fXa and the complex of TF:fVIIa on phospholipids membranes. About 80% of TFPI that is produced by endothelial cells remains associated with their membranes. The plasma concentration of TFPI is around 2.5 nM. Protein S is a vitamin K-dependent plasma protein that was previously discussed as having a critical role as cofactor in the activated protein C pathway. Protein S also serves as cofactor for TFPI in the inhibition of fXa [95, 97].

Endothelial cells also provided an anti-platelet surface. Endothelial cells and smooth muscle cells produce prostacyclin (PGI_2), a multifunctional molecule. PGI_2 initiates a series of events that lead to platelet production of cAMP with subsequent inhibition of platelet activation. The inhibition of platelet aggregation can be achieved at the level of the membrane receptors or by blocking intracellular signalling pathways. When exploiting this ability pharmacologically, inhibition at the level of platelet receptors offers higher specificity, while inhibition of signal transduction is more effective. NO is also well established for its anti-platelet activity. NO is synthesized by endothelial cells and has a vasorelaxant effect on vascular smooth muscle as well. NO is

secreted into the blood vessel lumen for interaction with blood cells [98]. The target of NO within platelets is the heme moiety of the soluble guanylyl cyclase (SGC) enzyme which increases the intracellular levels of cGMP [99].

Healthy endothelium expresses molecules with anticoagulant and antiplatelet properties. Under certain pathologic conditions, endothelial dysfunction can occur. Endothelial cell dysfunction is usually the result of excessive stimulation by cytokines or microbial agents. Under conditions of endothelial dysfunction the expression of the anticoagulant and anti-platelet molecules will decrease, resulting in these cells losing their thromboresistant properties. This could lead to inappropriate activation of platelets and thrombosis as well as myocardial infarct or stroke [100].

1.8.1 Fibrin Clot Formation and Fibrinolysis

The formation of a hemostatic thrombus requires the cleavage of fibrinogen to fibrin by the serine protease thrombin that is generated during blood coagulation. Fibrinogen is a 340 kDa protein that is present in high concentration within plasma. The exact mechanisms that form the basis of fibrin formation have been determined from studies that involved the addition of exogenous thrombin to purified fibrinogen. Fibrinogen consists of pairs of three different disulfide-linked polypeptide chains. The chain of events that lead to fibrin aggregation begins with thrombin mediated cleavages of fibrinogen N-terminal peptides. The structure of a fibrin clot formed *in vitro* will

depend on pH, and the concentrations of calcium, fibrinogen and thrombin. Studies *in vivo* have shown that the relationship between fibrin structure and the thickness of a clot is highly complex and depends on a number of variable properties of the fibrin fibers themselves. These include thickness, length, density, degree of branching and extent of cross-linking. Fibrin functions as both a cofactor and substrate for the fibrinolytic enzyme, plasmin. Therefore, the structure of a particular fibrin clot will influence its susceptibility to fibrinolysis [101].

Under physiological conditions, the processes of coagulation and fibrinolysis are precisely regulated by their respective control mechanisms. Both processes are co-ordinated in such a way as to maintain blood fluidity without loss of blood. The major fibrinolytic protease is plasmin. The active enzyme plasmin is cleaved from the circulating zymogen plasminogen by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (uPA) (Fig. 1-8). Endothelial cells are the major source of t-PA and expression is mostly found in blood vessels of small diameter (i.e. postcapillary venules and vasa vasorum). The endothelial lining of larger vessels such as the femoral artery and vein express much lower levels of t-PA. The release of t-PA from endothelial cells is regulated by a number of different mediators such as thrombin, histamine and shear stress. It is the major intravascular activator of plasminogen. The urokinase plasminogen activator is expressed by endothelial cells, macrophages, renal epithelium and some known tumor cells. Plasminogen has a much higher affinity for t-PA than for uPA. The reactions catalyzed by the plasminogen activators are subject to positive feedback. Plasmin is capable of cleaving both t-PA and uPA, converting them

from single-chain to double-chain polypeptides with higher enzymatic activity. Fibrin, the main substrate of plasmin is capable of regulating its own degradation by the simultaneous binding of plasminogen and t-PA to its surface, accelerating and localizing plasmin generation. In fact, the affinity of t-PA for its substrate plasminogen is much higher in the presence of fibrin. There are no published clinical examples of a complete deficiency of either tPA or uPA in humans [102]. Plasmin participates in the process of thrombus resolution by catalyzing the degradation of fibrin clots. The process of resolution of DVT resembles that of wound healing and also involves leukocyte invasion [103]. Plasmin activity is negatively regulated by the serpin family of proteins[102].

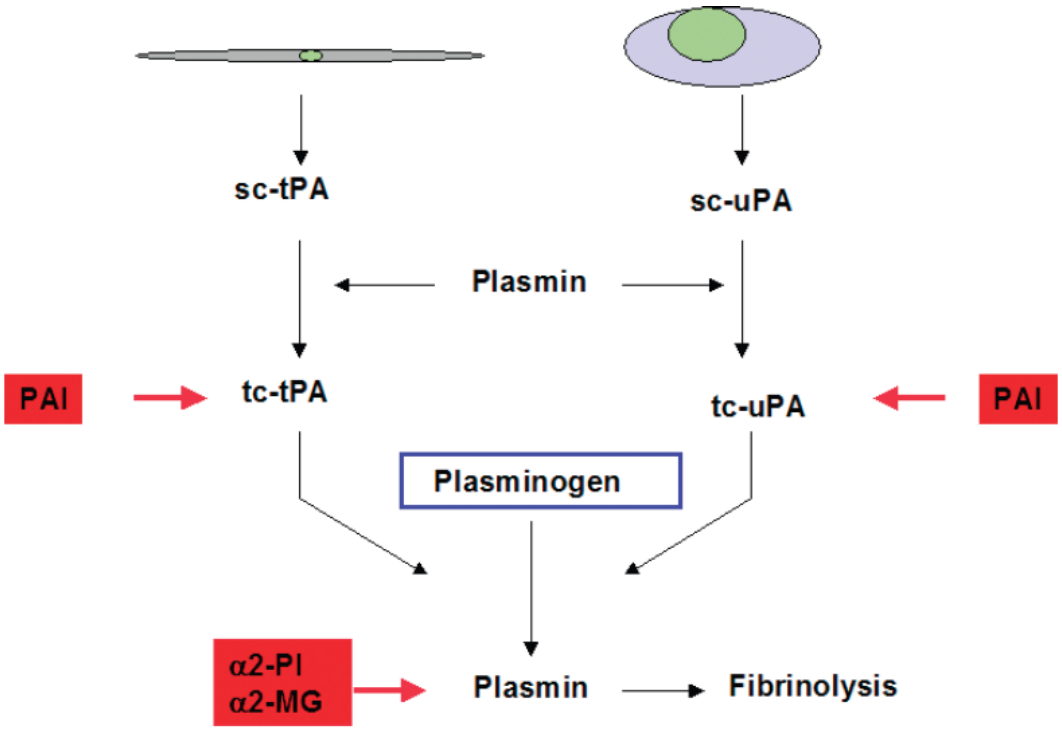


Figure 1-9. The Fibrinolytic System. The Zymogen plasminogen is converted to the active serine protease plasmin. This is achieved by the two-chain (tc) forms of t-PA and uPA, which can both be inhibited by plasminogen activator inhibitor-1 (PAI-1). Plasmin activity is regulated by the major α2-plasmin inhibitor (α2-PI) [102].

1.9 Murine Models of Venous Thrombosis

The Surgeon General's Call to Action to Prevent Deep Vein Thrombosis and Pulmonary Embolism stated in 2008 that "the disease disproportionately affects older Americans and we can expect more suffering and more deaths in the future as the population ages-unless we do something about it". The use of animal models will be invaluable if this trend is ever to be averted. Humans are in fact the only species with the propensity to spontaneously develop VTE. The use of murine models of DVT allows investigators to study the biological mechanisms that underlie the development of DVT as well as evaluate potential new therapies for its treatment. Mice do not spontaneously develop thrombosis and thus a thrombus must be induced experimentally [104]. There are numerous ways investigators have gone about this. Historically, the development of *in vivo* thrombosis models can be traced back to Virchow who published work on a canine model in 1846, triggering interest in the development of models that would mimic the human disease. The initial thrombosis models were developed and performed in larger mammals until the beginning of the era of transgenic mice, when these models began being adapted for use in the small rodents. The advantages of using mice for studies on thrombosis (as well as numerous other diseases) include the low maintenance cost, ease of manipulation and importantly, the existence of several genetically manipulated strains. Another advantage is the fact that it is usually impossible to obtain samples from a site of DVT in a human patient. Noteworthy differences between mice and humans that must be taken into account when analyzing experimental data obtained from murine models include body size, life span, genetic differences and blood vessel size. Despite these

differences, murine thrombosis models have greatly expanded the understanding of the disease. Large vessel models mostly involve the inferior vena cava (IVC) and the types of data one can obtain include thrombus size, immunohistochemistry and protein activity (i.e. TF). Some of the major large vessel models will be briefly discussed as well as their various advantages and disadvantages [105].

1.9.1 IVC Ligation

In this particular model, a non reactive proline suture is looped around the IVC (and major side branches) immediately caudal to the renal veins to produce complete stasis of blood. It is believed that thrombosis by this model is induced by a combination of stasis induced injury to the vessel wall and enhanced TF expression on endothelial cells and leukocytes. The IVC ligation model is useful for obtaining high yields of thrombus and vein wall tissue and for studies on the interactions between the thrombus and vein wall. A major limitation of this model regards the use of systemically injected therapeutic agents, whose effects will be greatly diminished due to the lack of blood flow to the affected area [105, 106].

1.9.2 Photochemical Injury

The photochemical injury model makes use of a systemically injected or topically applied photosensitizing dye called Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein). Rose Bengal is well documented for its ability to produce reactive oxygen species which are thought to be potent contributors to endothelial cell damage leading to thrombosis. The dye is inert until activated by a green light laser (543 nm). To induce thrombosis at a particular vascular site, the laser is shone on the vessel where the desired injury is to occur. The green light induces singlet oxygen radical formation at the target site. Rose Bengal is a useful tool. It has high photochemical efficiency and low systemic toxicity [107, 108].

1.9.3 Systemic Collagen/Epinephrine

The intravenous injection of a mixture of collagen and epinephrine induces widespread activation of platelets. Mice usually die within minutes as a result of extensive pulmonary embolism. This model has been used to evaluate the effects of antithrombotic agents and to study the consequences of knocking out different platelet receptors [82].

1.9.4 Mechanical Injury Models

There are several methods for the physical injury of a vessel in mice that lead to thrombosis. One such method already discussed involves ligation. Additional methods include perivascular electrical injury, placement of polyethelene cuffs, wire injury and mimicking mechanical injury by ferric chloride ($FeCl_3$) which will be discussed in detail in a subsequent section [108].

1.10 Gas6

1.10.1 Identification and Characterization of Gas6

In 1988, Schneider *et al.* published their findings on a set of genes induced during G0, a state of growth arrest in cells that can be initiated in the G1 phase of the cell cycle by removal of growth factors or serum from the growth medium. These G0 specific genes were negatively regulated by serum and growth factors and were henceforth known as the growth arrest specific genes or the *gas* genes [109].The sixth member in this set, *Gas6* was indeed originally identified by Schneider *et al*., but remained uncharacterized until 1993 when Manfioletti *et al.* confirmed that *Gas6* is induced following serum withdrawal in both murine (NIH3T3) and human (IMR90) fibroblast cell lines. Gas6 is a member of the vitamin K-dependent family of proteins [110] which includes the zymogens involved in blood coagulation such as prothrombin, factor VII, factor IX, factor X, protein S, protein C and protein Z [111]. The cDNA sequence of murine *Gas6* is 2,225 nucleotides in length and encodes a protein of 673 amino acids. The predicted amino acid sequence was compared with the complete protein sequence data bank available at the time,

revealing an approximate 43% amino acid homology with Protein S, one of the natural anticoagulants. This initially raised the possibility that Gas6 was the murine homolog of human protein S and prompted the researchers to screen a human lung fibroblast cDNA library with murine *Gas6*. A partial cDNA clone was isolated and was further used to screen a HeLa cDNA library. This screen revealed a full length cDNA clone of 2,461 nucleotides in length that encoded a protein of 678 amino acids. The protein was 81% homologous to murine Gas6 and 44% homologous to human protein S. Therefore Gas6 is similar to, but distinct from protein S and was officially added as a new member of the vitamin K-dependent family of proteins [110]. Interestingly, a subsequent FISH study revealed the chromosomal location of *Gas6* to be 13q34, a region shared by other vitamin K-dependent protein genes [112].

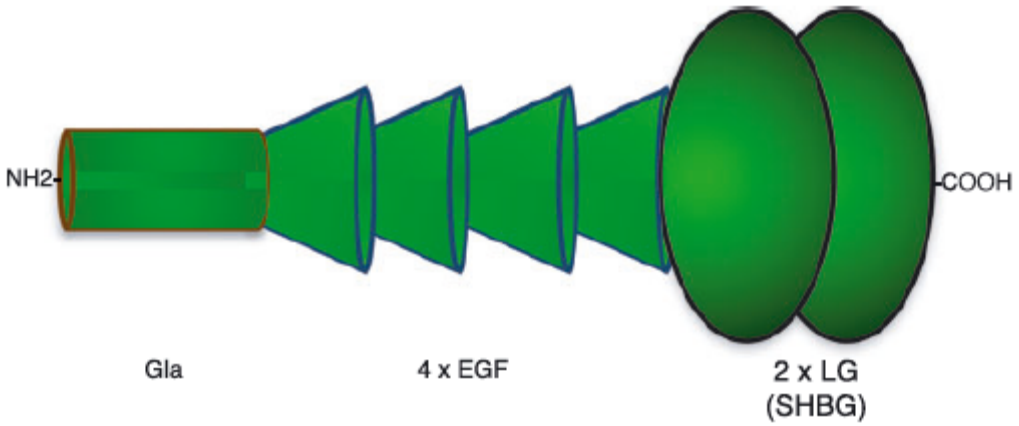


Figure 1-10.The domain organization of Gas6 and Protein S. Both of these proteins are composed of an N-terminal domain containing several γ-carboxyglutamic acid (Gla) residues. This is followed by four EGF-like repeats and a C-terminal domain that shares homology with the human sex hormone-binding protein [113].

The very amino terminus of Gas6 contains a conserved region of hydrophobicity that resembles a signal peptide for a secreted protein (i.e. protein S). Also within this region of the protein is the γ-carboxyglutamic acid (Gla) domain, characteristic of all proteins of the vitamin K-dependent family. In general, the Gla domains of the blood coagulation factors (and Gas6) are responsible for Ca^{2+}-dependent binding to phospholipid membranes. Four epidermal-like growth factor (EGF) repeats are present in Gas6. This region contains select hydroxylated residues that bind calcium ions with high affinity. Near this region in the homologous Protein S is a small span (Leu-Arg-Ser) that represents two thrombin sensitive cleavage sites. This consensus is absent in both human and murine Gas6, excluding it from thrombin sensitivity and a direct role in the blood coagulation cascade. It is also this region of Gas6 that shares the least homology to protein S (16%). Toward the carboxy terminal of Gas6 is a region that shares homology with the human sex hormone-binding protein (SHBP). This region is extensive in length, but Gas6 has not been shown to bind to any steroid-derived molecules (Fig. 1-10).

The expression of Gas6 mRNA can be found in a number of human tissues. They include lung, intestines, bone marrow and endothelial cells. Gas6 mRNA is expressed in numerous mouse tissues including heart, lung, stomach, kidney, muscle, brain, spleen, liver and testes [110].

1.10.2 Gas6 Receptors: TAM Receptor Tyrosine Kinases

Receptor Tyrosine Kinases (RTKs) are an important class of cell surface receptor that possess intrinsic protein-tyrosine kinase activity. The intracellular domains of RTKs possess the intrinsic tyrosine kinase activity and become activated once their extracellular domains are bound with ligand and undergo dimer- or oligomerization. The phosphorylated tyrosine residues can be bound by a variety of intracellular signaling molecules, activating a myriad of downstream signaling cascades. Following the completion of the human genome project, approximately 90 genes were identified as having protein tyrosine kinase activity. 58 of them encode for RTKs [114]. RTKs can be subdivided into 20 families based on structural characteristics. They all share a homologous domain that catalyzes tyrosine kinase activity. All known RTKs contain a single transmembrane domain that separates the intracellular catalytic domain form the extracellular receptor binding domain The extracellular moieties of RTKs share a variety of homologous regions such as immunoglobulin (Ig)-like or epidermal growth factor (EGF)-like domains. The catalytic domain of each RTK contains an ATP-binding pocket that catalyses autophosphorylation of the receptor and downstream phosphorylation of RTK substrates[115].

To summarize, the sequence of events leading to RTK activation are as follows. First, a monomeric RTK is bound by ligand in its extracellular domain. This is followed by a series of conformational changes that result in receptor dimerization (or oligomerization) and stabilization. Following this event is receptor autophosphorylation of the intracellular domains. Specific phosphorylated tyrosine residues on the receptor are

recognized by proteins that contain Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. These binding partners link RTKs to downstream signaling cascades [115].

RTKs are termed orphan receptors if their molecular ligands have yet to be identified. A family of three RTKs initially identified in the rat nervous system were designated Tyro 3, Tyro 7 and Tyro 12 [116]. Full length cDNAs encoding these RTKs have since been characterized. Tyro 3 has been named and will be henceforth referred to as Sky [117]. Tyro 7 has been named and will be henceforth referred to as Axl [118]. Finally, the human version of the third orphan, Tyro 12 has been named and will be henceforth referred to as Mer [119]. Together these RTKs are known as the TAM family (an acronym for Tyro3, Axl and Mer). They display differential expression patterns. Receptor based detection and affinity purification were used to identify the ligands for Axl and Sky as both Protein S and Gas6. In terms of Gas6 binding affinity for the TAM family of RTKs, the order is Axl > Sky > Mer. [120, 121]. The interaction between Gas6 and its receptors is unique in that Gas6 shares homology with the blood coagulation factors rather than growth factors that are the typical ligands of RTKs [122].

The TAM family of RTKs are structurally related. They are characterized by an extracellular domain that bears homology to cell adhesion molecules, with two immunoglobulin-like (IG) domains. This is followed by two fibronectin type-3 like domains (Fig 1-11).

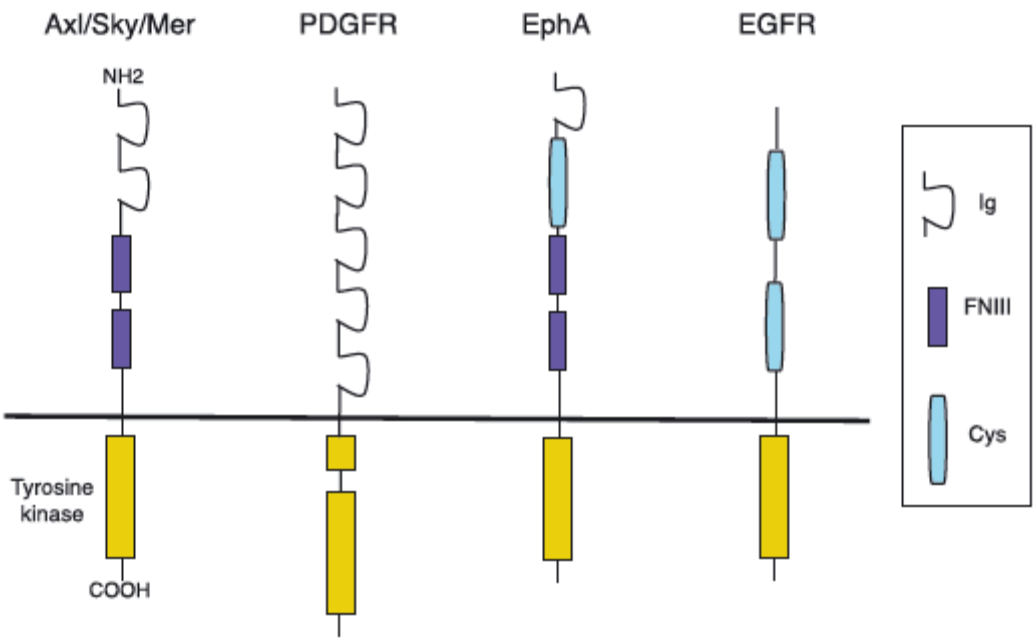


Figure 1-11. Domain organization of RTKs. The leftmost TAM receptors contain two Ig-like and two fibronectin type-3 (FNIII) like domains and share homology with other RTKs [113].

Gas6 mediated signalling depends on precise structural changes within the Gas6-receptor complex. To illustrate the importance of these structural changes is the example of the Gas6/Axl interaction. The site at which Gas6 binds to Axl is located wholly within the C-terminal domain of Gas6. Gas6 binds Axl with nanomolar affinity and 1:1 stoichiometry. Crystal structural analysis revealed that there are both major and minor contacts made between Gas6 and Axl and that both are required for receptor activation [122-124].

Following the identification of the Gas6/Axl interaction, the functions of Gas6 began to emerge. First, Gas6 was shown to be able to protect NIH3T3 fibroblasts from

apoptosis following serum withdrawal. It is also noteworthy that this pro-survival activity occurred independently of Gas6 mitogenic activity [125]. The mitogenic effects of Gas6 are not limited to fibroblasts. A role for Gas6 as well as its expression has been observed in numerous tissues. These roles will be discussed within the context of the particular TAM receptor involved.

1.10.3 Gas6/Axl Signalling

Axl is also known as UFO, ARK and Tyro7. Axl remained an orphan receptor until 1995, four years after its initial discovery [120, 126, 127]. The removal of orphan status can be attributed simultaneously to Varnum *et al.* and Stitt *et al.* They were the first to identify Gas6 as a molecular agonist of the Axl receptor. Varnum *et al.* purified an Axl stimulating factor from several cell types. The purified protein caused phosphorylation and downregulation of Axl. In addition, crosslinking studies revealed that the agonist bound to Axl with nanomolar affinity. A sequence analysis of the agonist ultimately revealed it as Gas6. Stitt *et al.* used receptor based affinity purification and arrived at the same conclusion. The findings from both groups were published within eight days of each other in 1995 [127]. Human Axl is composed of 894 amino acids and has a mass of 140 kDa. The distribution of amino acids on each side of the plasma membrane is roughly equal. The pattern of structural elements that make up the extracellular domain of Axl is also similar to several known cell adhesion molecules [127]. The downstream effects of Axl activation are diverse. Axl is involved in oncogenesis, cell survival,

spermatogenesis, immunity, platelet function and renal pathology [122]. Binding proteins of the Axl intracellular domain include Nck2, SOCS-1, C1-TEN and RanBPM [128].

The Gas6/Axl interaction quickly became a candidate system for the regulation of vascular function when Gas6 was detected and purified from the conditioned medium of vascular smooth muscle cells [129]. Another clue to the importance of the Gas6/Axl interactions in vascular function came from the cloning of Axl from balloon-injured rat carotid arteries. Both Gas6 and Axl were upregulated in the injured carotids [127]. It was subsequently shown that the Gas6/Axl system is activated in VSMCs following treatment with hydrogen peroxide. This has implications for the vascular response to injury caused by reactive oxygen species [130].

A well recognized downstream consequence of the Gas6/Axl interaction is cellular protection from apoptosis [127]. In endothelial cells, the protection offered by the Gas6/Axl interaction involves different intracellular signaling pathways. Of particular importance is the involvement of Akt. Akt is activated following phosphorylation of Ser-473 and Thr-308 and has been observed to mediate the antiapoptotic effects of VEGF and angiopoetin. The p65 subunit of NF-kB has also been found to participate in mediating cell survival downstream of Gas/Axl [131].

The Gas6/Axl interaction is involved in various other cellular and pathologic processes. Gas6 bound to Axl mediates the aggregation of 32D myeloid cells in culture, independently of Axl's tyrosine kinase activity. This is a heterotypic intercellular interaction between plasma membrane bound Gas6 and an Axl receptor on an adjacent cell. This study provided the first evidence that Gas6 can mediate cell-cell interactions [132]. Gas6 can act as a chemoattractant for primary VSMCs of human and rat origin. The chemotactic effect of Gas6 on VSMCs was attenuated by the addition of purified Axl extracellular domain to the experimental system, highlighting the specificity of Gas6/Axl binding in this context [133]. The type 5 early region IA gene (*E1A*) and its encoded protein, E1A, is the first viral gene expressed in a cell following infection by an adenovirus. EIA is a known immortalization oncogene, although it cannot immortalize cultured cells single handedly. E1A has tumor suppressing effects that can partially be explained by the fact that it mediates transcriptional repression of the transforming Axl. This effect is abrogated when Gas6 is added to the system and binds Axl [134]. This particular study was followed up with the observation that Akt is required for the Gas6/Axl mediated protection from apoptosis induced by E1A [135]. Axl was first shown to be expressed by human endothelium in a study that implicated Gas6/Axl signaling in the pathogenesis of rheumatoid arthritis [136]. As will be discussed shortly, Gas6 has emerged as a target in various diseases of the kidney. One example would be in a model of experimental glomerulonephritis, where it was demonstrated that the Gas6/Axl signaling pathway regulated proliferation of mesangial cells [137] in part through the STAT3 transcription factor [138].

Within human plasma, Gas6 is present in concentrations of approximately 0.2 nM, which is a thousand-fold lower than plasma levels of Protein S. Within plasma one can also find a soluble form of the Axl receptor. Axl, as well as Mer can be cleaved at the plasma membrane, yielding soluble molecules that consist of their respective extracellular portions. Interestingly, soluble Axl (sAxl) circulates in a complex with Gas6 in human serum and plasma. One can find sAxl in concentrations of 0.6 nM in serum and plasma, and apparently all plasma and serum Gas6 is bound to sAxl. From the standpoint of functionality, the existence of this soluble complex is likely to inhibit the ability of circulating Gas6 to activate TAM receptors [139]. Regardless, circulating levels of Gas6 and sAxl have been used as biomarkers in patients with severe sepsis, infection, systemic inflammatory response syndromes [140], large abdominal aortic aneurysms [141], critical limb ischemia [142] and systemic lupus erythematosus [143]. The levels of circulating Gas6 and sAxl positively correlate with disease severity in most cases.

1.10.4 Gas6/Sky Signalling

Sky is also known as Tyro3, Rse, Brt, Tif, etk2 and Dtk [144]. The structural basis for the Gas6/Sky interaction is similar to the interaction of Gas6 and Axl discussed above [145]. Gas6 was identified as a ligand for Sky in 1995 [146]. This interaction has been implicated in the stimulation of osteoclast function. Sky shows strong expression in primary osteoclasts [147] and it was later determined that the mechanism whereby Gas6 stimulates osteoclast function through Sky involves downstream activation of the

p42/p44 MAPK [148]. Sky, along with Gas6 and Axl were once implicated in the pathogenesis of human uterine leiomyoma. Sky is normally expressed in uterine myometrium tissues but was shown not to be involved in this particular tumor progression [149, 150].

1.10.5 Gas6/Mer Signalling

The RTK Mer is also known as MerTK, Nyk and Tyro12. Based on survey of the literature, the effects of the Gas6/Mer interaction are the least characterized among the three Gas6 receptors. It is also this receptor with which Gas6 has lowest affinity [121]. Mer has been implicated in macrophage clearance of apoptotic cells, cytokine release and of course, cell proliferation and survival. Novel among the interactions of Gas6 and its receptors is the finding that prolonged exposure of Mer with Gas6 induces the expression of a partial Mer glycoform that exists in lower amounts than fully glycosylated Mer. The partial N-glycosylated form of Mer was not found to be simply an intermediate of the fully glycosylated enzyme, but rather imparts functional consequence on the molecule. Gas6 influences Mer glycosylation which in turn determines the sub-cellular localization of Mer, the first of the TAM family of RTKs to be observed in the nucleus [151].

1.11 Functions of Gas6 within Organ Systems and Disease

Having now discussed a number of the biological effects attributable to Gas6 within the context of each one of its three receptors, it is important to also recognize the physiological and pathophysiological effects of Gas6 within the context of the different organ systems. In reviewing the literature, one can find relatively extensive findings on the various effects of Gas6 within the nervous system, kidney, vasculature and in oncogenesis. A review of these findings should serve to further emphasize the pleiotropic activities of Gas6.

1.11.1 Gas6 in the Nervous System

Gas6 is widely expressed in the CNS beginning at late embryonic stages and its levels remain high into adulthood [152]. The Gas6 receptors are also expressed in the developing nervous system [153]. Gas6 has been identified as a growth factor for human Schwann cells [154]. In mice, the Axl homolog is known as Ark (Adhesion related kinase) and signaling through this receptor has been shown to protect Gonadotropin-Releasing Hormone (GhRH) neurons from apoptosis [155]. In neurons of the hippocampus, Gas6 acts as a neurotrophic factor [144] while in cortical neurons, it can protect against apoptosis induced by amyloid-beta proteins (commonly associated with Alzheimer's disease) [156] and phospholipase A_2-IIA [157]. Finally, Gas6 promotes the survival and activity of oligodendrocytes in mice and humans [158-160].

1.11.2 Gas6 in the Kidney

Gas6 has been reported to have effects on renal cells and has been identified as a target for kidney disease. Gas6 has autocrine, mitogenic effects on cultured mesangial cells. One of the features of chronic rejection following kidney transplantation is the proliferation of mesangial cells. A rat model of chronic rejection following kidney transplantation revealed extensive Gas6 mRNA expression in kidney tissues that was also significantly increased in allografts. Any possible involvement of Gas6 in chronic rejection of kidney transplantations was also likely mediated through the Sky receptor [161]. This finding was followed up with a study on tissue samples from human renal allografts. Gas6 was widely expressed among the different allograft cells (glomeruli, tubules etc.) and was significantly increased in acute rejection [162]. An *in vivo* model of acute mesangial proliferative glomerulonephritis in rats also demonstrated increased expression of Gas6 and Axl in the kidney that correlated with disease severity [121]. The vitamin-K antagonist, Warfarin, successfully inhibited the mitogenic effects of Gas6 *in vitro* and disease progression in the *in vivo* model of glomerulonephritis. The role of Gas6 in a model of accelerated nephrotoxic nephritis (NTN) has also been observed. $Gas6^{-/-}$ mice are significantly protected against NTN disease progression [163]. A role for Gas6 in human nephritis was subsequently observed [164]. Diabetes mellitus patients often succumb to nephropathy, an early sign of which is glomerular hypertrophy. It was determined that the Gas6/Axl pathway contributes to glomerular hypertrophy in a streptozotocin-induced model of diabetes in rats [165]. The same authors later began to dissect the intracellular signaling pathways involved in Gas6 mediated glomerular

hypertrophy and found a role for the Akt/mTOR pathway [166]. In regards to human cancers, Gas6 and Axl have been shown to have mostly oncogenic effects. Generally, increased levels of Gas6 or Axl have correlated with disease progression and poor prognosis. Interestingly, it seems as though increased Gas6 correlates with a more favorable prognosis in a model of clear cell renal cell carcinoma. This seems to conflict with the observations made in regard to Gas6 and progression of other diseases of the kidney [167].

1.11.3 Gas6 and Cancer

Dysfunctional expression of the TAM receptors has been implicated in various human cancers. Dysfunctional expression can arise as a consequence of mutations, altered protein expression and gene amplifications, all of which promote the oncogenic potential of the TAM receptors. Axl is well established as having transforming properties. Increased expression of Axl has been observed in a wide range of human cancers including leukemia, colon cancer and breast cancer. Axl was originally cloned from patients with CML and had cell transforming potential when overexpressed. Axl overexpression regulates oncogenic processes such as invasiveness and metastasis [168].

The notion of targeting the Gas6/Axl system in human cancers has yielded conflicting results. It seems that in patients with glioblastoma or ovarian carcinoma, levels of Gas6/Axl correlated with poor prognosis while the opposite has been observed

in patients suffering from renal cell carcinoma [169]. Like Axl, Gas6 is overexpressed in a number of human cancers including gastric [170], ovarian [171], breast [172], thyroid [173] and endometrial [150]. Interest has grown as to how Gas6 contributes to oncogenesis. A novel process has been discovered whereby tumors promote self-growth by educating infiltrating leukocytes to increase production of mitogenic Gas6 [174]. In human prostate cancer, Gas6 increases the expression of Axl, which is a regulator of metastasis [175].

1.11.4 Role of Gas6 in Disorders of the Vascular System

Following the discovery of Gas6 as the ligand for Axl [120], Gas6 was shown to have both mitogenic and anti-apoptotic effects in VSMCs [176, 177]. This finding fostered interest among vascular biologists as to what other effects Gas6 might have on blood vessel function. Gas6 was demonstrated as a chemoattractant for primary VSMCs [133]. Murao *et al.* then showed that Gas6 induces the expression of scavenger receptors, the molecules that bind modified LDL on VSMCs which implicated a role for Gas6 in atherogenesis [178]. Gas6 and Axl are expressed by endothelial cells [110]. Regarding endothelial cell adhesiveness, Gas6 has been demonstrated to inhibit the adhesion of granulocytes [179] but promotes interactions between platelets and endothelial cells [180]. Gas6 expression is widespread among hematopoietic cells in general, but it does not serve as a growth factor for these cells [181].

1.11.4.1 Atherosclerosis

Atherosclerosis can be described as a form of chronic inflammation that results from the interactions of lipoproteins, macrophages, T-cells and cellular elements in the arterial wall [182]. Gas6 promotes the activation of endothelial cells and leukocytes and promotes the migration of VSMCs. These properties can be classified as pro-atherogenic. It was first reported in 2008 that Gas6 is detectable in all stages of both human and murine atherosclerosis. Within the plaque itself, Gas6 is expressed by endothelium, smooth muscle and by macrophages. Studies involving genetically modified mice revealed that the absence of Gas6 does not affect the number or size of initial and advanced plaques in $ApoE^{-/-}$ mice. The genetic ablation of Gas6 (in $ApoE^{-/-}$ mice) did however, result in altered plaque composition. These plaques contained increased amounts of smooth muscle cells and collagen. In addition, lipid cores were smaller and macrophages were less numerous. These findings established that Gas6 promotes atherosclerotic plaque instability by decreasing plaque fibrosis and increasing plaque inflammation. [183]. Subsequent studies would go on to identify Axl expression as being downregulated in human carotid plaques [184]. In human atherosclerosis, Gas6 is mainly found in VSMCs and is secreted in human atherosclerotic plaques and reduces the expression of inflammatory molecules [185].

1.11.4.2 Erythropoiesis

Erythropoiesis, the process of red blood cell generation is dependent on the differentiation of hematopoietic stem cells into erythtoblasts which mature into circulating erythrocytes. Erythropoiesis is regulated by a number of different cytokines, but central to the process is erythropoietin (Epo). Epo is used in the treatment of anemia that occurs secondary to conditions such as cancer and rheumatoid arthritis. It is not always effective, with many patients being completely refractory to treatment, even at high doses of Epo. It was observed that the embryonic livers of $Gas6^{-/-}$ mice were underdevelopped at E13.5. Embryonic liver is a major site of erythropoiesis in the embryo, prompting an investigation to delineate the role of Gas6 in this process. $Gas6^{-/-}$ mice were at a survival disadvantage in a model of acute anemia. Gas6 was found to act synergistically with Epo in maintaining the hematocrit in models of acute anemia. This finding lends to the possibility of using Gas6 in anemic patients who fail to respond to Epo treatment [186].

1.12 The Role of Gas6 in Thrombosis

In 2001, a study was published that described a novel role for Gas6. Gas6 deficiency was shown to cause platelet dysfunction and protect mice from experimentally induced thrombosis. At the time, other than sharing homology with Protein S, there were no other observations serving as precedence for exploring the role of Gas6 in the pathogenesis of thrombosis. Gas6 remained the only protein with a Gla domain that had not been reported to play a role in either hemostasis or thrombosis. After all, Gas6 lacks

the thrombin sensitive loop present in Protein S and is not directly involved in the blood coagulation cascade as discussed above. The investigators found that Gas6$^{-/-}$ mice were protected from several models of thrombosis and importantly, did not present a bleeding phenotype. Protection from thrombosis was explained by a deficiency in platelet aggregation and secretion in response to known platelet activators [187]. The original research presented within this dissertation is based largely on the scientific findings from this publication and will therefore be discussed in detail.

1.12.1 Platelet Dysfunction in Gas6$^{-/-}$ mice

The Gas6$^{-/-}$ mice were generated by deleting a number of critical regions from the *gas6* gene, including the translation initiation codon, and the regions encoding the signal peptide and Gla-containing module. Regarding hemostasis, Gas6$^{-/-}$ mice were phenotypically normal and bled the same amount as WT litter mates following tail clipping. The investigators found no differences in the plasma levels of fibrinogen, prothrombin, factors V, VIII, IX or X. Additionally, Gas6$^{-/-}$ mice showed no significant differences in the prothrombin times (PT), activated partial thromboplastin (aPTT) times or numbers of peripheral blood cells (erythrocytes, leukocytes and platelets).

The investigators used a number of thrombosis models in their study including venous stasis induced by ligation of the IVC, photochemical denudation of the carotid artery and platelet dependent thromboembolism induced by intravenous injection of

collagen and epinephrine. Thrombus weight was significantly lower in both the stasis and photoinjury models. Injection of collagen and epinephrine induces fatal pulmonary thromboembolism. With this model, Gas6 deficiency reduced mortality. The loss of Gas6 protected these mice from the development of extensive pulmonary thromboembolism as was observed in the WT mice (assessed by histological analysis). Taken together, these results show that $Gas6^{-/-}$ mice do not develop thrombosis as rapidly or to the same degree as WT mice.

A more mechanistic approach was then taken to explain the prothrombotic effects of Gas6. The protection offered by Gas6 deficiency could not be attributed to an increase in thrombolysis. Since platelets are known to play essential roles in venous and arterial models of thrombosis, they sought to examine the role of Gas6 in platelet function. It is important to acknowledge the fact that platelet count and ultrastructure are normal in $Gas6^{-/-}$ mice. Platelet aggregation experiments revealed significant functional defects in platelets harvested from $Gas6^{-/-}$ mice. Platelets from WT mice successfully aggregated in a dose-dependent manner when challenged with known platelet agonists such as ADP, thrombin or collagen. Platelet aggregation studies such as these had been validated previously [188]. In contrast to WT platelets, $Gas6^{-/-}$ platelets failed to aggregate in response to low doses of ADP (5.0 μM). $Gas6^{-/-}$ platelets did however, undergo the characteristic shape change and successfully aggregated when challenged with higher concentrations of agonists (i.e. 50 μM ADP).

At this point, it can be argued that these observations merit further investigation. The fact that the Gas6$^{-/-}$ platelets failed to aggregate only at lower agonist concentrations of ADP is a rather subtle defect, especially when compared to the dramatic thrombotic phenotype observed in these mice (60-85% smaller thrombi). This subtle defect lends to the possibility that other mechanisms are in play and that Gas6 from some source other than platelets may be contributing to thrombus formation *in vivo*.

Challenge with thrombin successfully caused the aggregation of WT and Gas6$^{-/-}$ platelets. However, ultrastructural analysis revealed that aggregates from Gas6$^{-/-}$ platelets were not as tightly packed, made fewer contact sites and were incompletely degranulated. Degranulation results in fibrinogen release from α-granules and Gas6$^{-/-}$ platelets displayed lower levels of surface fibrinogen and P-selectin, another marker of α-granule secretion. Platelet degranulation also results in the secretion of ADP from dense granules, which is necessary for the formation of stable macro-aggregates. ADP secretion from Gas6$^{-/-}$ platelets, as assessed by measuring the release of ATP, was significantly lower with respect to WT levels. There is a tight correlation between platelet aggregation and secretion. Both of these processes are impaired in the absence of Gas6, which is necessary to amplify the effects of known agonists [187].

To further confirm these results, that the platelet dysfunction in Gas6$^{-/-}$ mice was indeed the direct result of Gas6 deficiency, a series of experiments were performed that introduced the use of recombinant murine Gas6 (rGas6). *In vitro*, rGas6 was able to restore the defective aggregation of Gas6$^{-/-}$ platelets in response to low doses of ADP.

Importantly, *in vivo* administration of rGas6 to $Gas6^{-/-}$ mice also rescued the defects in thrombosis. Neutralizing antibodies directed against Gas6 protected WT mice from fatal pulmonary thromboembolism induced by collagen/epinephrine, implicating the specific targeting of Gas6 for the treatment of thrombosis [187].

1.12.2 Role of Gas6 Receptors in Thrombus Stabilization

The importance of not only Gas6, but the Gas6 receptors in experimental murine thrombosis was detailed in a subsequent publication from the same laboratory. Here the authors generated a series of knock-out mice with single deletions of Axl, Sky or Mer (as well as a triple mutant). Having previously established that Gas6 promotes the aggregation and secretion of platelets in response to known agonists, they sought to explore the downstream events that occur following Gas6 binding to its receptors on platelets.

Consistent with the observation made in the $Gas6^{-/-}$ mice, those with a triple deficiency in Axl, Sky and Mer do not present abnormal hemostasis under baseline conditions [189]. Here, Angellio-Scherrer *et al.* observed the effect of Gas6 receptor deletion in pathological thrombosis. The three single receptor knock-out strains, $Axl^{-/-}$, $Sky^{-/-}$ and $Mer^{-/-}$ mice, did not suffer from spontaneous bleeding, a characteristic shared

by the Gas6$^{-/-}$ mice. However, in contrast to the Gas6$^{-/-}$ mice, mice in each of the single receptor knock-out groups were prone to repetitive rebleeding following periods of transient hemostasis. In addition, a tail clipping model revealed that Gas6 receptor knock-out mice lost a higher volume of blood overall than WT littermates. When challenged with stasis induced thrombosis in the IVC, the average thrombus weight generated in the receptor knock-out mice was reduced by an impressive 90% compared to WT. A model of platelet depletion and reconstitution was used to confirm that the thrombotic phenotype observed was indeed due to altered platelet function [190]. *In vitro* aggregation experiments with Axl$^{-/-}$, Sky$^{-/-}$ and Mer$^{-/-}$ platelets showed that they responded in similar fashion to Gas6$^{-/-}$ platelets, namely failing to irreversibly aggregate in responses to low concentrations of the agonists ADP, collagen and U46619. They did however, aggregate in response to higher doses of these agonists. Additional experiments were designed in order to understand exactly how, at the molecular level, the Gas6 receptors contributed to platelet activation.

One of the initial events of platelet activation is the initial binding of secreted fibrinogen to the αIIbβ3 integrin in a process that depends on inside-out signaling. The initial binding of fibrinogen to αIIbβ3 following activation was normal in Axl$^{-/-}$, Sky$^{-/-}$ and Mer$^{-/-}$ platelets and thus could not serve to explain the signaling defect. The binding of fibrinogen to αIIbβ3 actually serves to convert this integrin from a receptor with low-affinity and low-avidity to one of high-affinity and high avidity and this phenomenon was unaltered by single ablation of any of the Gas6 receptors. Following the initial fibrinogen binding event is the beginning of platelet spreading and this is where defects began to

emerge in $Axl^{-/-}$, $Sky^{-/-}$ and $Mer^{-/-}$ platelets. Receptor ablated platelets bound fibrinogen coated surfaces less efficiently than WT and they did not spread out as extensively. Importantly is the fact that the spreading was slower, not completely abrogated and eventually spread to levels expected of WT platelets. Platelets deficient in Sky did not secrete dense granules as efficiently as WT platelets. It was determined that the Gas6 receptors influence inside-out signaling via the αIIbβ3 integrin. Upon ligand binding, select tyrosine residues that make up the cytoplasmic tail of β3 become phosphorylated and serve as docking sites for downstream binding partners. Treating WT platelets with Gas6 induced phosphorylation at Y-773 of the β3 integrin. Phosphorylation at this residue did not occur in platelets lacking any one of the Gas6 receptors and this could not be attributed to decreased expression of β3 as assessed by FACS analysis. Therefore signaling from the Gas6 receptors determines the degree to which total cell β3 phosphorylation occurs in both resting and activated platelets and this in turn has an effect on inside-out signaling via αIIbβ3. Gas6 induces tyrosine phosphorylation of its three receptors, but it seems as though the receptors can influence phosphorylation of each other. The absence of Sky prevented the ligand induced phorphorylation of Axl and Mer. Axl or Mer deficiency produced a similar effect on the remaining two receptors, revealing that the three Gas6 receptors cooperate in mediating their activation. The ablation of one receptor also resulted in lower expression of the other two. Gas6 was already known to activate the PI3K/Akt pathway and it was important to demonstrate that this occurs in platelets. In their experiments, Gas6 successfully induced phosphorylation of P13K and Akt in WT platelets but not in receptor knock-out platelets. Each of the

Gas6 receptors is independently necessary for Gas6 mediated signaling and in regard to platelets, the PI3K/Akt pathway is involved in regulating the activity of αIIbβ3 [190].

The thrombotic phenotype of the $Gas6^{-/-}$ mice implicated the targeting of platelet derived Gas6 for antithrombotic therapy in humans [190]. In an initial investigation on Gas6 signalling in human platelets, an anti-Gas6 antibody prevented human platelet rich plasma (PRP) from aggregating in response to ADP and a selective PAR-1 agonist, thus supporting the argument for clinical targeting of platelet derived Gas6 [191]. Subsequent immunologic analyses of Gas6 in human plasma and platelets challenged this idea. While Gas6 was once successfully detected in human plasma in the low sub-nanomolar range, the same authors failed to detect expression of Gas6 by human platelets and proposed that that any potential platelet amplifying ability that Gas6 had in humans would be from Gas6 derived from the circulation [192]. Further challenging the idea was the finding that physiological levels of Gas6 in human plasma from healthy subjects do not influence platelet activation *ex vivo* [193]. The controversy surrounding the expression and function of Gas6 in human platelets and plasma, especially when compared to the dramatic protection from thrombosis associated with Gas6 ablation in mice suggests that the role of Gas6 in thrombosis is complex. In order for Gas6 to ever be realized as a target for antithrombotic therapy in humans, much additional characterization of the protein within the context of the disease is necessary. Gas6 expression from vascular derived cells is documented, as is the role of the vascular wall in thrombosis. We sought to explore the role of vascular specific Gas6 in the pathophysiology of venous thrombosis to further characterize the protein and its potential as a target for antithrombotic therapy. The

experimental details and results of our investigation will be discussed in detail in Chapter 2. This will be preceded by a discussion on the background behind the methodologies chosen for our study.

1.13 Methodology

1.13.1 Ferric Chloride ($FeCl_3$) Model of Venous Thrombosis

We hypothesized that Gas6 from a source other than platelets contributes to thrombus formation *in vivo*. Angellilo-Scherrer *et al.*[187], the authors who originally identified the prothrombotic role of Gas6 did not explore the possibility of a contribution from vascular derived Gas6 in thrombus formation. The objective of the present work was to begin to answer the question as to whether or not vascular specific Gas6 contributes to thrombus formation *in vivo*. The initial step was the establishment of a working mouse model of venous thrombosis in the laboratory. Angellilo-Scherrer *et al.* have already demonstrated the thrombotic phenotype of the $Gas6^{-/-}$ mice using the models mentioned above.

We were interested in the contribution to thrombus formation by vascular Gas6, which necessitated the establishment of a murine model that is known to involve the vasculature. The ferric chloride ($FeCl_3$) model of thrombosis has classically been used for studies in the arteries of small animals. A topical solution of $FeCl_3$ was initially identified as a potent initiator of thrombus formation in a rat arterial model by Kurz *et al.* in 1990

[194]. Prior to the use of this form of chemical injury, researchers were accustomed to thrombosis models that involved delivering an electric current to the vessel with stainless steel electrodes. This model of electrically induced vascular injury successfully caused development of occlusive thrombi. Interestingly, it was observed during the course of these experiments that iron-containing electrodes corroded quickly, necessitating a constant current generator to maintain a uniform current. The use of electrodes made of other elements such as platinum, copper or brass were ineffective in inducing thrombus formation. Taken together, these observations led to the hypothesis that iron is directly involved in the injury of the vessel [195] and is what prompted Kurz *et al.* to substitute an electric current with application of a solution of $FeCl_3$ [194]. The model of $FeCl_3$ induced vascular injury was further refined over time. The majority of studies where it was used involved concentrations of $FeCl_3$ ranging from 10-60% which is considered high and made it difficult to evaluate the effectiveness of potential antithrombotic agents. Dose dependent studies in the mouse carotid artery revealed that lower concentrations of $FeCl_3$ in the range of 2.5% were effective in creating occlusive arterial thrombi [196]. The following year, the same laboratory that optimized the $FeCl_3$ model in mice, published an article detailing the use of $FeCl_3$ for injury of the IVC in factor IX deficient mice [197]. The usefulness of the refined model of $FeCl_3$ venous injury was further exemplified by a study to evaluate the use of the potato carboxypeptidase inhibitor, a natural small molecular weight inhibitor of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) activity *in vivo* [198]. Using the protocol established by Wang *et al*., the $FeCl_3$ model of venous thrombosis was successfully established and routinely used in the laboratory for

use in the Gas6$^{-/-}$ mice (Fig. 1-12). With this model, we able to successfully reproduce the thrombotic phenotype of the Gas6$^{-/-}$ mice as will be discussed in Chapter 2.

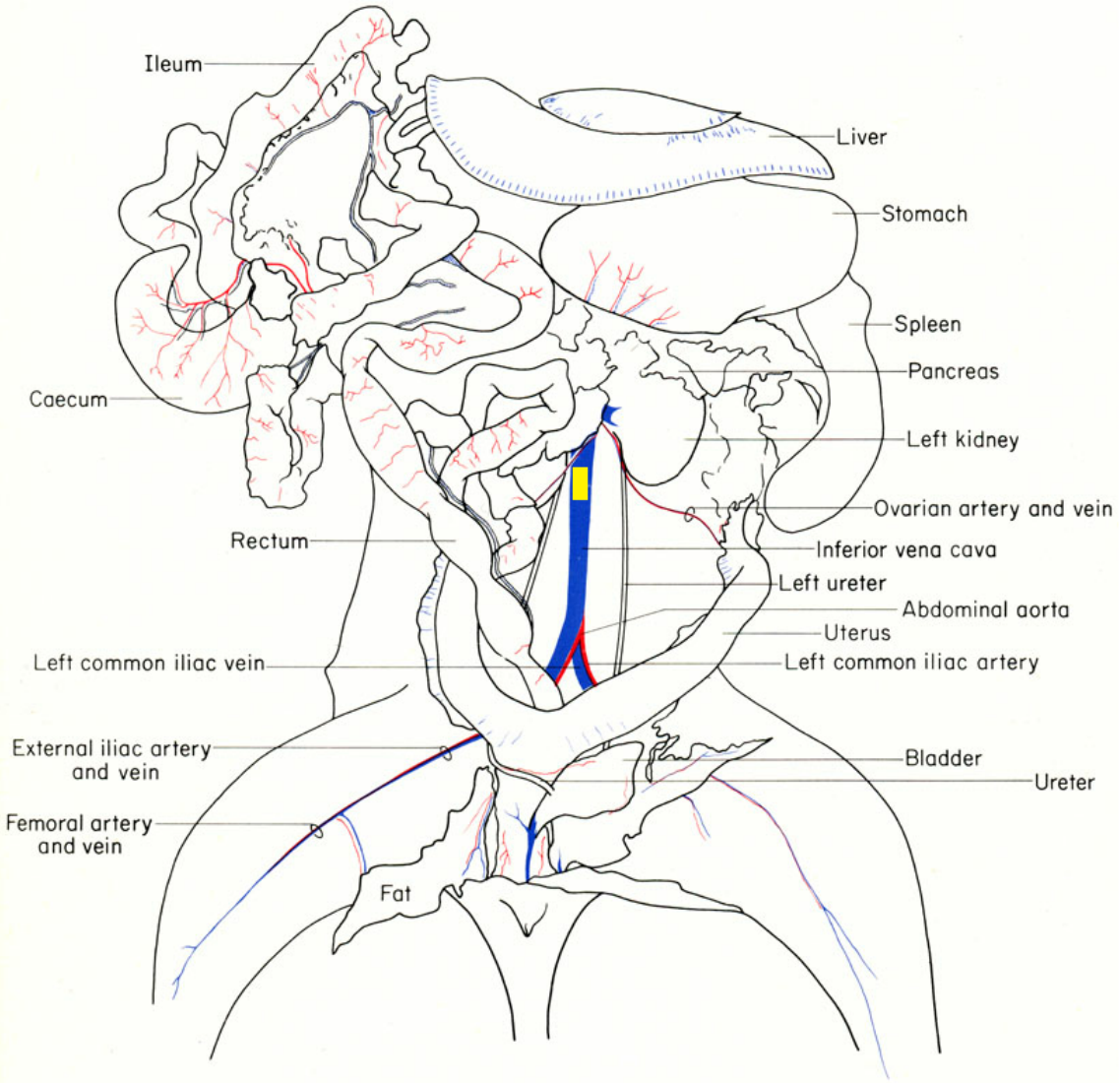


Figure 1-12. $FeCl_3$ model of venous thrombosis. Placed on the inferior vena cava is a small piece of Whatman filter paper saturated with a $FeCl_3$ solution (yellow). See Methods section in Chapter 2 for a detailed protocol (Cook, M.J., 1965).

The mechanism by which $FeCl_3$ causes endothelial injury, platelet aggregation and thrombus formation is thought to mimic a mechanical injury. This is because $FeCl_3$ causes endothelial cell denudation which exposes collagen and the subendothelium. The use of both genetically manipulated mice and pharmacologic agents have identified components of the coagulation cascade that participate in thrombus formation following injury by $FeCl_3$. For example, prolonged vessel occlusion times were reported in mice deficient in fIX, fXI and fXII, or in mice pretreated with a thrombin inhibitor, indicating roles for these coagulation enzymes in thrombus formation with this model. The involvement of platelets became apparent as important in mediating $FeCl_3$ induced thrombosis as mice deficient in platelet glycoproteins VI, β3, and P2Y1 had increased times to vessel occlusion. It has been observed that the platelet adhesion following application of $FeCl_3$ occurs more rapidly in venules than in arteries. It is likely that the diffusible ferric ions that mediate the damage [199] travel more rapidly through venule walls [196, 200]. TF derived from VSMCs is also critical to thrombus formation following injury by $FeCl_3$ [51].

A significant limitation to the use of $FeCl_3$ induced thrombosis is mice, is that the model is not necessarily reflective of heterogenous nature of thrombus development. It is accepted that murine hemostatic mechanisms differ in a vascular-bed specific manner. In addition, the injury to the vein wall produced by $FeCl_3$ injury is only reflective of a small number of deep vein thrombosis cases [105].

1.13.2 Models of Gas6 Chimerism

After successfully establishing a reproducible model of venous thrombosis by $FeCl_3$ injury, the next step was the establishment of experimental models that would allow for the separation of the contributions of vascular and hematopoietic derived Gas6 during thrombosis formation *in vivo*. We first employed a bone marrow transplantation (BMT) strategy to observe these individual contributions during thrombus formation. Gas6 is secreted by both cells of vascular and hematopoietic origin. In human tissues, Gas6 is detectable in bone marrow and endothelium [110]. To our advantage, Cui *et al.* published an article detailing the optimization of BMT protocols in mice. They performed a series of irradiation regimens in a variety of mouse strains. Certain strains of mice are radiosensitive and were not used as recipients in their experiments. Fortunately, radiosensitivity is not a concern when dealing with C57BL/6 mice. All WT and $Gas6^{-/-}$ mice used in our BMT experiments, as well as throughout the study are on a C57BL/6 background, eliminating a rather significant variable likely to influence survival rates. Using BMTs we were able to generate chimeric mice with selective ablations of Gas6 in the hematopoietic or vascular compartments.

In addition to the BMT model, we also chose a model of platelet depletion and reconstitution. Whereas, BMT experiments necessarily resulted in genetic manipulation of all cells of hematopoietic origin, the rendering of mice thrombocytopenic followed by re-infusion of platelet rich plasma (PRP) allowed us to examine the contributions of vascular Gas6 and platelet Gas6 to thrombus formation.

1.13.3 Tissue Factor Activity/ Endothelial Cell Activation

TF is the major cellular initiator of blood coagulation and is an important target molecule in our study. The rationale behind why TF was chosen as a downstream target of Gas6 signaling will be discussed in detail in the following chapter. A variety of methods to evaluate the expression of TF were used including immunofluorescence, qPCR and functional assays on both cells and whole tissue. As will be discussed in the following chapter, we have observed that Gas6 positively regulates TF, which is a marker of endothelial cell activation. We also begin to explore the possibility of the involvement of FoxO1 as a downstream mediator of Gas6 induced endothelial cell activation. We chose VCAM-1 as the marker of endothelial activation and these results will be discussed in Chapter 3

Chapter 2:

VASCULAR GAS6 CONTRIBUTES TO THROMBOGENESIS AND PROMOTES TISSUE FACTOR UPREGULATION FOLLOWING VESSEL INJURY IN MICE

2.1 Introduction

Venous thromboembolism (VTE) is a common cause of morbidity and mortality in clinical medicine, affecting 900,000 people per year in North America. The pathophysiology underlining VTE was first described by Virchow in 1853. Virchow's triad describes three mechanisms that account for VTE. VTE can be triggered by alterations in the levels of the circulating blood clotting factors (thrombophilia), changes in blood flow (i.e. stasis), and/or disruptions in the vascular wall [201]. Under normal physiological conditions the endothelial surface provides a thromboresistant surface due to the presence of various proteins, such as tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), and the endothelial cell protein C receptor (EPCR) [103]. However, physical (i.e. vascular damage) or functional (i.e. hypoxia) perturbation of the endothelium promotes thrombosis due to reduced expression of anticoagulants and the induction of the expression of the transmembrane procoagulant glycoprotein, tissue factor (TF) [202].

Gas6, the product of growth arrest specific gene 6 (*gas6*) is a member of the vitamin K-dependent family of proteins which includes blood coagulation factors II, VII, IX, and X and the anticoagulant factors, protein C and S, as well as protein Z [113]. Although Gas6 was discovered as sharing homology with protein S, it plays no role in the generation of fibrin and its role *in vivo* remains incompletely characterized [120, 203].

Originally identified in fibroblasts, Gas6 is expressed in various cell types, including endothelial cells [110], smooth muscle [129], and bone marrow cells [204]. Gas6 is a ligand for the TAM family of receptor tyrosine kinases, which include Axl, Sky (Tyro3) and Mer [205]. Gas6 has been shown to perform a wide range of biological functions that is reflected by a broad tissue expression profile. For example, Gas6 is found in plasma [140] as well as in hepatic [206], renal [207] and neural tissues [144, 156]. Gas6 and its receptors modify platelet activation and aggregation [187, 190], but the role of Gas6 in the interplay between platelets and other cell types, such as endothelial cells remains unclear. *In vivo*, Gas6 deficient mice are protected from lethal thromboembolism suggesting a prothrombotic role for Gas6 [187]. The phenotype is the result of a defect in platelet signalling with thrombus instability as a consequence. Interestingly these mice did not present a bleeding phenotype. Platelets from Gas6 deficient (-/-) mice showed defective platelet signalling when challenged with 5.0 μM ADP but not to other platelet agonists such as thrombin or collagen nor even to higher concentrations of ADP [187]. This subtle platelet defect suggests the existence of a discrepancy that could be explained by other mechanisms such as the contribution of

Gas6 from the vasculature to thrombus formation. Thus, we hypothesize that Gas6 from the vascular wall has a role in the pathophysiology of venous thrombosis.

2.2 Materials and Methods

Reagents

Human blood factors VIIa, X, Xa, Protein C, thrombin and antithrombin were from Haematologic Technologies Inc. Chromogenic substrates S-2765 and S-2236 were from diaPharma. FAST SYBR Green Master Mix and ribosomal RNA control reagents were from Applied Biosystems. Recombinant Taq DNA Polymerase was supplied by Invitrogen. RNeasy Mini kit and Quantitect Reverse Transcription Kit were from Qiagen. Custom primers for murine Gas6 PCR and tissue factor primers (human and murine) for qPCR were from Invitrogen and Applied Biosystems respectively. 18s RNA control reagents for qPCR were from Applied Biosystems as well. Proteinase K was from Fermentas. DMEM, DMEM/F12, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), penicillin/streptomycin and trypsin-EDTA were from Wisent. EBM and EGM-2 were from Clonetics. Purified anti-mouse CD102 (ICAM-2) antibody was from BD Pharmingen. Mouse monoclonal anti-TF antibody was from SantaCruz. The secondary antibodies, anti-sheep Alexa Fluor, anti-mouse 555- were from Molecular Probes. DAPI mounting medium was from Vector. RBC lysis buffer was from Sigma. Dynal M-450 Sheep anti-Rat beads, Dynal magnetic holder and phenol chloroform isoamyl mixture

were from Invitrogen. Collagenase A was from Roche. Finally, Rabbit anti-mouse thrombocyte serum was from Accurate Chemical and Scientific Corporation.

Mice

All experiments performed on mice were approved by the Animal Care Committee of McGill University. The $Gas6^{-/-}$ mice (on a C57BL/6 background) were donated by Dr. Peter Carmeliet (Laboratory of Angiogenesis and Neurovascular Link, Vesalius Research Center, Leuven, Belgium). The colony was maintained by the continuous crossing of heterozygous ($Gas6^{+/-}$) mice. It has been observed that male mice lacking all three Gas6 receptors do not produce sperm due to the progressive death of germ cells. This is attributed to the fact that the physiological expression of Axl, Sky and Mer by Sertoli cells is required for the trophic nurturing of germ cells. Although it has never been explored, it is possible that this finding explains why using $Gas6^{-/-}$ males for our breeding colony was inefficient [189]. The mice used in all experiments were male and aged between 8 and 12 weeks. Genotyping was performed by PCR amplification of the *Gas6* gene from total genomic DNA prepared by phenol-chloroform extraction of ear punch samples. The nucleotide sequences for the custom *Gas6* primers are as follows:

Gas6 sense: 5'-GAGTGCCGTGATTCTGGTC- 3',

Gas6 antisense: 5'-CCACTAAGGAAACAATAACTG- 3' and

Gas6 "new": 5'-ATCTCTCGTGGGATCATT – 3'

Thermal cycling was performed on a Perkin Elmer GeneAmp PCR System (2400). The thermal profile was as follows: 2 minutes at 94ºC, 35 cycles of: 94ºC for 30 sec, 57ºC for 30 sec and 72ºC for 30 sec followed by a final incubation at 72ºC for 10 minutes. PCR amplified DNA was resolved on ethidium bromide stained 1% Agarose gels (Invitrogen). A single band that migrates at 500 base pairs (bp) corresponds to a WT mouse, while a single band of 350 bp corresponded to a $Gas6^{-/-}$ mouse. In heterozygous mice, both bands are present.

Venous Thrombosis Model

The ferric chloride ($FeCl_3$) model of venous thrombosis was used as previously described [196, 198, 208]. Mice were anesthetised by inhalational isoflurane and placed in the supine position on a heating pad set to 37°C. Following a midline laparotomy, the intestines were exteriorized and placed to the left of the animal. A piece of gauze soaked in physiological saline was placed over the intestines in order to prevent desiccation. A section of IVC between the renal and left common illiac veins was cleared and separated from the adjacent section of aorta by dissection. A piece of Whatman filter paper (2.0 X 4.0 mm) was presaturated in a solution of 0.37M $FeCl_3$ in water and placed on the surface of the IVC such that the top of the filter paper was directly below the renal vein. The filter paper remained on the adventitial surface of the IVC for 3 minutes and was then removed and thrombus formation was allowed to proceed. 30 minutes following the initial application of filter paper, the thrombus alone or the entire section of IVC was removed for downstream processing. When the thrombus was to be weighed, it was

dissected out directly from the IVC in the mouse, and blotted dry before weighing. In most cases after the recording of weight, thrombi were placed in 200 µl of 100 mM TRIS buffer (pH 7.5) supplemented with 400 µg proteinase K for overnight digestion at 50ºC. The following morning, total thrombus protein content was measured by reading the optical densities of solutions at 280 nm [208]. Blank readings were taken as the TRIS buffer and proteinase K alone.

Bone Marrow Transplantation (BMT) Model

A BMT protocol has previously been optimized to maximize both animal survival and ablation of recipient marrow in C57BL/6 mice [209]. All recipient mice received a total body radiation dose of 12.0 Gy (2 doses of 6.0 Gy with a 4 hour interval). Recipient mice were anesthetised by intraperitoneal (i.p) injection of rodent cocktail (ketamine, xylazine, acepromazine) at a dose of 300 µl/25 g prior to each exposure. Following each exposure, mice were kept under a heating lamp for roughly 3 hours and were given i.p 1.0 ml injections of warm saline to aid recovery. Recipient mice were infused with donor bone marrow cells (BMCs) 24 hours following the second exposure. The isolation of BMCs was performed under sterile conditions. Donor mice were euthanized for harvesting of femurs and tibias. These bones were placed in DMEM supplemented with 10% FBS and antibiotics (penicillin/streptomycin) in a Petri dish on ice. The bones were cut at each end and flushed repeatedly (minimum 15 times) with a DMEM filled insulin syringe. The flushed marrow suspensions were filtered through a 100 µm cell strainer to remove cell clumps, pieces of bone and debris and collected in 50 ml falcon tubes and

centrifuged at 1500 rpm for 5 minutes. Erythrocytes were removed by resuspending the pellets in 2.0 ml of red blood cell lysis buffer as per the manufacturer's instructions. The cell pellets were washed 3 times in serum free DMEM and reconstituted such that each 300 µl i.v injection would contain 4 X 10^6 BMCs. Recipient mice were housed in sterile cages, with antibiotic containing food and allowed 1 month for recovery and hematologic reconstitution. Success of marrow uptake was determined by Gas6 PCR from DNA isolated from ear punch samples and peripheral blood cells [210] before and after transplantation.

Platelet Depletion/Reconstitution Model

To deplete endogenous platelets, mice were injected with rabbit anti-mouse thrombocyte serum (10 µL) intraperitoneally. Platelet depletion was allowed to proceed for 4 hours, following which a few microliters of blood was drawn from the saphenous vein of the animal and collected in EDTA primed Microvettes (Sarstedt). To confirm successful depletion of platelets, blood samples were analyzed on a Vet abc animal blood counter (Vet Novations). The average platelet count of mice under baseline conditions is established [211]. Platelet depletion was considered successful if the total level of circulating platelets decreased below 140,000 platelets per microliter of blood according to the manufacturer of the blood counter. Mice were then re-infused with platelets collected from a donor mouse. For preparation of plasma rich platelet (PRP), blood was collected by cardiac puncture in syringes primed with collection buffer (38 mM citric acid, 75 mM trisodium citrate, 100 mM dextrose) from a donor mouse. Approximately

1.0 mL of blood was collected from each donor mouse. The blood was then diluted in one volume of wash buffer (150 mM NaCl, 20 mM PIPES, pH 6.5) and centrifuged for 7 minutes at 60 g. The PRP was collected and centrifuged for an additional 10 minutes at 70 g. The supernatant was removed and platelets were resuspended in Walsh's buffer (137 mM NaCl, 20 mM PIPES, 5.6 mM dextrose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4, pH 7.4). The entire amount of PRP from a donor mouse was injected into a single recipient via tail vein injection. 10 minutes following the re-infusion of platelets, blood was once again collected via saphenous puncture (opposite leg) and analyzed by the blood counter. If the new circulating platelet count successfully rose to levels considered normal (140-600 X 10^3 platelets per microliter of blood) we proceeded to challenge the mice with $FeCl_3$.

Immunofluorescence microscopy

The venous wall with thrombi were removed, fixed in 4% paraformaldehyde for 4 hours and incubated in PBS +30% sucrose overnight at 4°C. Samples were embedded vertically in OTC compound (Tissue-tek, Sakura), and serial 5µm frozen sections were cut using a cryostat and transferred onto gelatin-coated slides. Nonspecific binding sites on the tissues sections were blocked with 10% BSA for 30 minutes at room temperature. Thereafter, sheep anti-mouse von Willebrand factor (Abcam) followed by an anti-sheep Alexa Fluor 568-nm (Molecular Probes) secondary antibody and rabbit anti-human

fibrinogen-FITC (Dako) were used to visualize thrombi in the inferior vena cava. Goat anti-mouse CD31, anti-mouse α−smooth muscle actin coupled to Cy3 (Dako) or mouse anti-mouse TF (Santa Cruz) antibodies were applied, followed by appropriate secondary antibodies, Alexa Fluor anti-goat 488- and anti-mouse 405-nm (Molecular Probes). Images were acquired with a Leica DM 2000 fluorescent microscope and the Infinity Capture software. Quantifications of tissue factor positive staining were done using ImageJ (NIH) by delimiting the signal between luminal side of the endothelial layer and the outer side of the smooth muscle cell layer.

Endothelial Cell Isolation

Although several methods for the isolation of murine lung endothelial cells (MLEC) have been described in the literature [212], the protocol we used is as follows. Mice were euthanized by cervical dislocation and the lungs were harvested and placed in RPMI containing antibiotics (penicillin/streptomycin). Under sterile conditions the lungs were cut with scissors into the smallest possible pieces and incubated in RPMI + 0.1% collagenase A for 1 hour at 37°C. The digested tissue suspensions were then passed through a 16G syringe 15 times and filtered through 100 μm cell strainers before centrifugation at 1000 rpm for 5 minutes. The supernatant was removed and the cell

pellet was resuspended in 10 ml of culture media (50% EGM-2/50% DMEM/F12 + 20% FBS + P/S) and plated in 75 cm^2 flasks precoated with 0.1 % gelatine. 24 hours after the initial plating of cells, the media was changed after white blood cells were displaced by firmly knocking on the flask. Cells were allowed to grow for 2-4 days until endothelial cell like colonies appeared.

To begin the immunomagnetic selection, the magnetic beads are washed three times (10 µl of beads/flask in 1.0 ml PBS+2% FBS) by vortexing and resuspending. To resuspend the beads, the 15 ml tube was placed in the magnetic holder and allowed to incubate for 1 minute for the beads to collect at the magnet. PBS was carefully removed for a total of three washes. The washed beads were resuspended in 160 µl PBS+2% FBS per 10 µl beads. Then, 10 µl anti-mouse CD102 (ICAM-2) antibody/ 10 µl beads was added to the suspension. The beads + antibody mixture was placed on a rocking platform for 2 hours at 4ºC. The beads were similarly washed and resuspended in 5.0 ml of culture media per 10 µl of beads and added to the flask of cells for incubation at 4ºC for 1 hour. On selection day 1, the cells were trypsinized and passed through the magnetic holder twice. The cells were allowed to incubate and reach confluence once again before being subjected to the day 2 selection, where they were passed through the magnetic holder but once. At this point the cells were considered passage 3 and were used for experiments. Purity of the cultured cells was assessed by morphology.

Tissue Factor Activity Assay on Endothelial Cell Monolayers

The following protocol was used as described in [213]. Cells were seeded in 24-well, gelatine (0.1%) coated plates at a density of 1.0 X 10^6 cells per ml of media (50% EGM-2/50% DMEM/F12). 24 hours following the seeding of cells, the monolayers were washed twice in wash buffer (10.0 mM HEPES, 0.15 M NaCl, 4.0 mM KCl, 11.0 mM dextrose, pH 7.5) and then overlayed with 250 µl of assay buffer (10.0 mM HEPES, 0.15 M NaCl, 4.0 mM KCl, 11.0 mM dextrose, 5.0 mM $CaCl_2$, 1.0 mg/ml BSA, pH 7.5) supplemented with 10.0 nM fVIIa. The cell monolayers were placed at 37°C for 5 minutes to allow TF:fVIIa complex formation. 175 nM fX was then added to each well and incubated at 37°C for 1 hour. The reaction was stopped by placing a 25 µl aliquot of the reaction mixture into 50 µl of stop buffer (TBS containing 1 mg/ml BSA and 10.0 mM EDTA). 50 µl of the stopped reaction mixture was mixed with 50 µl of chromogenic substrate S-2765 (1.7 mM). The plate was immediately placed in a microplate reader. The reaction was monitored by a 15 minute kinetic assay at 405 nm. The final rate of substrate cleavage values (V_{max}) were converted to the amount of fXa generated per minutes with reference to a standard curve constructed from serial dilutions of a stock solution of 1.0 µg/ml fXa (18).

Quantitative RT-PCR

Endothelial cells were seeded in 100 mm dishes and grown to confluence in 50% EGM-2/50% DMEM/F12 + 20% FBS. Cells were washed twice with PBS and incubated

overnight in low serum free media (EBM). They were then challenged with 1.5U/ml human thrombin for 4 hours. RNA was harvested using an RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions with a DNA removal step. Total RNA was quantified using a ND-1000 Nanodrop spectrophotometer (Nanadrop). 1.0 ng of total RNA was reverse transcribed with a QuantiTect Reverse Transcription kit (Qiagen) as per the manufacturer's instructions. Finally, 2.0 μl of a 1:80 dilution of cDNA was mixed with appropriate primers and SYBR green master mix. Thermal cycling was performed with a 7500 Fast Real Time PCR System from Applied Biosystems. The thermal profile was as follows: Holding Stage: 95°C for 20 seconds, Cycling stage: 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Amplification of TF mRNA was normalized to levels of endogenous 18s rRNA (Applied Biosystems). The primers sequences for murine TF are as follows:

mTF Forward: 5'-CTCCTCCTCCAGGTGATCG-3' and

mTF Reverse: 5'-GGGTTGCCACTCCAAAATTG-3' [214]

Data was calculated using the ΔCt method.

TF activity assay on IVC containing thrombus

This assay has been described in detail for use on mouse carotid artery homogenates [51, 215] and was adapted for use in IVC samples. Following $FeCl_3$ injury as described above, the IVC containing thrombus (between the renal and left common

illiac veins), was removed and immediately frozen in liquid-N_2 until processing. For homogenization, the liquid-N_2 frozen IVC samples were placed in Teflon capsules with a pestle (both liquid-N_2 chilled) and ground to power by placing the capsule in a Vari-Mix III homogenizer (Caulk/Dentsply) set to maximum speed for 20 seconds. Tissue powder was collected and resuspended in 200 µl of 50 mM TRIS, pH 8.0. The samples were diluted 1:3 in TRIS and placed on ice until assayed. In 96-well plates, 30 µl of dilute homogenate was mixed with 90 µl of TRIS buffered saline containing 1 mg/ml BSA, 3.0 nM human fVIIa, 100 nM human fX, 8.3 mM $CaCl_2$ and 0.33 mM S-2765). The reaction was allowed to proceed for 45 minutes at 37ºC. The reaction was stopped by the addition of 50 µl of 50% acetic acid and changes in optical density (relative to a blank sample) at 405 nm were measured in a micro plate reader (SpectraMax Plus 384, Molecular Devices). The endpoint OD-405 values were converted to amount of TF in solution with a standard curve constructed from serial dilutions of a solution of lipidated recombinant TF (American Diagnostica).

Activated Protein C (APC) Assay

Endothelial cells were isolated and grown as described above. Cells were seeded in 0.1% gelatine coated 6-well culture plates and grown to confluence, at which point they were washed twice with Hank's Balanced salt solution (HBSS) supplemented with 3.0 mM $CaCl_2$, 0.6 mM $MgCl_2$ and 0.5% human serum albumin. The washed monolayers were then overlayed with HBSS containing 100 nM protein C and 10.0 nM thrombin. Plates were incubated for 30 minutes at 37˚C. The reaction was stopped by the addition

of antithrombin (AT) (100 nM final concentration) and EDTA (0.4 M final). It was necessary to add AT to the reaction because thrombin is capable of cleaving the chromogenic substrate S-2236 and would produce false positive signals when trying to measure APC levels alone. The plates were incubated at room temperature on a gentle shaker for 10 min. 50 µl of the stopped reaction mixture was placed in wells of 96-well plate. To each well, 50 µl of a solution of S-2366 in ECA buffer (20.0 mM HEPES, 150 mM NaCl, 5.0 mM $CaCl_2$, pH 7.5) was added and the reaction was read in kinetic mode at 405 nm for 5 minutes. Standard curves were made from serial dilutions of a solution of APC in HBSS [216].

Statistical Analysis

Data are presented as mean ± SEM. Differences within groups were assessed by one-way analysis of variance followed by a *post-hoc* Student Newman-Keuls test. A value of $p < 0.05$ was considered statistically significant.

2.3 Results

$Gas6^{-/-}$ mice are protected against 0.37M $FeCl_3$ injury to the IVC.

The authors that originally described the role of Gas6 in thrombus formation used 3 different models to demonstrate the $Gas6^{-/-}$ phonotype. These models included ligation

of the IVC, photochemical denudation of the carotid artery and intravenous injection of collagen and epinephrine [187]. We opted to use a thrombosis model that had previously been unused in studying the $Gas6^{-/-}$ mice. We chose the model of 0.37% $FeCl_3$ injury in the IVC for our experiments because here thrombus formation is dependent on contributions from both vascular and hematopoietic derived factors [200]. Again, a major objective of this study was to discern the individual contributions of Gas6 from each of these compartments. It also seemed necessary to determine whether the same phenotype would be observed, namely if $Gas6^{-/-}$ mice were protected against injury by 0.37M $FeCl_3$. Following vessel injury by 0.37M $FeCl_3$, $Gas6^{-/-}$ mice show an average 69% reduction in thrombus weight with respect to WT mice (Fig 2-1). This value correlates strongly with the data obtained by Angelillo-Scherrer *et al*. [187]. WT mice subjected to injury by 0.37M $FeCl_3$ developed thrombi with a weighted average of 2.9 ± 0.5 mg while thrombi produced in $Gas6^{-/-}$ mice weighed only 0.9 ± 0.3 mg ($n=7$, $p<0.05$). This data demonstrates another useful and reproducible thrombosis model which can be used to study thrombosis in the $Gas6^{-/-}$ mice. It effectively allowed us to reproduce the published phenotype that $Gas6^{-/-}$ are protected against experimentally induced venous thrombosis and allowed us to pursue the experiments that follow.

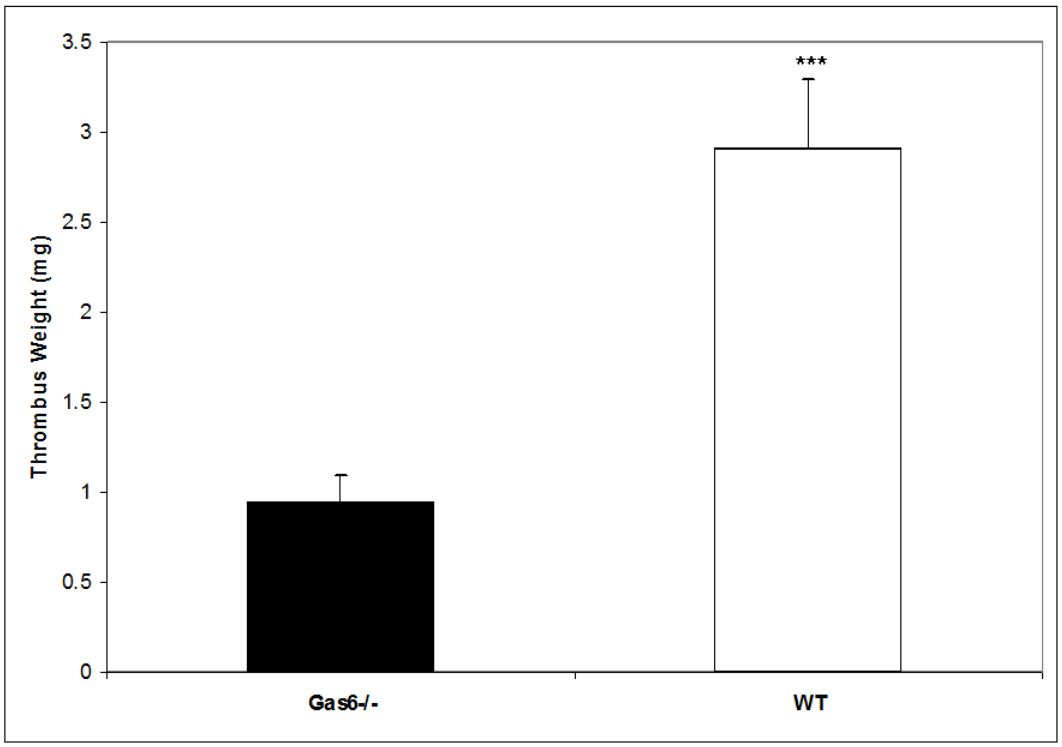


Figure 2-1. The Thrombotic Phenotype of Gas6$^{-/-}$ mice. Gas6$^{-/-}$ mice are protected against thrombosis in the IVC following injury by 0.37M $FeCl_3$.

WT mice with a selective ablation of Gas6 in the hematopoietic cell compartment develop thrombi of intermediate weight.

In order to assess if Gas6 expressed by vascular cells contributed to thrombus formation separately from the contributions of hematopoietic Gas6, we used a BMT approach. For BMT experiments, donor mice refer to those which were sacrificed for the purpose of bone marrow cell isolation from the femurs and tibias, while recipient mice were those which were subjected to irradiation as described in the methods section. Four

groups of mice were generated for this series of experiments. There were two control groups: WT donor and WT recipient (WT →WT) and $Gas6^{-/-}$ donor and $Gas6^{-/-}$ recipient ($Gas6^{-/-}$→ $Gas6^{-/-}$). Following the one month recovery period, post-irradiation, all surviving mice appeared healthy and weighed roughly the same as they did pre-irradiation. Animal survival rates per experiment ranged between 70-75%. The two experimental groups included $Gas6^{-/-}$ donor and WT recipient ($Gas6^{-/-}$→WT) and WT donor and $Gas6^{-/-}$ recipient (WT→ $Gas6^{-/-}$). The control groups WT→WT and $Gas6^{-/-}$→ $Gas6^{-/-}$ were used to verify any possible effects that the BMT protocol might have on thrombus formation following injury with 0.37M $FeCl_3$. It would appear that BMT did have an overall affect on thrombus weight as thrombi produced in irradiated control group mice were on average, smaller than those produced in WT or $Gas6^{-/-}$ mice that had not undergone BMT (compare Figures 1 and 2a). $Gas6^{-/-}$→ $Gas6^{-/-}$ mice produced clots weighing 0.317 ± 0.08 mg, while WT→WT mice produced clots weighing 2.483 ± 0.392mg. Despite the average thrombus weights being somewhat lower following BMT, the differences in weight between WT→WT and $Gas6^{-/-}$→ $Gas6^{-/-}$ thrombi remained statistically significant ($n=8$, $p<0.05$).

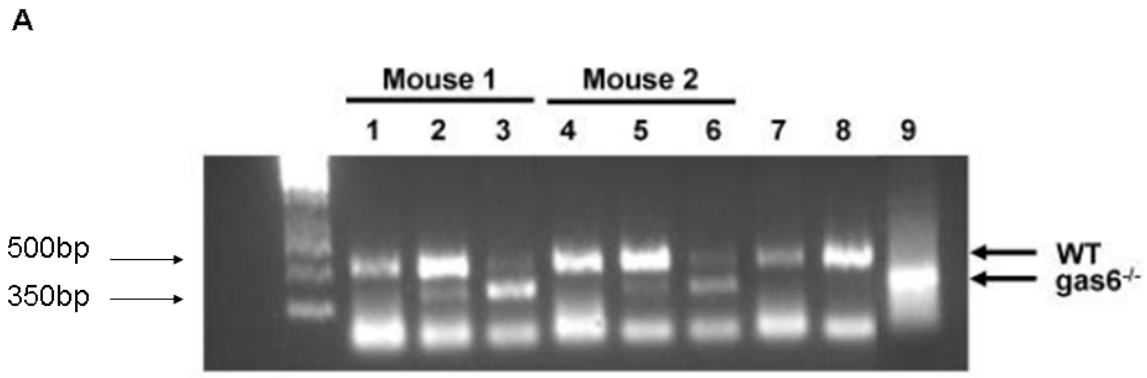
Thrombi developed in $Gas6^{-/-}$→WT mice following $FeCl_3$ injury weighed less (1.057 ± 0.105 mg) than those in WT→WT mice ($n=8$, $p<0.05$), and more than those from $Gas6^{-/-}$→ $Gas6^{-/-}$ mice ($n=8$, $p<0.05$) (Fig 2-2B). The partial reduction in thrombus weight in this experimental group indicated partial protection from $FeCl_3$ injury. In addition, the data lent to the possibility that Gas6 from a source other than the myeloid compartment (i.e. platelets) is involved in thrombus formation. We hypothesized that the

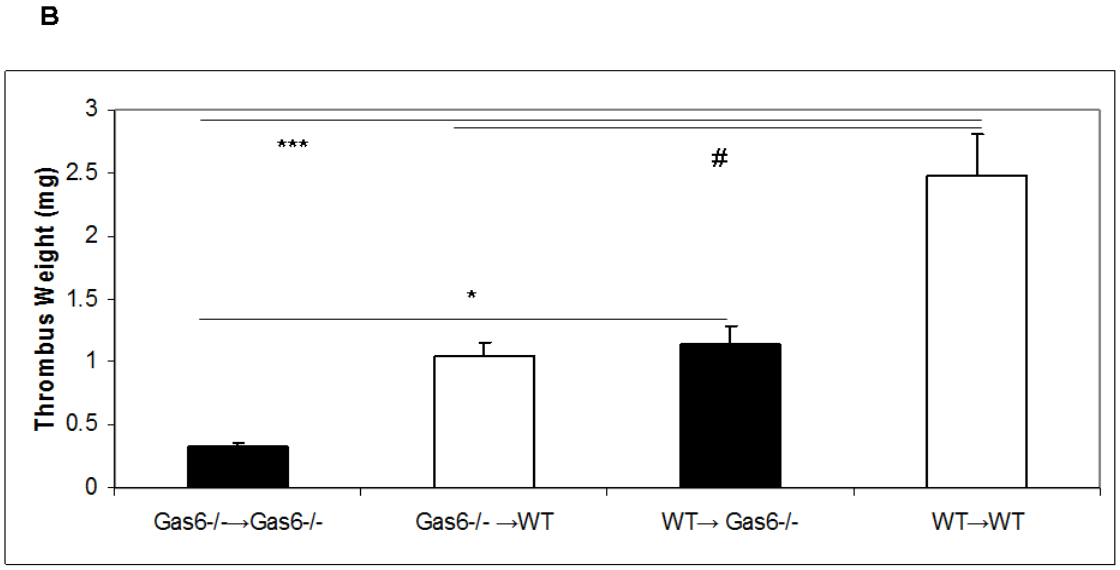
venous vasculature represents a potential source of this Gas6. The second experimental group, WT→ Gas6$^{-/-}$ mice produced thrombi weighing 1.13 ± 0.178 mg, which was roughly equal to those produced in Gas6$^{-/-}$→WT mice suggesting an approximately equal contribution to thrombus formation from myeloid derived and vascular derived Gas6.

To confirm the trend in thrombus weight observed in the BMT experiments, the weighed thrombi were placed in a TRIS buffer containing 400 µg of proteinase K and incubated overnight in a water bath set to 50ºC in order to determine the total thrombus protein content. The following morning the samples were briefly centrifuged and the optical densities (280 nm) of solutions (normalized to buffer with proteinase K alone) were measured. The average OD-280 from Gas6$^{-/-}$→ Gas6$^{-/-}$ thrombi was 0.652 ± 0.12 and those from WT→WT thrombi measured 1.5 ± 0.127. The differences in OD between these control groups is statistically significant ($n=8$, $p<0.05$). In addition, ODs from Gas6$^{-/-}$→WT thrombi (0.929 ± 0.0748) were statistically smaller than WT→WT ($n=8$, $p<0.05$) but not statistically bigger than clots from Gas6$^{-/-}$→ Gas6$^{-/-}$ mice ($n=8$, $p<0.1$) (Fig 2-2C). The OD values from experimental group WT→ Gas6$^{-/-}$ measured 1.11 ± 0.04. This value was statistically significant compared to Gas6$^{-/-}$ →Gas6$^{-/-}$, but not to WT→ WT.

The cumulative data from BMT experiments suggest that the contribution by Gas6 to thrombus formation comes from both myeloid and vascular derived sources. To verify the success of bone marrow cell uptake in each transplanted animal, DNA was extracted from both ear punch samples and blood (saphenous vein) before and after

BMT. A representative experiment is shown in Figure 2-2A. Comparing lanes 2 and 3 shows a strong Gas6$^{-/-}$ signal in the blood (lane 3) of a WT recipient post-irradiation (lane 2).





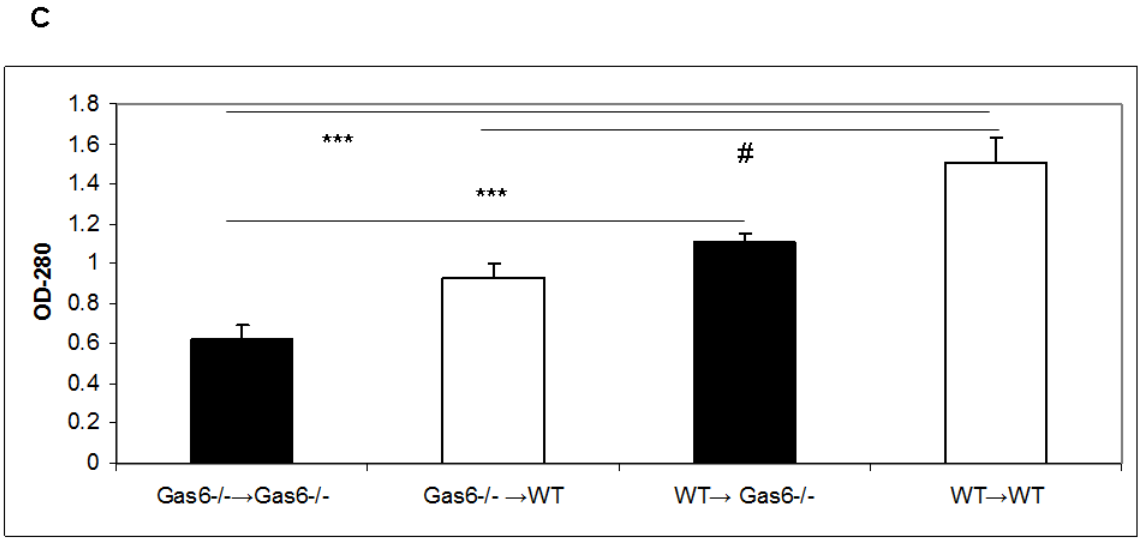


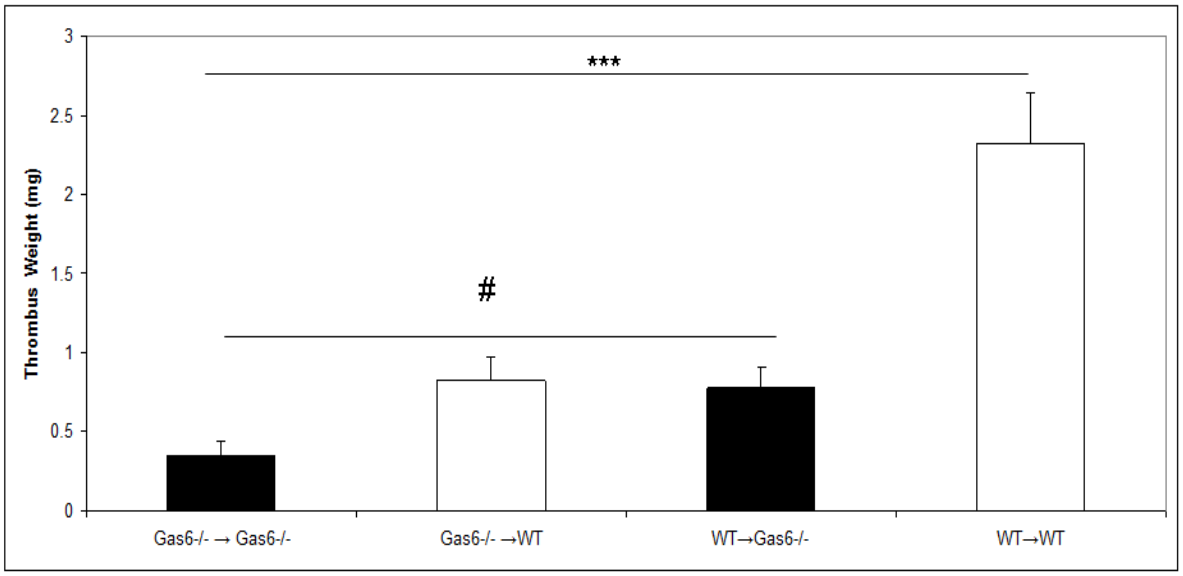
Figure 2-2. Bone Marrow Transplantation Experiments. (A) Representative agarose gel showing genotyping of blood and ear tissue before and after BMT. **(B)** Mice with a selective ablation of Gas6 in the hematopoietic or vascular compartment develop thrombi of intermediate weight following injury by $FeCl_3$. **(C)** Total protein content of thrombi confirms the trend observed in thrombus weight.

The Contribution of Platelet and Vascular Derived Gas6 to Thrombus Formation in vivo

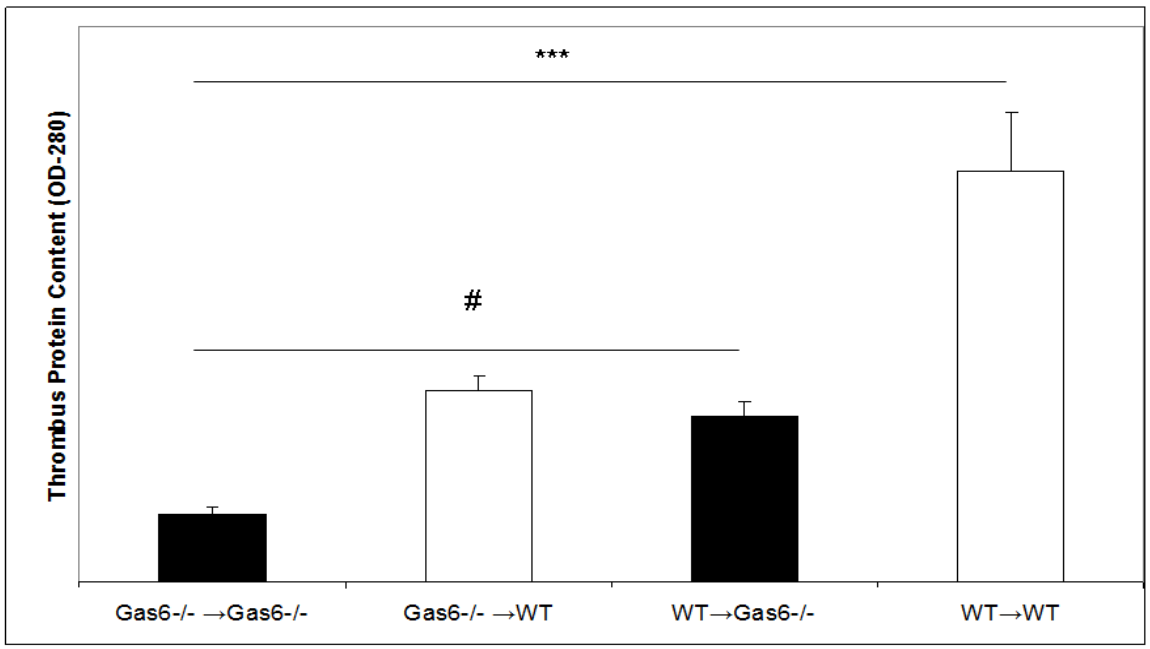
Our BMT experiments yielded results that offer the possibility that the thrombotic phenotype of the Gas6$^{-/-}$ mice is the result of more than a defect in platelet signalling, which is the only existing scientific evidence to explain this phenomenon thus far [187]. We therefore sought to evaluate the contributions of vascular versus of platelet derived Gas6 in thrombus formation, which in contrast to the BMT experiments described above involved contributions by all bone marrow derived cells. To address this issue, we utilized a platelet depletion/reconstitution model to examine thrombus formation *in vivo* in different cohorts of mice (WT→WT, Gas6$^{-/-}$→WT, WT→Gas6$^{-/-}$ and Gas6$^{-/-}$→Gas6$^{-/-}$) that were challenged with 0.37 M $FeCl_3$ in the IVC. Here we use the same donor → recipient notation but a donor mouse in these experiments is one sacrificed for collection of PRP, while a recipient mouse is one that underwent platelet depletion and re-infusion. WT and Gas6$^{-/-}$ mice were successfully rendered thrombocytopenic by injection of a rabbit anti-mouse thrombocyte serum and reconstituted with donor PRP as described in methods. After induction of thrombosis in the IVC, thrombus weight was evaluated in the 4 groups of mice. We found that chimeric mice (WT mice with Gas6$^{-/-}$ platelets or Gas6$^{-/-}$ mice with WT platelets) developed thrombi of intermediate size (0.82 ± 0.15 mg or 0.78 ± .13 mg, respectively) when compared to the control WT→WT (2.35 ± .32 mg) and Gas6$^{-/-}$→Gas6$^{-/-}$ mice (0.35 ± 0.09 mg). Gas6$^{-/-}$→WT and WT→Gas6$^{-/-}$ produced significantly smaller thrombi than WT→WT (n=4-5, $p<0.05$) (Figure 2-3A).

As with our BMT experiments, the thrombi produced in mice that had undergone platelet depletion and reconstitution were digested overnight for measurement of total thrombus protein content. These data are confirmatory and display the trend observed in thrombus weight. OD-280 from thrombi generated in $Gas6^{-/-} \rightarrow Gas6^{-/-}$ mice was 0.145 ± 0.0175 which was significantly lower than the values from WT→WT which was 0.88 ± 0.128 ($n=4$, $p<0.05$). As expected, the OD-280 values produced from the thrombi of chimeric mice were intermediate to those of the controls. OD-280 values for $Gas6^{-/-} \rightarrow$WT thrombi were 0.41 ± 0.034 and for WT→$Gas6^{-/-}$, the values were 0.36 ± 0.032, a significant increase from $Gas6^{-/-} \rightarrow Gas6^{-/-}$ samples ($p<0.05$, $n=4$-5) (Fig.2-3B).

A


Thrombus Weight (mg)
3
2.5
2
1.5
1
0.5
0
***
#
Gas6-/- → Gas6-/-
Gas6-/- →WT
WT→Gas6-/-
WT→WT

B


Thrombus Protein Content (OD-280)
***
#
Gas6-/- →Gas6-/-
Gas6-/- →WT
WT→Gas6-/-
WT→WT

C

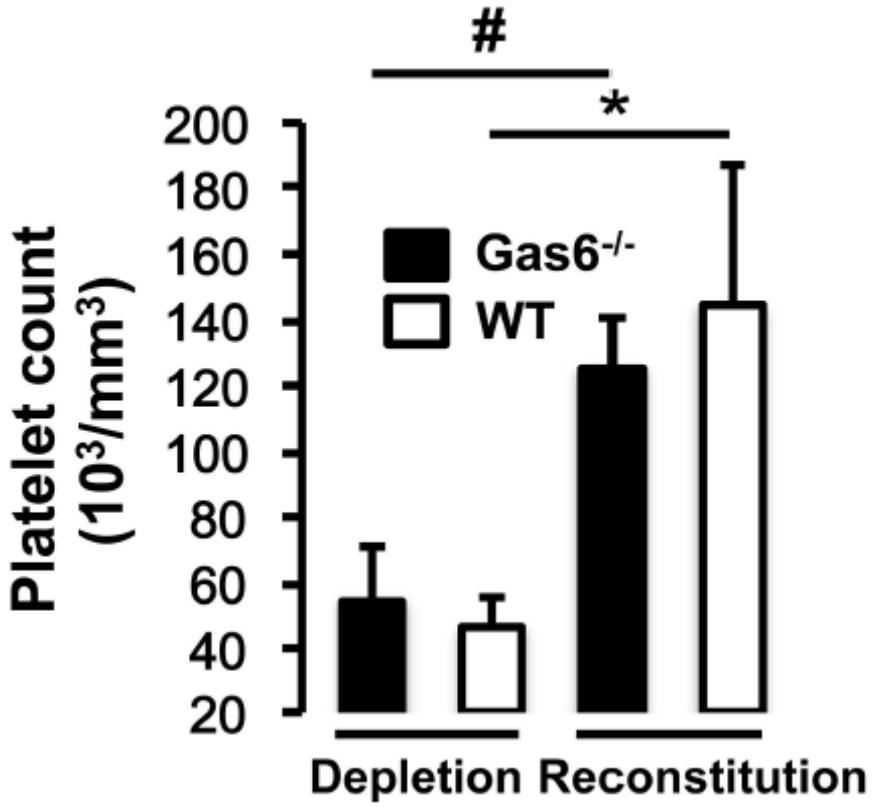


Figure 2-3. Platelet Depletion and Reconstitution Experiments. (A) Thrombus weight in mice that have undergone platelet depletion/reconstitution. Chimeric mice produced thrombi of intermediate size with respect to either control group following $FeCl_3$ injury. **(B)** Thrombus protein content confirms the trend observed by thrombus weight. **(C)** Platelet count in WT (white) and $Gas6^{-/-}$ (black) mice depleted following injection of the anti-thrombocyte serum. Following reinfusion with PRP platelet levels rose again to physiological levels.

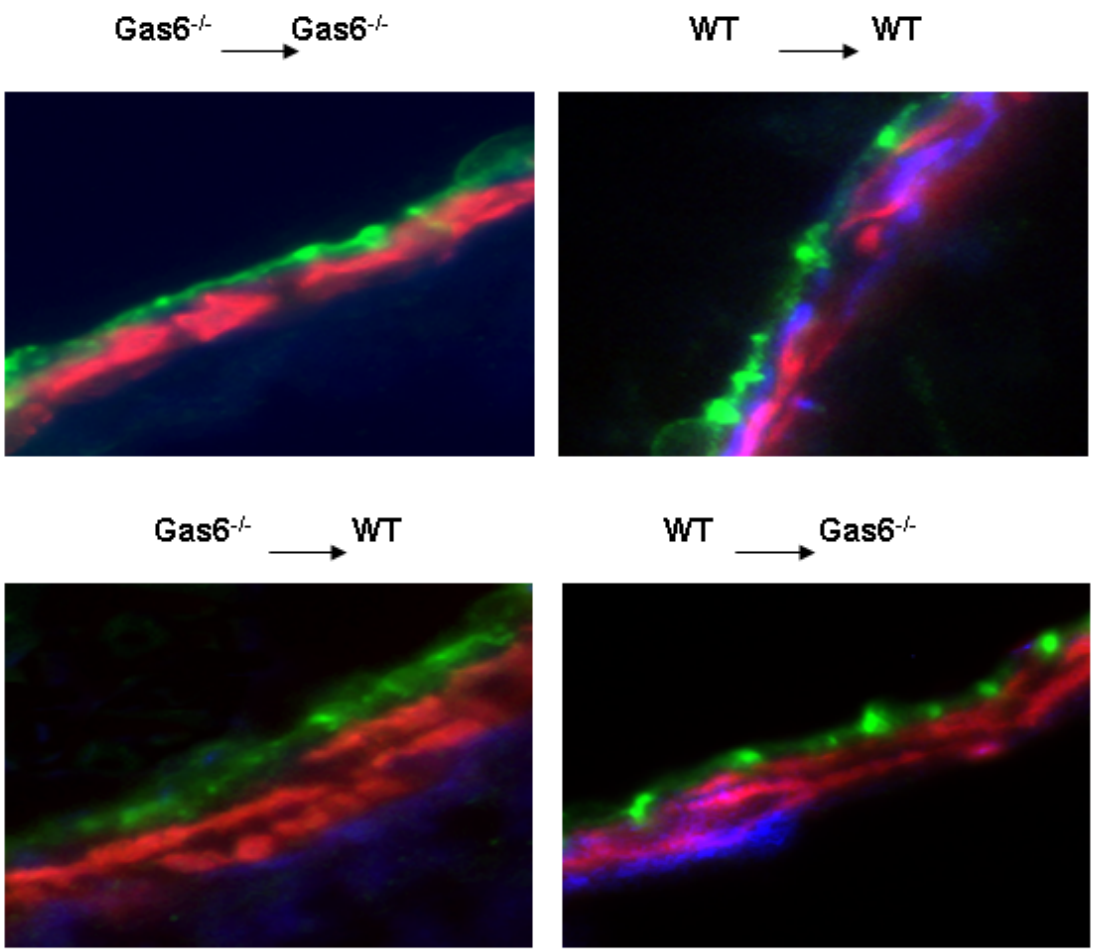
Vascular TF upregulation is blunted in $Gas6^{-/-}$ mice

Up to this point, we have described by two individual models that Gas6 from the vascular wall contributes to thrombogenesis. To understand possible molecular mechanisms that underlie this phenomenon, we began by observing the effect of vascular Gas6 on TF expression. We hypothesized that vascular Gas6, being prothrombotic, would

promote TF upregulation in the vessel wall thereby contributing to thrombogenesis. We observed TF expression in the inferior vena cava (IVC) by immunofluorescence microscopy and by a specific functional assay. Under baseline conditions the endothelium from the IVCs of WT and Gas6$^{-/-}$ mice display nearly undetectable levels of TF. When the vessels were injured with 0.37M $FeCl_3$ (following platelet depletion and reconstitution), TF is robustly upregulated on the WT vasculature. The same cannot be said in Gas6$^{-/-}$ mice, where TF induction was blunted (Figure 2-4A). Endothelial cells in cross-section were located by an antibody against PECAM-1, a known marker of the cell type [217].

To assess the functional pool of TF in the IVC following $FeCl_3$ injury, sections of IVC between the renal and illiac veins were removed, immediately placed in liquid N_2 and stored until ready to assay, when they were homogenized as described in the methods section. IVC homogenates were tested for TF activity by a specific functional assay. The OD values obtained were converted to pM TF by a standard curve constructed by serial dilutions of a solution of a lipidated recombinant TF. Here we show that Gas6$^{-/-}$ homogenates of the IVC contain significantly less TF (33.8 ± 2.74 pM TF) than those from WT mice (61.9 ± 6.04 pM TF) (Fig 2-4B).

A



TF + CD31 (PECAM-1) + αSMA

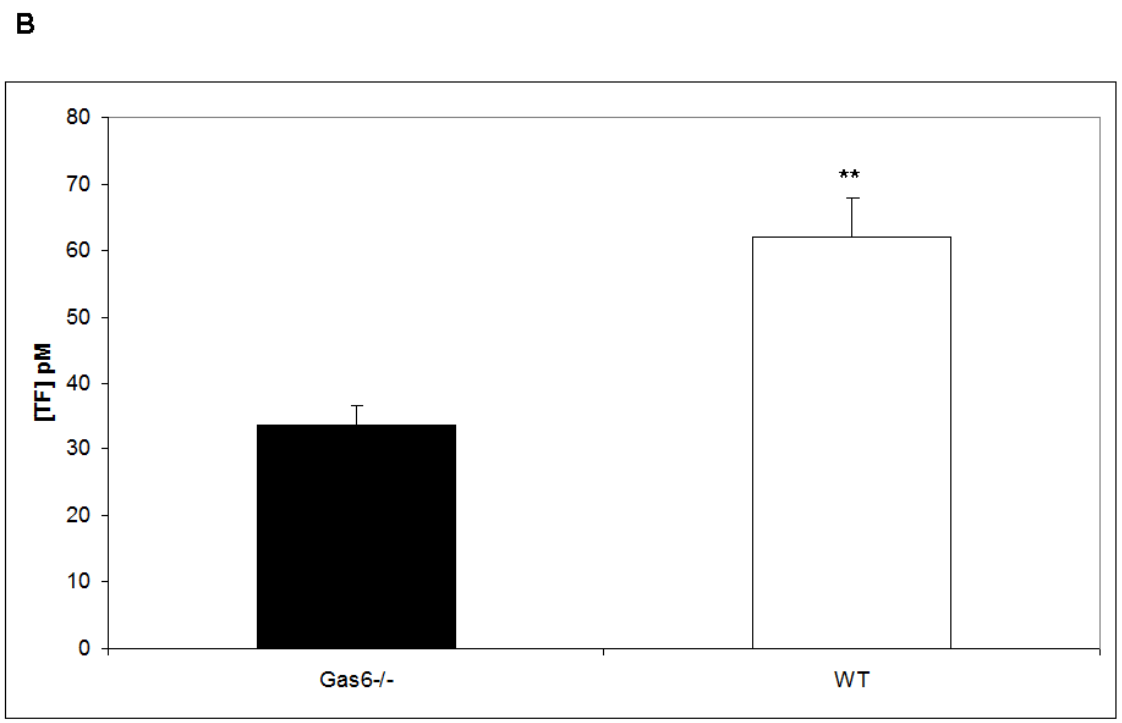


Figure 2-4. TF induction in the IVC during thrombosis. (A) TF antigen is expressed on vascular cells following injury by 0.37 M $FeCl_3$. Following platelet depletion and reconstitution, TF induction is blunted in the IVC of $Gas6^{-/-}$→$Gas6^{-/-}$ mice. Tissue sections were triple stained for TF (blue), PECAM-1 (green) and α-smooth muscle actin (red) **(B)** Homogenates of IVC tissue from $Gas6^{-/-}$ mice contain a significantly lower pool of active TF with respect to WT samples following injury by 0.37 M $FeCl_3$.

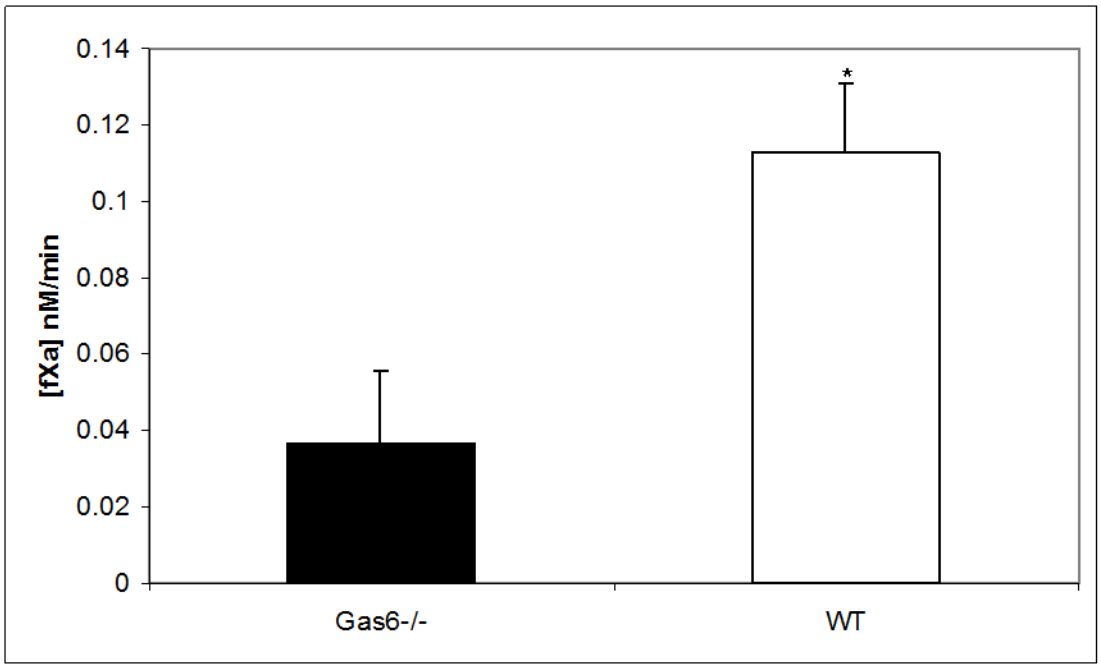
$Gas6^{-/-}$ endothelial cells display reduced levels of surface tissue factor activity in vitro.

As supporting evidence for our results obtained *in vivo*, we sought to evaluate the ability of $Gas6^{-/-}$ endothelial cells to support TF expression and activity *in vitro*. The following experiments were performed on endothelial cells harvested from murine lung tissue. Murine lung endothelial cells (MLEC) were isolated as described in the methods section. To assess the active pool of surface TF on MLEC, WT and $Gas6^{-/-}$ cells were

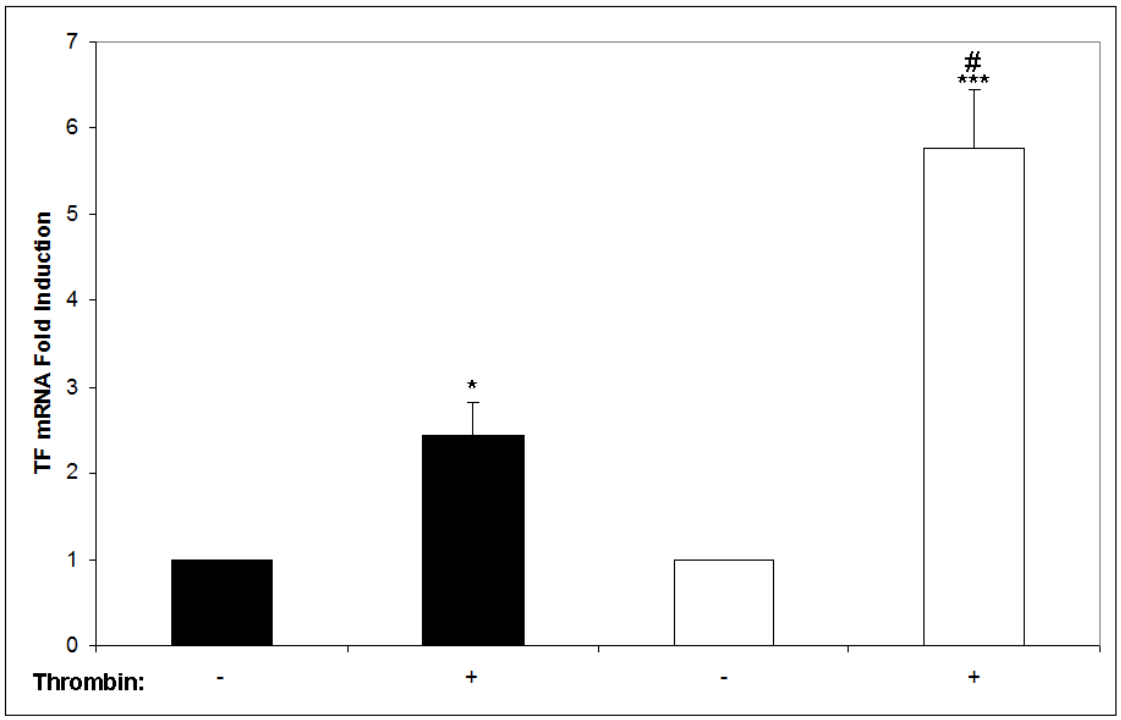
plated at equal densities (1 X 10^6 cells/ml) in 24-well plates. 24 hours after plating, the cells were washed and assayed as described in methods. WT MLEC generated 0.9 ± 1.0 nM fXa/min while Gas6$^{-/-}$ MLEC supported significantly less, 0.047±0.8 nM fXa/min (n=5 $P<0.05$) (Fig. 2-5A).

TF mRNA levels were measured following a 4hr incubation with 1.5U/ml human thrombin. In WT MLEC challenged with human thrombin, TF mRNA was upreguled 5.8 ± 0.66 fold while in Gas6$^{-/-}$ MLEC upregulation was reduced 2.4 ± 0.38 fold (Fig. 2-5B).

A


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
[fXa] nM/min
*
Gas6-/-
WT

B


7
6
5
4
3
2
1
0
TF mRNA Fold Induction
*
#
***
Thrombin:
-
+
-
+

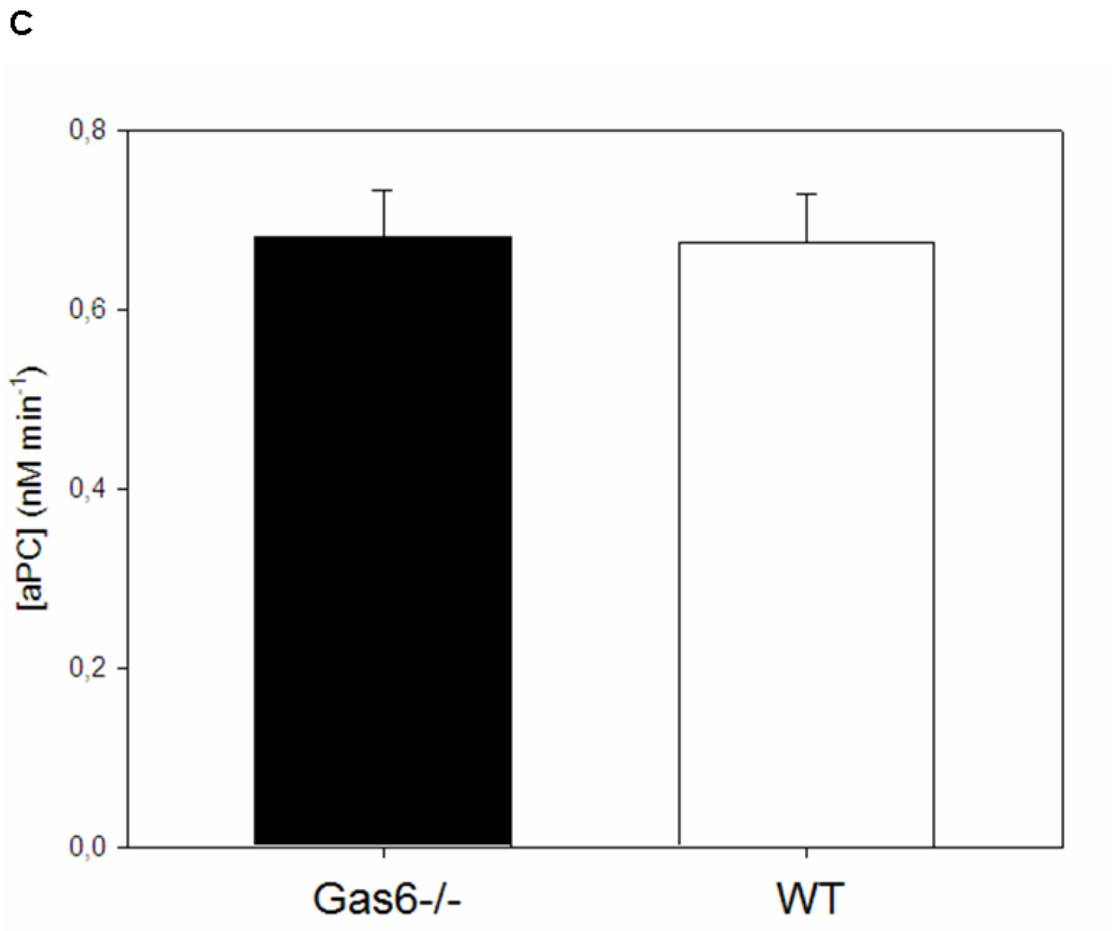


Figure 2-5. ***In vitro*** **assessment of TF and TM activity. (A)** Monolayers of Gas6$^{-/-}$ endothelial cells support significantly less TF activity. **(B)** TF mRNA induction by thrombin is blunted in Gas6$^{-/-}$ endothelial cells. **(C)** Activated Protein C assays shows that levels of surface TM are unaffected by the ablation of Gas6.

Resting Levels of Endothelial Thrombomodulin are Unaffected by Gas6 Ablation

The ablation of Gas6 from MLEC clearly had an effect on the ability of these cells to support TF expression and activity. We also sought to verify whether surface levels of thrombomodulin (TM) were different in the two cell strains. As TM is an established antithrombotic protein, we hypothesized that endothelial ablation of the

prothrombotic Gas6 would increase surface TM levels, in contrast to TF. When plated at the same cell density, WT and Gas6$^{-/-}$ MLEC displayed identical levels of APC activity (n=3, p=NS) (Fig 2-5C).

Recombinant Human Gas6 blunts Thrombin Mediated Upregulation of TF in Human Endothelial Cells

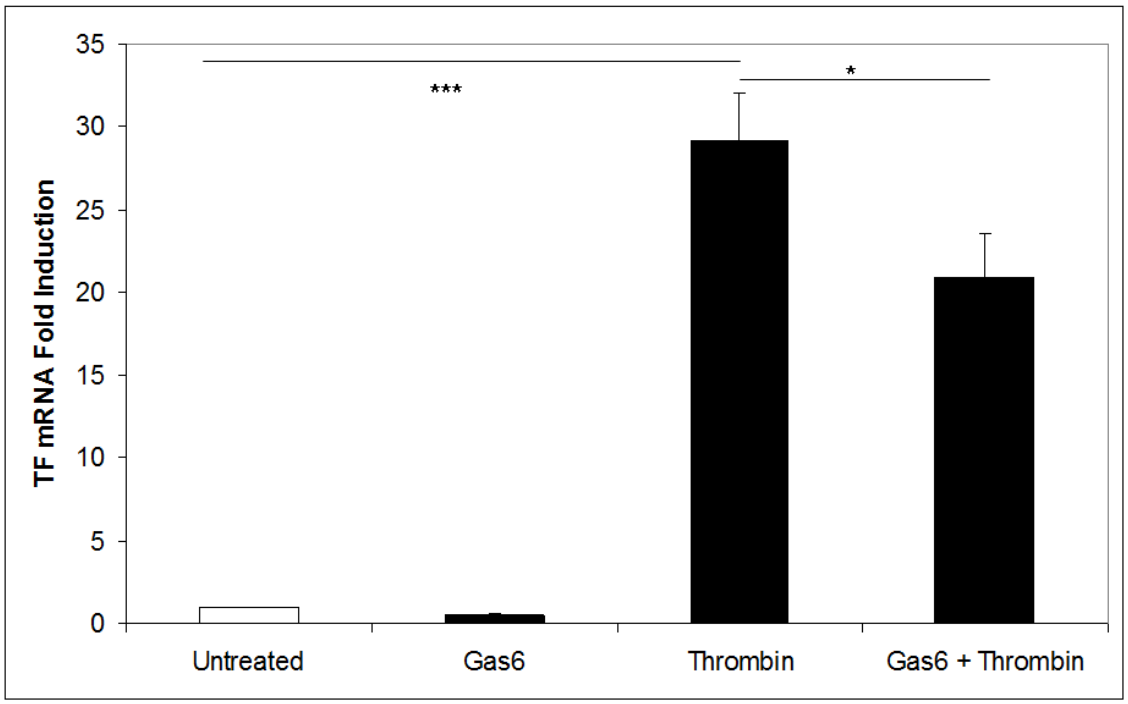
Thrombin (fIIa) induces TF mRNA, antigen and activity in human endothelial cells, an observation made in 1985 by Galdal *et al.* Human Umbilical Vein Endothelial Cells (HUVECs) supported a 2.5 fold induction of TF activity following a 4 hour incubation with human thrombin [218]. As discussed in Chapter 1 within the context of Virchow's triad and the involvement of the vascular wall in the initiation of thrombotic events, endothelial cells are extremely sensitive to their microenvironment. In one study, it was observed that the preincubation of HUVECs with different mediators alters the "set-point" or response capacity to thrombin of these cells. The investigators incubated HUVECs in the presence of various mediators associated with sepsis such as TNF-α and LPS. When the cells were primed under sepsis like conditions, the ability of a subsequent dose of thrombin to induce TF expression was blunted [219]. Given these observations, we hypothesized that Gas6 could also potentially modulate thrombin mediated TF induction. We hypothesized that Gas6 would amplify the response to thrombin, which is along the lines of Gas6 amplifying the response of known agonists in platelet signalling [187]. Although not a significant consideration during the performance of these

experiments, it is interesting to note that elevated plasma Gas6, like TNF-α and LPS, is also associated with sepsis [220].

Here, HUVECs were grown on gelatine-coated plates until confluence. At this point, complete growth medium (EGM-2) was removed, cells were washed twice with PBS and placed in low serum media (EBM-2 + 0.5% FBS) for 16 hours. The cells were then treated in the absence or presence of 100 ng/ml recombinant human Gas6 and in the absence or presence of 1.5 U/ml thrombin. In HUVECs treated with thrombin alone for 2 hours, TF mRNA was induced 29.2 ± 2.9 fold. When HUVECs were first incubated in the presence of 100 ng/ml Gas6 for 15 minutes, washed and then treated with thrombin, the effect of thrombin was blunted, inducing TF mRNA by 20.9 ± 2.6 ($p<0.05$) (Fig. 2-6A). The fact that Gas6 blunted thrombin's ability to induce TF mRNA expression is inconsistent with our murine data, both *in vivo* and *in vitro* which shows that Gas6 positively contributes to TF expression in endothelial cells. The results obtained here are more along the lines of those obtained when HUVECs were incubated with the sepsis associated mediators [219]. This phenomenon could in part be explained by the opposing effects of Gas6 and thrombin on Akt phosphorylation. It is known that Gas6 induces rapid phosphorylation of Akt in HUVECs [111]. Thrombin on the other hand has been observed to inhibit Akt phosphorylation by activation of PKCδ in endothelial cells [221]. Suppression of TF expression has been linked to increased Akt phosphorylation [222] and this might explain the effect of an acute treatment with Gas6 on thrombin mediated TF induction.

These results obtained by qPCR were validated with a TF functional assay as described in Methods. Untreated HUVECs or those treated with Gas6 alone supported very little generation of factor Xa (0.0045 ± 0.0008 nM/min factor Xa for untreated versus 0.006 ± 0.003 nM/min factor Xa for Gas6 treated). As expected, when HUVECs were treated with 1.5 U/ml thrombin for 5.5 hours, this induced a robust induction of TF activity (0.043 ± 0.0076 nM/min factor Xa) which corresponds to an approximate 10-fold induction over the untreated cells. Interestingly, despite the fact that Gas6 treatment alone induced a minor increase in TF activity, when HUVECs were treated with Gas6 for 15 minutes prior to thrombin challenge, the induction of TF activity was blunted (0.026 ± 0.007 nM/min fXa). These experiments involved an acute treatment of Gas6 (15 minutes), prior to thrombin stimulation. A series of similar experiments were also performed whereby the cells were allowed to incubate in the presence of Gas6 for 16 hours (not shown). The results were the same. Even a prolonged exposure to Gas6, blunted the capacity of thrombin to incuce TF expression in HUVECs. The use of exogenously added Gas6 to HUVECs represented an alternative approach to explain the prothrombotic effect of Gas6 on endothelial cells (i.e. TF induction). It should be noted that this approach is different from the experiments done on MLEC from WT and $Gas6^{-/-}$ mice. This approach or the use of human versus murine cells lines could explain the discrepancy in the results.

A


TF mRNA Fold Induction
35
30
25
20
15
10
5
0
***
*
Untreated
Gas6
Thrombin
Gas6 + Thrombin

B

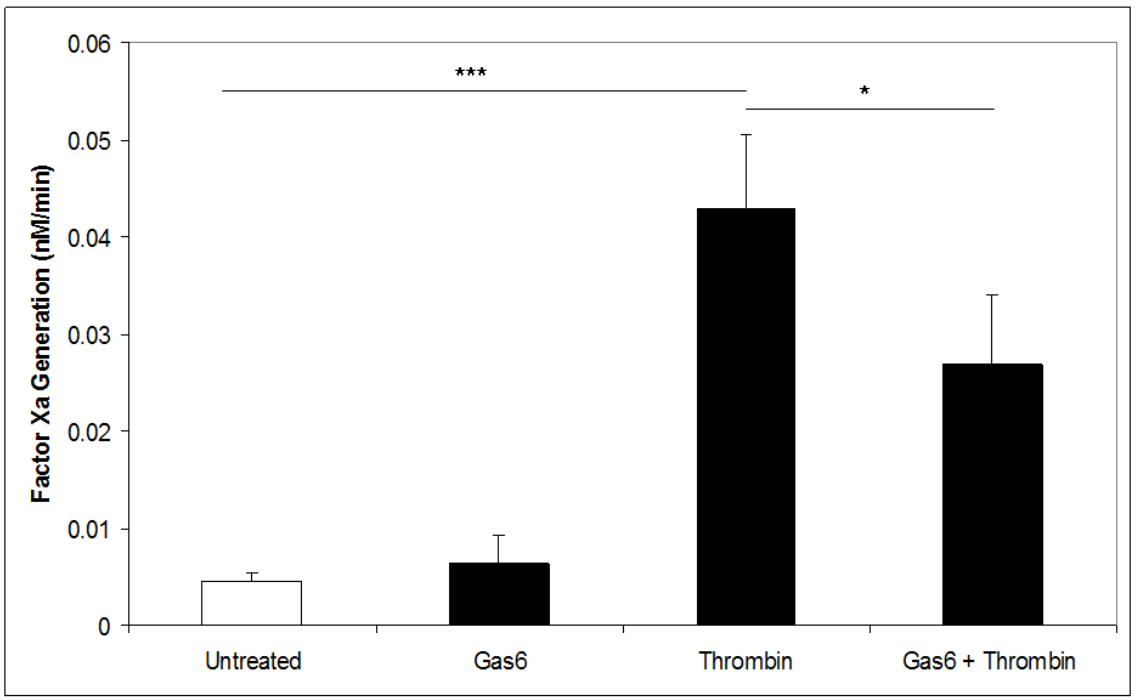

Factor Xa Generation (nM/min)
0.06
0.05
0.04
0.03
0.02
0.01
0
***
*
Untreated
Gas6
Thrombin
Gas6 + Thrombin

Figure 2-6. Preconditioning of HUVECs with Gas6 blunts thrombin mediated TF induction. (A) HUVECs pre-treated with Gas6 for 15 minutes before thrombin challenge were hyporesponsive to TF mRNA induction. **(B)** Gas6 also blunts thrombin mediated upregulation of TF activity.

2.4 Discussion

Venous thromboembolism is a leading cause of morbidity and mortality in the Western world [105]. The disease risks include, but are not limited to obesity, sepsis, pregnancy, cancer and chemotherapy [223]. In 2001, the mechanisms underlying a novel role for the vitamin K-dependent protein Gas6 in thrombus formation were published. $Gas6^{-/-}$ mice were shown to be protected from experimentally induced thrombosis. The observed phenotype was explained by a defect in platelet aggregation and secretion. Additionally, hemostasis is normal in these mice [187]. A subsequent study by the same group examined the roles of the Gas6 receptors, Axl, Sky and Mer in platelet aggregation. Mice with functional losses of any one of these receptors were phenotypically similar to the $Gas6^{-/-}$ mouse in terms of thrombogenesis [190]. Taken together, these observations implicate the possibility of targeting Gas6 for antithrombotic therapy. Careful examination of the data obtained by Angellio-Scherrer *et al.* in the 2001 publication reveals what is arguably a discrepancy between the thrombotic phenotype of the $Gas6^{-/-}$ mice and the platelet defect observed to explain said phenotype. The $Gas6^{-/-}$ mice were challenged with various models of thrombosis and were protected against all of these interventions. The authors also observed, following a series of *in vitro*

experiments, that platelets derived from Gas6$^{-/-}$ mice are defective in the ability to completely aggregate and secrete in response to known platelet agonists. Importantly however, is the fact that Gas6$^{-/-}$ platelets only failed to aggregate in response to one agonist, ADP, and at only the concentration of 5.0 μM. It is this specific observation that lends to the possibility that other mechanisms are involved to explain the thrombotic phenotype of the Gas6$^{-/-}$ mice. Gas6, in addition to being expressed by murine platelets is also expressed by cells of the vasculature. As discussed above, the vasculature has a critical role in thrombus formation and we hypothesized that vascular derived Gas6 contributes to thrombus formation *in vivo*.

To begin, we sought to a) establish a reproducible model of venous thrombosis in the laboratory that is dependent on contributions from both the hematopoietic and vascular compartments and b) reproduce the thrombotic phenotype of the Gas6$^{-/-}$ mice. We chose the 0.37M $FeCl_3$ injury model which is dependent on platelets, coagulation proteins and vascular derived TF for proper thrombus formation [200]. We were able to successfully reproduce the thrombotic phenotype of the Gas6$^{-/-}$ mice using this model, which had up to this point never been used to study this strain of mice (Fig 2-1). The $FeCl_3$ model of vessel injury has traditionally been used in arterial models of thrombosis but has emerged as applicable for use in veins as well [196-198, 208].

With the model of venous thrombosis firmly established and the thrombotic phenotype of the Gas6$^{-/-}$ mice successfully reproduced, we began to explore the possibility of a contribution by vascular derived Gas6 to thrombus formation *in vivo*. To

do this, we used a BMT approach, allowing us to manipulate the genotype of hematopoietic stem cells and those derived from them. We generated groups of chimeric mice with selective ablations of Gas6 in either the hematopoietic or vascular compartments. Our mice were on a C57BL/6 background and were therefore radioresistant, an important consideration when undertaking these kinds of experiments [209]. As detailed in the results section, we generated four cohorts of mice. Two control (WT→WT and $Gas6^{-/-}$ → $Gas6^{-/-}$) and two experimental ($Gas6^{-/-}$ →WT and WT→ $Gas6^{-/-}$) groups. Following a month long period for recovery and hematologic reconstitution, the mice in each group were challenged with injury by 0.37M $FeCl_3$ for assessment of thrombus weight. We found that in the two experimental groups, thrombus weights were intermediate to those obtained in the control groups. These data suggested that Gas6 from both the vascular and hematopoietic compartments contributed to thrombus formation *in vivo*. An obvious weak point of this experiment was that it did not give any indication as to which vascular and which hematopoietic cells expressing Gas6 are actually contributing to thrombus formation. Survey of the literature reveals the presence of hematopoietic cells within thrombi. Following venous thrombosis, an inflammatory response occurs in both the vein wall and thrombus which leads to thrombus amplification. Initially there is neutrophil infiltration which is followed by monocytes and macrophages. This event is facilitated by the action of cytokines, chemokines and inflammatory factors such as TNF-α [224]. Leukocyte infiltration has also been observed in arterial thrombi. For example, a recent clinical study found leukocyte infiltration in the thrombi from patients with acute coronary syndrome (ACS) who suffered from plaque rupture. These thrombi contained activated neutrophils and macrophages. In addition

these activated leukocytes stained positively for TF and PAI-1 and thrombus size correlated with the levels of these antigens, suggesting they contribute to thrombus propagation [87]. Gas6 expression has also been detected in inflammatory cells such as macrophages [225]. To answer the question as to whether Gas6 expressing leukocytes contribute to thrombus propagation will require further research. Furthermore, techniques that allow for specific manipulation of the leukocytes of interest will be required.

It was necessary to perform additional experiments that would serve to confirm the results obtained by BMT and verify the individual contributions of Gas6 from both vascular and hematopoietic origin. We therefore made use of an additional model, one that allowed focus on the separation of vasculature derived Gas6 and Gas6 from platelets alone, as the role of Gas6 in platelet activation is established. We established a model of platelet depletion and reconstitution. Murine platelet depletion models have been useful in studying pathophysiological conditions such as transfusion-related acute lung injury [226], thermal injury and cerebral infarct [227] to name a few. Models of platelet depletion have also been used in larger mammals such as sheep [228] and rabbits [229]. In our experiments, recipient mice were rendered thrombocytopenic by i.p injection of a rabbit anti-mouse thrombocyte serum. Whole blood was collected from donor mice and PRP was prepared. The full complement of PRP from a donor mouse was used to reconstitute the platelets of a single recipient mouse. Following i.v injection of a full complement of PRP, circulating platelet levels rose to levels in the range considered normal for mice (i.e. 140-600 X 10^6 platelets/ μl of blood). As in the BMT experiments, we similarly generated four cohorts of mice. Following platelet depletion and

reconstitution and injury by $FeCl_3$, the phenotype of the $Gas6^{-/-}$ mice remained the same. The thrombi generated in $Gas6^{-/-}\rightarrow Gas6^{-/-}$ mice were significantly smaller than those generated in the WT → WT mice. In the two chimeric groups, thrombus weight was intermediate to both control groups, indicating that platelet-derived and vascular-derived Gas6 contribute to thrombus formation *in vivo*. The rabbit anti-mouse thrombocyte serum did have a rather profound effect on overall thrombus weight in these experiments. In general they were much looser and weighed less than thrombi produced in any other experiment performed over the course of this study.

We chose to examine TF as a target molecule in our study based on a few observations. First, the prospect of TF being regulated by Gas6 has been briefly mentioned [190], but no published data exists to support this statement. Nor were any model systems described that led the authors to mention this particular observation. The idea that TF represented a potential downstream target of Gas6 signalling partially lies in the fact that Gas6 promotes the activation of endothelial cells in an *in vivo* model [180], a work showing that Gas6 promotes the upregulation of ICAM-1 and VCAM-1 on endothelial cells and promotes their interaction with both leukocytes and platelets. Thus, Gas6 promotes endothelial cell activation and TF is of course, a well established marker of activated endothelial cells [230]. The present study provides evidence that Gas6 positively regulates TF expression *in vivo* in murine vascular cells and *in vitro* in endothelial cells from murine lung tissue. We propose that this observation could partially explain the contribution to thrombus formation by vascular derived Gas6 that we observed in both BMT and platelet depletion and reconstitution experiments. It is

unlikely that the BMT protocol had any effect on vascular TF and the ability to mount a coagulation response after $FeCl_3$ injury. A previous work established that the composition of TF in the vascular wall is unaltered by BMT [51].

TF plays a key role in several models of thrombosis, including a stasis model where it is rapidly upregulated [231] and the $FeCl_3$ injury model [51]. Our current understanding of $FeCl_3$ induced thrombosis is that it mimics mechanical trauma to a vessel, causing endothelial cell denudation and collagen exposure. The response to $FeCl_3$ mediated injury will also differ in a vascular bed specific manner as has been recently reviewed [200]. Here, we demonstrated that TF upregulation occurs on venous endothelial cells following $FeCl_3$ induced thrombosis. By immunofluorscence microscopy, TF induction is robust on WT endothelial cells following 0.37M $FeCl_3$ injury. Consistent with the study by Tjwa *et al.* [180] showing that Gas6 contributes to activation of the endothelium, we have observed a blunted response to $FeCl_3$ injury on endothelial cells of the $Gas6^{-/-}$ IVC, which displayed less TF staining. We validated these observations by assaying the functional pool of TF on homogenates of the IVC that had undergone $FeCl_3$ injury. The homogenates prepared from $Gas6^{-/-}$ mice supported significantly less TF activity than those from WT mice.

To add supporting evidence to our results obtained *in vivo*, that Gas6 is prothrombotic and promotes TF upregulation, we began the isolation of endothelial cells from mouse lung tissue for *in vitro* experiments. By a functional assay, MLECs isolated from $Gas6^{-/-}$ mice supported significantly less TF activity on their surface. At the level of

mRNA, thrombin mediated induction TF was significantly reduced in Gas6$^{-/-}$ cells indicating that these cells are hyporesponsive to endothelial activation by thrombin which is once again consistent with published observations [180].

Unfortunately, these data do not firmly establish the link between vascular Gas6 induced TF expression and thrombus weight. Additional experiments will be required to prove that vascular Gas6 induced TF supports thrombogenesis *in vivo*.

An interesting, although somewhat disappointing observation made over the course of this study was the fact that HUVECs treated with recombinant human Gas6 (rhGas6) had a reduced capacity to mount an activation response to thrombin. When we pretreated these cells with 100 ng/ml rhGas6 for different time periods, the upregulation of TF mRNA and protein activity by thrombin was reproducibly blunted. Therefore, we could not use human cells to support our observations that Gas6 has a prothrombotic effect on endothelial cells. It could be that, these results are specific to the times of rhGas6 incubation chosen. Also, we did not attempt siRNA mediated knock-down of Gas6 in human cells before thrombin challenge. It is also possible that human endothelium may simply respond differently to Gas6. After all, it is not unheard of for human and murine endothelial cells to have differing signalling responses to stimuli [232]. This would seemingly make it even more important to understand the role of Gas6 in platelets and vascular cells in mice if it is to ever one day be targeted for antithrombotic therapy in humans.

Gas6 has become known for having roles in other disorders of the hemovascular system. For example, Gas6 is proatherogenic in both mouse and humans [183, 233], and has the potential to stimulate erythropoiesis in anaemic patients who have become hyporesponsive to Epo treatment Unfortunately, over the past decade, little progress has been made in the advancement of our knowledge of the role of Gas6 in venous thrombosis. There have been reports that effectively challenge the potential targeting of platelet derived Gas6 for antithrombotic therapy in humans. An initial supporting finding was the observation that when incubated with an anti-Gas6 antibody, PRP from humans is defective in aggregation and secretion in response to ADP and the selective PAR-1 agonist, SFLLRN [191]. Gas6 mRNA was detected in human platelets [187, 234] but subsequent immunologic analyses of Gas6 in human plasma and platelets challenged this idea. In one study, Gas6 was detectable in human plasma in the low sub-nanomolar range, but the same authors failed to detect expression of Gas6 by human platelets and proposed that that any potential platelet amplifying ability that Gas6 had in humans would be from Gas6 derived from the circulation [192]. Another finding was that physiological levels of Gas6 in human plasma from healthy subjects do not influence platelet activation *ex vivo* [193]. Further complicating the matter is recent data from Cosemans *et al*., who investigated the issue once again as to whether or not human platelets expression physiologically relevant amounts of Gas6. These authors were able to detect Gas6 in human platelets at concentrations of 1.0 μg/L whereas the amount of Gas6 in human plasma was 20 fold higher, and thus the major source of Gas6 in human blood. The authors attributed their successful detection of platelet Gas6 to more sensitive

detection methods. It would also seem that the small amounts of platelet Gas6 are stored in α-granules and become released upon thrombin stimulation. Interestingly, the Gas6 receptor Axl was predominantly found in platelet granules while at rest and at the plasma membrane following thrombin stimulation. Despite the presence of Gas6 in human platelets, it was still predominantly plasma derived Gas6 that contributed to human platelet aggregation and stabilization [235].

In order for the targeting of human Gas6 for antithrombotic therapy to become a reality, it will become necessary to fully understand the controversial role of blood derived Gas6 in thrombus formation. Addition sources of Gas6 include the endothelium, bone marrow, vascular smooth muscle and macrophages. It will also be important to determine the roles of Gas6 from these additional sources to thrombus formation. The present work provides evidence that the specific targeting Gas6 derived from the vasculature represents a potential antithrombotic therapy. Also, targeting of the Gas6 protein itself should not be the only focus in regards to Gas6 and venous thrombosis. It is also necessary to understand the downstream signaling events that occur in platelets and vascular cells following stimulation by Gas6. Gas6 binding to TAM receptors on platelets activates PI3K/Akt[190] which also represents a therapeutic target.

A member of the Forkhead superfamily of transcription factors, FoxO1 has emerged as a downstream target in Gas6 mediated signaling in endothelial cells [111]. FoxO1 is also known for its role in promoting endothelial cell activation and induction of the adhesion molecule VCAM-1 following stimulation by VEGF [236, 237]. Given that

Gas6 is also involved in VCAM-1 induction, we derived a hypothesis that Gas6 dependent induction of VCAM-1 is dependent on FoxO1. This hypothesis is explored in the following chapter.

Chapter 3:

GAS6 REGULATES THROMBIN MEDIATED UPREGULATION OF VCAM-1, A FOXO1 DEPENDENT GENE

3.1 Forkhead (FOX) Transcription Factors

The first member of the FOX family of transcription factors was identified in *Drosophila* and was named *fork head.* The gene encoded for a nuclear protein involved in *Drosophila* embryogenesis [238]. Following the discovery of the first forkhead (FOX) gene (now classified as FOXA according to contemporary nomenclature), it became apparent that this family of transcription factors have critical and diverse roles in developmental processes. The forkhead genes are evolutionarily conserved and have been identified in organisms ranging from yeasts to humans. The products of the forkhead genes represent a subgroup of the helix-turn-helix class of proteins and each member contains the characteristic 100-amino acid winged helix domain [239]. Following the finding of the first forkhead gene in *Drosophila*, over 100 members of this gene family have been identified. The sheer number of gene sequences that have emerged from numerous laboratories necessitated a strict system of nomenclature to classify these genes and their encoded proteins. This issue was first raised in 1998 at the first international

meeting on Forkhead/Winged Helix Proteins. FOX family classification has since undergone some revision.

In addition to the conserved winged helix domain, FOX family proteins contain transactivation or transrepression domains that are highly divergent. Phylogenetic analysis currently reveals 17 subclasses for all known FOX proteins. The phylogenetic tree continues to be updated. Each subclass is designated by a letter (A-Q) and within each subclass individual proteins are designated by an Arabic numeral (i.e. FoxO1). Interestingly, the number of FOX proteins has increased over the course of evolution as more of them are expressed in vertebrates than invertebrates. The chromosomal locations of the FOX genes are not random. Eight clusters of FOX genes have been identified in the human genome, with at least an additional 20 genes scattered throughout. In humans, FOX genes are necessary for proper formation of a number of different organs and mutations in certain FOX genes can manifest as serious clinical conditions, such as severe T-cell immunodeficiency and impaired intellectual development [239, 240].

3.2 The FoxO Subclass

A rather well established characteristic of the O-class of FOX proteins is their involvement in the insulin/PI3K/Akt signaling pathway. Mammals express four different forms of the FoxO family of transcription factors. They are FoxO1, FoxO3, FoxO4 and FoxO6. For the sake of completion, FoxO2 is identical to FoxO3 and FoxO5 has been

assigned as the fish ortholog of FoxO3. The *FOXO* genes were first identified in humans. The family members FoxO1, FoxO3 and FoxO4 were found at chromosomal translocations in patients diagnosed with rhabdomyosarcomas and acute myeloid leukemias [241]. With the exception of FoxO6, the FoxO isoforms are known downstream targets of the PI3K/Akt signaling pathway (Fig 3-1).

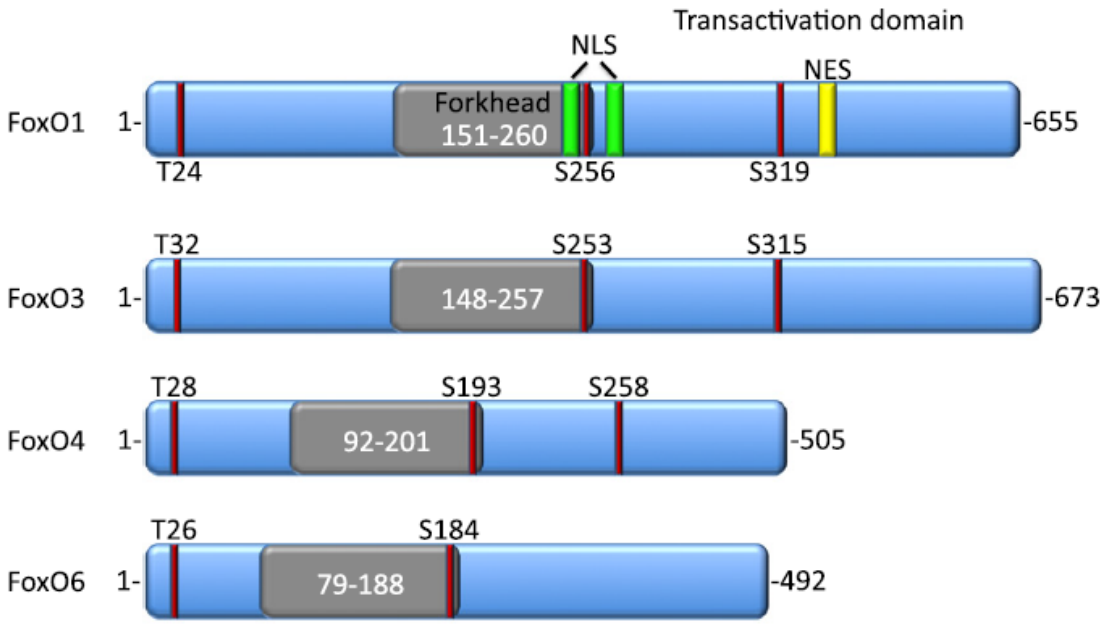


Figure 3-1. Mammalian FoxO proteins. Depicted are the conserved Akt phosphorylation sites, Forkhead domains, and nuclear localization signals (NLS) and nuclear export signals (NES) [242].

Akt phosphorylates FoxO isoforms at specific sites following growth factor stimulation of the cell. Phosphorylation of FoxO transcription factors results in their translocation from the nucleus to the cytoplasm, thereby inhibiting their transcriptional activity. While the PI3K/Akt system is the major regulator of FoxO activity, it is broadly accepted that the FoxO transcription factors are a convergence point of various signaling cascades that result from either growth factor stimulation or oxidative stress. Other

known intracellular regulators of FoxO function include JNK [243], Erk [244] and Sirt1 [245, 246]. FoxO transcription factors are subject to a number of post-translational modifications that influence their activity. In addition to phosphorylation, these include acetylation, and mono- or polyubiquitination. These modifications affect the subcellular localization, stability, DNA binding capacity and transcriptional activity of FoxO proteins. FoxO transcription factors regulate genes involved in glucose metabolism, cell cycle arrest, apoptosis, differentiation and DNA repair. For example, FOXOs initiate apoptosis by initiating transcription of FasL, the ligand for the Fas-dependent cell death pathway [247]. They can also initiate cell cycle arrest through the upregulation of $p27^{kip1}$ or GADD45. FOXOs are involved in cell stress resistance through the upregulation of two enzymes involved in the detoxification of reactive oxygen species, MnSOD and catalase. The upregulation of GADD45, along with DDB1 also implies a role for FOXOs in mediating the repair of damaged DNA [241].

Different model organisms have been used to elucidate the functions of FoxO transcription factors within the context of the organism as a whole. Invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster* each express only one isoform of FoxO (named DAF-16 and dFoxO, respectively). From these models we have learned about the importance of FoxO factors in organismal metabolism and lifespan. It seems that in worms, DAF-16 regulates the production of hormones that coordinate the metabolism and lifespan of a number of tissues within the organism. Consistent results were observed in flies overexpressing dFoxO, revealing the critical roles for FoxO factors in metabolism and longevity [245].

Mammals express the aforementioned isoforms of the FoxO transcription factors, and they display complementary and overlapping functions during development and within adult tissues. In adult mice and humans, the expression pattern of FoxO1 is similar, with levels being highest in adipose tissue, the uterus, ovaries and lowest in skeletal muscle and the spleen [245].

3.3 Regulation of FoxO Transcription Factors

The master regulator of mammalian FoxO proteins is Akt. The first evidence that Akt regulates mammalian FoxO proteins was published in 1999 [248]. The study focused on FoxO3 and showed that Akt can phosphorylate FoxO3 at all predicted sites both *in vivo* and *in vitro* and that this event resulted in nuclear exclusion of the protein. Another important finding from this work was the identification of the participation of nuclear export enzyme 14-3-3 [249], a now established facilitator of FoxO3 shuttling. Subsequently, we learned that Akt does in fact phosphorylate and similarly regulate both FoxO4 [250] and FoxO1 [251]. The three regulatory Akt phosphorylation sites are present among all mammalian FoxO proteins (except FoxO6, which only has 2) and are evolutionally conserved. The phosphorylated residues on FoxO proteins serve as docking sites for 14-3-3. In regards to FoxO1, these phosphorylation sites are Thr-24, Ser-256 and Ser-319 [242].

3.4 The Pleiotropic Effects of FoxO Factors on Endothelial Cell Biology

Within endothelial cells, FoxO1 and FoxO3 are the predominantly expressed isoforms [252]. FoxO transcription factors have been demonstrated as having important roles in endothelial cell biology. For example, it has been observed that FoxO factors have anti-angiogenic properties. The proliferation and migration of endothelial cells is attenuated by FoxO factors. Paik *et al.* have demonstrated through deletion of *FoxO1*, *FoxO3* and *FoxO4* in mice causes overproliferation of endothelial cells, resulting in hemangioma formation and premature death of the animal [253]. Potente *et al.* showed FoxO transcription factors have a suppressive effect on endothelial nitric oxide synthase (eNOS) expression [252], whereas they promote the expression of the inducible NOS (iNOS) in response to oxidative stress. The fact that FOXOs have these effects on endothelial NO production implicated a role in endothelial dysfunction considered as conducive to the development of atherosclerosis. It has recently been confirmed that the deletion of the three FOXO isoforms in endothelial cells protects mice from vascular dysfunction and atherosclerosis [254].

FOXOs are involved in regulation of endothelial cell apoptosis in different ways. FoxO1 mediates the protective effects exerted on endothelium by Endothelin-1 (ET-1). ET-1 promotes the phosphorylation and nuclear exclusion of FoxO1 downstream of PI3K/Akt [255]. FoxO3 has the ability to induce endothelial cell apoptosis through the

activation of matrix metalloproteinases (MMPs). The overexpression of FoxO3 induced the production of endothelial MMP-3 and detachment from the underlying matrix [256]. In endothelial cells, FOXO factors regulate the expression of known mediators of apoptosis and cell survival such as BCL-2, BAD, BAX and BAK [255].

3.5 Regulation of VCAM-1

3.5.1 VCAM-1

Vascular adhesion molecule-1 (VCAM-1) is a 110 kDa glycoprotein expressed by activated endothelium. The VCAM-1 promoter was originally cloned and characterized in cultured endothelial cells and has been valuable in understanding the molecular mechanisms that underlie endothelial cell activation following treatment with thrombin [257, 258]. The overall function of VCAM-1 *in vivo* is in immune surveillance and inflammation where it mediates leukocyte recruitment in a number of diseases. VCAM-1 is induced by cytokines, reactive oxygen species, turbulent shear stress, high levels of glucose and by microbial products. The VCAM-1 promoter has binding sites for NF-κB, SP-1 and AP-1, to name a few. VCAM-1 mediates the transendothelial migration of leukocytes, which is a three step process. The first step is low affinity rolling of leukocytes on the endothelium which is followed by leukocyte arrest by high affinity binding. Leukocytes then transmigrate through the endothelial cell layer. The expression of VCAM-1 carries importance with respect to cardiovascular diseases. For example, it is the first adhesion molecule expressed before the development of an atherosclerotic

plaque [259]. A role for VCAM-1 in the pathogenesis of venous thrombosis has been proposed. Elevated levels of the soluble form of VCAM-1 have been detected in the serum of patients with venous thrombosis [260]. However, it is not yet clear what this relationship entails [261].

3.5.2 Regulation by Thrombin

The serine protease thrombin is involved in blood coagulation, cell proliferation and inflammation. Stimulation by thrombin induces the expression of several genes in cultured endothelial cells via the Protease-Activated Receptors (PARs) [262]. Over a decade has passed since we learned that thrombin mediates the induction of VCAM-1 antigen and promoter activity in cultured endothelium. Downstream of PAR activation, some of the signalling pathways involved in VCAM-1 induction include PI3K, PKC and p38 MAPK [257].

3.5.3 Regulation by FoxO1

In 2006, a novel class of FoxO1 responsive genes was identified in endothelial cells. The treatment of endothelial cells with VEGF induced phosphorylation of FoxO transcription factors via the PI3K/Akt pathway. As predicted, VEGF treatment also

resulted in nuclear exclusion of FoxO proteins and the inactivation of their transcriptional activity. The known FoxO target gene, $p27^{kip1}$ was downregulated and cells entered a state of increased proliferation [263]. Abid *et al.* have identified a subset of VEGF responsive genes that are FoxO1-dependent, while falling outside the classical pathway of FoxO mediated gene regulation as discussed above. To demonstrate this phenomenon, they used tools that allowed for the modulation of the levels of endogenous FoxO1. Adenoviral constructs were designed for endothelial cell transduction in order to overexpress either WT FoxO1 or a constitutively active mutant form of the protein. This triple mutant (TM) is characterized by 3 point mutations (T24A, S256A and S315A) that abolish the phosphorylation sites. The TM is therefore not receptive to nuclear exclusion. Endogenous FoxO1 levels were also manipulated by siRNA mediated knock down.

The VEGF-responsive genes that depended on FoxO1 activity were divided into two classes from data obtained using the above techniques in combination with microarray analysis. These classes were defined as follows. Class I genes belong to the so-called "classic" pathway, meaning their expression is repressed following nuclear exclusion of FoxO1. The new Class II genes are induced by VEGF and depend not only on initial nuclear exclusion of FoxO1 but on an additional event where FoxO1 re-enters the nucleus and interacts with other transcription factors (Fig. 3-2). The nuclear re-entry and partner binding induces the VEGF-dependent gene expression. Among the Class I genes were BTG-1, GADD45A and $p27^{kip1}$ that demonstrated the classic agonist (VEGF) mediated inhibition of expression. The Class II genes were found to be highly induced by VEGF in the TM-FoxO1 containing cells. In addition, the use of siRNA directed against

FoxO1, inhibited VEGF mediated induction of genes in this second class. The Class II genes included bone morphogenic protein 2 (BMP2), endothelial specific molecule-1 (ESM-1), decay accelerating factor (DAF) and matrix metalloproteinase 10 (MMP-10). VCAM-1 was identified as a Class II, FoxO1 dependent gene as well [237].

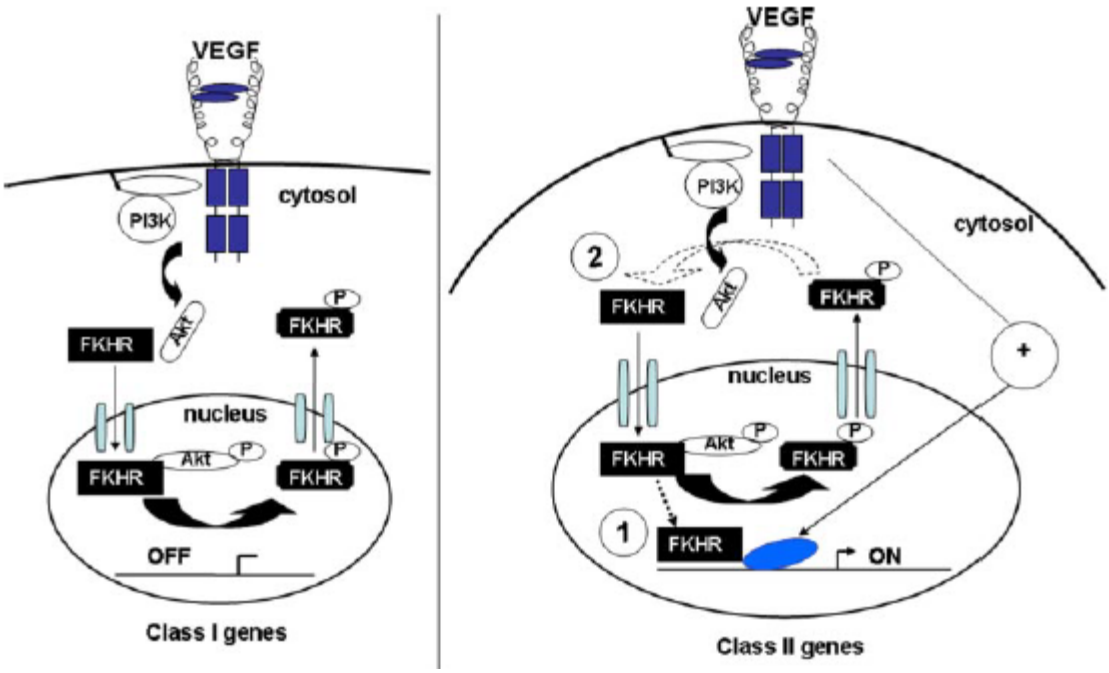


Figure 3-2. Regulation of Class I and Class II FoxO1 Dependent gene expression. (Left) Class I FoxO1 (FKHR) dependent genes are regulated by the so-called 'classic' pathway whereby agonist (VEGF) binding at the cell surface activates PI3K-Atk and subsequent nuclear exclusion of FoxO1, shutting gene expression off. **(Right)** In the Class II pathway, the same initial events resulting in FoxO1 nuclear exclusion occur. This is followed by a second event involving FoxO1 returning to the nucleus and interacting with an additional non-FoxO1 transcription factor to turn on expression of the Class II gene [237].

3.5.4 Regulation by Gas6

As was discussed in the preceding chapter, Gas6$^{-/-}$ endothelial cells have lower responsiveness to activation. This was first demonstrated both *in vivo* and *in vitro* by Tjwa *et al*. [180], who showed that the expression of the endothelial cell activation markers ICAM-1 and VCAM-1 were blunted in the absence of Gas6. In addition to adhesion molecules, TNF-α challenged endothelial cells express the inflammatory cytokines IL-1β and IL-6. This phenomenon was also blunted by Gas6 ablation. Therefore, endothelial cell activation and optimal expression of VCAM-1 following inflammatory stimulus is dependent on Gas6 [180].

To consolidate thus far, regulation of the thrombin inducible gene VCAM-1 in endothelial cells is dependent on both FoxO1 following growth factor stimulus (VEGF) [237] and Gas6 following inflammatory stimulus (TNF-α) [180].

3.6 Gas6 Regulation of FoxO1

Our lab has identified a role for FoxO1 in the protection from apoptosis by Gas6. Prior to this, the activation of the PI3K/Akt system downstream of Axl had already been known to be involved in Gas6 mediated protection from apoptosis [131, 264]. When this observation was coupled with the established role of VEGF and PI3K/Akt in mediating FoxO1 dependent cell survival, a logical hypothesis was derived. It was predicted that protection from apoptosis by Gas6 is mediated in part by FoxO1 transcriptional inactivation downstream of PI3K/Akt. The hypothesis indeed proved correct. Gas6 was

shown to induce time-dependent phosphorylation of FoxO1 in an Akt dependent manner. The importance of the involvement of Akt in mediating this phosphorylation event was revealed by experiments with the PI3K inhibitor Wortmannin. As predicted by the phosphorylation data, Gas6 treatment also resulted in nuclear exclusion of FoxO1 in a PI3K dependent manner. Endothelial cell survival experiments showed that the anti-apoptotic effects of Gas6 are dependent on phosphorylation and subsequent nuclear exclusion of FoxO1 [111].

In summary, expression of VCAM-1 in endothelial cells is regulated by both FoxO1 and Gas6. In addition, Gas6 regulates FoxO1 activity. Together, these observations led to the formulation of the hypothesis that the ability of Gas6 to promote endothelial cell activation/VCAM-1 induction is mediated in part by FoxO1.

The work presented within this chapter serves as a beginning point in describing the signaling molecules involved in the ability of Gas6 to mediate endothelial activation and induction of VCAM-1. Our rationale for having chosen FoxO1 as likely being involved has been described above. We hypothesize that Gas6 modulates thrombin mediated induction of VCAM-1 via FoxO1.

3.7 Methods

Reagents

Antibodies directed against FoxO1, pFoxO1, and histone H3 were from Cell Signalling. Antibodies directed against GAPDH and β-actin were from Santacruz. 4-15% gradient gels for SDS-PAGE and nitrocellulose membranes were supplied by Bio-Rad. Dharmafect 4 reagent, short interfering (si) RNA for mouse FoxO1 and the non targeting sequence were from Dharmacon. EBM-2, EGM-2, DMEM/F12, FBS, Penecillin/Streptomycin were from Wisent. FAST SYBR Green Master Mix and 16S ribosomal RNA control reagents were from Applied Biosystems. RNeasy mini kit, Qiashredders and Quantitect Reverse Transcription Kit were supplied by Qiagen.

Isolation and Propagation of Endothelial Cells

Endothelial cells from WT and Gas6$^{-/-}$ mouse lung tissue were isolated and propagated in culture as described in detail in Chapter 2. For all experiments, endothelial cells were used between passages 3 and 4. For all experiments, complete media was removed, confluent cells were washed twice with PBS and subjected to overnight serum starvation by culturing in EBM. Cells were then treated with 1.5U/ml human thrombin for the indicated time points, depending on the particular experiment.

Protein Lysate Preparation and SDS-PAGE

Cells were washed twice with ice-cold PBS and lysed with 100 µl of whole cell lysis buffer [50mM Tris-HCl (pH 7.5), 150mM NaCl, 50mM NaF, 0.1mM EDTA (pH 8.0), 1% Igepal] supplemented with 1M DTT, 100mM Na_3VO_4 and Complete Protease Inhibitor Cocktail (Roche). Whole cell lysates were collected by scraping and centrifuged for 15-20 minutes at 16,000 X g in a Beckman Centrifuge cooled to 4ºC. The supernatants were quantified for protein content by the Bradford assay (Bio-Rad). For Thrombin induced FoxO1 phosphorylation and siRNA mediated knock-down experiments, 100 µg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Western Blot analysis was performed with the following antibodies and dilutions: anti-FoxO1 (1:200), anti-pFoxO1 (1:200), anti-GAPDH (1:1000), anti-H3 (1:2000) and anti-β-actin (1:1000).

Cellular Fractionation

Separation of cytoplasmic and nuclear fractions was carried out using a modified version of a protocol developed by PANOMICS. Cells were washed twice with ice-cold PBS. Cell monolayers were overlayed with 100 µl of Buffer A [10 mM HEPES (pH 7.9), 10mM KCl, 10mM EDTA] supplemented with 100 mM DTT, Complete Protease Inhibitor Cocktail and 40 µl of 10% IGEPAL/ ml of buffer. The cells overlayed with Buffer A were placed on ice and shaken on a rocking platform for 10 minutes at 150 rpm. Cells were then collected by scraping and centrifuged at 15,000 X g for 3 minutes at 4ºC. The supernatant was collected as the cytoplasmic fraction and stored at -80ºC until use. The pellets were resuspended (by vortexing) in 50 µl of Buffer B [20 mM HEPES (pH

7.9), 0.4M NaCl, 1mM EDTA, 10% Glycerol] supplemented with the same protease inhibitors as Buffer A. At this point the resuspended pellets in eppendorf tubes were placed horizontally on ice and placed on a rocking platform for 2 hours. A final centrifuge step was then performed: 1500 x g for 5 minutes at 4ºC. The supernatant was collected as the nuclear fraction. Cytoplasmic and nuclear fractions were quantified by the Bradford assay. 10 µg of protein was loaded onto 4-15% gradient gels (Bio-Rad) for SDS-PAGE analysis.

siRNA-mediated knock-down of FoxO1

WT MLEC were seeded in 6-well, gelatine coated plates and incubated in media containing 50% EGM-2 and 50% DMEM/F12 + 20% FBS. No antibiotics were present for knock-down experiments as suggested by the manufacturer. When the cells reached confluence, they were treated with 100 nM siRNA against murine FoxO1 or a non-targeting sequence according to the manufacturer's protocol. Non-transfected control samples were prepared as well. The cells in transfection medium were allowed to incubate for 32 hours before serum starved in EBM2 overnight. Cells were then challenged with thrombin for 30 minutes or 4 hours.

Quantitative RT-PCR

Total RNA was isolated with an RNA extraction kit from Froggabio-Geanaid according to the manufacturer's instructions. A genomic DNA digestion step was included using DNAse from Fermentas. The purified RNA was qnantified with a ND-1000 Nanodrop spectrophotometer (Nanadrop). 1.0 ng of total RNA was reverse transcribed with a qScript cDNA synthesis kit from Quanta-VWM. Finally 2.0 µl of 1:80 dilutions of cDNA were prepared and mixed with primers and SYBR Green FastMix from Quanta-VWR. Thermal cycling was performed on a 7500 Fast Real Time PCR System from Applied Biosystems. The thermal profile was as follows: Holding Stage: 95°C for 20 seconds, Cycling stage: 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Amplification of VCAM-1 mRNA was normalized to levels of endogenous 16s rRNA as calculated using the ΔCt method.

Densitometric analysis

For all Western blots, nitrocellulose membranes were exposed using a ChemiDoc imaging system (Bio-Rad). Densitometric analysis was performed using the Quantity One software.

Statistical Analysis

All numerical data is expressed as the mean ± SEM. Statistical significance was calculated using a paired student's t-test. P-values lower than 0.05 were considered significant.

3.8 Results

FoxO1 negatively regulates the expression of VCAM-1 mRNA

It has been observed that VCAM-1 is a Class II FoxO1 dependent gene downstream of VEGF [237]. In that particular study, siRNA mediated knock-down of FoxO1 blunted the ability of VEGF to induce VCAM-1 mRNA. Here, it was necessary to determine if FoxO1 is involved in VCAM-1 mRNA induction downstream of thrombin in murine endothelial cells. WT endothelial cells were seeded in 6-well plates and transfected with siRNA (FoxO1 and non-targeting) as described in methods. Efficient knock-down of FoxO1 was observed in these cells by 24 hours and was maximal at 48 hours. The levels of endogenous FoxO1 began to rise again by 72 hours, as expected (Fig. 3-3A). At roughly 32 hours post-transfection, the cells were subject to overnight serum starvation and thrombin challenge for 30 minutes or 4 hours. Knock-down of FoxO1 by siRNA resulted in increased basal levels of VCAM-1 mRNA by 2.63 ± 0.54 fold (n=4) compared to the non-targeting siRNA. FoxO1 therefore is a transcriptional repressor of VCAM-1. Unexpectedly, when FoxO1 is knocked down, thrombin seems to cause a time dependent decrease in VCAM-1 mRNA levels. (Fig. 3-3B).

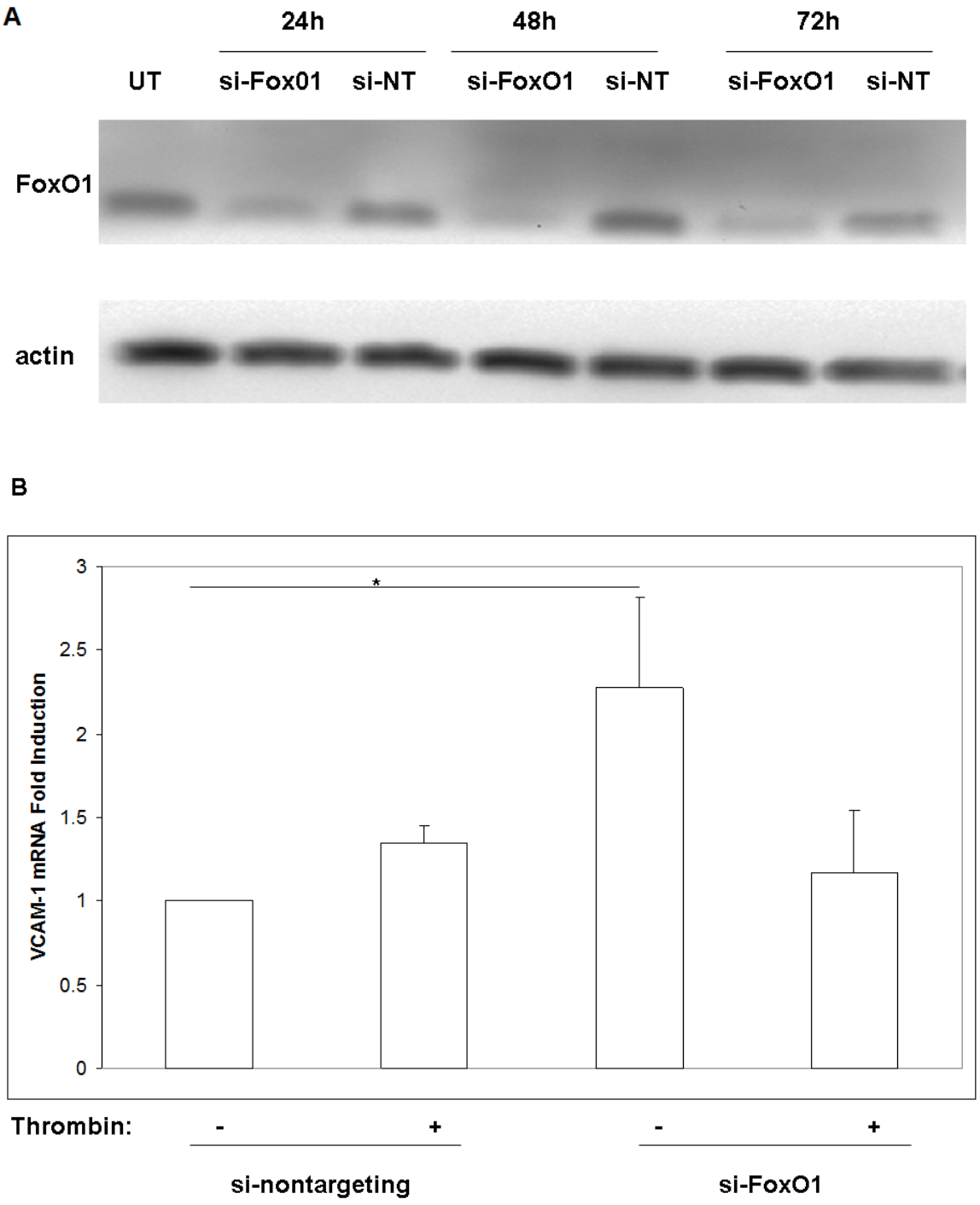
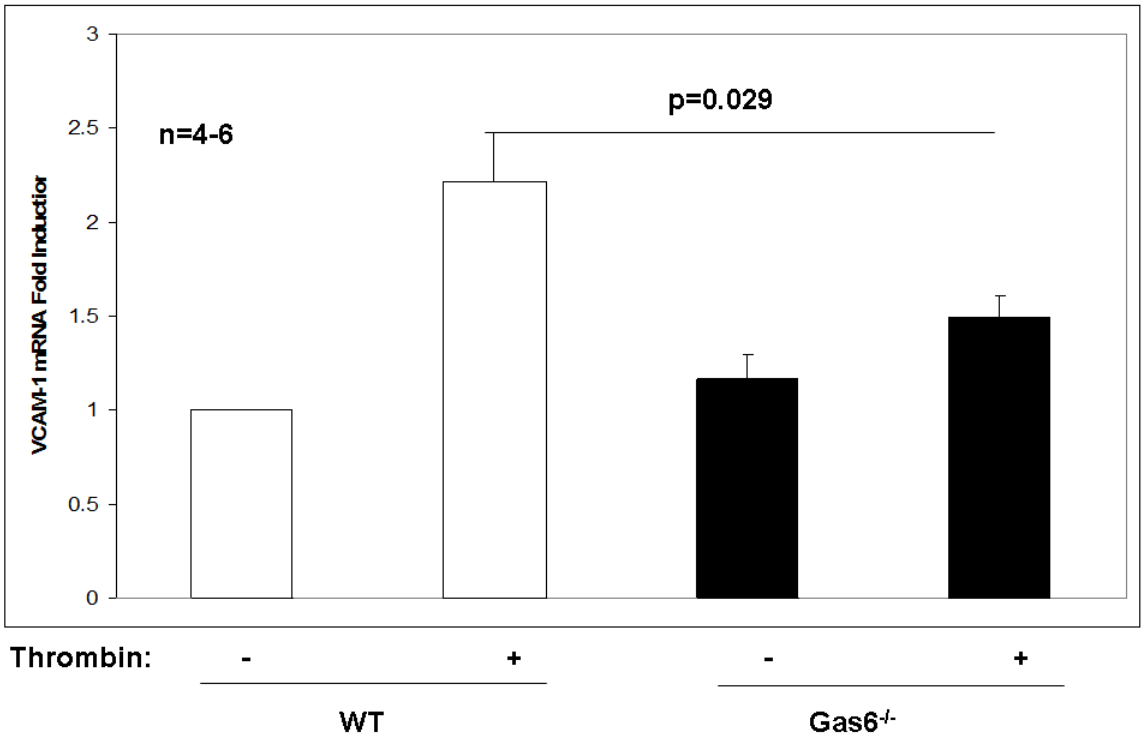

A
24h
48h
72h
UT
si-Fox01
si-NT
si-FoxO1
si-NT
si-FoxO1
si-NT
FoxO1
actin
B
VCAM-1 mRNA Fold Induction
3
2.5
2
1.5
1
0.5
0
*
Thrombin:
-
+
-
+
si-nontargeting
si-FoxO1

Fig 3-3. The effect of FoxO1 knock-down on VCAM-1 mRNA expression. (A) Representative western blot showing specificity of siRNA and efficient knock-down of FoxO1 by 24 hours in WT endothelial cells. **(B)** Knock-down of FoxO1 results in increased basal levels of VCAM-1 mRNA (indicated by -). In thrombin treated cells deficient in FoxO1, the increased levels of basalVCAM-1 mRNA are reduced.

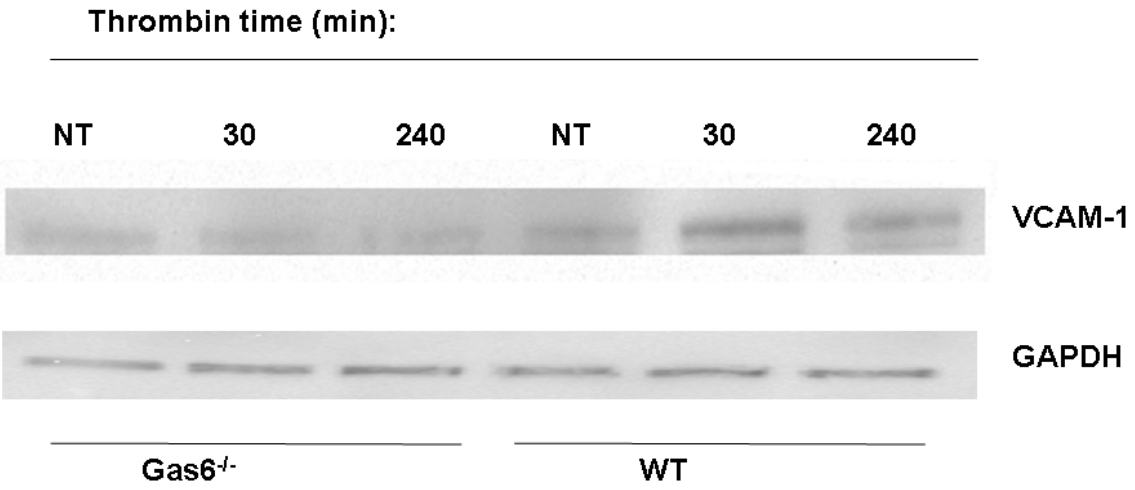
Gas6 is required for thrombin mediated induction of VCAM-1

Confluent monolayers of WT and Gas6$^{-/-}$ MLEC were serum starved and challenged with 1.5U/ml thrombin for 4 hours. At the 30 minute time point, thrombin induced VCAM-1 mRNA by 2.22 ± 0.25 fold in WT cells and by 1.49 ± 0.11 fold in Gas6$^{-/-}$ cells ($n=4$, $p<0.05$) (Fig 3-4A). The blunted induction of VCAM-1 was confirmed at the level of protein expression. A representative western blot experiment demonstrating the attenuation of thrombin mediated VCAM-1 induction is shown in Fig.3-4B and is accompanied by a densitometric quantification (Fig.3-4C) These data demonstrate the attenuation of thrombin induced endothelial cell activation in the absence of Gas6.

A


3
2.5
2
1.5
1
0.5
0
VCAM-1 mRNA Fold Induction
n=4-6
p=0.029
Thrombin:
-
+
-
+
WT
Gas6-/-

B


Thrombin time (min):
NT
30
240
NT
30
240
VCAM-1
GAPDH
Gas6-/-
WT

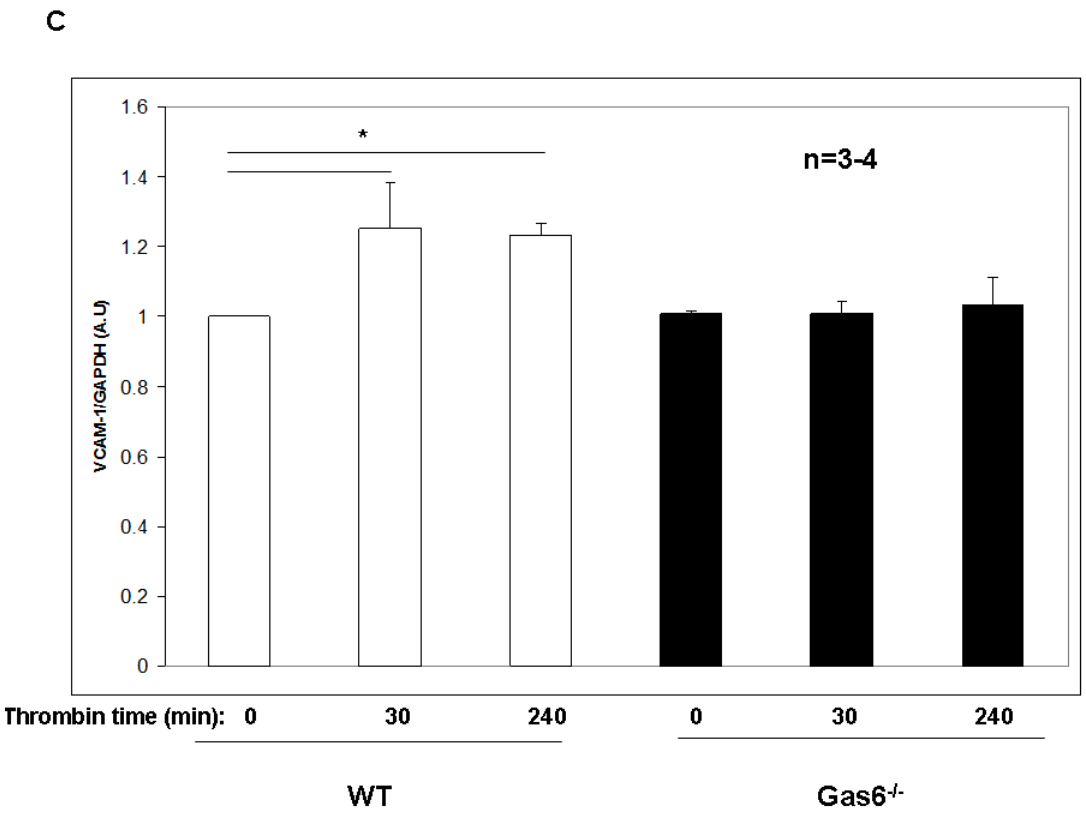


Figure 3-4. Induction of VCAM-1 mRNA by Thrombin is Blunted in Gas6$^{-/-}$ Endothelial Cells. (A) In WT endothelial cells (white), VCAM-1 is strongly induced at 30 minutes post-thrombin treatment. VCAM-1 induction is lower in Gas6$^{-/-}$ endothelial cells (black). **(B)** Representative immunoblot demonstrating the blunted induction of VCAM-1 in Gas6$^{-/-}$ endothelial cells. **(C)** Densitometric quantification of the immunoblot experiments performed in (B).

Thrombin dependent phosphorylation of FoxO1 is blunted in Gas6$^{-/-}$ endothelial cells

FoxO1 has three phosphorylation sites: Thr-24, Ser-256 and Ser-316. FoxO1 has been shown to become phosphorylated following the treatment of cells with a number of agents including insulin [265], VEGF [236], hydrogen peroxide, [266]. Recently it has been observed that thrombin induces time dependent phosphorylation of FoxO1. In the

same study, it was determined that FoxO1 is a downstream mediator of thrombin mediated proliferation of smooth muscle cells and expression of genes involved in cell cycle regulation. It was also observed that in smooth muscle, this occurs via PAR-1 followed by activation of PI3K/Akt [267]. Here, endothelial cells from WT or $Gas6^{-/-}$ mouse lung tissue were isolated and cultured as described in methods. In WT cells, thrombin induced time-dependent phosphorylation of FoxO1 by 15 minutes and was maximal at 30 minutes. In WT endothelium, the P-FoxO1/FoxO1 ratio was 1.941 ± 0.44 at 30 minutes. A representative experiment from WT cells is shown in Fig. 3-5A. The band for pFoxO1 appears as a doublet at 82 kDa. When this experiment was repeated in $Gas6^{-/-}$ cells, thrombin induced phosphorylation of FoxO1 was maximal at 30 minutes as well, but the response was lower overall than in WT cells. In $Gas6^{-/-}$ endothelial cells, the pFoxO1/FoxO1 ratio was reduced to 1.31 ± 0.14 (n=5-6, p=0.1) (Fig. 3-5B). Thrombin induced FoxO1 phosphorylation decreased after 30 minutes, but it seems that phosphorylation rose again at 120 minutes.

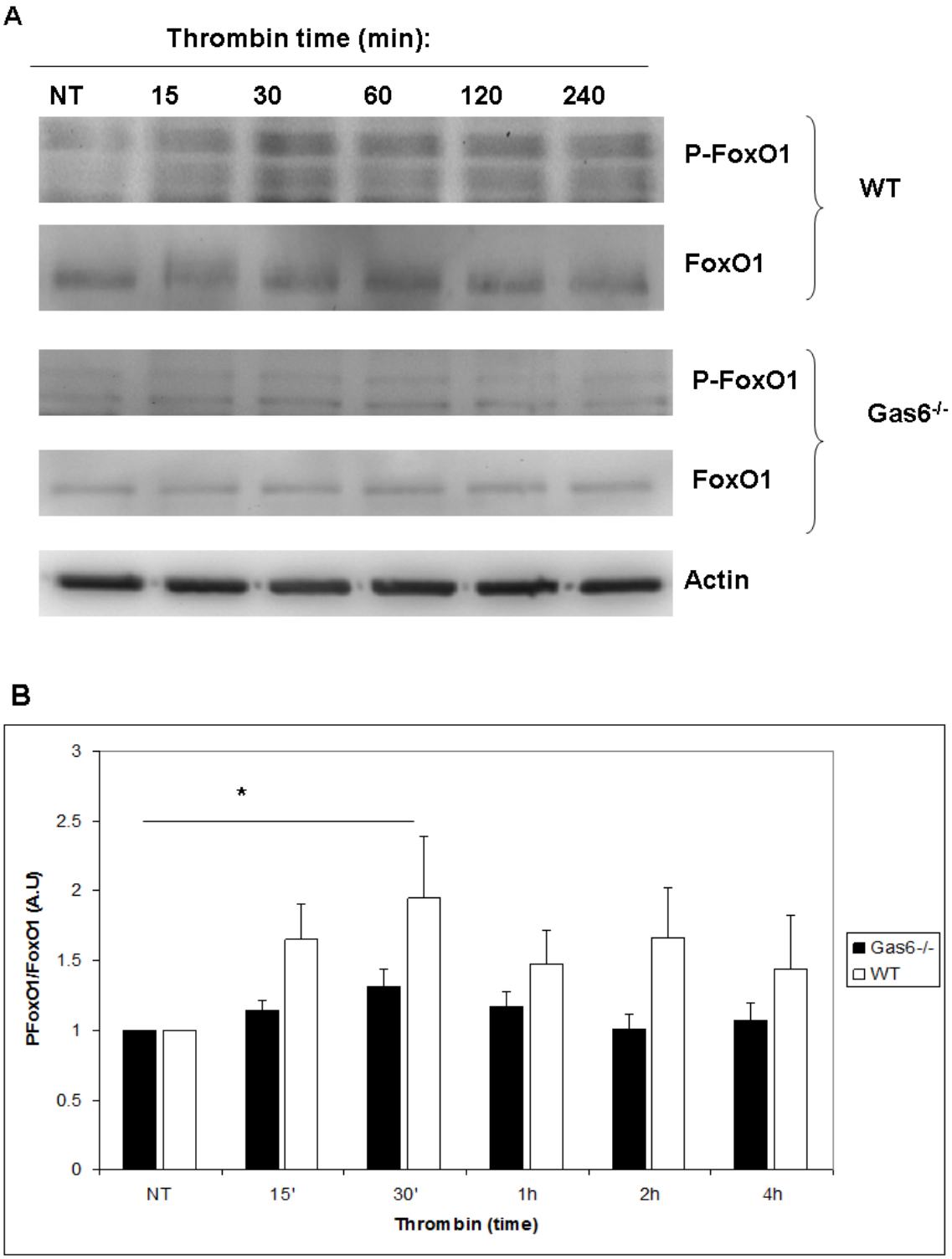


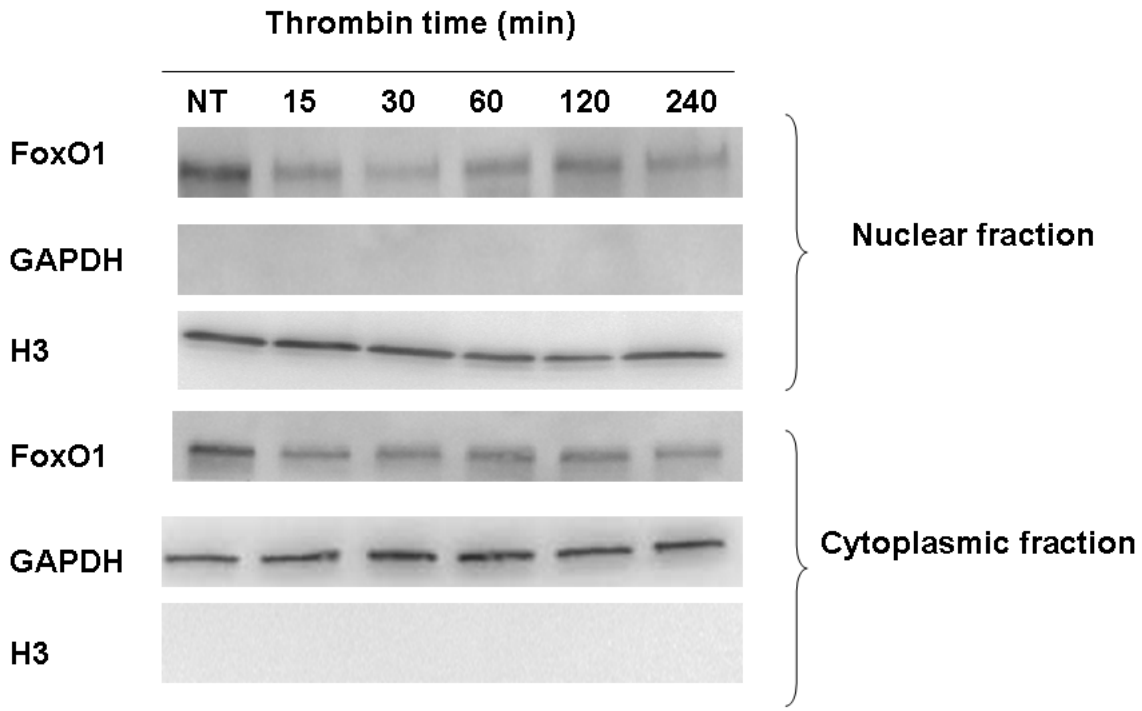
Figure 3-5. Thrombin induced phosphorylation of FoxO1 is blunted in the absence of Gas6. (A) Representative immunoblot showing maximal phosphorylation of FoxO1 (appears as a doublet) at 30 minutes following treatment with thrombin in WT and Gas6$^{-/-}$

cells. **(B)** Quantification of the pFoxO1/FoxO1 ratio shows FoxO1 phosphorylation occurs to a lesser degree in Gas6$^{-/-}$ endothelial cells.

Thrombin induced nuclear exclusion of FoxO1 is blunted in Gas6$^{-/-}$ endothelial cells.

Thrombin induced phosphorylation of FoxO1 that was maximal at 30 minutes. This prompted us to observe the subcellular location of the protein over the course of thrombin treatment. As expected, thrombin induced nuclear exclusion of FoxO1 that was maximal at 30 minutes. This was observed by immunoblot analysis of nuclear and cytoplasmic fractions from WT and Gas6$^{-/-}$ endothelial cells. Consistent with the phosphorylation data, we observed maximal nuclear exclusion of FoxO1 at 30 minutes post thrombin treatment. A representative experiment from WT cells, including controls for both cytoplasmic and nuclear fractions is shown in Fig 3-6A. At 30 minutes post thrombin treatment the Nuclear/Cytoplasmic (N/C) ratio of FoxO1 in WT cells was 0.64 ± 0.08 compared to untreated ($n=3$, $p < 0.05$). In addition, in the Gas6$^{-/-}$ cells, the N/C ratio at 30 minutes was 0.98 ± 0.01 indicating a degree of nuclear retention of FoxO1 compared to WT cells ($n=3$, $p < 0.05$) (Fig 3-6B).

A


Thrombin time (min)
NT
15
30
60
120
240
FoxO1
GAPDH
H3
Nuclear fraction
FoxO1
GAPDH
H3
Cytoplasmic fraction

B

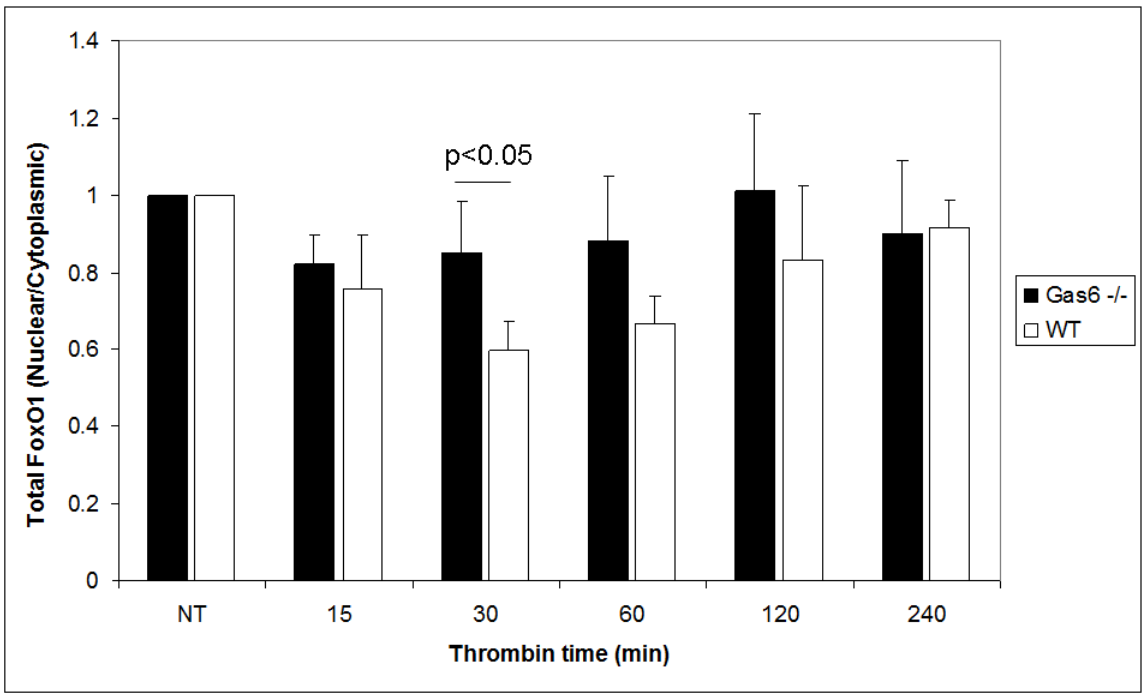

1.4
1.2
1
0.8
0.6
0.4
0.2
0
p<0.05
Total FoxO1 (Nuclear/Cytoplasmic)
NT
15
30
60
120
240
Thrombin time (min)
Gas6 -/-
WT

Figure 3-6. Thrombin induced nuclear exclusion of FoxO1 is blunted in Gas6$^{-/-}$ endothelial cells. (A) Representative immunoblots from cellular fractionation experiments show subcellular location of FoxO1 over the course of thrombin treatment. **(B)** Quantification of the nuclear/cytoplasmic ratio of FoxO1 show nuclear retention in the absence of Gas6.

3.9 Discussion

The present work serves as a starting point in understanding the signalling pathways involved in Gas6 mediated induction of VCAM-1 on endothelial cells. We hypothesized that the transcription factor FoxO1 is partially responsible. The induction of VCAM-1 is an important event in the inflammatory response of many disease states [259]. Gas6 has the ability to promote the induction of VCAM-1 in response to inflammatory stimuli. When treated with TNF-α, VCAM-1 induction was blunted in Gas6$^{-/-}$ cells [180]. By qPCR, we observed the same phenomenon when Gas6$^{-/-}$ endothelial cells were challenged with thrombin. We hypothesized that FoxO1 is involved in activation of endothelial cells downstream of Gas6. Based on published data from our lab, Gas6 affects the transcriptional activity of FoxO1 via Akt and protects endothelial cells from apoptosis in a FoxO1 dependent manner [111].

As is the case with Gas6, FoxO1 has recently emerged as a mediator of inflammatory responses. For example, FoxO1 promotes the induction of TLR4 [268] and IL-1β [269] in macrophages, the proinflammatory cytokines MCP-1 and IL-6 in adipocytes [270], and of course, VCAM-1 in endothelial cells [237]. In regards to our

hypothesis, thrombin is known to induce the expression of endothelial VCAM-1 [257]. More recently, however was the identification of thrombin as being able to regulate FoxO1 signalling in VSMCs [267].

We began by determining if FoxO1 is involved in thrombin mediated upregulation of VCAM-1. To do so, we observed the effect of FoxO1 knock-down on the thrombin response to VCAM-1 induction. FoxO1 was reproducibly knocked down by 24-48 hours, with levels beginning to increase by 72 hours. In cells that were deficient in FoxO1, basal levels of VCAM-1 mRNA were induced implicating the role of FoxO1 as transcriptional repressor in mediating induction of VCAM-1. Also, by qPCR we have observed that VCAM-1 induction by thrombin is blunted in $Gas6^{-/-}$ cells.

We have therefore shown that the levels of endothelial VCAM-1 mRNA are dependent on both FoxO1 and Gas6. We then began a series of experiments in which we observed thrombin mediated signalling of FoxO1 in WT and $Gas6^{-/-}$ endothelial cells. Consistent with the recent work by Mahajan *et al*., thrombin induces time dependent phosphorylation of FoxO1 in endothelial cells [267]. In our cells, FoxO1 phosphorylation was rapid, being induced by 15 minutes and was maximal at 30 minutes. Interestingly, the response to thrombin in $Gas6^{-/-}$ endothelial cells was blunted. While the same overall trend in FoxO1 phosphorylation was observed, the effect of thrombin was lower in $Gas6^{-/-}$ endothelium. Arguably the most significant consequence of FoxO1 phosphorylation is its capacity to bind the 14-3-3 proteins resulting in their accumulation in the cytoplasm, thereby shutting off their transcriptional ability.

Having shown that thrombin induced phosphorylation of FoxO1 was lower in Gas6$^{-/-}$ endothelial cells, the following step was to evaluate the subcellular location of FoxO1 over the course of thrombin treatment. We hypothesized that lower phosphorlyation of FoxO1 would impart a degree of nuclear retention in the Gas6$^{-/-}$ cells. Consistent with the phosphorylation experiments, thrombin induced nuclear exclusion of FoxO1 which was maximal at 30 minutes and in Gas6$^{-/-}$ endothelium, nuclear exclusion occurs to as lesser degree.

At the time of writing, this study has only begun to reach the stage where focus will turn to the VCAM-1 promoter. It will be necessary to demonstrate by chromatin immunoprecipitation (ChIP) assays the physical interaction between FoxO1 and the VCAM-1 promoter. It will be necessary to observe this interaction over the course of thrombin treatment and to determine if Gas6 ablation does in fact alter these binding kinetics. At this point we can only propose a plausible mechanism that will serve to explain how FoxO1 mediates the ability of Gas6 to promote VCAM-1 induction. Here we can propose that thrombin stimulation downstream of the PAR receptor creates a scenario which leads to nuclear exclusion of FoxO1 likely via Akt. As FoxO1 is a nuclear repressor of VCAM-1, its exclusion from the nucleus should serve to increase the activity at the VCAM-1 promoter. In the Gas6-/- cells we propose that initial exclusion of FoxO1 from the nucleus is lower resulting in lower promoter activity. We can also hypothesize that in WT endothelial cells, thrombin and secreted Gas6 cooperate to exclude FoxO1 from the nucleus. This is plausible based on the observations that thrombin induces

secretion of Gas6 from intracellular stores in platelets [187, 190] and that Gas6 is sufficient in causing nuclear exclusion of FoxO1 [111]. Based on these observations, we can also hypothesize that thrombin induces secretion of Gas6 from endothelial cells and synergistically mediates (with thrombin) nuclear exclusion of FoxO1. Obviously, this cannot occur in Gas6$^{-/-}$ endothelial cells and explains why FoxO1 phosphorylation and nuclear exclusion is lower in these cells.

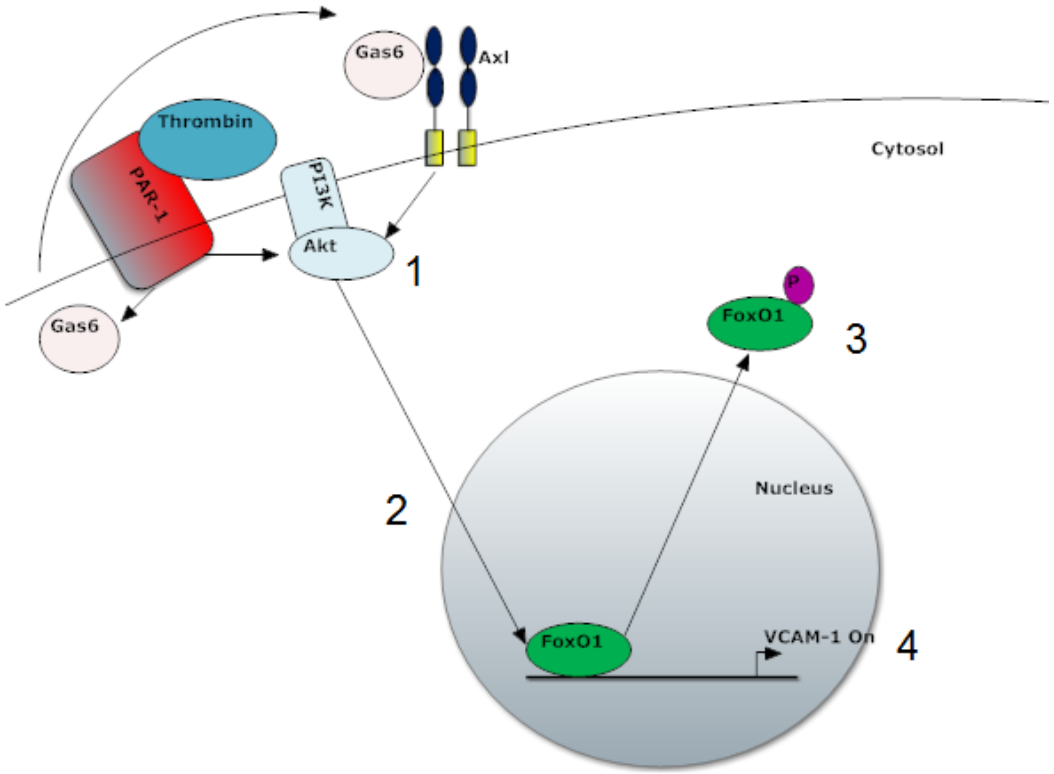


Figure 3-7. Proposed Model of FoxO1 dependent VCAM-1 Induction by Thrombin and Gas6. In this proposed model, (1) thrombin activates PAR-1 which leads to nuclear exclusion of FoxO1 following activation of PI3K/Akt. Gas6 similarly activates PI3K/Akt downstream of Axl which leads to nuclear exclusion of FoxO1. (2) Phosphorylated Akt travels to the nucleus and phosphorylates FoxO1. (3) FoxO1 which acts as a repressor of the VCAM-1 promoter is excluded from the nucleus in its phosphorylated form. (4) Transcription of the VCAM-1 gene. Included is the possibility that Gas6 synergizes with the signalling cascade induced by thrombin by converging at the level of PI3K/Akt

activation. Thrombin may also induce Gas6 secretion, causing further nuclear exclusion of FoxO1. The lack of secreted Gas6 in the medium of $Gas6^{-/-}$ cells may explain the lower levels of pFoxO1 observed in our experiments.

CHAPTER 4:
GENERAL DISCUSSION AND CONCLUSIONS

Gas6 is unique among the vitamin-K dependent family of proteins in that it has no direct role in blood coagulation. Another feature that distinguishes Gas6 from its vitamin-K dependent family members is that it serves as the molecular ligand for a family of receptor tyrosine kinases, thus exerting effects typically associated with growth factors [187]. Following its identification in the late 1980s [109] and characterization in the early 1990s [110], Gas6 has emerged as having numerous and diverse roles among the different organs systems and in disease. Gas6 has been of particular interest to the vascular biologist since its expression was found in vascular smooth muscle and endothelial cells [177]. Subsequently, an article published within the pages of *Nature Medicine* detailed the unique ability of Gas6 to promote platelet aggregation. Mice with a genetic ablation of Gas6 were protected against various forms of experimental thrombosis and in addition did not present a bleeding phenotype. These observations had important clinical implications. Venous thromboembolism, which manifests as deep vein thrombosis and pulmonary embolism is a significant cause of clinical morbidity and mortality. Anticoagulation therapy is used as prophylaxis and for treatment of DVT/PE. An example of anticoagulation therapy would be the drug Warfarin that acts by inhibiting the vitamin-K dependent coagulation factors II, VII, IX and X [271]. One of the inherent risks of anticoagulation therapy is the increased risk of bleeding. The fact that Gas6

ablation protected mice from thrombosis without the hemorrhagic side effect makes it an ideal candidate target for antithrombotic therapy. Over a decade has passed since this idea was proposed, but unfortunately there has been little advancement in our understanding of the role of Gas6 in the pathophysiology of venous thrombosis. We have since come to learn the importance of the Gas6 receptors in thrombus stabilization [190] and of Gas6 in endothelial cell activation [180]. There have been attempts, some successful, some not, at detecting Gas6 in human platelets and plasma and determining what role it has in human platelet activation. As previously discussed this remains a controversial issue.

A careful examination of the work by Angellilo-Scherrer *et al*., that described the platelet defect in the $Gas6^{-/-}$ mice which manifests as protection from thrombosis, reveals a discrepancy between the dramatic clinical phenotype versus a rather subtle platelet defect. According to their data, the $Gas6^{-/-}$ mice were protected against all forms of experimental thrombosis. To explain this phenomenon was the observation that $Gas6^{-/-}$ platelets were unable to completely aggregate in response to the agonist ADP at only the concentration of 5.0 μM[187]. This particular observation led to our hypothesis that other mechanisms are involved to explain the phenotype. Given the observations of the role of Gas6 in aspects of vascular cell biology [131, 264], we hypothesized that vascular specific Gas6 contributes to thrombogenesis *in vivo*.

We established two models of Gas6 chimerism to explore this possibility. Using bone marrow transplantations and a model of platelet depletion and reconstitution, we were able to show that Gas6 from both the vasculature and hematopoietic compartments

contribute to thrombogenesis. We also showed that Gas6 positively regulates the expression of TF on vascular cells using both *in vivo* and *in vitro* techniques.

Two important findings from the research in Chapter 2 are that vascular Gas6 contributes to *in vivo* thrombogenesis and promotes the upregulation of TF. It must be stressed that these are two independent findings. Our data does not necessarily prove that TF induction by Gas6 is the mechanism whereby vascular Gas6 contributes to thrombogenesis. At this point, we can only hypothesize that it does. Further research will be required to consolidate these findings. Although this study ends with a rather important question that remains unanswered, we have nonetheless provided scientific evidence warranting the continuing characterization and study of vascular Gas6 and its potential targeting for antithrombotic therapy in humans.

Clinical targeting of Gas6 will obviously pose a new set of challenges. Both venous thromboembolism and arterial thrombosis can lead to life threatening events. These include pulmonary embolism and myocardial infarction, respectively. Venous thrombi are formed under conditions of low shear and are composed mainly of fibrin and trapped erythrocytes with few platelets. In contrast, arterial thrombi which are formed under high sear are formed of platelet aggregates and contain less fibrin. Therapies designed to inhibit arterial thrombogenesis focus on inhibiting platelet function, while therapies targeting venous thrombogenesis involve the coagulation cascade. There are limitations to the use of these therapies. Therefore, new antithrombotic drugs must be developed to overcome these limitations [272]. An important consideration in the

development of an antithrombotic drug is that it be targeted to a potential site of thrombus development as prophylaxis or to the site of an already existing thrombus. The data presented within this thesis raises the possibility of Gas6 derived from vascular cells (i.e endothelium) as a potential target for future antithrombotic therapies. The endothelium is a key target for anti-thrombotic intervention and the main goal of targeting the endothelium is for the purpose of prophylaxis. One can envision a small inhibitor of Gas6 that is chemically conjugated to an antibody directed against molecules that are expressed constitutively on the endothelial cell surface such as PECAM-1 or ICAM-1. Such a construct would also ideally be infused locally as to avoid accumulation in the pulmonary circulation [273].

There remain several unanswered questions that could serve as a focus of future experiments in the laboratory. For example, selective ablation of Gas6 from endothelial cells in mice using a Cre-lox recombinase strategy could add further insight to the source of Gas6 that is responsible for promoting thrombogenesis by $FeCl_3$ injury. It may also be interesting to explore the possibility that Gas6 regulates the expression of other proteins involved in blood coagulation. The data generated within this dissertation shows that TF activity is positively regulated by Gas6 but perhaps Gas6 also regulates the expression of TFPI or enzymes of the fibrinolytic system. Our data seems to suggest Gas6 regulates *de novo* synthesis of TF in endothelial cells. It may also be worthwhile to design experiments based on studying the direct interaction between activated platelets and endothelial cells. It is known that activated platelets signal directly to endothelial cells via CD40 on platelets and CD40L on endothelial cells, triggering an endothelial

inflammatory response [274]. Given the established role of Gas6 in activation of platelets, it is possible that this represents an additional mechanism whereby Gas6 triggers TF induction on endothelial cells.

In 2008, Tjwa *et al*. reported that endothelial cells with genetic ablation of Gas6 have a reduced ability to mount an activation response to inflammatory stimuli [180]. In Chapter 2 we were able to demonstrate that Gas6 ablation impairs the ability of endothelial cells to respond to thrombin stimulation. With these findings in mind, it is surprising that there have been virtually no attempts at understanding why this occurs and through which signalling cascades. In Chapter 3, we introduced the possibility of FoxO1 being a downstream mediator of Gas6 induced endothelial cell activation. Our reasoning for pursuing this hypothesis stems from various sources. Expression of the endothelial cell activation marker VCAM-1 has been shown to be regulated by both Gas6 [180] and FoxO1 [237]. Furthermore Gas6 regulates the transcriptional activity of FoxO1 downstream of PI3K/Akt activation [111]. We thus proposed a hypothetical signalling axis whereby Gas6 modulates thrombin mediated induction of VCAM-1 via the transcription factor FoxO1. As a means of maintaining consistency with the published observation that TNF-α induced expression of VCAM-1 is blunted in $Gas6^{-/-}$ endothelial cells, we devised an experimental system where we would observe the thrombin mediated shuttling dynamics of FoxO1 in WT and $Gas6^{-/-}$ endothelial cells for evidence that would support our hypothesis.

Thrombin was very recently shown to time-dependently phosphorylate FoxO1 in vascular smooth muscle and contribute to cell proliferation [267]. Here, we have shown that thrombin also phosphorylates FoxO1 in a time-dependent manner in murine endothelial cells. Additionally, FoxO1 phorphorylation resulted in its nuclear exclusion. These events downstream of thrombin are blunted in $Gas6^{-/-}$ endothelial cells. The blunted induction of phosphorylation and nuclear exclusion in the $Gas6^{-/-}$ could be the result of the lack of secreted Gas6 into the culture supernatant. Exogenous Gas6 has been shown to mediate both phosphorylation and nuclear exclusion of FoxO1 [111]. Therefore it is possible that FoxO1 is a convergence point downstream of both thrombin and Gas6 and that they act synergistically to phosphorylate and exclude it from the nucleus.

The work presented here adds to our body of knowledge about Gas6, its role in thrombus formation and the signalling cascades that mediate its functions. While it will be some time before Gas6 is completely understood, and targeting it for antithrombotic therapy is realized, this work should bring us closer to achieving these goals and hopefully serves as a point for others to build on.

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