ABERRANT AND ALTERNATIVE SPLICING OF VON WILLEBRAND FACTOR

by

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von Willebrand disease (VWD) is the most commonly inherited bleeding disorder in humans and is the result of quantitative deficiencies or qualitative defects of von Willebrand factor (VWF). VWD can be caused by a wide variety of mutations all throughout the VWF gene, the majority of which are missense changes. Approximately 10% of pathologic VWF mutations are thought to disrupt the process of VWF splicing leading to VWD; however, this primarily acknowledges canonical splice site mutations, where the nucleotides are most integral and highly conserved to accomplish proper splicing. We hypothesized that pathologic splicing of VWF is likely an under-recognized mechanism of VWD, caused by variation outside of canonical splice sites.

This thesis characterized three VWF mutations acting through pathologic splicing defects using plasma and patient-derived blood outgrowth endothelial cells (BOEC). In Family 1 it was found that the c.3538G>A mutation in exon 26 induces transcription of three in-frame aberrant splice forms (1) skipping exon 23, (2) skipping exon 26, (3) skipping exons 23 & 26 together, leading to Type 1 VWD. In Family 2, the canonical c.5842+1G>C mutation causes VWD through production of intracellularly retained in-frame VWF, skipping exons 33-34. The affected family also produces a transcript skipping exon 33 which was found in three normal BOEC lines at 13±0.2%. Family 3 has an intronic branch site c.6599-20A>T mutation which causes Type 1 VWD through nonsense mediated decay (NMD) of the premature termination codon (PTC) containing VWF transcript skipping exon 38. In-frame aberrant transcripts were found to be decreased under high shear stress, whereas PTC-inducing transcripts were increased.

The aberrant splice forms identified in these families were further characterized in a heterologous cell expression model using human embryonic kidney (HEK)293T cells for VWF expression, secretion, functionality and intracellular trafficking. All mutants except for skipping exon 33 showed reduced expression and secretion and most colocalized with the endoplasmic reticulum. In-frame skipping of exons 33-34 was the only mutant that was able to multimerize and retain functional platelet, collagen, and Factor VIII (FVIII) binding without co-expression of wildtype (WT) VWF.

Alternative splicing is known to occur in approximately 95% of mammalian multi-exon genes; however, no alternative splice variants have been described for VWF thus far. We hypothesized that given the large size of the
VWF gene alternative splice variants likely exist. This thesis investigated the possibility of alternative VWF splicing in normal endothelial cells (EC)s using mRNA from BOEC, human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) grown in unstimulated static conditions or after stimulation by estrogen, histamine, plasma, DDAVP, and 15 dynes/cm² laminar flow. The expression profiles of these EC varied by cell line; however, shear stress appeared to consistently modulate the expression of both in-frame and PTC-inducing alternative splicing transcripts.

The results presented in this thesis provide insight into VWF biology and biosynthesis, through the removal of skipped exons and VWF trafficking, and gleans insight into potential alternative VWF splice forms which may be regulated by shear stress and act to control VWF expression or serve other functions in vivo.
Co-Authorship

Dr. Paula James supervised research and assisted with manuscript writing and editing.

Dr. Georges-Etienne Rivard, Dr. Mary-Frances Scully, and Dr. Man Chiu Poon helped organize patient phlebotomy.

Research technologist Angie Tuttle performed VWF multimer, VWF:RCo and FVIII:C assays.

Jeff Mewburn and Matt Gordon provided technical assistance acquiring confocal imaging and flow cytometry data.

All other work is my own.
Acknowledgements

Now that this adventure is coming to a close, I find myself looking back on it with a much different perspective than when I began. I have found myself noticing how much I have changed over these past five years and recognizing all the people who have helped me grow along the way. I have overcome a great deal of self-doubt and uncertainty, and I know I would not be here without the continued support of many generous and loving people.

My supervisor, Dr. Paula James, has always encouraged me in my passions, both academic and personal, helping me to have a positive outlook, not only in my scientific endeavours, but also in myself. Thank you Paula, for all that you have done.

I have learned many lessons and made many great friendships throughout these five years. To all my friends old and new, thank you for your camaraderie and your support.

Last but certainly not least, my family has always encouraged my academic pursuits, with my father’s iconic early words of “stay in school”, little did they all know how much it would stick, but here I am twenty-five years later and still in school. My husband, Benjamin, has been an unwavering support to me, lending his ears and his shoulders to my woes and doubts, reminding me that I’m stronger than I think I am, that I will get through this, and that I can make it to the light at the end of the tunnel. My mother and sister have always been my cheering squad and they supported me through all the ups and downs. To all of my family who have travelled this long and winding road with me, thank you for always believing in me, even when I did not.
Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

(Lindsey Gail Hawke)

(August, 2015)
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<th>Full Form</th>
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<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ADAMTS-13</td>
<td>a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 13th member</td>
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<td>Ang-2</td>
<td>angiopoietin-2</td>
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<td>APC</td>
<td>activated protein C</td>
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<tr>
<td>ASBT</td>
<td>apical sodium dependent bile acid transporter</td>
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<tr>
<td>aVWS</td>
<td>acquired von Willebrand syndrome</td>
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<tr>
<td>BAT</td>
<td>bleeding assessment tools</td>
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<td>BBP</td>
<td>branchpoint binding protein</td>
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<td>β-gal</td>
<td>betagalactasidase</td>
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<td>BOEC</td>
<td>blood outgrowth endothelial cell</td>
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<td>bp</td>
<td>base pairs</td>
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<td>BRCA1</td>
<td>breast cancer early onset 1 gene</td>
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<td>BS</td>
<td>bleeding score</td>
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<td>bovine serum albumin</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator</td>
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<td>CK</td>
<td>cysteine knot</td>
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<td>CPT</td>
<td>cell preparation tubes</td>
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<td>cTNT</td>
<td>striated muscle-specific cardiac tromonic T</td>
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<td>DAPI</td>
<td>4'-6-diamidino-2-phenylindole</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
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<td>deoxyribonucleic acid</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>ECFC</td>
<td>endothelial colony forming cells</td>
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<td>EDTA</td>
<td>ethylenediaminetetracetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>ERKs</td>
<td>extracellular signal-regulated kinases</td>
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<td>ESE</td>
<td>exonic splicing enhancer</td>
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<td>epithelial splicing regulatory proteins</td>
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<td>FIXa</td>
<td>activated factor IX</td>
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<td>fragile X mental retardation gene</td>
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<td>HMWM</td>
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<td>hnRNP</td>
<td>heterogeneous nuclear ribonucleoprotein</td>
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<td>human microvascular endothelial cells</td>
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<td>ICAM-1</td>
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<td>c-Jun N-terminal kinases</td>
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SDS  sodium dodecyl sulfate
SF   splicing factor
SMN2 survival motor neuron 2
SNP  single nucleotide polymorphism
snRNP small nuclear ribonuclear protein
SR   serine rich
SSRE shear stress responsive elements
TF   tissue factor
TNFα tumor necrosis factor α
TRIS trisaminomethane
TSP-1 thrombospondin-1
ULMWM ultralarge molecular weight multimers
VCAM-1 vascular cell adhesion molecule-1
VE-cadherin vascular endothelial cadherin
VEGF vascular endothelial growth factor
VEGFR2 vascular endothelial growth factor receptor 2
VWD von Willebrand disease
VWF von Willebrand factor
VWF human von Willebrand factor gene
VWF:Ag von Willebrand factor antigen
VWFpp von Willebrand factor propeptide
VWF:RCo von Willebrand factor ristocetin cofactor activity
WBP Weibel-Palade body
WT  wildtype
CHAPTER 1
A General Introduction to Splicing, von Willebrand Factor (VWF) & von Willebrand Disease (VWD)

1.1 Splicing: The Ins & Outs

Splicing is a critical and strictly regulated cellular process. Generally, deoxyribonucleic acid (DNA) sequences contain regions of protein coding information (exons) interspersed throughout noncoding sequences (introns). When a gene is to be expressed, both introns and exons are transcribed into a pre-mRNA sequence; however, these intervening intronic sequences need to be removed and the exons joined together in a cohesive mRNA sequence. This process is known as splicing. A complex series of biochemical reactions between the nucleotide sequences and multiple different proteins which bind to them are involved in the splicing process. Specific highly conserved splice site sequences are found at the junctions between introns and exons. The 5’ splice site, known as the donor site, is located at the junction between an exon and an intron, with the intron usually beginning with a GT dinucleotide (Mount 1982). The 3’ splice site, or acceptor site, is located at the junction between an intron and an exon (Jian et al. 2014), with the intron generally ending in an AG dinucleotide (Mount 1982) (Figure 1.1). These most highly conserved dinucleotide sequences are integral canonical nucleotides that are surrounded by several bases of variable conservation which mark the donor and acceptor splice sites (Mount 1982). Approximately 99% of introns follow the GT-AG rule for intronic ends; however introns beginning with the dinucleotide AT and ending in AC have been shown as well (Jackson 1991; Burset et al. 2000). Upstream of the acceptor site, there are semi-conserved polypyrimidine sequences and further upstream there is a region known as the branch site. This branch site is generally found up to 30-50 nucleotides upstream of an exon and the 3’ splice site and contains an integral adenosine nucleotide known as the invariant A (Yeo et al. 2004, Jian et al. 2014). Many other nucleotide sequences, usually 4-18 nucleotides long, known as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic
splicing silencers (ISSs) also contribute to proper splicing of the pre-mRNA through trans-acting elements that bind to these regions (Blencowe 2000, Gravely 2000, Caceres & Kornblihtt 2002, Woodley & Valvarcel 2002, Cartegni et al. 2002, Fairbrother et al. 2002) (Figure 1.1). A complex of trans-acting elements known as the spliceosome is responsible for mediating splicing reactions. This complex ribonucleoprotein megaparticle is comprised of five small nuclear ribonucleoproteins (snRNPs): U1, U2, U4, U5, U6 and two ancillary factors U2AF65 and U2AF35. RNA-dependent ATPases and helicases aid in making the spliceosome catalytically active allowing the spliceosome to perform two transesterification reactions required to excise the introns and join the remaining exonic sequences. Initially the branch site is bound by the branchpoint-binding protein (BBP), the U1 snRNP binds the 5' splice site and the two ancillary U2AF spliceosome proteins bind the 3' splice site and the polypyrimidine tract (Wahl et al. 2009) (Figure 1.1). The bound U1 snRNP and several other trans-acting splicing factors such as SF3 and SF3b promote the binding of the U2 snRNP to the invariant A in the branch site forming a complex known as the pre-spliceosome (Zhuang et al. 1988). A tri-snRNP complex comprised of U4, U5 and U6 snRNPs integrates into the pre-spliceosome mediated by splicing factor SPF30 (Meister et al. 2001; Rappsilber et al. 2001) and initiates a series of RNA:RNA rearrangements directed by RNA helicases in the DExD/Hbox family (Staley & Guthrie 1999). As U4 is removed from the complex, the U6 snRNP is able to displace the U1 snRNP from the donor site and interact with U2 which associates the donor and branch sites, and a transesterification reaction forms a lariat with the intron (Tazi et al. 2009). The U5 snRNP then uses non-canonical interactions to form a new phosphodiester bond between the last nucleotide of the upstream exon and the first nucleotide of the downstream exon which releases the intron from the sequence completing the splicing reaction (Tazi et al. 2009). Upon completion of the splicing reaction, more proteins comprising the exon junction complex are deposited at the site of the joined exons and the mRNA is transported to the cytoplasm (Dreyfuss et al. 2002, Maquat 2004, Tange et al. 2004) (Figure 1.1). Just as
Figure 1.1 Splicing of Pre-mRNA. Splice Site= SS; BP= Branch Point, ESE= Exonic Splicing Enhancer, ESS= Exonic Splicing Silencer, EJC= Exon Junction Complex. The trans-acting spliceosome U1-6 snRNPs, auxiliary protein U2AF65, and ESEs and ESSs work to remove the intervening intronic sequences through forming a lariat with the intron and linking exonic sequences. A) Pre-mRNA sequences are transcribed. B) Splicing enhancers & silencers bind their cis-sequences helping 3’, 5’ SS and branch site recognition. U1 binds the 5’SS, U2AF65 binds the 3’SS and polypyrimidine tract, and U2 binds the branch site. C) The U4, U5, U6 complex incorporates into the spliceosome. D) As U4 is removed, U6 displaces U1 at the 5’ SS associating with U2 and forming an intron lariat. E) Exon ligation is facilitated by U5 interactions. F) An EJC is deposited at the ligation site.
there are some non-canonical splice site sequences, a different set of splicing machinery that is 100 fold less abundant exists to recognize these uncommon splice site sequences. This pathway reflects the commonly used spliceosome machinery but uses different snRNPs (U11, U12, U4atac, U6atac, and U5) (Patel & Steitz 2003).

Other than the spliceosome, trans-acting elements involved in splicing generally fall into two classes: serine rich (SR)-proteins, and heterogeneous nuclear ribonucleoproteins (hnRNPs). These trans-acting proteins are present in vast numbers and their relative stoichiometry is important when determining splicing outcomes for many genes (Smith & Valcarcel 2000). SR-proteins have a domain rich in serine and arginine, an RNA-binding domain, and a protein interaction domain (Tazi et al. 2009). These proteins commonly interact with low specificity to enhancer sequences surrounding splice sites through their RNA binding domain and help to recruit the snRNPs of the spliceosome. The low specificity of these enhancer elements enables them to bind to a variety of exonic and intronic sequences while their protein binding domains help localize the core elements of the spliceosome in RNA:RNA interactions with the RNA, and other trans-acting proteins. There are well known interactions between SR-proteins and the U1 snRNP binding to the 5’ donor site, as well as recruiting U2AF to the polypyrmidine tract upstream of the 3’ acceptor site. On the other hand, hnRNPs generally interact with silencing elements of the RNA through their RNA-binding domain and act to block exon recognition (Zhu et al. 2001). These negative regulators of splice site recognition may compete with enhancer elements for nearby binding sites (Zhu et al. 2001).

Splicing can occur in different manners depending on how the spliceosome recognizes the splice sites. Knowledge of most splicing mechanisms were elucidated in yeast where the introns are short and interrupted by larger exons; when this is the case, the spliceosome can recognize the conserved splice sites and form directly around the intron (Talerico & Berget 1994). This process is called intron recognition. This schematic however is not always applicable in human genes where introns tend to be longer and
exons are shorter sequences therefore the spliceosome tends to form around the exons in a process known as exon recognition (Berget 1995). Both intron and exon recognition models require interactions from additional trans-acting proteins such as splicing-associated SR proteins, which bridge the sequences between neighbouring splice sites and bring spliceosome components together (Gravely 2000).

Length of introns and exons plays into splicing in more ways than determining the recognition system used by the spliceosome. The process of splicing has been found to occur as a co-transcriptional process where nascent transcripts were found to contain splicing loops using electron microscopy (Beyer & Osheim 1988). Pausing of RNA-polymerases while transcribing long exons, particularly the terminal exon, allows for co-transcriptional splicing of the preceding introns (Oesterreich et al. 2010). This pausing of RNA-polymerase and recruitment of additional splicing factors whether enhancer or silencer proteins can have an influence on splicing patterns which can lead to the common process of alternative splicing. The rate at which introns are removed is dependent on the rate to which splice sites are exposed after transcription and the binding of appropriate trans-elements which help in the recognition of the splice sites (Kornblihtt et al. 2013). Additionally, long introns can be processed through recursive splicing, a process by which cryptic splice sites throughout the intron are used to remove the intron in smaller chunks as the splicing machinery gains access to the splice site sequences (Sibley et al. 2015).

1.1.1 In Silico Splicing Analyses

As knowledge of genetic structure, splicing factors, and the spliceosome grows, so does our ability to predict splicing events. Currently there are many software programs that can be used to perform in silico analyses of genetic sequences to estimate splicing outcomes and the potential for genetic mutations to illicit changes to splicing patterns. Training sets of true splice sites are used to generate frequency tables which assess the probability of each base at each position in a sequence belonging to a splice site (Staden 1984, Harr et al. 1983). The score of potential sites are determined by summing the scores of individual
bases within a defined proximity. Newer technologies incorporate the use of decision trees to aid in
discrimination between true splice sites and false sites in the neighbouring vicinity (Thanaraj & Robinson
2000); however, when calibrated to identify 95% of true sites, approximately six false positives are also
identified per kilobase (kb) (Thanaraj & Robinson 2000). Detecting the -2, -1, +1, +2 positions of the intron-
exon boundaries generally have a sensitivity between 86-92% and specificity of 73-93% across the main
splicing analysis programs such as NNSplice, Max Ent Scan, Gene Splicer, and Splice Site Finder Like
(Jian et al. 2014). Both NNSplice and Max Ent Scan retain a sensitivity >80% for nucleotides outside of
these 4 canonical nucleotides (Jian et al. 2014).

1.1.2 Alternative Splicing

Alternative splicing is a fundamental process where multiple mRNA variants are produced off of a
single gene and were discovered in adenoviruses alongside the process of splicing (Chow et al. 1977;
Berget et al. 1977). This mechanism is a key crossroad of expression regulation between transcription and
translation which is known to affect approximately 95% of mammalian genes (Pan et al. 2008; Barash et al.
2010). This process is particularly integral in vertebrates, which have a similar number of protein-coding
genes as invertebrates; therefore alternative splicing is likely responsible for their added complexity.
Distributions of alternative splicing isoforms are known to be tissue specific and dependent on
developmental stage (Barash et al. 2010). In mechanocytes, cells which respond to mechanical signals
such as endothelial cells, alternative splicing has been found to be controlled by mechanical stressors (Liu
& Tang 2013). Mechanical stretching has shown to regulate alternative splicing of insulin-like growth factor
1 (IGF-1), inducing its mechano-growth factor (MGF) splice form in osteoblasts, neurons, cardiac muscle
The complexity of the splicing process described above holds true, and in fact facilitates the process of alternative splicing. Due to the variability in consensus splice site sequences, each site has a varying strength for being chosen as the site for splicing, where strong sites are those most adapted to the consensus sequences and are more efficiently recognized. Constitutive splicing generally occurs at strong splice sites. The degree to which a weak splice site is used depends on the context in which it is found, such as its proximity to a strong site. Alongside this variable sequence, the interactions with trans-acting enhancer and silencer elements play a strong role in the determination of which sites are chosen.

There are four main methods of alternative splicing that can occur: (1) exon skipping where exons are removed along with their flanking introns, (2) alternative 5' splice site selection, or (3) alternative 3' splice site selection, which can either include a portion of an intron, or exclude a portion of the exon at either end of the exon, and (4) intron retention, where the full intron is retained in the mature mRNA molecule (Sugnet et al. 2004, Kim et al. 2007) (Figure 1.2). Additional splicing variants are possible with alternative transcription start sites and poly-adenylation sites (Breitbart et al. 1987, Suzuki et al. 2001, Black 2003) (Figure 1.2). Exon skipping is the most prevalent type of alternative splicing in higher eukaryotes (Kim et al. 2007). These alternatively produced protein isoforms can have variable functions; for example, alternative splicing of the calcium activated potassium channel gene, Slo, is thought to produce ~500 different mRNA variants through changes in exon inclusion which each contribute to the sensitivity of sound recognition and assessment in the hair cells of the inner ear (Black 1998, Graveley 2001). Alternative splice forms can also exhibit opposite effects. For example, splicing of the FAS gene’s sixth exon determines whether the transcript will produce a membrane-bound pro-apoptotic ligand by including exon 6, or if excluded from the mRNA sequence, soluble apoptosis antigen-1 is produced which exhibits a pro-survival phenotype (Sehgal et al. 2014).
Splice site selection is strongly influenced by bound trans-acting proteins. It has been shown that cellular concentrations of splicing factors and whether there is an abundance of negative regulators such as hnRNP A1 or positive splicing regulators such as ASF/SF2 can determine which splice sites are used and thus which splice isoforms are produced (Mayeda & Krainer 1992). When concentrations of hnRNP A1 and ASF/SF2 were varied in the presence of beta-globin pre-mRNA, different 5’ splice sites were selected for; an abundance of hnRNP A1 initiated splicing at the distal 5’ site, whereas an absence of hnRNP A1 selected for use of the proximal 5’ sites (Mayeda & Krainer, 1992). The ratio between these two splicing trans-factors also modifies alternative splicing of adenovirus E1A and SV40 pre-mRNA by regulating the use of three different 5’ splice sites during viral infection (Berk and Sharp, 1978; Perricaudet et al. 1979). Differences in splicing patterns can occur depending on the cell/tissue type processing the pre-mRNA and alterations in splice forms can play a role in cell type differentiation (Kalsotra & Cooper 2011). The polypyrimidine tract-binding protein (PTB) prevents differentiation of neural cells by promoting exon 10 skipping of its neuronal homologue nPTB which introduces a premature termination codon (PTC) and targets nPTB for NMD in non-neuronal cells allowing for neural-specific expression of constitutive nPTB (Boutz et al. 2007, Spellman et al. 2007). Splicing of cell adhesion protein CD44 is also affected by cell type. In epithelial cells, epithelial splicing regulatory proteins (ESRPs) interact with the CD44 transcript and lead primarily to inclusion of 10 variable exons and producing the CD44v splice variant, whereas in mesenchymal cells not expressing ESRPs, these variable exons are excluded, creating the CD44s splice form (Kalsotra & Cooper 2011).
There are many selective pressures that act on splice variants and alternative exons. A strong selective pressure to maintain the open reading frame and prevent the introduction of a PTC would explain why 63-72% of alternative exons maintain the original reading frame (Koren et al. 2007). However, despite the risk for the introduction of PTCs and thus production of potentially deleterious truncated proteins with dominant negative effects, approximately 30% of splicing events with alternative exons disrupt the reading frame (Koren et al. 2007). These transcripts may have persisted throughout evolution, due to the regulatory process of nonsense mediated decay (NMD) which is responsible for degrading mRNA transcripts containing PTCs and responsible for keeping these transcripts at low or even non-existent levels (Lewis et al. 2003, Wollerton et al. 2004). However, the fact that these PTC containing transcripts are known in the
transcriptome may indicate that the role of NMD degradation is not as ubiquitous as previously assumed (Baek & Green 2005, Blencowe 2006, Pan et al. 2006). Alternative exons which introduce PTCs are found more commonly in adult tissues possibly as a mechanism to decrease expression compared to embryonic tissues where mRNA expression is activated (Barash et al. 2010). Many alternatively spliced transcripts that cause frame shifts are translated into proteins such as the fragile X mental retardation (FMR1) gene whose exon 14 skipping frame shift variant alters the localization of the protein from the cytoplasm to the nucleus (Sittler et al. 1996). Exon skipping in apical sodium dependent bile acid transporter (ASBT) mRNA also produces truncated proteins; however the biological role of these truncated proteins remains undetermined (Lazaridis et al. 2000). The persistence of these frame shifting aberrant splice forms may act as a way to shut off or down-regulate protein production (Vithana et al. 2007, Wilkie et al. 2008).

While alternative splicing is essential for genetic diversity, when this process goes awry it can lead to disease; as well, disease states have been found to change patterns of alternative splicing. Diseases that arise from mis-regulated alternative splicing are known as spliceopathies. For example, exon 10 is alternatively spliced in the MAPT gene which produces the microtubule-associated tau protein (Cooper et al. 2009). When the 1:1 ratio of transcripts including and excluding exon 10 is disrupted, the balance between 4R-tau and 3R-tau isoforms become unbalanced and tau proteins become hyperphosphorylated and aggregate forming neurofibrillary tangles which are common elements in neurodegenerative disorders such as Alzheimer’s disease (Cooper et al. 2009).

1.1.3 Aberrant Splicing

Genetic mutations located in the specific splice site sequences can lead to changes in splicing of those genes which can cause pathologic consequences. Up to 25% of synonymous substitutions can affect normal splicing, as can non-synonymous and termination codon changes (Pagani et al. 2005). These splicing mistakes are known as aberrant splicing and have been implicated in many disease states,
including myelodysplastic syndromes, and neurodegenerative pathologies such as Alzheimer’s disease and Parkinson’s disease (Papaemmanuil et al. 2011, Mills & Janitz 2012). Splicing mutations of tumour suppressor genes have been found in a variety of cancers. Familial adenomatous polyposis can be caused by induction of exon 4 skipping, or use of an alternate 3’ splice site for exon 8 of the adenomatous polyposis coli (APC) tumor suppressor gene (Charames GS et al. 2002, Neklason DW et al. 2004). Exon 18 skipping and alternate 3’ splice site selection have occurred in the breast cancer 1, early onset (BRCA1) gene and lead to breast and ovarian cancers (Hoffman et al. 1998, Lui et al. 2001). Mutations to exonic splicing signals, even single nucleotide changes, can have hugely detrimental effects. Single nucleotide changes affecting splicing of the survival motor neuron 2 gene (SMN2) have led to spinal muscular atrophy, a fatal motor neuron degenerative disease which is particularly lethal in infancy (Lefebvre et al. 1995). The single nucleotide C>T change at the 6th nucleotide of exon 7 of SMN2 causes greatly increased skipping of exon 7 through the destruction of an ESE site for ASF/SF2 and the strengthening of and ESS for hnRNP A1 (Cartegni & Krainer, 2002; Kashima & Manley 2003; Kashimia et al. 2007). Aberrant splicing is also a common cause for cystic fibrosis due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes the transmembrane chloride channel leading to improper function of the secretory epithelium. Lengthening of the polypyrnmidine tracts in the 3’ splice site of intron 8 increases the degree of exon 9 skipping due to increased binding of hnRNP TDP-43 (Buratti et al. 2004).

Mutations to the essential proteins of the splicing machinery are not commonly reported, likely because these mechanisms are so overarching, that defective splicing elements are often incompatible with life (Tazi et al. 2009). There are some exceptions however; retinitis pigmentosa has been caused by mutations in several splicing factors including PRPF31/U4-61k, PRPF8, PRPF3, and RP9 (Wilkie et al. 2008, Vithana et al. 2001, Boon et al. 2007). Mutations to the splicing factor SF3B1 have been linked to myelodysplastic syndromes presenting with ring sideroblasts (Papaemmanuil et al. 2011). It is thought that
mutations linked to splicing are vastly underestimated and often uninvestigated; many mutations thought to be neutral or missense changes are being found to have links to splicing alterations.

1.2 von Willebrand Factor (VWF)

von Willebrand factor (VWF) is a large multimeric glycoprotein important for both primary and secondary hemostasis. It promotes platelet adhesion and aggregation at sites of vascular injury as well as acting as an integral chaperone for the coagulation factor VIII (FVIII) (Sadler 1998). When individuals have a deficiency or dysfunction in VWF they can present with an inherited bleeding disorder known as von Willebrand disease (VWD). Production of VWF will be discussed below.

1.2.1 VWF: From Gene to Protein Biosynthesis

The von Willebrand factor (VWF) gene is encoded on 178 highly polymorphic kilobases (kb) on the short arm of chromosome 12 (12p13.3) which comprises 52 exons and 51 intronic regions (Mancuso et al. 1989; Gallinaro et al. 1990). These exonic regions vary in length from 40bp (exon 50) to 1.3kb (exon 28) with intronic stretches ranging from the 97bp comprising intron 29 to intron 6 which contains 19.9kb (Mancuso et al. 1989). Chromosome 22 (22q.11-13) contains a partial copy of the VWF gene known as the VWF pseudogene which reflects the unprocessed exons 23-34 with ~97% sequence homology (Mancuso et al. 1991) and can complicate analysis of VWF mutations.

VWF is produced in endothelial cells (ECs) and megakaryocytes (MKs); transcription and traditional constitutive splicing of VWF condenses the sequence to approximately an 8.5kb mRNA molecule which can then be translated into the 2813 amino acid (AA) pre-pro-VWF polypeptide in the rough endoplasmic reticulum which is then shuttled to the endoplasmic reticulum (ER) (Mancuso et al. 1989, Wagner 1990, Gallinaro et al. 1990). The pre-proVWF protein is comprised of a 22AA signal peptide, a
741AA propeptide (VWFpp), and 2050AA mature VWF protein and is divided into many domain assemblages which are further divided into small subunits: von Willebrand D (VWD), 8-cysteine (C8), trypsin inhibitor-like (TIL), fibronectin type 1-like domains (E), and a unique D4N module in the D4 assembly (Zhou et al. 2012) (Figure 1.3). The cysteine knot (CK) domain is at the carboxyl (C)-terminal end of the VWF and contains 11 cysteine residues which participate in intermolecular pairing and stabilizing VWF dimers (Katsumi et al. 2000, Zhou & Springer 2014). Across these domains there are many binding sites for proteins that associate with VWF both intracellularly and when encountered in circulation: such as FVIII, platelet glycoproteins receptors (Gp) GpIbα and GpIIb-IIIa, and collagen.

The pre-pro-VWF monomer is comprised of approximately 8% cysteine residues, which is higher than most proteins (Marti et al. 1987, Mancuso et al. 1989, Sadler 1998, Lenting et al. 2015). The degree to which these cysteines are paired is currently a point of contention in the literature; some studies state that all cysteines are paired whereas other mass spectrometry data suggests cysteines at positions 889, 898, 2448, 2451, 2453, 2490, 2491, 2528, and 2533 remain as free thiols (Wagner et al. 1984, Sadler 1998, Xie et al. 2000, Schooten et al. 2005, Choi et al. 2007, Shapiro et al. 2014). The existence of these free thiols may be vital to the accurate folding and secretion of VWF as they sequentially engage in disulfide bonds throughout VWF synthesis and assembly (Shapiro et al. 2014, Lenting et al. 2015). Some of the disulfide bonds are then susceptible to reduction in circulation which may vary the functional properties of VWF based on the oxidative state of the VWF (Li et al. 2008, Lenting et al. 2015).
Figure 1.3: The current annotated domains in VWF, their functions, and cleavage sites. The re-annotated domains of VWF are shown in grey relation to the historical D domain assemblies above. VWF-VWF interacting domains are shown in green and intermolecular binding domains are shown in orange. This figure is adapted from Zhou et al. 2012.
Once in the ER, pre-proVWF is subject to proteolytic cleavage of the signal peptide by signal peptidases and the remaining proVWF peptide undergoes a series of post-translational modifications (Wagner 1990). N-linked glycosylation occurs at 12 sites throughout the mature VWF protein and 4 sites in the VWFpp which is essential in determining the structure and function of VWF (Titani et al. 1986, McKinnon et al. 2010). Glycosylation is known to influence protein folding and disulfide bond formation and deter aggregation of the protein (Federici et al. 1984, Titani et al. 1986, Wagner et al. 1986, Berkowitz et al. 1988). Dimerization of VWF monomers occurs through three intermolecular disulfide bonds in the CK domains of the proVWF monomers; these bonds are quite stable and not vulnerable to reduction when released into circulation (Sadler 1998, Shapiro et al. 2014, Lenting et al. 2015). Transport out of the ER is a strictly regulated process dependent on adequate protein dimerization and glycosylation; if these processes are impaired either naturally or through induced impairment using tunicamycin then monomeric and dimeric proVWF are retained in the ER (Wagner et al. 1986).

After release from the ER, the proVWF is transported to the Golgi apparatus where the VWF is further glycosylated with O-linked oligosaccharides at 10 sites primarily clustered just 5’ to the A1 and A2 domains (Marti et al. 1987, Zhou et al. 2012) and high-mannose oligosaccharide processing occurs (Wagner 1990). VWF N- and O-linked oligosaccharides are also modified through the addition of ABO blood group oligosaccharides throughout the mature VWF subunit (Matsui et al. 1992). Approximately one or two N-linked glycans are modified with blood group determinants per VWF subunit; this comprises ~13% of VWF’s N-linked glycans (Lenting et al. 2015). Only 1% of O-linked glycans on VWF are subject to deposition of blood group determinants; which works out to approximately one O-linked glycan per ten VWF monomers (Lenting et al. 2015). Over 90% of endothelial VWF’s glycan structures are capped with sialic acid (Matsui et al. 1992, Canis et al. 2010, Canis et al. 2012), and some of the O-linked glycans are capped with two or three sialic acids residues (Canis et al. 2010). Glycosylation patterns are known to vary
based on cell type and this holds true between endothelial- and platelet-derived VWF (McGrath et al. 2013, Lenting et al. 2015). VWF of platelet origin has 50% less sialic acid deposition and is not glycosylated with the A and B blood group antigens despite the presence of the H-antigen glycosylation (McGrath et al. 2013). VWF is also subject to sulfation. Pro-Xxx-Arg/Lys/His motifs are located upstream of five asparagine (Asn)-linked carbohydrate chains of the mature VWF subunit which may lead to sulfation of these Asn residues (Canis et al. 2012, Carew et al. 1990). Asn384 and Asn468 can also be subject to sulfation as the VWF dimers pass through the trans-Golgi network (Carew et al. 1990).

In the acidic pH of the trans-Golgi, VWF dimers assemble into “bouquet-like” structures where the A2 and A3 domains, as well as the D4 assembly resemble the “flowers” and the C domains represent the “stem” of the “bouquet” (Zhou et al. 2011, Zhou et al. 2012). (Figure 1.4) These pH based conformational changes to the VWF dimers facilitates multimerization through inter-chain disulfide bonds at the amino (N)-termini of the mature VWF subunits which can reach sizes of greater than 20,000kDa (Mancuso et al. 1989, Rosenberg et al. 2002). The VWFpp and the D’ domain are required for the proper alignment of VWF dimers allowing this interdimer disulfide bonding of the D3 domain (Mayadas & Wagner 1989); when the VWFpp is absent or mutated, multimerization does not occur (Wise et al. 1988). It is proposed that the two CXXC sequences (Cys-Gly-Leu-Cys) in the VWFpp are classic disulfide oxidoreductases which are required for disulfide bond formation of multimers in the Golgi (Mayadas & Wagner 1992, Purvis & Sadler 2004). Dimers are linked forming multimers of varying sizes which can comprise greater 60 VWF subunits (Wagner 1990, Sadler 1998). Finally, furin mediated cleavage of the VWFpp occurs at AA 763 and the VWFpp remains non-covalently associated with the mature VWF protein facilitated by the low NaCl, high CaCl₂, and low pH of the cellular environment (Wagner et al. 1986, Lenting et al. 2004). VWFpp is required for the localization of VWF multimers to their storage organelles, Weibel-Palade bodies (WPBs) in ECs,

![Diagram of VWF dimeric bouquet formation](image)

**Figure 1.4: The dimeric bouquet formation of VWF** under the acidic conditions of the Golgi from Zhou et al. 2012.

In the trans-Golgi, VWF is packaged into newly forming organelles and the tightly packed VWF tubules which assemble into ministacks causing projections in the trans-Golgi network which bud off forming immature WPBs (Valentijn et al. 2008, Ferraro et al. 2014, Lenting et al. 2015). This tubule formation allows for a 100-fold compaction of VWF the ministacked immature WPB (Lenting et al. 2015). These immature WPB ministacks appear to fuse when their contents are similar, which may contribute to the heterogeneity of WPBs and increasing in length increments of 0.5 µm (Valentijn et al. 2008, Ferraro et al. 2014, Lenting et al. 2015). Multimerization of VWF continues after separation of the immature WBPs from the trans-Golgi network (Wagner & Marder 1984). The adaptor protein (AP-1), Discs Large-1 (Dlg1) protein, and a clatherin coat are required for these immature WPBs to form into the characteristic cigar-shaped structure of WPBs (Valentijn et al. 2013, Nightengale & Cutler 2013, Philippe et al. 2013). These nascent WPBs anchor to filamentous actin via a complex containing Rab27a, myosin Va (MyoVa), and a myosin-Rab27a interaction protein (MyRIP) and undergo maturation (Rojo et al. 2011).
1.2.2 VWF Storage and Secretion

1.2.2.1 Storage Organelles

WPBs are cigar shaped, membrane bound organelles that are 0.1-0.2 µm wide and 4 µm long and characterized by longitudinal striations (Weibel & Palade 1964, Wagner et al. 1982). These striations are caused by the acidic environment of the WPB propelling the D1 and D2 domains of the VWFpp and the D'D3 domains in the mature VWF arrange into tightly packed helical tubules which can be visualized by electron microscopy (EM) (Wagner et al. 1982, Wise et al. 1988). These tubules compress VWF by 100-fold allowing for proper intracellular storage and secretion (Michaux et al. 2006, Metcalf et al. 2008). As VWF is characteristic of WPBs, both the VWFpp and mature VWF are required for the formation of these organelles; the VWFpp is an intra-molecular chaperone for VWF mediated through non-covalent association between the Arg416 AA of the VWFpp and Thr869 AA of the VWD3 domain of the D'D3 assembly directing VWF to WPBs (Haberichter et al. 2003, Valentijn et al. 2011, Zhou et al. 2012). VWFpp can traffic to WPBs in the absence of mature VWF; however, the reverse is not true indicating that it is the VWFpp which is required for WPB synthesis (Haberichter et al. 2000). Many other proteins also accompany VWF in the WPB, most of which are participants in hemostatic functions, modulation of vascular tone, angiogenesis, as well as inflammation (Rondaij et al. 2006, Metcalf et al. 2008). Proteins such as P-selectin, CD63, interleukin-8 (IL-8), osteoprotegerin (OPG), angiopoietin-2 (Ang-2), endothelin-1, and eotaxin are also found in WPBs, however the population of WPBs appears to be quite heterogeneous based on organelle contents (Valentijn et al. 2011).

In platelets, VWF is stored in α-granules which, like WPBs discussed above, form by P-selectin precursor granules budding off the trans-Golgi network of the MK (Harrison & Cramer 1993). Unlike WPBs, the formation of these P-selectin precursor granules do not require VWF and VWFpp (Blagoveshchenskaya et al. 2002, Blair & Flaumenhaft 2009). These granules increase as MKs mature and ~50-80 α-granules are
packaged into each platelet during thrombopoiesis (Blair & Flaumenhaft 2009). Ranging in size from 200-500nm, α-granules comprise approximately 10% of the platelet volume (Blair & Flaumenhaft 2009). This unique secretory organelle acquires its contents through two mechanisms, either: 1) biosynthesis in the MK or vestigial synthesis in platelets, (eg. VWF and platelet factor 4 (PF4)) or 2) endocytic and pinocytic internalization of proteins by either the MK or the platelet (eg. Fibrinogen and IgG) (Harrison & Cramer 1993). A vast number of proteins are found in α-granules including cellular mitogens and promotores of angiogenesis (eg. platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF)), anti-angiogenic molecules (eg. Angiostatin, PF4), adhesive proteins (eg. VWF & thrombospondin (TSP)), coagulation factors (eg. Factor V), and protease inhibitors (eg.α-2-macroglobulin) (Harrison & Cramer 1993, Whiteheart 2011). The inner lining of α-granules contains many important receptors such as glycoprotein alpha IIb beta 3 (GPαIIbβ3), and P-selectin (Harrison & Cramer 1993). As with WPBs, heterogeneity exists in the α-granule population as well; molecules with opposing properties were thought to segregate into granule subsets (Italiano et al. 2008, Ma et al. 2005); however, it has been noted that α-granule contents appear to be randomly assigned, not separated based on molecular properties (van Nispen tot Pannerden 2010). As within WPBs, SNARE proteins play a large role in trafficking α-granules to fusion with the platelet membrane as well as syntaxins 2 and 4 (Blair & Flaumenhaft, 2009, Sudhof & Rothman, 2009). The contents of α-granules are released upon platelet activation at regions of vascular injury and serve functions involving hemostasis, wound healing, and inflammation.

1.2.2.2 Stimulants of WPB Exocytosis

The release of VWF from WPBs into circulation can be stimulated based on a variety of stressors, such as response to vascular injury and generation of thrombin (Richardson et al. 1994); stress and exercise induce exocytosis through activation of the beta-adrenergic receptor (Prentice et al.1972, Small et al.1984). Additionally, exposure to 1-desamino-8-D-arginine vasopressin (DDAVP), an analog of the
hormone vasopressin is known to induce release of VWF from WPB through indirect activation of the vasopressin V2 receptor which has been found on lung microvascular endothelial cells but is not commonly present on human endothelial cells (Kaufmann et al. 2000). DDAVP binds to and activates circulating monocytes which in turn release platelet-activating factor (PAF) responsible for triggering VWF release from EC WPBs through cyclic-AMP (c-AMP) mediated signaling (Hashemi et al. 1990, Hashemi et al. 1993). Due to this indirect association with most ECs, *in vitro* ECs do not respond to direct DDAVP stimulation unless transduced to express the V2 receptor (Wang et al. 2013).

*In vitro* stimulated exocytosis of WPBs can be achieved through exposure to a number of secretagogues which direct exocytosis through several pathways: A) Stimulation of ECs with epinephrine or forskolin induce WPB release through c-AMP signaling responses similar to DDAVP, vasopressin, or PAF stimulation (Vischer et al. 1997, Hegeman et al. 1998). B) The common *in vitro* stimulus phorbol 12-myristate 13-acetate (PMA) is believed to facilitate phosphorylation of WPB exocytotic proteins through activation of protein kinase C (Morgan et al. 2005). And finally, C) When stimulated by divalent, cationic, calcium secretagogues, for example calcium ionophore, thrombin, or histamines, ECs experience an increase in intracellular Ca\(^{2+}\) which causes immediate exocytosis of WPBs and thus VWF release (Kaufmann et al. 2000, Hamilton & Sims 1987, Birch et al. 1992). These variable stimulation pathways exude different effects on the endothelial cells, allowing for differential secretion of WPBs (Rondaij et al. 2006).

1.2.2.3 Secretion Mechanisms

In platelets VWF is trafficked to α-granules and released upon platelet activation (Blair & Flaumenhaft 2009). Endothelial cells however secrete VWF in two manners, either constitutively or through triggered release of the storage organelles; the proportion to which each of these pathways is used remains unclear. Many studies have found that the majority of circulating VWF was secreted constitutively and the
remainder is trafficked into WPBs to be released into circulation en mass in response to vascular injury (Sporn et al. 1986, Giblin et al. 2008, Valentijn & Eikenboom 2013, Nightingale & Cutler 2013, van den Biggelaar et al. 2014). Other studies however, have also shown that the bulk of VWF is trafficked to WPBs prior to release and smaller amounts are constitutively released (Tsai et al. 1991, Giblin et al. 2008).

Variation of WPB exocytosis has also been observed in vivo, both in the manner in which the WPBs fuse with the plasma membrane, as well as the degree of release observed from the WPB. Three main mechanisms have been observed: A) The exocytosis of VWF through fusion of a single WPB with the plasma membrane, spilling its contents into circulation (Nightingale et al. 2011). The basal release of single WPBs in this manner is unlikely to produce long, cell-anchored VWF strings which recruit platelets, as these strings appear to only form under situations of EC stimulation in vivo or in vitro (Lenting et al. 2004, van Schooten et al. 2008). B) Many WPBs have been shown to merge intracellularly forming a 'secretory pod' which can then fuse with the plasma membrane of the cell and release its combined cargo (Valentijn et al. 2010). This is known as 'multigranular exocytosis' (Valentijn et al. 2010). C) More selective release of WPB contents has been shown as well, where WPB fusion with the plasma membrane is a transient event and a small pore is formed in the membranes allowing selective WPB contents to be released into circulation such as the smaller molecules CD63 and IL-8, or eotaxin-3 (Babich et al. 2008, Nightengale & Cutler 2013, van den Biggelaar et al. 2014). This model, known as the 'lingering kiss', would retain larger proteins such as VWF and P-selectin within the WPB, and may be explained by deacidification of the WPB upon exposure to the extracellular environment, collapsing the tubular structure of the WPB VWF, and preventing VWF secretion (Babich et al. 2008).

The process of WPB exocytosis occurs over a series of steps which can be visualized where WPBs initially traffic to the perinuclear, microtubule-organizing center (MTOC) region of the cell (Romani de Wit et al. 2003). SNARE and Rab family proteins target and traffic WPBs to actin filaments where they
concentrate into discrete patches, likely due to the fusion of WPBs into secretory pods (Romani de Wit et al. 2003, Valentijn et al. 2010, Lowenstein et al. 2005). Lone WPBs and secretory pods can then travel to the cell membrane and fuse before the release of VWF concentrated VWF multimers into circulation (André et al. 2000, Dong et al. 2002). While perinuclear clustering in the MTOC occurs regardless of the secretory pathway, variation in the degree of WPB release observed in ECs based on the secretagogue encountered by the cell (de Wit & van Mourik 2001, Rondaij et al. 2006). In response to secretagogues which act through increases in intracellular Ca\(^{2+}\), such as thrombin, WPBs are released quickly both from the MTOC and the periphery of the cell. Stimulants enacting release through c-AMP mediated pathways, such as epinephrine, drive a selective release mediated by dynein and dynactin where many WPBs remain clustered in the MTOC, and only WPBs from the periphery are released (de Wit & van Mourik 2001, Rondaij et al. 2006).

When VWF is initially secreted from WPBs, it is as ultra large molecular weight multimers (ULMWM) which are quite prothrombotic (André et al. 2000, Dong et al. 2002). These ULMWM are much more adhesive than VWF that has been passing through the circulation (Sporn et al. 1986). During exodus from the EC the ULMWM will bind to EC which in combination with the shear stress applied by passing blood will cause the partial unfolding of the VWF, unveiling the tyrosine(Y)1605-Methionine(M)1606 site in the A2 domain (Furlan et al. 1996). In vitro trans-membrane P-selectin has shown to bind VWF after release; however, in vivo studies suggest P-selectin may be less relevant, though integrin α\(_v\)β\(_3\) may be involved in fastening VWF to the EC membrane (Furlan et al. 1996, Huang et al. 2009, Wang et al. 2012). It is at this newly exposed Y1605-M1606 site that VWF is cleaved by A disintegrin-like and metalloprotease with thrombospondin type 1 motifs 13 (ADAMTS-13) from ULMWM into circulating HMWM (Furlan et al. 1996).
1.2.3 VWF Interactions and Functions in Circulation

VWF multimers and VWFpp are secreted in a 1:1 molar ratio due to the non-covalent bonds linking them in the intracellular environment. Upon release from their storage organelles they are no longer exposed to the low pH conditions of the WPB or α-granule and the VWF multimers dissociate from the VWFpp (Wagner et al. 1987). This release of the VWFpp is essential for the tightly packed tubules of VWF multimers to unfold into functional VWF strings (Michaux et al. 2006). This pattern of dissociation may not hold true for all VWFpp; some VWFpp molecules may remain linked to VWF multimers which can decrease the accessibility of the VWF A1 domain for interactions such as Gp1bα binding from platelets (Madabhushi et al. 2012).

Once in circulation, the mature VWF multimers are subject to multiple intermolecular relationships and are involved in many biological functions. VWF D’ and D3 domains bind the essential coagulation protein, Factor VIII (FVIII), also known as anti-hemophilic factor (Ruggeri & Ware 1993, Goodeve 2010). FVIII is a key cofactor in the intrinsic pathway of the coagulation cascade which downstream forms a fibrin clot over vascular injuries (Graw et al. 2005). Deficiencies in FVIII lead to the X-linked bleeding disorder Hemophilia A (Graw et al. 2005). In circulation, 95% of FVIII is bound to VWF which acts as a chaperone increasing the half-life of FVIII by protecting it from cleavage by activated protein C (APC), and early clearance, as well as transporting FVIII to sites of vascular injury (Ruggeri & Ware 1993, Goodeve 2010). Although each VWF monomer in circulation is able to bind a FVIII molecule, in vivo this interaction appears to be dampened to only a 50:1 ratio of VWF:FVIII due to the low concentrations of FVIII in the plasma (Lenting et al. 1998). Interactions between VWF and FVIII can be hampered by mutations in their binding sites (the acidic light chain of the FVIII a3 and C2 domains, and the D’ or D3 domain of VWF) (Foster et al. 1987, Takahashi et al. 1987, Spiegel et al. 2004). In instances where the VWFpp does not detach from the mature VWF, the interaction with FVIII is impaired from binding to the D’D3 domains (Leyte et al. 1991,
Bendetowicz et al. 1998, Casonato et al. 2003). The VWF:FVIII complex is dissociated through cleavage of FVIII by thrombin; this releases activated FVIII (FVIIIa) from the complex which can then perform its hemostatic cofactor functions in the coagulation cascade, interacting with activated Factor IX (FIXa) to activate Factor X (FX) to FXa (Hamer et al. 1987).

The A3 domain of VWF is also able to bind to collagen when exposed to the sub-endothelial matrix at sites of vascular injury. This interaction helps facilitate vascular integrity at these injured sites because the shear stress applied by the blood flow elongates the VWF multimers exposing the active Gp1b platelet binding site in the A1 domain of VWF enabling the accumulation and aggregation of activated platelets at the site of injury (Ross et al. 1995). Many types of collagen are found in the subendothelial matrix of the vessel wall (Ross et al. 1995). Type I, III, and VI collagen are the most likely to interact with VWF. Collagen type VI is responsible for the initial interactions between the VWF A1 domain and the superficial subendothelium which allows for platelet adhesion under low shear stress conditions (Hoylaerts et al. 1997, Romijn et al. 2003, Lisman et al. 2006). On the other hand, if vascular injury exposes deeper layers of the subendothelial matrix, VWF interacts through its A1 and A3 domains with type I and III collagen which strengthens the effect of platelet adhesion to the injury and aggregation of platelets at the wound site to form a platelet plug (Hoylaerts et al. 1997, Romijn et al. 2003, Bernardo et al. 2004, Lisman et al. 2006).

Platelet binding is one of the integral hemostatic functions of VWF. As VWF binds to collagen exposed at sites of vascular injury the HMWM unravel under shear stress exposing two sites for binding to the platelet receptors; the platelet receptor Gplbα (Gplb-IX-V complex) binds the A1 domain of VWF and the αIIbβ3 receptor (Gpllb-IIIa complex) binds to the VWC4 domain (Savage et al. 2002, Ruggeri 2003, Zhou et al. 2012). Binding to collagen and exposure to shear stress causes a conformational change to VWF’s A1 domain increasing its binding affinity for platelet Gplbα (Ruggeri 2003). Once bound to VWF, platelets are able to roll across the vascular surface and tether to sites of vascular injury using E- and P-
selectins and integrin αIIbβ3 (Ruggeri 2003). Tethered platelets are activated and consequently bind more VWF and fibrinogen via their αIIbβ3 receptor furthering platelet recruitment and aggregation at sites of injury (Savage et al. 2002).

The A1 domain of VWF binds many additional ligands, osteoprotegerin (OPG), a member of the tumour necrosis-factor (TNF) receptor superfamily, is one such ligand. OPG is involved in bone remodeling and plays a role in EC survival (Reid & Holden 2009). VWF and OPG are known to associate in WPBs (Zannettino et al. 2005) and platelet α-granules (Chollet et al. 2010). The complex between OPG and VWF appears to form during VWF’s passage through the Gogli network; both proteins are trafficked to WPBs and can be found in association in circulation (Zanettino et al. 2005, Shahbazi et al. 2007). OPG may play an indirect role in regulating the size of VWF through its interactions with the adhesive glycoprotein thrombospondin-1 (TSP-1) (Pimanda et al. 2002).

TSP-1, while interacting with OPG, also interacts with VWF in the A3 domain (Pimanda et al. 2002); TSP-1 is released from α-granules alongside VWF, and plays a role in cell adhesion (Bonnefoy et al. 2008). The significance of this interaction between VWF and TSP-1 has yet to be elucidated; however, TSP-1 may reduce the disulfide bonds linking VWF multimers and act as a limiter to VWF size (Xie et al. 2001). As ADAMTS-13 is known to bind to the A3 domain under shear stress conditions, TSP-1 may be a competing ligand for ADAMTS-13 binding preceding cleavage of VWF multimers (Wang et al. 2010).

The A1 domain of VWF is also known to associate with heparin (Fujimura et al. 1987, Adachi et al. 2006) which may inhibit VWF’s integral interaction with GpIbα affecting the platelet binding; this is of particular interest for individuals who are on heparin therapy (Sobel et al. 1991). Heparin’s interaction with VWF also includes the FVIII binding region in the D’ and D3 assemblies.
A major regulator of angiogenesis, Ang-2, co-localizes in WPBs with VWF. It is presently unclear at what point during biosynthesis Ang-2 associates in the A1 domain of VWF; however, this interaction can persist regardless of the conformation of VWF and this association is maintained once these proteins are released from the WPBs (McKinnon et al. 2011). As VWF and Ang-2 remain associated in circulation, this may explain correlations between hemostatic and angiogenic processes.

1.2.4 VWF Catabolism and Clearance

1.2.4.1 Catabolism

As VWF is released from ECs as ULMWM, it binds to P-selectin on the surface of ECs unfolding to expose the cleavage site at the Tyrosine(Y)1605-Methionine(M)1606 in the A2 domain allowing cleavage by ADAMTS-13 (Furlan et al. 1996). Once released from the EC surface VWF circulates through the plasma in a globular form in which this cleavage site in the A2 domain is inaccessible to cleavage by ADAMTS-13 (Tsai 1996, Feys et al. 2009). Despite the inaccessibility of the ADAMST-13 cleavage site, the C-terminal domain of this metalloprotease is still able to bind the C-terminus of VWF (D4-CK domains) in its globular form (Feys et al. 2009, Zanardelli et al. 2009). This initial binding of ADAMTS-13 to globular VWF may ensure the close proximity of ADAMTS-13 to the VWF HMWM when the cleavage site is unveiled as VWF unfolds. When VWF binds to ECs or subendothelial matrix at sites of vascular injury the HMWM unfold under the shear stress of the circulation. ADAMTS-13, which may be initially bound to the C-terminal domain of VWF, is now able to access the cleavage site and catabolize VWF HMWM regulating hemostatic events. ADAMTS-13 is also known to bind to the A3 domain under flow conditions (Lopez & Dong 2004).

1.2.4.2 Clearance

As a circulating protein, VWF is subject to the effects of oxidation, catabolism by ADAMTS-13, glycation and many other environmental effects, because of these alterations, proteins must be cleared
from circulation in a timely and regulated manner (Lenting et al. 2015). Multimeric VWF for example has a half-life of 8-12 hours, circulating at levels of approximately 10 µg/mL, whereas VWFpp has a much shorter half-life of ~2 hours and circulates at lower levels of 1 µg/mL (Montgomery & Zimmerman 1978, Borchiellini et al. 1996, Vischer et al. 1997). There is a great inter-individual variation in VWF half-life, much of which is thought to relate to glycosylation of the VWF, particularly the additions of ABO blood group oligosaccharides to N-linked glycosylation sites during VWF biosynthesis (Gill et al. 1987, Gallinaro et al. 2008). It appears as though the presence of A or B antigens has a protective effect against VWF clearance represented in these individuals having 25% higher circulating VWF:Ag levels than blood type O individuals (Gill et al. 1987, Gallinaro et al. 2008). Single nucleotide polymorphisms (SNPs) in CLEC4M have been linked to VWF:Ag levels in circulation, which more recent studies have shown may be through a clearance effect (Hannah et al. 2003, Rydz et al. 2013).

Though the clearance mechanism of VWF has not been fully elucidated, it is thought to be cleared primarily by macrophages in the liver and spleen (Lenting et al. 2004, van Schooten et al. 2008, Casari et al. 2013). VWF can bind and be endocytosed by macrophages quite efficiently, through receptor mediated binding, and possibly also through macropinocytosis (van Schooten et al. 2008, Castro-Nunez et al. 2012, Casari et al. 2013, Casari et al. 2013); however, it is still possible that non-macrophage cells participate in VWF clearance. When unfolded due to shear-stress, binding sites for the macrophage lipoprotein receptor (LRP-1) are unveiled increasing VWF’s capacity to be endocytosed by macrophages (Castro-Nunez et al. 2012, Rastegarlari et al. 2012). This LRP-1 based pathway appears to significantly contribute to basal VWF clearance as VWF levels increase and its half-life is increased ~2 fold when LRP-1 is deficient (Rastegarlari et al. 2012). In its globular, usually sialylated form, VWF can be recognized by the potential clearance receptors SCARA5, CLEC4M on sinusoidal ECs, and Siglec-5 on the surface of macrophages; however, whether these receptors affect VWF levels directly or indirectly remains to be determined (Pegon et al.
2012, Casari et al. 2013, Rydz et al. 2013, Lenting et al. 2015). When VWF becomes desialylated in the presence of a pathogen infection, galactose residues are exposed, transforming VWF into a ligand for yet another receptor, the asialoglycoprotein receptor (ASGPR) which is found on both macrophages and hepatocytes (Ellies et al. 2002, Grewal et al. 2008).

1.3 von Willebrand Disease (VWD)

von Willebrand disease (VWD) is an autosomally-inherited congenital bleeding disorder caused by quantitative deficiencies or dysfunction of VWF (Bloom 1991). VWD was initially described by Dr. Erik von Willebrand while investigating a severe bleeding phenotype in a family from the Åland islands archipelago. Dr. von Willebrand called this disease “pseudo-hemophilia” recognizing that although there were similarities to the bleeding phenotype of hemophiliacs, there were distinctions, including the autosomal heritability of what we now know to be VWD (von Willebrand 1931).

VWD is currently recognized as the most commonly inherited bleeding disorder in humans, with approximately 1% of the population having this disease; however, the symptomatic prevalence is only 0.1% (Rodeghiero & Castaman 1987, Bowman et al. 2010). VWD is characterized by prolonged and excessive mucocutaneous bleeding, VWF abnormalities, as well as, a history of affliction within the family (James et al. 2007). Three general types of VWD have been distinguished and recognized by the International Society on Thrombosis and Hemostasis (ISTH) (Sadler et al. 2006). Quantitative deficiencies in VWF are the hallmark of Types 1 and 3 VWD, while Type 2 VWD is assigned when VWF is qualitatively abnormal (Sadler et al. 2006).
1.3.1 Diagnosis of VWD

Laboratory analysis for VWD includes VWF:Antigen (VWF:Ag) to measure VWF levels, VWF:Ristocetin Cofactor assay (VWF:RCo) to assess binding of patient VWF to normal formalin-fixed platelets, and FVIII coagulant activity (FVIII:C) testing of patients’ plasma. Plasma levels of VWF are quantified using the VWF:Ag enzyme-linked immunosorbant assay (ELISA). The average VWF concentration in the normal population is 1 IU/mL which equates to approximately 10 µg/mL (Montgomery & Zimmerman 1978, Borchiellini et al. 1996, Vischer et al. 1997); however, normal levels can range between 0.50 IU/mL-1.50 IU/mL. VWF:Ag levels below 0.30 IU/mL are generally considered pathologic, though different diagnostic laboratory values vary slightly between clinics (Nicols et al. 2008). When assessing functional platelet binding, the VWF:RCo assay uses the agonist ristocetin to expose the GpIbα site in VWF and samples are mixed with normal platelets to measure platelet agglutination. In some European and Canadian centers, this assay is being replaced by GpIbα-binding ELISAs which can measure platelet:VWF interactions independent of ristocetin antagonism, which can be especially useful as SNPs affecting ristocetin binding, and not platelet binding have been known to give falsely decreased VWF:RCo results (Flood et al. 2010). FVIII:C is measured due to the interlinking effects of VWF on FVIII. Additional assessment of VWF functionality can be helpful in further distinguishing between different VWF subtypes discussed below. This can be accomplished through multimer analysis to visualize the distribution of multimers. To evaluate a patient’s platelet functionality the ristocetin induced platelet agglutination (RIPA) assay can be used.

Diagnosis of Type 1 VWD can be complicated due to the wide range of normal VWF levels that exist in the general population, as well as the milder phenotype that frequently presents. Individuals may not recognize that their bleeding is excessive or abnormal. To this end, bleeding assessment tools (BATs) are used to quantify bleeding symptoms and determine if they are abnormal (Tossetto et al. 2006, Tossetto
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et al. 2008, Bowman et al. 2008, Marcus et al. 2011). The hope is that these BATs will reach the general public or be employed in general practitioners’ offices before referral to a hematologist. An electronic version of the condensed MCMDM-1VWD BAT, the eBQ, has been produced for use in studies (Kaur et al. 2014). Hopefully, BATs will soon be widely available electronically so patients can more readily assess whether their bleeding symptoms are abnormal before consultation with a physician.

1.3.2 Type 1 VWD

Of the three VWD subtypes, Type 1 VWD is the most predominant in human populations, occurring in ~80% of VWD cases (Ruggeri & Zimmerman 1987, James et al. 2007). This mild form of VWD presents with mild to moderate mucocutaneous bleeding such as epistaxis, easy bruising, excessive bleeding from minor wounds and oral cavities. Type 1 VWD is generally inherited in an autosomal dominant manner with varying VWF levels due to incomplete penetrance and variable expressivity of VWF mutations; however there are rare cases in which Type 1 VWD has been inherited in a recessive manner (Eikenboom et al. 1993, Kadir et al. 1999).

Type 1 VWD is defined by a partial reduction in the quantity of normal circulating VWF with VWF:Ag levels between 0.05 and 0.50 IU/mL (James et al. 2007, Nichols et al. 2008, Goodeve 2010). Some testing centers have an upper cut off VWF:Ag value of 0.30 IU/mL (Nicols et al. 2008). The diagnosis of Type 1 VWD can be difficult due to the extensive variability in VWF levels across the normal population (0.50-1.5 IU/mL), as well as the aforementioned variable expressivity and incomplete penetrance of Type 1 VWD mutations (Kadir et al. 1999). Diagnosis is commonly based on VWF:Ag and VWF:RCo level which can be affected by various physiological states such as age (Kadir et al. 1999, Albanaz et al. 2015), estrogen levels and supplements (Kadir et al. 1999), pregnancy (Sie et al. 2003), exercise (Rock et al. 1997, Stakiw et al. 2008), ethnicity (Miller et al. 2001), and genetic factors such as ABO blood group as discussed previously (Gill et al. 1987).
Many types of mutations are causative of Type 1 VWD and missense mutations are the most common (~70%) (Smith et al. 2010, van Loon et al. 2012, Rydz et al. 2013); however approximately 30-35% of Type 1 cases remain without an identified genetic cause (Cumming et al. 2006, Goodeve et al. 2007, James et al. 2007). Splicing mutations have been attributed to ~10% of cases; however they are predominantly canonical splice site mutations and are rarely investigated further. Mutations that cause Type 1 VWD generally lead to defective synthesis, storage, or secretion of VWF, as well as, intracellular retention, degradation, or increased clearance of secreted aberrant proteins from circulation (Goodeve 2010). The large proportion of mutation-negative VWD cases suggest that mutations in VWF promotor, the deep intronic regions, other within other genes may also cause pathologic variation in VWF levels. The contribution of other genes to VWF levels is presently being investigated; however, ABO blood group remains the gene most highly associated with circulating VWF levels (Smith et al. 2010, van Loon et al. 2012, Rydz et al. 2013).

1.3.3 Type 2 VWD

Type 2 VWD has four further subdivisions of VWD representing qualitatively abnormal VWF caused by mutations to VWF’s various functional domains.

1.3.3.1 Type 2A VWD

Type 2A VWD, is the most common subset of Type 2 VWD and is generally inherited in an autosomal dominant manner. This subtype is characterized by diminished or a lack of VWF HMWM which causes a significant decrease in VWF-platelet interactions (Ruggeri & Zimmerman 1980, Sadler 1998, Israels & Israels 2002). VWF:Ag is usually decreased as mutations cause some intracellular retention and FVIII:C levels may be in the normal range or slightly decreased; however, VWF:RCo functional assessment will be significantly impaired (Ruggeri & Zimmerman 1980, Jacobi et al. 2012). Mutations causative of Type
2A VWD are generally caused by single amino acid substitutions in the A2 domain which produce a decreased proportion of HMWM either through increased cleavage by ADAMTS-13 and subsequent clearance, or impaired assembly in the Golgi. Type 2A mutations have also been found in the A1, D2, and D3 domains (Sadler 1998, Israels & Israels 2002). This absence in HMWM can be caused by a defect in intracellular transport involving defective storage and secretion of large multimers, or an increased susceptibility to proteolytic cleavage once the HMWM are released into the plasma by ADAMTS-13 (Sadler 1998, Israels & Israels 2002).

1.3.3.2 Type 2B VWD

Type 2B VWD is caused by autosomal dominant mutations in the platelet binding regions of the VWF A1 domain which cause a marked gain-of-function phenotype where the mutant VWF has a higher affinity for platelet GpIbα binding (Ruggeri & Zimmerman 1980, Ruggeri et al. 1980). This excessive platelet binding causes increased clearance of VWF:platelet complexes from the circulation, which often times leads to these patients suffering from thrombocytopenia (Ruggeri & Zimmerman 1980, Ruggeri et al. 1980, Sadler 1998). This VWF subtype often presents with decreased VWF:Ag and VWF:RCo with a loss of HMWM, while the RIPA may be abnormal, showing increased platelet binding at low ristocetin concentrations.

Platelet-type VWD (PT-VWD) is a rare autosomal dominant bleeding disorder resembling type 2B VWD (Miller & Castella 1982, Weiss et al.1982). Instead of mutations to the platelet-binding region of VWD, PT-VWD is caused by mutations in the Gplbα gene (GP1BA). These mutations result in an increased binding affinity of Gplbα for the A1 domain of VWF (Miller et al. 1991, Russel & Ross 1993); therefore, as with Type 2B VWD, PT-VWD displays spontaneous interactions between platelets and VWD leading to their increased clearance from circulation (Russel & Ross 1993). Distinguishing between these diseases is
best accomplished through genetic testing as their phenotypes present so similarly (Franchini et al. 2008. Othman 2011).

1.3.3.3 Type 2M VWD

Autosomal dominant single amino acid substitutions in the A1 domain and GP1b binding site which cause an inactivation of platelet binding lead to Type 2M VWD. This subtype exhibits a normal multimer distribution, a moderate reduction of VWF:Ag levels, and significantly reduced VWF:RCo values due to the loss of platelet binding interactions (Mancuso et al. 1996, Sadler 1998).

1.3.3.4 Type 2N VWD

The VWD subtype 2N develops from inheritance of autosomal recessive missense mutations which inactivate the FVIII binding site on VWF, the D’ and D3 domains (Mazurier et al. 1990). The decrease in circulating FVIII levels that result from these mutations mimic hemophilia A and occasionally cause misdiagnosis of this disease (Mazurier et al. 1990, Sadler 1998). Patients afflicted with Type 2N may have normal VWF:Ag and VWF:RCo levels, and their FVIII levels will be significantly reduced to between 0.05 to 0.40 IU/mL (Mazurier et al. 1990).

1.3.4 Type 3 VWD

Finally, Type 3 VWD, the most severe form of this disease is indicated by absent or virtual absence of VWF protein resulting in moderate to severe mucocutaneous bleeding symptoms as well as hematomas and hemarthroses (Eikenboom 2001, James et al. 2007, Goodeve 2010). These dramatic deficiencies of VWF cause a corresponding decrease in circulating FVIII due to the requirement of VWF as a chaperone for FVIII, protecting it from proteolysis (Keeney & Cumming 2001). Fortunately, this severe bleeding disorder is rare, affecting 0.11-5.3 individuals per million, with these higher instances being found in countries where consanguinity is more common among parents of individuals with Type 3 VWD.
(Eikenboom 2001). Inheritance is usually autosomal recessive with affected individuals presenting with homozygous or compound heterozygous for VWF mutations commonly causing null alleles; that being said, these mutations have also exhibited co-dominance in many instances, where carriers of Type 3 mutations also present with an increased bleeding phenotype (Castaman et al. 2006, Montgomery 2006, Bowman et al. 2013).

1.3.5 Acquired von Willebrand Syndrome (aVWS)

Acquired von Willebrand syndrome (aVWS) is a rare bleeding pathology caused by structural or functional alterations in VWF that are not inherited or linked to genetic defects in the VWF gene (Tiede 2012). Elderly patients are most commonly affected with aVWS which can develop from lymphoproliferative, cardiovascular, myeloproliferative, or autoimmune diseases, as well as other underlying diseases (Tiede 2012). These pre-existing illnesses can cause aVWS through increased clearance or inhibition of VWF by other pathologic proteins or pathologic increases in shear stress in the bloodstream can cause shearing of HMWM of VWF and increased proteolysis by ADAMTS-13 (Tiede 2012).

1.3.6 Treatment of VWD

As VWD presents with such a wide range of severities, treatment of the disease must be individualized. Treatment of mild VWD often involves administration of 1-deamino-8-D-arginine vasopressin, commonly known as desmopressin (DDAVP), either intranasally, subcutaneously, or intravenously. DDAVP is used to increase levels of VWF and FVIII in the circulation by stimulating the release of VWF from WPBs (Castaman et al. 2003). Consecutive doses of DDAVP are avoided as they become less effective when WPBs become depleted. This treatment works well for minor bleeding incidences in individuals who are able to respond which normally limits the use of this treatment to Type 1
VWD cases and some Type 2 cases (Castaman et al. 2008). Individuals afflicted with Type 2 A VWD have a variable response to DDAVP therapy due to their qualitatively defective VWF not forming HMWM (Sadler 1998, Israels & Israels 2002). DDAVP is contraindicated in cases of Type 2B so as to not increase the risk of thrombocytopenia in these patients. Unfortunately, this treatment is ineffective in Type 3 VWD patients due to their absence of endogenous VWF. As an anti-diuretic, fluid intake must be restricted for 24 hours after DDAVP treatment to decrease the risk of hyponatremia, which can lead to nausea, headache, seizures and possible comas (Sharman & Low 2008).

If the patient does not produce enough of their own VWF or DDAVP treatment is ineffective or contraindicated, a common approach to VWD management is to replace the lack of endogenous VWF with plasma-derived concentrates of VWF and FVIII (Thompson et al. 2004, Federici 2007). These are viable options for instances of surgical and long-term prophylaxis, or on demand treatment of bleeding symptoms (James & Hawke 2014). To ensure safe and effective treatment plasma-derived concentrates are treated with virucidal methods and should provide sufficient amounts of active VWF to correct primary hemostasis and stabilize the patient’s endogenous FVIII. There are many concentrates on the market employing a variety of virucidal and purification techniques and providing varying concentrations of VWF and FVIII (Federici & James 2012, James & Hawke 2014). Unfortunately, it has been found that 7.5-9.5% of Type 3 VWD patients taking plasma derived factor concentrates develop anti-VWF antibodies, thereby decreasing the efficacy of these treatments, and possibly resulting in anaphylaxis upon subsequent exposures to concentrates (James & Hawke 2014). Patients with anti-VWF antibodies may respond to injections of FVIII to control hemostasis (James & Hawke 2014, Mannucci & Federici 1995).

Clinical trials are currently underway for recombinant VWF as an alternative to these plasma-derived concentrates (Mannucci et al. 2013, James & Hawke 2014). Additionally, use of recombinant interleukin 11 (IL-11) has been shown to sustain increases of VWF and FVIII by upregulating VWF mRNA
expression and subsequent VWF release (Olsen et al. 2003, Ragni et al. 2013). Recombinant IL-11 treatment would benefit patients who are unresponsive to DDAVP, or enhance the efficacy of DDAVP when used concomitantly as these treatments increase factor levels through different mechanisms (Olsen et al. 2003, Ragni et al. 2013, James & Hawke 2014).

1.4 Endothelial Cells (ECs)

The endothelium is an integral component of the vascular wall and in the average adult it comprises approximately 7 square meters (Cines et al. 1998). Endothelial cells (ECs) comprise the lining of the luminal surface of the vasculature, acting as a tightly regulated interface between the blood stream and the surrounding tissue, regulating trafficking of blood components and nutrients, controlling neovascularization, vascular tone, the extravasation of leukocytes, and hemostasis (Lin et al. 2000, Aird 2006). Significant EC heterogeneity has been witnessed throughout the vasculature where the cellular structure and gene expression profiles of ECs are highly dependent on the vascular bed to which they belong (Chi et al. 2003). This heterogeneity of ECs is thought to be regulated by two main forces: the cellular microenvironment, and epigenetic markers affecting gene expression (Torres-Vazquez et al. 2003, Aird 2012).

Structural differences have been noticed, as some vascular beds such as those found in large arteries, have tight EC junctions, and more permeable endothelium tend to be found in venules to allow for extravasation of leukocytes under inflammatory conditions (Aird 2007). ECs lining the straight segments of the vasculature appear to align in an elongated fashion in the direction of blood flow, whereas vascular curvatures and bifurcations are lined with smaller, disorganized ECs with a cobblestone morphology (Aird 2006). Structural differences between vascular beds extend to the intracellular level as well, as ECs of the pulmonary artery produce an increased number of WPBs (Yamamoto et al. 1998).
As well as structural differences, ECs of different vascular beds are known to have different gene expression profiles. At sites prone to the development of atherosclerotic lesions, curvatures and bifurcations of the arteries, the endothelium will have enhanced expression of vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) relative to other arterial ECs (Nakashima et al. 1998). Differentiated ECs can respond differently to various stimuli as well; in mice it has been shown that the endothelium of the inferior vena cava become more permeable when exposed to tumour necrosis factor alpha (TNFα), whereas no such effect is seen in the endothelial cells of the aorta (Eriksson et al. 2005).

As the luminal lining of the vasculature, the endothelium plays a major role in hemostasis by expressing pro- and anti-coagulant molecules to varying degrees in response to environmental stimuli (Stern et al. 1991). In its natural, quiescent state, the endothelium presents an anticoagulant phenotype through the production of endothelial nitric oxide synthase (eNOS), and the activated protein C/protein S pathway, and the expression of the anti-coagulant mediator endothelial protein C receptor (EPCR) (Stern et al. 1991, Laszik et al. 1997). However, when endothelial cells are in a more activated state or exposed to disrupted blood flow, they are known to express tissue factor (TF), a promotor of the coagulation cascade (Lupu et al. 2005). A vast amount of the endothelial phenotype presented is determined by an element of their microenvironment known as shear stress.

### 1.4.1 Endothelial Cells and Shear Stress

The force a fluid moving over a solid surface exerts on the surface, in the direction of fluid movement, is known as shear stress (Resnick et al. 2003). When considering blood flowing through the circulatory system, the blood is exerting shear stress on the ECs lining the blood vessel walls. The degree of shear stress relates to a number of features of the blood and the vessels, and is proportional to the
viscosity of the blood, the rate of flow, and the inverse cube of the vessel radius and can be measured in dynes/cm$^2$ (force/area) (Boon & Horrevoets 2009).

1.4.1.1 The Endothelial Mechanosensory Complex

Shear stress is detected by the ECs mechanosensory complex of transmembrane proteins involving platelet-endothelial cell adhesion molecule (PECAM-1), vascular endothelial cadherin (VE-cadherin), and vascular endothelial growth factor receptor 2 (VEGFR2) (Shay-Salit et al. 2002, Osawa et al. 2002, Newman & Newman 2003, Tzima et al. 2005, Conway & Schwartz 2012, Zaragoza et al. 2012). PECAM-1 is the major component responsible for detecting the shear stress being exerted on the ECs and these signals can be instigated through manipulation of magnetic beads coated with PECAM-1 specific antibodies (Osawa et al. 2002, Chiu et al. 2008). PECAM-1 knock out mice are shown to have dramatically reduced responses to shear and decreased nitric oxide-dependent responses (Bagi et al. 2005, Conway & Schwartz 2012) and in pairing with the remaining components of the mechanosensory complex these signals are transmitted intracellularly. VE-cadherin is an indirect activator of the shear stress response, implicated in transmitting PECAM-1 signaling into the cell, aiding in the association between PECAM-1 and VEGFR2 (Tzima et al. 2005, Liu et al. 2008). Mechanosensory for applied to PECAM activates and recruits src family kinases (Chiu et al. 2008, Conway & Schwartz 2012); as VE-cadherin mediates the PECAM-1 interaction with VEGFR2, VEGFR2 is transactivated by src family kinases. Activated VEGFR2 mediates the activation of phosphoinositide-3-kinase (PI(3)K) and endothelial nitric oxide synthase (eNOS) within 15 seconds of shear onset (Tzima et al. 2005, Conway & Schwartz 2012).

Intracellularly the effects of shear stress are mediated by a vast number of kinases such as src family kinases, PI(3)K, extracellular signal-regulated kinases (ERKs), c-Jun amino terminal kinases (JNKs), p38 mitogen-activated kinase, and AKT serine/threonine kinases, as well as many transcription factors such as Kruppel-like factor 2 (KLF2) and nuclear factor of kappa light chain gene enhancer in B cells (NF-
κB) (Tzima et al. 2005). These factors mediate a wide range of downstream effects with PI(3)K being particularly involved with integrin activation (Tzima et al. 2005)

1.4.1.2 Effects of Laminar and Oscillatory Shear Stress

Depending on the consistency in direction of blood flow, different types of shear stress will be exerted on the endothelium and the type of flow exerted on the EC will play a role in determining its EC phenotype (Boon & Horrevoets 2009). When flow is unidirectional this is known as laminar flow, which occurs in straight vessels experiencing uninterrupted blood flow (Resnick et al. 2003). This laminar flow occurs throughout the vasculature at a variety of speeds; in small veins the shear stress is 0.1-5 dynes/cm²; 5-10 dynes/cm² is found in large veins and small arteries, and 10-40 dynes/cm² in large arteries (Resnick et al. 2003). When laminar flow reaches rates of >60 dynes/cm² this shear stress is known to have pathologic consequences and rates this high are commonly found in stenotic vessels or individuals with hypertension (Resnick et al. 2003). When healthy levels of laminar flow are exerted on ECs, an anti-inflammatory, anti-coagulant phenotype is induced through the increased expression of the transcription factor Kruppel-like factor 2 (KLF2), the potent vasodilator and inhibitor of platelet aggregation, eNOS, and the anti-coagulant molecule thrombomodulin (TM) (Ranjan et al. 1995, Dekker et al. 2006, Rabelink et al. 2010, Egorova et al. 2012) (Figure 1.5).

Oscillatory shear stress occurs when blood flow rapidly changes direction and because of these directional shifts the net shear stress can vary from negative values, to 0 dynes/cm² to positive values of 40 dynes/cm², and in cases of hypertension shear stress values are increased (Resnick et al. 2003). This type of flow is represented in the vascular curvatures or at sites of bifurcations (Resnick et al. 2003). This oscillatory flow provokes an EC phenotype that is prone to atherosclerotic lesions, pro-coagulant, and pro-inflammatory (Resnick & Gimbrone 1995, Resnick et al. 2003) by causing disorganization of the EC’s
cobblestone morphology, and inducing the expression of TF, VCAM-1 and ICAM-1 which can bind the pro-inflammatory transcription factor NF-κB (Resnick & Gimbrone1995) (Figure 1.5).

**Figure 1.5: Effects of Laminar and Oscillatory Flow on the Endothelium.** Figure adapted from Resnick et al. 2003.

1.4.1.3 Shear Stress and Splicing

Mechanosensory signaling has been shown to modulate not only gene expression in mechanocytes such as endothelial cells, smooth muscle cells and fibroblasts (Huan & Tang 2013, Murphy & Hynes 2014). Interestingly, many of these shear stress responsive genes have alternative splicing variants, the regulation of which has also been shown to be altered through shear stress signaling pathways in mechanocytes (Huan & Tang 2013, Murphy & Hynes 2014). Vascular endothelial growth factor (VEGF) splice forms vary with the degree of shear stress (Houck et al. 1991, Seko et al. 1999, Saygili et al. 2011). Under high shear stress, matrix bound splice forms of VWF are produced; whereas under low shear stress stimulation, soluble variants predominant stimulating outgrowth of the vasculature (Maes et al. 2004). Insulin-like growth factor (IGF-I) splicing produces the MGF splice form in response to mechanical

It is hypothesized that regulation of alternative exons may be driven by regulation of Ca\(^{2+}\) signaling triggered by mechanical stimulation such as shear stress, affecting the enhancing and silencing splicing factors and machinery of the spliceosome (31, Huan & Tang 2013). This splicing effects of the Ca\(^{2+}\) signaling may be coupled with phosphorylation pathways or depolarization (Huan & Tang 2013). There are many different phosphorylation signaling pathways regulated by mechanotransduction and protein phosphorylation is integral to alternative splicing (Huan & Tang 2013). Phosphorylation is involved in spliceosome assembly and phosphorylation of SR proteins is involved in splice site selection and identification by these splicing factors (Huan & Tang 2013). Depolarization is known to increase the amount of hnRNP A1, a splicing silencer, in neurons, which reduced inclusion of alternative exons of other splice factors and snRNPs (34, Huan & Tang 2013).

1.5 Studying von Willebrand Disease In Vitro

*In vitro* investigation of VWD has changed dramatically over the past few decades. As ECs are a primary producer of VWF, the protein of interest when studying VWD, they would be considered the gold standard for assessing VWD *in vitro*. Initially, human umbilical vein endothelial cells (HUVEC) were the cells of choice in this regard as they were easily accessible and readily produced endogenous VWF with biosynthesis techniques reflective of *in vivo* conditions (Booyse et al. 1981, Ewenstein et al. 1990, Federici.
et al. 1993). The downside to these ECs is that while normal HUVEC were in ample supply, it is harder to access HUVEC carrying VWD mutations of interest for further studies. With this, \textit{in vitro} study of VWD turned to the use of heterologous cell models such as AtT-20 cells, COS cells, or human embryonic kidney (HEK)293T cells to investigate specific mutations in both homozygous and heterozygous conformations through transient and stable cell transfections. These heterologous cell lines are ideal as they do not produce any endogenous VWF therefore any background ‘noise’ is not an issue in quantification and visualization of transfected mutant VWF. The use of HEK cells became more popular as it was noted that these cells also process the transfected VWF more similarly to ECs, packaging the VWF into pseudo-WPBs capable of regulated secretion (Micheaux et al. 2003).

More recently it was shown that ECs could be derived in the outgrowth of cultured peripheral blood samples (Lin et al. 2000). These blood outgrowth endothelial cells (BOEC) have been characterized as true ECs; they are positive for endothelial cell surface markers such as platelet endothelial cell adhesion molecule (PECAM) and VE-cadherin, and are known to produce VWF, P-selectin, and Ang-2 which they contain in WPBs (van Beem et al. 2009). As well, this endothelial phenotype can be maintained over multiple passages (Lin et al. 2000). In the literature, these cells have also been referred to as endothelial colony forming cells (ECFCs) and late outgrowth endothelial progenitor cells (EPC). \textit{In vitro} investigations using EC models have taken to evaluating these cells under various shear stress conditions and observing changes in the endothelial cell phenotype. This is also applicable to the study of VWD as ECs are in a more physiologically relevant environment when subjected to shear stress than when grown in static culture. BOEC under shear stress have shown a mature, yet undifferentiated, EC transcriptional response; not quite reflective of either arterial or venous endothelium (Egorova et al. 2012). Regardless of shear stress, BOEC show high expression of CD44 which may reflect their undifferentiated status as well as enabling these cells to navigate to the endothelial monolayer in circulation by binding to hyaluronic acid of the glycocalyx in...

While it is not yet clear exactly which endothelial bed BOEC reflect, they have been seen to recapitulate the donor’s phenotype in terms of VWF expression (Wang et al. 2013). This ex vivo representation of the patient condition is especially useful in the study of the pathogenesis of VWD as it is now possible to isolate and culture BOEC from patients with VWD and observe and manipulate a model of their endothelium (Berber et al. 2009, Starke et al. 2011, Starke et al. 2013). The intricacies of VWD, such as the mechanisms behind patient VWF biosynthesis, trafficking and WPB secretion can now be observed. Additionally, these cells could provide a useful first step in testing novel therapies for VWD, or other diseases involving endothelial dysfunction.

1.6 Overall Thesis Objectives

Aberrant splicing of von Willebrand factor is known to cause approximately 10% of VWD cases (James et al. 2007, Corrales et al. 2011); however this assessment primarily accounts for mutations to consensus splice site sequences and we hypothesize this mechanism is likely under-recognized. This study aims to identify and characterize the effect exonic and intronic sequence variants that are causative of VWD have on VWF splicing. Secondly, as alternative splicing is a predominant mechanism of diversity in multi-exon genes, we hypothesize there are unrecognized alternative splice variants of VWF in the normal population due to the large size of the VWF gene.
1.6.1 Specific Objective 1: Genotypic & Phenotypic Investigation of Families with Potential Splicing Mutations

This study aimed to determine if splicing mutations are the genetic basis of bleeding pathology across three families diagnosed with VWD, genotypic and phenotypic investigation of three putative splicing mutations will evaluate the effect of exonic, consensus splice site and intronic changes on the highly regulated process of VWF splicing. The functionality of their circulating plasma VWF will be assessed and blood outgrowth endothelium grown in culture to evaluate mechanisms of VWF biosynthesis. The presence of aberrantly spliced VWF in the regions of BOEC RNA surrounding their genomic mutations will be evaluated and the VWF splice variants will be quantified from the RNA of BOEC grown under static conditions as well as after short-term exposure to high levels of laminar shear stress.

1.6.2 Specific Objective 2: Characterization of Aberrant VWF Splicing Variants

Aberrant VWF splice variants are the genetic basis of VWD in an acknowledged 15% of cases; however, the explicit pathologic mechanisms are not commonly elucidated. Chapter 3 aimed to assess the mechanisms by which the aberrant splice forms identified in Chapter 2 lead to VWD, and their effect on VWF functionality, by fully characterizing VWF synthesis, secretion, functionality (platelet, FVIII and collagen binding and multimerization), and intracellular trafficking of the mutant VWF using a heterologous human embryonic kidney cell (HEK) 293(T) cell model.

1.6.3 Specific Objective 3: Identification of Alternative VWF Splicing Variants in Normal BOEC

As ~90% of human genes are subject to the protein diversifying process of alternative splicing, it remains curious that such a large gene as VWF would not be subject to such a common and beneficial mechanism. Chapter 4 aimed to elucidate the possible alternative splicing of VWF in normal ECs, and whether such splicing is subject to regulation by biochemical and biomechanical stressors or heterogeneity.
between EC types. To accomplish this, a targeted approach to alternative splicing of VWF was undertaken with RNA from three normal BOEC lines, HUVEC and HMVEC with and without exposure to biochemical and biomechanical stressors.
CHAPTER 2
Patients’ Phenotype, Genotype & Ex Vivo Investigation

2.1 Summary

Approximately 10% of VWD cases are caused by splicing mutations the majority of which are located in the canonical bases of the intron-exon splice sites (James et al. 2007). This is not the only location where splicing mutations can occur, and therefore the proportion of cases caused by splicing mutations may in fact be much higher. In silico analyses can be used as a preliminary method to determine whether putative mutations may induce changes in splicing patterns. This chapter investigates three cases where putative splicing mutations were identified by in silico analyses. Patient VWD phenotypes were assessed using plasma samples and through the ex vivo analysis of patient-derived and normal BOEC.

The first family under investigation was found to have the exonic mutation c.3538G>A which is predicted to cause loss of recognition of the exon 26 donor site. This family has moderate Type 1 VWD with decreased VWF:Ag and VWF:RCo and normal multimers. The reduction in VWF levels is reflected in the BOEC ex vivo phenotype which showed intracellular retention of VWF. Three aberrant transcripts were found in this family alongside WT VWF: exon 23 skipping, exon 26 skipping, and skipping of exons 23 & 26 together.

The second family’s VWD was found to be caused by a canonical splice site mutation c.5842+1G>C which causes combined skipping of exons 33-34. This mutation causes Type 1 VWD with mildly decreased VWF:Ag, and when co-inherited with the nonsense mutation c.3939G>A causes Type 3 VWD with severely decreased VWF:Ag and VWF:RCo in patient T151. BOEC from Family 2 show diffuse VWF staining which colocalizes with the ER, but they also form punctate Weibel-Palade bodies (WPBs) which show colocalization with P-selectin and Ang-2. A transcript skipping exon 33 was also found in this
family as well as several normal controls. Exon 33 skipping would cause a frame-shift, introducing a premature termination codon (PTC) and target this transcript for nonsense-mediated decay (NMD).

The third family under investigation was the intronic mutation c.6599-20A>T which is predicted to abolish the acceptor site of intron 37 causing mild Type 1 VWD in this family; this is thought to be due to the loss of the invariant A of the branch site. Sequencing of the BOEC RNA showed VWF transcripts skipping exon 38 at low levels and WT VWF as both patients are heterozygous for the c.6599-20A>T mutation. VWF transcripts skipping exon 38 would also introduce a PTC and be targeted for NMD. BOEC from these affected siblings showed colocalization of VWF with the ER as well as WPB inhabitants Ang-2 and P-selectin. This chapter characterizes the disease phenotype in three affected families, identifying three VWF splicing mutations which cause VWD through exon skipping, leading to intracellular retention or degradation of the patient VWF.
2.2 Introduction

von Willebrand disease (VWD) is the most common bleeding disorder affecting humans and it is caused by either quantitative deficiencies or qualitative defects in the integral hemostatic protein von Willebrand factor (VWF). VWD can be divided into three main subtypes: Type 1, Type 2, and Type 3, the most common of which is Type 1 VWD, reflecting approximately 80% of VWD cases (Goodeve 2010). Many individuals affected with VWD have causative mutations to the VWF gene though in many instances no VWF mutations have been found. Despite the prevalence of Type 1 VWD, causative mutations have not been determined for approximately 35% of afflicted individuals across three multicenter cohort studies (James et al. 2007, Cumming et al. 2006, Eikenboom et al. 2006, Goodeve et al. 2007).

For many of the putative mutations that have been found, the pathogenic mechanism has yet to be unraveled. The pathobiology of the apparently mutation-negative cases could be explained by a variety of possibilities including: 1) missed mutations because of primer SNPs, 2) partial VWF gene deletions, duplications, or inversions missed by direct sequencing, 3) mutations in loci outside of VWF which reduce VWF levels, and/or 4) VWF changes outside of consensus splice sites causing aberrant splicing (James et al. 2007). It is possible that some mutations are pathogenic only if combined with other factors (James et al. 2007). Along with these issues: incomplete penetrance, variable expressivity, and the effect of ABO blood group complicate investigations of the genetic basis of Type 1 VWD (Goodeve 2010). Additionally, VWF levels are known to be affected by age, ethnicity, stress levels, hormones, and exercise (Kadir et al. 1999, Miller et al. 2001, Stakiw et al. 2008).

2.2.1 Pathologic VWF Mutations

Mutations causing VWD have been found all throughout the expansive VWF gene (Mancuso et al. 1989). Missense mutations are the most prevalent in Type 1 VWD; however, insertions, deletions, frame
shifts, nonsense and splicing mutations have also been identified (Faustino & Cooper 2003, Goodeve 2010). Of the 123 index cases in the Canadian Type 1 VWD Study, four had consensus splice site mutations considered to be the basis of their disease (James et al. 2007). Mutations outside of consensus splice sites that affect splicing could explain the pathogenic mechanism for some VWD cases for which the mechanism was previously unknown.

2.2.1.1 VWF Splicing Mutations

Mutations affecting VWF splicing have been described in large cohort studies though the majority of mutations suspected of affecting splicing are located in the highly conserved canonical nucleotides (Cumming et al. 2006, Goodeve et al. 2007, James et al. 2007, Bowman et al. 2013). The majority of these splicing mutations are not further investigated with mRNA studies. Exon 26 skipping may be the most commonly described aberrant splicing of von Willebrand factor having been caused by the exon 26 mutations c. 3538G>C (James et al. 2004), a synonymous c.3390C>T mutation, and a canonical splice site mutation, c.3380-2A>G (Pagliari et al. 2013) all of which describe Type 2A VWD in their affected patients. The c.1534-3C>A mutation in acceptor site of exon 14 has been described to cause a variety of splicing outcomes: skipping of exon 14 which introduces a premature termination codon (PTC), the inclusion of 62 bp of intron 13 through the activation of a cryptic splice site, as well as retention of WT splicing at the mutated acceptor site (Gallinaro et al. 2006). In many cases of aberrant splicing mutations the specific splicing changes can be disguised by effective NMD which upon transcriptome analysis shows no detectable transcript from the mutated allele (Plate et al. 2010, Corrales et al. 2011).

2.2.2 Assessment of VWD

Diagnosis of Type 1 VWD can be complicated due to the wide range of normal VWF levels that exist in the general population, as well as the milder phenotype that is presented. Individuals may not
recognize that their bleeding is excessive or abnormal. To enable quantification of bleeding symptoms and determine if they are abnormal bleeding assessment tools (BATs) are used. While most are meant to be expert administered, the hope is that these BATs will become accessible to the general public and be employed by general practitioners before referral to a hematologist. Type 1 VWD poses other complications in that definitively causative mutations can be difficult to identify and in vitro investigation of these putative mutations may be required to determine whether they are truly pathogenic of VWD in these individuals.

2.2.3 Endothelial Cell Models of VWD

As issues with VWF production or function are the main cause of VWD, and VWF is primarily synthesized in endothelial cells, this is a good place to start an in vitro investigation into the pathologic mechanisms underlying VWD. Since circulating endothelial cells are sparse in the peripheral blood of normal adults, human umbilical vein endothelial cells (HUVEC) have often been used in the study of the pathogenesis of VWD; however access to HUVEC containing VWF mutations of interest is rare (Booyse et al. 1981, Ewenstein et al. 1990, Federici et al. 1993). To that end, the observation that endothelial outgrowth occurs from cultured peripheral blood (Lin et al. 2000) has become highly advantageous. These blood outgrowth endothelial cells (BOEC) are known to produce VWF, contain WPBs, maintain their endothelial phenotype over multiple passages (Lin et al. 2000), and have been seen to recapitulate the patient’s endothelial phenotype and VWD, making them an excellent model to investigate the molecular pathogenesis of specific mutations (Berber et al. 2009, Starke et al. 2011, Starke et al. 2013, Wang et al. 2013).
2.3 Objectives

This chapter aimed to investigate the molecular pathogenesis of VWD across three families with mutations with potential to affect the VWF splicing mechanism. This was accomplished through identification of their causative mutation, disease phenotype, and ex vivo investigation of their endothelium using a BOEC model.

2.4 Materials and Methods

2.4.1 Patient Selection & Genotyping

Patients were selected from index cases and their affected family members from the Type 1 VWD Study (James et al. 2007) and Canadian Type 3 VWD Study (Bowman et al. 2013) when putative mutations were thought to have potential to affect VWF splicing. Three families were included based on their mutations, with two affected family members from each family. One family had a consensus splice site mutation, the most likely type of mutation to affect splicing, the other families had either exonic or intronic mutations less commonly expected to disrupt splicing. All participants gave informed consent and approval for this study was granted by the Research Ethics Board of Queen’s University, Kingston, Canada. Peripheral venous blood samples were collected to obtain plasma, genomic DNA, platelet RNA and BOEC from these patients (detailed methods below).

2.4.1.1 DNA Analysis

Genomic DNA was isolated from whole blood samples anti-coagulated with EDTA. Five millilitres (mL) of blood was mixed with 5 mL of low salt TKM1 buffer (10 mM Tris HCl pH 7.6, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA). Cells were lysed with 1.25 mL of 10% Nonidet P-40 and the solution was mixed well by inversion before centrifugation at 2200 rpm for 10 minutes at room temperature. The supernatant was
discarded; the nuclear pellet was saved and washed with TKM1 buffer. Centrifugation and washes were repeated until most RBCs were removed. The pellet was then gently resuspended in the high salt buffer TKM2 (10 mM Tris HCl pH 7.6, 10 mM KCl, 10 mM MgCl2, 0.4 M NaCl, 2 mM EDTA). After the addition of 50 µL of 10% SDS, the solution was incubated at 50°C for 20-30 minutes, until the pellet was fully dissolved. After mixing in 0.4 mL of 5 M NaCl, the solution was centrifuged at 12,000 rpm for 5 minutes. The supernatant was then added to 2.4 mL of 100% ethanol to allow for the precipitation of DNA strands at room temperature. The resulting DNA strands were added to 0.2 mL of buffer TE (10 mM Tris HCl, 1 mM EDTA; pH 8.0) and incubated at 65°C for 5 minutes uncovered to allow the ethanol to evaporate, then covered and left to incubate for several hours. Extracted DNA was quantified through spectrophotometry and each exon and 1.5kb of the VWF promotor was amplified by polymerase chain reaction (PCR) including ~50nts of the intron/exon boundaries and flanking intronic sequences (Primer sequences in Appendix B). Sanger sequencing of each region was performed and chromatograms were evaluated by two experienced technologists to confirm their putative VWF splicing mutations.

2.4.2 Phenotypic Assays

The frequency and severity of these patients' bleeding symptoms including mucocutaneous bleeding, muscle hematomas and hemarthroses, were evaluated using the Condensed MCMDM-1VWD BAT (Bowman et al. 2008) which was expert administered. Bleeding scores were determined through summation of all bleeding symptoms experienced by an individual. VWD status was confirmed through VWF:Antigen (VWF:Ag) enzyme-linked immunosorbant assay (ELISA) measuring plasma VWF levels; VWF activity was assessed using the ristocetin cofactor assay (VWF:RCo) which measures VWF interactions with normal platelets. FVIII coagulant activity (FVIII:C) was measured level in a one stage assay and VWF multimer analysis was completed on a 1.4% SDS-Page gel. The capacity of the patients'
plasma VWF to bind types 1 and 3 collagen, and FVIII (Advate®) was also assessed using ELISA based assays.

2.4.2.1 VWF:Antigen (VWF:Ag) ELISA

An ELISA was used to detect the amount of VWF in the plasma of each of the patients. The plates were coated overnight at 4°C with the primary antibody, a polyclonal rabbit anti-human von Willebrand factor A0082 (DAKO). The plate was then washed 3 times using a wash/dilution buffer (10 mM Na₂HPO₄, 500 mM NaCl, 1% Tween 20; pH 7.2) and the plasma samples which were diluted 1:50 (vol/vol) in this same buffer were added to the plates. A standard curve was created using normal reference plasma (CCNRP-10, Precision Biologic) and serial dilutions beginning at 1:20 (vol/vol). Samples were incubated for at least 2 hours at room temperature to bind to the primary antibody on the plate before washing the plate (as above). The detection antibody was horseradish peroxidase (HRP) conjugated polyclonal rabbit anti-human von Willebrand factor P0226 (DAKO) which was diluted 1:8000 (vol/vol) in wash buffer and added to the plates for a 1 hour room temperature incubation. The plate was then washed again (as above) before the addition of the colour reagent [15 mL citric acid-phosphate buffer (0.0347 M citric acid, 0.0667 M Na₂HPO₄; pH 5), 2 o-Phenylenediamine (OPD) 5mg tablets, 6.2 µl 30% hydrogen peroxide (H₂O₂)] which was incubated at room temperature until the appearance of the standard curve. The development of the ELISA was then stopped through the addition of 1 M H₂SO₄ and the plate was read at 492 nm on the plate reader (Versamax).

2.4.2.2 VWF:Ristocetin Cofactor Assay (VWF:RCo)

VWF’s ability to bind platelets and induce platelet aggregation was measured using the ristocetin cofactor assay (VWF:RCo). Firstly, the aggregometer (Chrono-log) was calibrated using standard curves of normal and abnormal reference plasma (CCNRP-10 and ARP1-10, Precision Biologic), and normal, formalin fixed, lyophilized platelets (Helena Laboratories) were reconstituted with Trisaminomethane
(TRIS)-buffered saline. Platelets were incubated for 4 minutes in cuvettes before the addition of ristocetin (American Biochemical and Pharmaceutical Corporation) 2 minutes prior to the introduction of the patient plasma. Once the plasma had been added to the sample cuvette, the aggregometer measured the increase in transmission of light through the sample as a reflection of the degree to which the platelets had aggregated.

2.4.2.3 FVIII Coagulation Assay (FVIII:C)

Measurements of coagulation time and FVIII level were conducted using a one stage FVIII coagulation assay. Standard curves for coagulation time were created using normal and abnormal reference plasma (CCNR-10 and ARP1-10, Precision Biologic). Patient plasma was then mixed with FVIII-deficient plasma and the coagulation time was measured. The FVIII level is then calculated by the STA Compact Coagulation Analyzer (STAGO) based on the patient plasma’s ability to normalize the partial thromboplastin time (PTT) of the FVIII-deficient plasma.

2.4.2.4 Multimer Analysis

Multimer analysis was conducted on patient plasma using 1.4% separating sodium dodecyl sulphate (SDS)-agarose gel electrophoresis and chemiluminescent detection including a normal pooled reference plasma control (CCNR-10, Precision Biologic) and a Type 2A VWD abnormal reference control (ARP1-10, Precision Biologic).

2.4.2.5 Collagen Binding ELISA

Patient plasma VWF was assessed for its ability to bind collagen using a collagen binding ELISA. The plate was coated with a blend of bovine collagen type I (95%) and type III (5%) (ICN) and incubated with 3% bovine serum albumin (BSA) to block non-specific binding. Standard curves were made using normal pooled reference plasma control (CCNR-10, Precision Biologic) and plasma samples were
incubated over 3 hours and detected with an HRP conjugated polyclonal rabbit anti-human von Willebrand factor P0226 (DAKO). The ELISA was developed using the colour reagent from the VWF ELISA (Section 2.3.2.1) and 1M H₂SO₄ and read at 492nm on the plate reader (Versamax).

2.4.2.6 FVIII Binding ELISA

Patient plasma VWF was assessed for its ability to bind FVIII using a FVIII binding ELISA. The plate was coated with rabbit anti-human von Willebrand factor (A0082, DAKO) Standard curves using normal pooled reference plasma control (CCNRP-10 and ARP1-10, Precision Biologic), and plasma samples were diluted in TBST (50 mM Tris, 100 mM NaCl, 0.1% BSA, 0.05% Tween20, pH8.0) buffer with 3% BSA to block non-specific binding and incubated for 2 hours at room temperature. Endogenous FVIII was removed from immobilized VWF during an hour incubation with TBST containing 0.35 M CaCl₂ after which 1.25IU/mL FVIII (Advate®) was applied to bind the VWF. Bound FVIII was detected using HRP conjugated anti-human FVIII (#F8C-EIS-D, Affinity Biologicals) and measured using the same colour reagent and quantification protocol as the VWF ELISA (Section 2.3.2.1).

2.4.3 Ex Vivo BOEC Investigation

2.4.3.1 BOEC Isolation

To isolate BOEC from the patients as well as normal individuals, ~48mL of peripheral blood collected in heparin-sulphate anti-coagulant Cell Preparation Tubes (CPT)™ (BD Biosciences) were centrifuged at room temperature for 30 minutes at 1600 RCF. The serum and mononuclear cells (MNC) were collected and subjected to a series of centrifugation and washing steps with PBS supplemented with 10% (vol/vol) fetal bovine serum (FBS) before being seeded onto rat-tail type 1 collagen coated 6-well tissue culture plates at a density of 3-5x10⁷ MNC per well using Endothelial Growth Medium-2 (EGM-2) (CC-3156, Lonza) supplemented with 10% FBS, 1% penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL)
amphotericin (25 µg/mL) (15240-062, Invitrogen), and the EBM-2 BulletKit™ (CC-3162, Lonza) (complete EGM-2 media, cEGM-2). Twenty-four hours after seeding, cells were washed with cEGM-2 to remove non-adherent cells and debris, and then cEGM-2 was replaced; this process was repeated daily for 7 days, and media was changed every other day thereafter. BOEC colonies appeared between days 9 and 23 in culture. (Martin-Ramirez et al. 2012). BOEC were used between passage four to ten.

2.4.3.2 BOEC Characterization

Once established these BOEC were characterized as true ECs through: A) their cobblestone morphology when grown in confluent monolayers, B) staining for cell surface markers assessed by flow cytometry as well as, C) immunofluorescent (IF) staining and confocal microscopy for intracellular VWF.

To confirm their endothelial cell phenotype, these cells were assessed for the presence of CD31 (platelet endothelial cell adhesion molecule, PECAM-1), and CD144 (VE-cadherin), and/or CD146 (melanoma cell adhesion molecule, MCAM), as well as assessing the absence of the monocyte CD14 and CD45 (protein tyrosine phosphate receptor type C, PTPRC) surface markers. To assess these markers fluorescently conjugated antibodies were used for direct immunofluorescence as follows: FITC CD31, PE CD144, PE CD 146, PerCp Cy5.5 CD14, PE-Cy5 CD45 (Ebioscience). Positive controls for the endothelial cell phenotype CD31, CD144, and CD146 were assessed by staining human umbilical vein endothelial cells (HUVEC) and human acute monocytic leukemia cells (THP1) were used as a control for the monocyte markers CD14 and CD45. Additionally, isotype control antibodies were also used to account for nonspecific binding and samples were analyzed using an Epics Altra HSS flow cytometer (Queen’s Cytometry and Imaging Facility; The Cancer Research Institute at Queen’s University, Kingston, Canada).

To visualize the intracellular VWF, confluent BOEC monolayers were seeded 5x10⁵ cells per well onto glass coverslips coated with rat tail collagen type I in 6-well tissue culture plates. Twenty-four hours after seeding the BOEC were fixed with BD cytofixperm (554714, BD Biosciences), permeabilized with 1%
Triton-X, and stained. Polyclonal rabbit anti-human VWF (1:500, A0082, DAKO) and anti-rabbit immunoglobulin-FITC (F0054, DAKO) were used to visualize intracellular VWF, alongside a negative control rabbit immunoglobulin fraction (X0936, DAKO) isotype control for non-specific binding. Phalloidin-TRITC (P1951, Sigma) and DAPI (D9542, Sigma) were used to stain for actin filaments and nuclei respectively. When possible, staining of additional WPB proteins such as P-selectin (1:50 sc-6941, Santa Cruz) and Ang-2 (1:100, AF623, R&D Systems) was conducted to determine if the VWF was co-localizing to WPBs. The endoplasmic reticulum was stained with goat anti-calnexin (1:100, sc-6465, Santa Cruz), and mouse anti-Lamp-1 (1:100, MAB4800, R&D Systems) was used to stain the lysosomes. Secondary antibodies for the organelles included donkey anti-goat IgG-Rhodamine (sc-2094, Santa Cruz), and Alexa-fluor 568 donkey anti-mouse IgG (A10037, Invitrogen).

Slides were visualized at room temperature using the Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification; images were captured with a Hamamatsu Orca high resolution camera, and Image J software was used for analysis (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University, Kingston, Canada).

2.4.3.3 BOEC & VWF Secretion

The capacity of the BOEC to produce and secrete VWF constitutively and after stimulation was evaluated using the in vitro secretagogue phorbol 12-myristate 13-acetate (PMA) (P8139, Sigma). BOEC were seeded onto rat tail collagen type 1 coated 6-well plates at a density of 1x10⁶ cells per well in cEGM-2 and left to incubate for 24 hours after which the medium was switched to Opti-MEM reduced serum media (31985-070, Life Technologies) supplemented with 100 nM CaCl₂. Twenty-three hours later, half the wells were treated with 160nM PMA for 1 hour and the rest remained as unstimulated controls. Media was collected and cells were lysed after 24 hours in Opti-MEM. Secreted and intracellular VWF was quantified by VWF:Ag ELISA.
2.4.3.4 BOEC & Shear Stress

BOEC were subjected to laminar flow inducing high levels of shear stress to observe whether shear stress affects the splicing of VWF. BOEC were grown to confluence on 0.2 µluer slides (80161, Ibidi) and subjected to laminar flow at a shear stress at 50 dynes/cm² for 48 hours using an Ibidi pump system. Cells were then harvested and RNA was then extracted using an RNAqueous®-Micro Kit (AM1931, Ambion) due to the small number of cells present on the slides.

2.4.4 Platelet RNA Extraction

Peripheral blood was collected, anti-coagulated with sodium citrate, and processed within 24 hours of venipuncture. Platelet rich plasma (PRP) was obtained through centrifugation at 150 RCF for 20 minutes at 18°C which was then collected, avoiding the interface to prevent leukocyte contamination. PRP was then centrifuged at 2000 RCF for 15 minutes at 18°C to obtain a platelet pellet which was resuspended in 750 µl of 75% Trizol (Invitrogen), incubated for 5 minutes before the addition of 200 µl of chloroform. The solution was then vigorously shaken for 15 seconds and incubated another 10 minutes at room temperature which was followed by centrifugation at 20,000 RCF for 15 minutes at 4°C. The upper aqueous phase of the solution containing the platelet RNA was then added to 500µl of isopropanol, mixed and left to precipitate at -80°C overnight. Subsequently, the sample was centrifuged at 20,000 RCF for 20 minutes at 4°C, the supernatant was removed, and the pelleted RNA was washed with 75% ethanol, before being centrifuged again at 15,000 RCF for 5 minutes at 4°C. The ethanol was then removed and the pellet is air dried before resuspension in 20 µl of diethylpyrocarbonate (DEPC)-treated water (10813-012, Life Technologies) and quantified by spectrophotometry.
**2.4.5 In Silico Splicing Analysis**

Patient mutations were subjected to *in silico* analyses to predict whether they may affect the normal splicing pathways of the VWF gene using the web-based program Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), as well as Alamut 2.0. These programs assess the strength of the consensus splice site sequences with and without mutations, and Human Splicing Finder was used to infer how ESEs may be affected by the change.

**2.4.6 RNA Evaluation for Aberrant VWF Splicing**

BOEC RNA was extracted using the QIAamp RNA Blood Mini Kit (52304, QIAGEN) for each of the patients. Platelet and BOEC RNA were reverse transcribed (RT) to visualize any variation in splicing products in the RNA. Splicing of the VWF gene was evaluated through RT-PCR of the gene in 9 overlapping segments (exons 1-9; 8-17; 16-21; 20-28; 28-31; 30-36; 35-41; 40-45; 44-52) (Primer Sequences in Appendix B). No-template controls were used as negative controls. These segments were visualized on an 1% agarose gel, and any bands of variant sizes were sequenced to identify the splicing alterations.

The ratio of wildtype (WT) VWF and aberrant splicing variants within each patient’s RNA samples from BOEC grown under static and high shear stress conditions were quantified with Superscript III® Platinum® SYBR® green one step qRT-PCR (Invitrogen) according to the manufacturer’s protocol. Primer sequences can be found in Appendix B. No-template controls were used as negative controls. To allow for positive controls and standard curves for absolute quantification, plasmids containing the exon skipping sequences being probed for, as well as WT VWF were constructed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). Absolute quantification was conducted using standard curve analysis of serial dilutions of these plasmids allowing the comparison of the number of transcripts of each splice variant between BOEC grown in static conditions and those under high shear stress.
2.5 Results

2.5.1 Patient Genotype & Phenotype

2.5.1.1 Patient Selection and Mutation Identification

Six individuals from three families carrying mutations with potential splicing effects underlying their VWD were enrolled in this study (Figure 2.1). Affected family members from Family 1, V69 and V70, were both heterozygous for the exonic mutation c.3538G>A in exon 26 of VWF. Sequencing of the genomic DNA of Family 2 identified heterozygosity for a VWF consensus splice site mutation c.5842+1G>C in the mother, T152, and compound heterozygosity in her son T151 who had both the canonical c.5842+1G>C mutation as well as a nonsense mutation c.3939G>A in exon 28. Siblings from Family 3 were both found to be heterozygous for the intronic mutation c.6599-20A>T in intron 37. This mutation is thought to be a branch site mutation and therefore integral to proper splicing surrounding intron 37.

2.5.1.2 von Willebrand Disease (VWD) Phenotype

Phenotypic testing was conducted on each of the participants to confirm the diagnosis of VWD if VWF:Ag and VWF:RCo levels lower than the normal range (0.5-1.5 IU/mL) and abnormal BS ≥4 was documented using the MCMDM-1VWD BAT (Table 2.1). Five of the participants (V69, V70, T152, V447, and V449) were confirmed to have Type 1 VWD with VWF:Ag between 0.05-0.50 IU/mL with correspondingly decreased VWF:RCo. Patient’s T152, V447, and V449 exhibited the full range of high molecular weight multimers (HMWM); V69 and V70 however showed a slight reduction in these important HMWM which may indicate a Type 2A phenotype (Figure 2.2) Some multimer degradation from possible freeze/thaw during shipping of the plasma may be affecting the multimers from patients V69 and V70 (Figure 2.2); however, multimers from concentrated V70 BOEC media show a full range of HMWM. The remaining patient’s (T151) diagnosis of Type 3 VWD was confirmed as he exhibited significant reductions.
in VWF:Ag and VWF:RCo <0.05 IU/mL and a FVIII:C <0.10 IU/mL, and an absence of VWF multimers (Figure 2.5).

**Figure 2.1: Patient phylogenies, phenotypes, and mutation status.** Patients included in this study are outlined with green boxes. Normal VWF:Ag, VWF:RCo and FVIII:C fall between 0.05-1.5 IU/mL. Normal bleeding score (BS) ≤4.  A) Family 1 with moderate Type 1 VWD in a father and daughter. B) Family 2 with a mild Type 1 VWD mother and her compound heterozygote son affected with Type 3 VWD. C) Family 3 with two siblings exhibiting mild Type 1 VWD.
The first family, containing patients V69 and V70, exhibited significant mucocutaneous bleeding with bleeding scores of 17 and 11 respectively and this was reflected by their low VWF:Ag (0.13 and 0.27 IU/ml), VWF:RCo (0.12 and 0.17 IU/mL) and FVIII plasma levels (0.15 and 0.45 IU/mL) (Table 2.1). Their plasma VWF had a normal collagen binding ability, but a decreased capacity to bind FVIII 0.53±0.20IU/mL and 0.75±0.09IU/mL of normal pooled plasma (NPP) respectively (Table 2.1). This decreased functionality may reflect the location of their mutation in exon 26 and splicing defects in the surrounding area which is involved in the VWF:FVIII interaction and may lend insight into their decreased plasma FVIII levels.

T151 exhibited a strong Type 3 bleeding phenotype with a BS of 29 and effectively negligible factor levels: VWF:Ag 0.02 IU/ml, VWF:RCo 0.04 IU/mL, and FVIII:C of 0.01IU/mL. As expected, his Type 1 mother, T152, had higher, but still abnormal, factor levels: VWF:Ag 0.49 IU/mL, VWF:RCo 0.42 IU/mL, and FVIII:C of 1.16 IU/mL. Collagen binding was minimal in the Type 3 patient; however, T152’s plasma VWF reflected normal collagen binding (1.29 IU/mL) and FVIII binding (1.86±0.19) abilities relative to NPP (Table 2.1).

Family 3 subjects V447 and V449 presented with similar mild reductions in factor levels with VWF:Ag levels of 0.41 and 0.44 IU/mL and VWF:RCo of 0.38 and 0.44 IU/mL respectively (Table 2.1). FVIII:C levels for both subjects were in the normal range (0.51 and 0.75 IU/mL respectively) and their plasma VWF retained its full capacity to bind both collagen and FVIII relative to NPP (Table 2.1). Despite their similarities in factor level, the bleeding scores in this family were divergent; a higher bleeding score of 11 was found in the sister V447 which is likely due to the increased number of bleeding challenges women face compared to men.
Table 2.1: Patient von Willebrand disease phenotypes where BS ≥4 indicate symptomatic abnormal bleeding. The normal range for VWF:Ag, VWF:RCo, FVIII:C, collagen binding, and FVIII binding are between 0.50-1.50 IU/mL.

<table>
<thead>
<tr>
<th>Putative Splicing Mutation</th>
<th>Patient ID (Sex)</th>
<th>Bleeding Score (BS)</th>
<th>VWF:Ag (IU/mL)</th>
<th>VWF:RCo (IU/mL)</th>
<th>FVIII:C (IU/mL)</th>
<th>VWF: Collagen Binding (IU/mL)</th>
<th>VWF:FVIII Binding (IU/mL)</th>
<th>Multimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.3538 G&gt;A</td>
<td>V69 (F)</td>
<td>17</td>
<td>0.13</td>
<td>0.12</td>
<td>0.15</td>
<td>0.92</td>
<td>0.53±0.20</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>V70 (M)</td>
<td>11</td>
<td>0.27</td>
<td>0.17</td>
<td>0.45</td>
<td>0.85</td>
<td>0.75±0.09</td>
<td>Normal</td>
</tr>
<tr>
<td>c.5842 +1 G&gt;C</td>
<td>T151 (M)</td>
<td>29</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td>0.04</td>
<td>0.76±0.39</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>T152 (F)</td>
<td>8</td>
<td>0.49</td>
<td>0.42</td>
<td>1.16</td>
<td>1.29</td>
<td>1.86±0.19</td>
<td>Normal</td>
</tr>
<tr>
<td>c.6599-20 A&gt;T</td>
<td>V447 (F)</td>
<td>11</td>
<td>0.41</td>
<td>0.38</td>
<td>0.51</td>
<td>1.63</td>
<td>1.73±0.15</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>V449 (M)</td>
<td>4</td>
<td>0.44</td>
<td>0.44</td>
<td>0.75</td>
<td>1.25</td>
<td>1.60±0.10</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Figure 2.2: Patient Plasma Multimers. Lane 1: abnormal Type 2A control; Lane 2: normal pooled plasma control; Lane 3: V69 plasma; Lane 4: V70 plasma; Lane 5: T151 plasma; Lane 6: T152 plasma; Lane 7: V447 plasma; Lane 8: V449 plasma; Lane 9: NPP
2.5.2 BOEC Ex Vivo Analyses

BOEC were successfully isolated from all of the affected individuals, except V69, as an *ex vivo* model to evaluate the mechanisms behind their VWD. Constitutive VWF expression levels (VWF in media / VWF from media and lysates) from the patient BOEC were reflective of the VWF levels in the patient’s plasma, all of which were less than half the expression seen from normal BOEC (p<0.01). BOEC from patient V70 expressed 30±14% the amount of VWF expressed from normal controls which resembles their 0.27 IU/mL plasma VWF:Ag (Figure 2.3). T151 had minimal VWF expression, at levels 1.5±2% as echoed in his negligible plasma VWF levels, and his Type 1 VWD mother showed moderate VWF expression 52±13% of normal BOEC which is reflective of her mild symptoms (Figure 2.3). BOEC from Family 3 exhibited a similar phenotype *ex vivo* with constitutive expression levels of 43±3% and 47±3% for V447 and V449 respectively (Figure 2.3). This supports the theory that BOEC are an excellent model reflective of patient phenotype *ex vivo*.

![Figure 2.3: Constitutive and Induced VWF Expression from BOEC](image)

**Figure 2.3: Constitutive and Induced VWF Expression from BOEC.** BOEC constitutive VWF expression from unstimulated cells as well as PMA stimulated release of BOEC WPBs N=3 (* p<0.05; **p<0.01; ***p<0.001). A line under the significance stars indicates a significant increase in expression between unstimulated and PMA stimulated BOEC; no line under the significance, indicates significantly different unstimulated expression than the normal BOEC.
The BOEC from all the Type 1 individuals were responsive to PMA stimulus, which replicates the patients’ responses to DDAVP in vivo (V70, V449 p<0.05) (T152 p<0.01); however, the Type 3 BOEC from T151 showed a negligible increase in VWF release with PMA stimulation as expected (Figure 2.3). BOEC from V70 showed significant intracellular retention of VWF, T151 and V447 had significantly reduced intracellular VWF relative to the normal BOEC, and T152 and V449 had lysate levels in the normal range (Figure 2.4).

Immunofluorescent staining of the V70 BOEC showed a mixture of diffuse staining as well as punctate and rounded WPB; many of the cells also exemplified the intensity of intracellular VWF retention observed in the lysates (Figure 2.4; Figure 2.5B). T151’s BOEC contained very few rounded WPBs and some diffuse staining (Figure 2.5C). While the Type 1 T152 BOEC showed more WPBs, they often appeared rounded rather than the rod-shape characteristic of these organelles (Figure 2.5D). Patients V447 and V449 BOEC were unremarkable from normal BOEC (Figure 2.5A), exhibiting elongated WPBs as well as some diffuse staining which is more prominent within V447’s BOEC (Figure 2.5E-F).

**Figure 2.4: Lysate VWF from BOEC** show that V70 BOEC exhibit significant intracellular retention compared to normal BOEC as measured by VWF:Ag ELISA. N=3 (* p<0.05; **p<0.01; ***p<0.001 lysate levels compared to normals).
**Figure 2.5: Normal and patient BOEC IF staining.**

A) Normal BOEC with normally distributed rod shaped WPBs. B) Patient V70 with punctate VWF as well as diffuse VWF staining throughout the cells. C) Type 3 patient T151 with few punctate WPBs. D) Patient T152 BOEC show more classic, but also rounded WPBs as well as some diffuse staining E) Patient V447’s VWF appears diffuse within the cell as well as localized to punctate WPBs F) VWF staining in patient V449 appear normal with many rod-shaped WPBs.

Polyclonal rabbit anti-human VWF (1:500, A0082, DAKO) and anti-rabbit immunoglobulin-FITC (F0054, DAKO) were used to visualize intracellular VWF in green. Phalloidin-TRITC (red, P1951, Sigma) and DAPI (blue, D9542, Sigma) were used to stain for actin filaments and nuclei respectively. A negative control rabbit immunoglobulin fraction (X0936, DAKO) isotype was used to control for non-specific binding. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
To assess for VWF retention during the biosynthesis process, BOEC were immunofluorescently stained for VWF and for organelle markers such as the ER (calnexin), other cohabitants of the WPBs (Ang-2 and P-selectin), as well as a lysosome marker (lamp-1). VWF from normal BOEC was colocalized primarily with Ang-2 and P-selectin, which indicated the majority of VWF produced in these cells was being trafficked to WPBs as would be expected of normally functioning endothelium (Figure 2.6). Some colocalization was seen between VWF and the ER in normal BOEC, which was likely due to VWF currently being synthesized in the ER, and not abnormal retention in this organelle (Figure 2.6). Additionally, little VWF from normal controls was found to be colocalized with lysosomal marker lamp-1 again indicating that VWF produced in these cells was likely stored in WPBs or secreted, rather than being degraded in the lysosomes (Figure 2.6).

Unfortunately this analysis was not completed for all the patient BOEC lines due to loss of the cell lines in a liquid nitrogen tank failure; however, it was completed for patient T151 from Family 2, and patients V447 & V449 from Family 3. VWF from patient T151 BOEC appeared to colocalize primarily with calnexin in the ER, but additional punctate granules could be seen within the BOEC, and can reside in WPBs with P-selectin or Ang-2 (Figure 2.7). This intense ER colocalization may be due to retention of the mutant VWF in the ER, unfortunately, due to the compound heterozygous status of patient T151 it was unclear which of his three transcripts would be prone to ER retention. There was also colocalization seen between the anti-lamp-1 stain of the lysosome and T151’s VWF indicated that their defective VWF was being degraded in the lysosomes rather than secreted from their ECs. In the patients V447 and V449, we saw a similarly strong colocalization with the ER which indicated that their aberrantly spliced VWF skipping exon 38 may be withheld in the ER (Figure 2.8; Figure 2.9). Interestingly, within the patients from the 3rd family, there appeared to be more colocalization between punctate VWF staining and Ang-2 rather than P-selectin (Figure 2.8; Figure 2.9).
**Figure 2.6: Colocalization of normal BOEC VWF with endothelial compartments.** Rabbit anti-human VWF (1:500, A0082, DAKO) was used to visualize intracellular VWF in green. Endothelial compartments were stained in red: WPBs were identified by goat anti-P-selectin (1:50, sc-6941, Santa Cruz) and goat anti-Ang-2 (1:100, AF623, R & D), ER was stained with goat-anti-calnexin (1:100, sc-6465, Santa Cruz) and lysosomes with mouse-anti-Lamp-1 (1:100, MAB4800, Santa Cruz). Secondary antibodies included anti-rabbit immunoglobulin-FITC (F0054, DAKO), donkey anti-goat IgG-R (sc-2094, Santa Cruz), and Alexa-fluor 568 donkey anti-mouse IgG (A10037, Invitrogen). Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 2.7: Colocalization of T151 BOEC VWF with endothelial compartments. Rabbit anti-human VWF (1:500, A0082, DAKO) was used to visualize intracellular VWF in green. Endothelial compartments were stained in red: WPBs were identified by goat anti-P-selectin (1:50, sc-6941, Santa Cruz) and goat anti-Ang-2 (1:100, AF623, R & D), ER was stained with goat-anti-calnexin (1:100, sc-6465, Santa Cruz) and lysosomes with mouse-anti-Lamp-1 (1:100, MAB4800, Santa Cruz). Secondary antibodies included anti-rabbit immunoglobulin-FITC (F0054, DAKO), donkey anti-goat IgG-R (sc-2094, Santa Cruz), and Alexa-fluor 568 donkey anti-mouse IgG (A10037, Invitrogen). Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 2.8: Colocalization of V447 BOEC VWF with endothelial compartments. Rabbit anti-human VWF (1:500, A0082, DAKO) was used to visualize intracellular VWF in green. Endothelial compartments were stained in red: WPBs were identified by goat anti-P-selectin (1:50, sc-6941, Santa Cruz) and goat anti-Ang-2 (1:100, AF623, R & D), ER was stained with goat-anti-calnexin (1:100, sc-6465, Santa Cruz) and lysosomes with mouse-anti-Lamp-1 (1:100, MAB4800, Santa Cruz). Secondary antibodies included anti-rabbit immunoglobulin-FITC (F0054, DAKO), donkey anti-goat IgG-R (sc-2094, Santa Cruz), and Alexa-fluor 568 donkey anti-mouse IgG (A10037, Invitrogen). Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 2.9: Colocalization of V449 BOEC VWF with endothelial compartments. Rabbit anti-human VWF (1:500, A0082, DAKO) was used to visualize intracellular VWF in green. Endothelial compartments were stained in red: WPBs were identified by goat anti-P-selectin (1:50, sc-6941, Santa Cruz) and goat anti-Ang-2 (1:100, AF623, R & D), ER was stained with goat anti-calnexin (1:100, sc-6465, Santa Cruz) and lysosomes with mouse anti-Lamp-1 (1:100, MAB4800, Santa Cruz). Secondary antibodies included anti-rabbit immunoglobulin-FITC (F0054, DAKO), donkey anti-goat IgG-R (sc-2094, Santa Cruz), and Alexa-fluor 568 donkey anti-mouse IgG (A10037, Invitrogen). Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
2.5.3 *In Silico* Analyses

The *in silico* analyses for Family 1 (V69 and V70)'s exonic mutation predicted that c.3538G>A would weaken the exon 26 donor site and introduce an exonic splicing silencer site. Additionally, this mutation was predicted to weaken a SRp40 enhancer site and remove a SF2/ASF enhancer site (Figure 2.10). Therefore, this mutation is predicted to affect splicing based on changes to enhancer and silencer motifs combined with a weakening of the exon 26 donor site (Figure 2.10).

![In silico analysis](image)

**Figure 2.10: In silico analysis of c.3538 G>C Enhancers.** This mutation causes a weakening of a SRp40 site and the loss of a SF2/ASF site [red arrows]. The different splicing enhancer elements are represented by different coloured bars: SC35 [blue], SRp40 [green], and SRp55 [yellow]. The mutation is circled in red.

The consensus splice site mutation c.5842+1 G>C in Family 2 was strongly predicted to influence splicing due to its location in a canonical splice site. This mutation is said to abolish the ability to recognize the donor splice site for exon 34 and the removes binding sites for two splicing enhancer proteins SC35 and SF2/ASF (Figure 2.11). This mutation also introduces a new splicing silencer site.
Figure 2.11: Changes in splicing enhancer elements surrounding Family 2’s c.5842+1G>C mutation. (c.5842+1G>C) between wildtype and T152’s mutated allele [red box]. The different splicing enhancer elements are represented by different coloured bars: SF2/ASF [red], SF2/ASF (IgM-BRCA1) [pink], SC35 [blue], SRp40 [green], and SRp55 [yellow]. The mutation is circled in red.

Family 3 (V447 & V449)’s intronic mutation c.6599-20 A>T was predicted to lead to an inability to identify the acceptor splice site and branch site for intron 37. Therefore, this mutation is likely to cause aberrant splicing through what may be the loss of the invariant A. As well, a splicing silencer site is removed two new ones are created. A binding site for the splicing enhancer protein SC35 is thought to be abolished and an enhancer site for the serine rich protein SRp55 is created in close proximity to the mutation (Figure 2.12).
2.5.4 RNA Evaluation for Aberrant VWF Splicing

Total RNA was isolated from the BOEC and VWF mRNA was reverse transcribed to identify any aberrant band patterns reflective of variant splicing in the patient RNA, particularly around the region of their individual mutations. RNA from patient V70’s BOEC grown under static conditions contained four VWF splice forms: WT VWF, skipping of exon 23 (23del), skipping of exon 26 (26del), and VWF which skipped both exons 23 and 26 (23/26del). All of these additional transcripts are in frame changes which would likely result in continued production of the aberrant VWF protein. When exon 23 is skipped, no additional changes to the amino acid (AA) sequence are incurred other than the missing 47 residues; however, when exon 26 is skipped either singly or in conjunction with exon 23, a Proline is exchanged with an Arginine at the junction of exon 25 and now exon 27. RNA was extracted from BOEC grown under static conditions, and after 48 hours of exposure to high shear stress (50 dynes/cm²) and the ratios at which these transcripts
were present was quantified (Table 2.2). Under static conditions there was an abundance of the aberrant transcripts particularly the exon 26 skipping transcript which comprised 64±3% of the VWF RNA compared to 2±0.6% WT VWF. When shear stress was applied to these BOEC, the proportion of WT VWF mRNA produced by the BOEC increased significantly to 98±1% of the total VWF transcripts (p<0.01) (Table 2.2).

BOEC from both subjects from Family 2 (T151 and T152) had two additional VWF splice forms present alongside normally spliced VWF mRNA when cultured under static conditions. Transcripts were found to skip solely exon 33 (33del) or both exons 33 and 34 (33-34del). Skipping of only exon 33 would cause a frame shift and introduce a PTC in exon 34 likely targeting this transcript for NMD, whereas VWF skipping both exons 33 and 34 (33-34del) retains the proper reading frame and would translate into a truncated VWF protein lacking the 74 amino acid residues in the D4N domain. Interestingly, analysis of heterozygous SNPs indicated that the 33del transcript was in fact transcribed from these patient’s normal alleles and was found to comprise 13±0.3% of the VWF mRNA across 6 normal BOEC lines when RNA was isolated under static culture conditions. When subjected to 50 dynes/cm² shear stress the ratios of these transcripts shifted in favour of the frame-shifting 33del transcript which increased from 22±11% of T152’s VWF under static conditions to 68±13% under high shear stress (Table 2.2). A similar increase of the 33del transcript from 13±0.3% to 34±3% was observed in normal BOEC subjected to shear stress.

Family members V447 and V449 BOEC contained primarily WT VWF with minimal levels of a transcript skipping exon 38 (38del) which would substitute an Alanine residue for a Valine at the site where exon 37 joins exon 39, and introduce a downstream PTC in exon 40. The likely targeting of this aberrant transcript for NMD rather than completion of protein translation may explain why it was present at low levels of 2±0.85% and 3±0.7% of the total VWF for V447 and V449 respectively (Table 2.2). When subjected to high rates of shear stress at 50 dynes/cm², the proportions of these transcripts (WT and exon 38 skipping) shift dramatically, reversing their dominance to 60±5% and 98±22% for V447 and V449 respectively (Table
This pattern is similar to what we observed with the other PTC-inducing transcript, skipping exon 33, which was also increased with shear stress.

Table 2.2: Quantification of variant VWF splice forms N=3; * p<0.05; ** p<0.01; *** p<0.001 comparing transcripts ratios between BOEC grown under static conditions vs under 48 hour exposure to shear stress at 50 dynes/cm². n/a = cells would not remain adherent for the duration of the assay.

<table>
<thead>
<tr>
<th>Patient (mutation)</th>
<th>Splice form</th>
<th>% of VWF BOEC RNA Under Static Conditions</th>
<th>% of VWF BOEC RNA Under 50 dynes/cm² Shear Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>V70 (c.3538G&gt;A)</td>
<td>WT</td>
<td>2±0.6</td>
<td>98±1 **</td>
</tr>
<tr>
<td></td>
<td>23del</td>
<td>22±7</td>
<td>0±0 **</td>
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<tr>
<td></td>
<td>26del</td>
<td>64±3</td>
<td>2±1 **</td>
</tr>
<tr>
<td></td>
<td>23/26del</td>
<td>11±8</td>
<td>0±0.5 *</td>
</tr>
<tr>
<td>T151 (c.5842+1 G&gt;C; c.3939G&gt;A)</td>
<td>WT</td>
<td>41±8</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>33del</td>
<td>15±3</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>33-34del</td>
<td>44±4</td>
<td>n/a</td>
</tr>
<tr>
<td>T152 (c.5842+1 G&gt;C)</td>
<td>WT</td>
<td>51±7</td>
<td>22±9 **</td>
</tr>
<tr>
<td></td>
<td>33del</td>
<td>22±11</td>
<td>68±13 **</td>
</tr>
<tr>
<td></td>
<td>33-34del</td>
<td>27±5</td>
<td>10±1 **</td>
</tr>
<tr>
<td>V447 (c.6599-20 A&gt;T)</td>
<td>WT</td>
<td>97±31</td>
<td>40±3 *</td>
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<tr>
<td></td>
<td>38del</td>
<td>2±0.85</td>
<td>60±5 *</td>
</tr>
<tr>
<td>V449 (c.6599F-20 A&gt;T)</td>
<td>WT</td>
<td>97±31</td>
<td>2±2 *</td>
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<tr>
<td></td>
<td>38del</td>
<td>3±4</td>
<td>98±22 *</td>
</tr>
<tr>
<td>Normal BOEC N=3</td>
<td>WT</td>
<td>87±0.9</td>
<td>65±3 *</td>
</tr>
<tr>
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<td>23del</td>
<td>0±0</td>
<td>0±0</td>
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<td></td>
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<td>0±0</td>
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<tr>
<td></td>
<td>23/26del</td>
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<tr>
<td></td>
<td>33del</td>
<td>13±0.3</td>
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</tr>
<tr>
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<tr>
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<td>38del</td>
<td>0±0</td>
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</table>
2.6 Discussion

In this chapter we aimed to elucidate whether three VWF mutations were pathologic and causative of VWD through the mechanism of aberrant splicing. To accomplish this, patient plasma samples were collected and BOEC were derived from affected family member from three VWD families. Their underlying VWF mutations were confirmed through genetic sequencing of the VWF gene. Patients’ diagnosis of either Type 1 or Type 3 VWD was confirmed through phenotypic testing of the patient plasma analyzing their VWF:Ag, VWF:RCo, FVIII:C and their multimerization status of their circulating VWF. Additionally, plasma VWF was assessed for its ability to bind collagen and FVIII. The VWF mutations investigated were distributed throughout the VWF gene (Figure 2.13). BOEC were cultured for ex vivo analysis of the patients’ endothelium and VWF. VWF expression and intracellular localization were evaluated and BOEC were exposed to 48 hours of shear stress at 50 dynes/cm² to assess any changes in VWF mRNA splice forms between BOEC grown in static culture and under high levels of shear stress.

Figure 2.13: Splicing mutations’ locations throughout the VWF gene. Family 1’s c.3538G>A mutation is in the multimerization and FVIII binding regions of the D3 domain. Family 2’s c.5841+1G>C is in the D4N region of the D4 assembly possibly affecting ADAMTS-13 docking on VWF. Family 3’s intronic mutation c.6599029A>T is located at the end of the D4 assembly.
2.6.1 Family 1: c.3538G>A and VWD

Assessment of VWF splicing from the exonic 26 mutation c.3538G>A in our second family was evaluated previously in our lab using patient platelet mRNA (James et al. 2004). In the absence of splicing complications, this mutation would cause G1180R, which cause no appreciable expression or multimerization defects (James et al. 2004). Analysis of the patients’ platelet VWF mRNA identified two aberrant splice forms from this mutated allele which exhibit skipping of exon 26, or co-skipping of exons 23 and 26, both of which introduce a P1127R amino acid change where exon 25 is ligated to exon 27 (James et al 2004). Both of these aberrant splice forms showed decreased secretion and a loss of multimerization when transfected alone, but multimerization was restored through co-transfection with WT VWF (James et al. 2004).

The location of the mutation c.3538G>A and subsequent exon skipping in Family 1 is located in the D'D3 assembly, and therefore the FVIII binding region of VWF which may explain their decreased FVIII:C relative to their VWF:Ag level. FVIII (Advate ®) binding of the patients’ plasma VWF was also decreased in this family, the lack of significance may be due to their heterozygous mutation status allowing their VWF to retain a moderate capacity to bind FVIII despite co-expression of three in-frame splicing variants. VWF multimerization also occurs in this region of VWF and therefore the VWF encoded from the mutated allele may struggle to form functional high molecular weight multimers, despite the multimer output from the V70 BOEC displaying a full range of multimers. Since affected family members are heterozygous for this exonic change, they would still produce VWF from their normal allele, which may reflect the functionality of their plasma VWF. All the aberrant splice variants observed in this family’s BOEC RNA are in frame changes and would be translated into essentially full-length VWF monomers with the exception of the missing exons. The effect of these missing exons on the functionality of the aberrant VWF remains to be determined. The observation of significant intracellular retention of VWF in BOEC of patient V70 indicates that these
aberrant splice forms may be retained within the endothelium, either on their own, or may simultaneously withhold co-produced WT VWF within the cells. This would explain the moderate VWD phenotype and decreased VWF:Ag levels of 0.13 and 0.27 IU/mL. The patient BOEC also show a decrease in VWF expression and increased intracellular retention as measured by VWF:Ag ELISA. Upon IF staining of the BOEC, they appeared to contain more VWF than the average normal BOEC (Figure 2.8). Unfortunately we were unable to successfully establish a BOEC line on patient V69 to compare the effect of this splicing mutation across affected family members.

Application of shear stress at 50 dynes/cm$^2$ caused a significant shift in the distribution of the splicing transcripts within V70’s BOEC RNA. Under static conditions, the aberrant splice forms prevailed with the BOEC only producing 2±0.6% normally spliced, WT, VWF. However this shifted dramatically when BOEC were cultured under conditions of high shear stress to 98±1% WT VWF. This could perhaps be evidence of the endothelium being in a more native environment while subjected to flow as in vivo endothelium are constantly exposed to flowing blood exerting some degree of shear stress on the cells.

2.6.2 Family 2: c.5842+1 G>C and VWD

Two different types of VWD were present in Family 2. Heterozygosity for the canonical consensus splice site mutation c.5842+1 G>C in the first nucleotide after exon 34 causes Type 1 VWD in the mother, T152, leading to mild decreases in VWF:Ag, VWF:RCo and normal multimers and FVIII:C binding through introduction of exons 33-34 skipping. Collagen binding and FVIII (Advate ®) binding of the mother’s plasma was unremarkable from the normal pooled plasma controls. BOEC from this individual appear to form elongated, cigar shaped WPBs; however, colocalization with other WPB markers was unable to be confirmed for this patient. It is likely that this localized VWF is being stored in normal WPB which would also host P-selectin and Ang-2, as this patient also produces normally spliced VWF, has a very mild laboratory phenotype. T152 is also responsive to DDAVP therapy in clinic, indicating a releasable pool of
stored VWF in the patient’s endothelium. When subjected to shear stress, again there was a shift in the distribution of WT and aberrant transcripts in this individual, except this time, instead of favouring production of the WT transcripts, significant increases of VWF skipping exon 33 were observed, which would introduce a premature termination codon (PTC) into the VWF sequence in exon 34.

Compound heterozygosity for this splicing mutation as well as the nonsense mutation c.3939 G>A caused a significant bleeding phenotype (BS: 29) confirms Type 3 VWD in patient T151. As to be expected with Type 3 VWD, his laboratory levels reflected his high bleeding score, showing negligible VWF:Ag, VWF:RCo, and FVIII:C as his FVIII would have no VWF to chaperone it in circulation and protect it from degradation. Interestingly, the minimal amounts of circulating VWF in his plasma were still able to bind FVIII to such a degree that its binding capacity was not significantly different from normal plasma. The lack of response to PMA in vitro corresponds with this patient’s lack of response to DDAVP. Release of patient WPBs after stimulation with thrombin or IL-11 would be interesting to investigate as these stimuli act through different pathways than DDAVP and PMA stimulation to release VWF from ECs (Olsen et al. 2003, Ragni et al. 2008, Ragni et al. 2011, Ragni et al. 2013). Intracellular staining of his BOEC do show VWF synthesis which is diffuse, colocalizing primarily with the ER, as well as a few small punctate granules which show co-storage for both P-selectin and Ang-2. These WPBs are much more rounded than what are generally found in BOEC from normal individuals which may speak to the mutant VWF’s inability to form tightly packed tubules responsible for the characteristic cigar shape of the WPB. It is likely that the VWF from the c.3939G>A allele is primarily retained in the ER of the endothelial cells due to its lack of an appropriate C-terminal end for dimerization, whereas the aberrantly spliced products may be trafficked to WPBs, since more characteristic WPBs are observed in his mother who only has the splicing mutation affecting her VWF production.
It is interesting that this splice site mutation would cause concurrent skipping of exons 33 and 34 as the mutation is located at the 3' end of exon 34. This may indicate that exon 34 contains binding sites for splicing elements that are integral for the identification of exon 33, and distinguishing it from an intron. This co-skipping of exons 33 and 34 retains the proper reading frame of VWF allowing for the production of VWF only truncated by two exons which comprise the D4N region of VWF as annotated by Zhang et al. (2012). These two exons have been noted to be responsible for the initial binding of ADAMTS-13 allowing it to be posed for VWF proteolysis when VWF is expanded under conditions of shear stress (Zanardelli et al. 2009). It would be interesting to assess the efficiency to which ADAMTS-13 cleaves VWF in this family to determine if the loss of these two exons decreases the effectiveness of VWF cleavage.

2.6.3 Exon 33 Skipping: Aberrant or Alternative

Possibly the most interesting finding from this chapter is that the transcript skipping exon 33 alone in Family 2 is in fact not encoded from their mutated allele. When the presence of this transcript was found in normal controls, analysis of heterozygous polymorphisms in the patients showed us that this transcript was in fact produced as a splicing variant from their normal allele. During transcript quantification this transcript was found consistently across six normal BOEC lines grown under static conditions comprising 13±0.3% of the VWF. This poses the question as to whether this transcript is in fact an aberrant splice product or perhaps an alternative splicing variant of VWF. There are several possibilities for the production of this transcript. The skipping of exon 33 introduces a PTC into exon 34 which would likely target this transcript for nonsense mediated decay (NMD) unless it was an alternative splice variant using a different 3' termination site. Production of alternate splice variants incorporating PTCs into the sequence is a known cellular mechanism to down-regulate protein production though degradation of the aberrant transcripts (Pimentel et al. 2014). This protein if produced could serve other functions in circulation. The synthesis and functionality of this protein will be investigated in the following chapter.
2.6.4 Family 3: c.6599-20 A>T and VWD

The siblings of the third and final family investigated in this chapter displayed quite similar laboratory phenotypes for Type 1 VWD with mild decreases in VWF:Ag and VWF:RCo and normal FVIII:C, multimers and collagen and FVIII binding capacities. Despite their close laboratory values, the bleeding score of V447 (BS:11) was much higher than V449 (BS:4) indicating a more severe bleeding phenotype is experienced by V447. This is likely explained primarily based on gender as V447 is female and therefore would experience more bleeding challenges through heavy menses, childbirth and the post-partum period, challenges to the hemostatic process that men do not face.

Fortunately we were able to establish BOEC lines from both of these siblings and investigate their Type 1 VWD ex vivo. This also enabled us to confirm the presence of the exon 38 skipping transcript that is produced from the VWF mutation c.6599-20 A>T, as the concentration was too low in platelet RNA to sequence the aberrant band. This aberrant transcript causes a frame shift and introduces a PTC 42AAs downstream in exon 40, likely targeting this transcript for NMD. As seen previously, the BOEC from this family also displayed a mixture of diffuse VWF staining which colocalized with the ER of these cells, as well as punctate and elongated WPB where the VWF colocalized with both WPB markers P-selectin and Angiopoietin-2. Again this may be explained by the heterozygous status of these patients, where normal VWF may be trafficked to the WPBs while VWF aberrantly skipping exon 38 may be retained in the ER and degraded by the ECs. These patients are responsive to DDAVP treatment for their bleeding symptoms and this can be seen with V449’s significant increase in VWF release after stimulation of the BOEC with PMA; a similar, yet statistically insignificant increase was observed from V447’s BOEC upon stimulus.

When subjected to shear stress at 50 dynes/cm², the PTC-inducing aberrant transcripts skipping exon 38 are increased from 2-3% of VWF to ~60-98% of the patient VWF across both patients’ BOEC. This is the same pattern observed with the PTC-inducing transcript skipping exon 33 in the patients from Family
2, as well as the normal population. This increase in PTC-inducing transcripts after the introduction of high levels of shear stress is curious, particularly within the normal population as it has been proposed that there is a selective pressure against the production of transcripts which introduce PTCs (Kim et al. 2007). VWF expression is thought to increase under the effects of shear stress (Hough et al. 2008), and it is possible that the increase in transcripts that would be targeted for NMD could be a way to regulate this increased expression of VWF, and ensure that VWF is not produced in excess, which could lead to thrombotic complications.

2.6.5 BOEC Splicing and Shear Stress

The degree to which the aberrant transcripts were produced across all the patients’ BOEC was highly influenced by the application of shear stress to the cells. This indicates that these aberrant splice forms may be produced at different levels from cell to cell, and vascular bed to vascular bed depending on the extracellular environment and the degree of shear stress the endothelium is under. As we know, shear stress responsive elements (SSREs) play a role in transcriptional regulation, and this observation may advise that these changes in transcription and gene expression profiles may extend to influences on pre-mRNA splicing. This may be due to SSREs present in the promotor regions of various components of the spliceosome, auxiliary proteins, or the many enhancer and silencer proteins involved in the complex process of splicing. Additionally changes in expression and phosphorylation of splicing factors and spliceosome components in response to shear stress will play a role in these alterations in VWF splicing and splice site selection. We have noted that transcripts introducing a PTC, such as the skipping of exon 33 or 38 may be upregulated by high levels of shear stress, whereas in-frame splicing variants (skipping exon 23, 26, or exons 23 & 26) have been shown to decrease under subjection to high shear stress. Both of these mechanisms may cause a trend toward production of WT VWF as transcripts encoding PTC are degraded possibly to down-regulate expression, and in-frame aberrant transcripts are under-produced.
This significant difference in transcript distribution with the application of high shear stress indicates that while shear stress is known to affect many aspects of gene expression, SSREs may be regulating these expression changes, at least in part by influencing the splicing patterns within the endothelium. Shear stress has been shown to modulate alternative splicing of endothelial fibronectin; where in conditions of disturbed flow and low shear stress alternative fibronectin splice forms EIIIA and EIIIB are more commonly produced (Murphy & Hynes 2014). The same mechanisms which modulate this mechano-induced alternative splicing, which have yet to be fully elucidated, may be participating in the regulation of aberrant splicing in these patients.

The shear stress applied in these experiments was quite high; 50dynes/cm² is around the highest physiologic shear stress encountered by the endothelium before shear stress becomes pathologic at 60 dynes/cm² in instances of hypertension or stenotic vessels. This degree of shear was chosen as a proof of principle, under the assumption that if shear stress affected splicing, this effect would be observed most significantly under high levels of shear stress. To gain a more comprehensive view of how shear stress affects splicing of VWF a variety of slower, more commonly encountered shear stresses (5-20 dynes/cm²) should be evaluated to see if similar changes in splicing are seen. The question remains- are these effects simply based on whether shear stress is occurring in an “on” or “off” fashion, or are the changes more gradual, being affected by the degree or direction of flow?

2.6.6 Inferences & Next Steps

This chapter has identified six aberrant VWF splicing variants across three families: exon 23 skipping, exon 26 skipping, exons 23 & 26 skipping together, skipping exon 33, skipping exons 33 & 34, as well as exon 38 skipping. We have also seen that the degree to which these aberrant splice forms are produced is related to the microenvironment of these cells, and stimulation by high levels of shear stress in particular. These dramatic shifts of transcript proportions based on flow could indicate that individuals with
VWD caused by pathogenic splicing mutations may exhibit a more variable VWD phenotype over time, with aging, or with situations such as hypertension or atherosclerosis, due to these shifts in transcript production either in favour of mutant transcripts or WT VWF.

The next step in fully elucidating the mechanism behind the VWD in these families is to characterize the synthesis, secretion and functionality of these VWF variants using a heterologous cell model as this allows the evaluation of each of the identified splice forms individually without co-production of wildtype VWF or other aberrant splice forms. Otherwise BOEC would need to be isolated from individuals homozygous for these genetic changes, or transcript specific siRNAs employed to knock down interfering transcripts. Chapter 3 will assess the expression and functionality of these aberrant splice forms using a heterologous HEK293(T) cell model.
CHAPTER 3
In Vitro Characterization of Aberrant VWF Splice forms

3.1 Summary

Elucidation of the molecular mechanisms underlying particular von Willebrand disease (VWD) mutations can be instrumental in gaining further understanding into von Willebrand factor (VWF) and endothelial cell (EC) biology, as well as the molecular pathogenesis of VWD. This chapter investigates the synthesis, secretion, and functionality of the VWF aberrant splicing variants identified in Chapter 2 using a heterologous HEK293T cell expression system. Most of these aberrant transcripts showed a reduction in VWF secretion when expression vectors were transfected into HEK293T cells. Exon 23 skipping (23del), exon 26 skipping (26del), exons 23 and 26 skipping together (23/26del), and exon 38 skipping (38del) had dramatically decreased VWF expression when transfected to mimic homozygosity (p<0.001). This effect of reduced expression appeared to be caused by dominant negative retention of WT VWF by 23del and 23/26del VWF as expression remained significantly decreased to less than half of WT in mock heterozygous co-transfections with WT VWF expression vectors (23del p<0.05; 23/26del p<0.001), and lysates of VWF were significantly increased (p<0.05). The variant VWF mimicking exon 33 skipping (33del) was expressed easily from transfected cells both in homozygous and mock-heterozygous conditions at levels similar to that of WT VWF. Truncated VWF lacking both exons 33-34 (33-34del) was also able to be released from the HEK293T cell; however, significantly less VWF was released than from cells transfected with WT VWF (p<0.05). When transfected in a mock homozygous state, all of the aberrant VWF splicing mutants were not responsive to PMA stimulated release from the HEK293T cells. This may be due to improper storage within the cell, ER retention, or defective pseudo-Weibel Palade bodies (pWPBs) if the mutant is actually trafficked to the storage organelles. VWF functionality of these mutants VWF splice forms
was significantly reduced, when measuring VWF:RCO, FVIII binding, and collagen binding ability, particularly when the aberrant splice forms were transfected in the homozygous state (p<0.05). VWF released from co-transfections with WT had increased functionality, likely due to the release of WT VWF. Interestingly, homozygous VWF skipping exon 33, not only retained FVIII binding ability, but had a greater affinity for FVIII than WT (p<0.05). No VWF multimerization was observed in homozygous mutant transfections, with the exception of 33-34del, an in-frame change that produced band-shifted multimers. Multimerization was restored in most samples by co-transfection with WT VWF, with the exception of 23del where the expressed VWF remained unmultimerized. To help elucidate the cause for the decreased expression of many of these mutants, transfected cells were immunofluorescently stained for VWF and intracellular compartments involved in VWF biosynthesis and colocalization was observed and quantified. WT VWF as well as all the splicing mutants showed colocalization with the endoplasmic reticulum (ER), and 23del VWF had increased colocalization with lysosomes and decreased colocalization with pseudo-WPB marker Rab27a. This increased lysosomal colocalization was also observed in cells transfected with 26del and 38del VWF. VWF skipping exons 33-34 displayed more punctate localization compared to the diffuse staining seen with most of the transfected mutants.

These VWF splicing mutants are thought to cause VWD through different mechanisms. Transcripts lacking exon 23 (23del and 23/26del) appear to cause dominant intracellular retention of mutant and WT VWF possibly in the ER. Skipping of exon 26 or 38 (26del or 38del) may cause decreased expression through intracellular VWF degradation by lysosomes. Exon 33 skipping appears to be constitutively expressed without being hung up in the cell, and while 33-34del can be expressed from the cells at reduced amounts, this may be a trafficking difference between HEK293T cells and the native EC producers of VWF, as VWF expression was very low in patient T151 who was compound heterozygous for this mutation.
3.2 Introduction

von Willebrand factor (VWF) is an essential hemostatic protein which plays a role in both primary and secondary hemostasis, through its roles in platelet activation and aggregation as well as its capacity as a molecular chaperone for coagulation factor VIII (FVIII) in circulation, protecting it from premature degradation and transporting FVIII to sites of vascular injury (Ruggeri & Ware 1993, Goodeve 2010). This large multimeric glycoprotein is highly adhesive, is involved in many molecular interactions, and contains a vast number of functional domains. When the VWF gene is mutated, this can lead to quantitative deficiencies in circulating VWF or qualitative defects in the VWF protein leading to the bleeding disorder von Willebrand disease (VWD). Changes to the CK domain affect the initial dimerization of the VWF monomers whereas alterations in the 741aa propeptide (VWFpp) and the D’D3 assembly can affect VWF’s ability to form full high molecular weight multimers (HMWM). These HMWM are essential for the full platelet aggregation and wound healing properties of VWF as they are able to more successfully elongate over sites of vascular injury under shear stress, more successfully enabling platelet plug formation. Mutations to the D’D3 assembly can also affect FVIII’s ability to bind VWF and can cause Type 2N VWD which can have similar symptoms to hemophilia due to the low FVIII levels resulting from their lack of chaperone in circulation. If the A1 or C4-C5 domains are altered, VWF may lose ability to bind platelets through either the GpIbα or GpIIb-IIIa receptors. Collagen binding of VWF can be affected by changes in either the A1 or A3 domains.

In vitro investigation of von Willebrand disease (VWD) has changed dramatically over the past few decades as accessibility to patient blood outgrowth endothelial cells (BOEC) has increased. Prior to the use of BOEC to investigate the patient phenotype, a heterologous cell model was derived using human embryonic kidney (HEK) 239T cells transfected with VWF expression vectors to evaluate the effect of putative VWF mutations on VWF synthesis, secretion, and function. The use of HEK cells has proven to
benefit the *in vitro* investigation of VWF and VWD far more than previously used cell lines such as COS cells because HEK cells are uniquely able to from pseudo-WPBs which are capable of stimulated exocytosis, also recruit Weibel-Palade bodies (WPB) proteins such as CD63 and P-selectin which are found in the membrane of endothelial cell (EC) WPBs (Micheaux et al. 2003). Unfortunately, there are limitations to this model in that heterologous cell systems cannot replicate *in vivo* post translational modifications such as specific glycosylation patterns (Haberichter et al. 2010). Co-transfections into heterologous cell systems may not replicate the heterozygous or compound heterozygous states *in vivo* precisely due to varying ratios of each plasmid entering a particular cell. Additionally, these non-native cells may not be equipped with intracellular chaperones or signaling pathways which are active in the native EC environment, and would influence the biosynthesis and trafficking of VWF *in vitro* (Haberichter et al. 2010).

Despite the shortcomings of the heterologous cell model, they can be advantageous to determine whether a genetic change is pathologic. This is particularly useful when investigating the VWF gene which is quite polymorphic and what may appear at first glance to be a synonymous polymorphism can actually have pathologic consequences causing VWD in some instances. Determination of the functional outcomes of genetic changes, especially those which appear synonymous could be of great importance to the study of VWD as many cases including ~35% of the Type 1 population across three cohorts remain without an identified causative mutation (Cumming et al. 2006, Eikenboom et al. 2006, James et al. 2007). Transfection of expression vectors containing changes to the VWF cDNA into HEK293T cells can allow for inference into the mechanisms by which a genetic change affects protein synthesis, storage, secretion or functionality.
3.3 Objectives

In this chapter, we aimed to characterize the pathologic mechanisms through which the aberrant splice variants identified in the previous chapter act to cause VWD. To accomplish this a heterologous HEK293(T) cell system was used to assess synthesis and secretion of aberrant splice form expression vectors, and the produced VWF was functionally assessed using VWD phenotypic assays.

3.4 Materials and Methods

3.4.1 Heterologous Expression System: Human Embryonic Kidney (HEK) 293T Cells in Culture

3.4.1.1 Expression Plasmids

Expression vectors reflecting each of the identified aberrant splice forms were created using QuikChange II site-directed mutagenesis (200522, Agilent Technologies) of a full length WT VWF cDNA-pCIneo expression vector. The regions directly surrounding the mutations were removed by restriction enzyme digest and sub-cloned into a WT vector to ensure the integrity of the vector backbone. Mutagenesis primers can be found in Appendix B.

3.4.1.2 VWF Expression of Aberrant Splice Forms

Transfected HEK293T cells were used to model the splice forms. These cells were plated on poly-L-lysine coated 10 cm plates and grown in complete DMEM medium (cDMEM) (DMEM medium (11965-092, Invitrogen), supplemented with 10% (vol/vol) fetal bovine serum (FBS) (1283-020, Life Technologies), 1% penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL) (15140-122, Life Technologies)). Aberrant splice form vectors were expressed in HEK293T cells through calcium phosphate mediated transfection to mimic both a homozygous and heterozygous state (50% mutant: 50% WT) (Appendix A5). Co-transfections with beta-galactosidase (β-gal) plasmids were performed to control for cell number and transfection efficiency.
After 17 hours in the transfection cocktail, the cells were washed with PBS with 1 mM EGTA and medium was replaced with Opti-MEM reduced serum media (31985-088, Life Technologies). Seventy-two hours after transfection, media and lysates were collected and media were concentrated using Centricon Plus-70 centrifugal filter units (UFC710008, EMD Millipore) for further functional analyses.

3.4.1.3 Proteasomal Degradation of Aberrant Splice Forms

To determine if aberrant splice forms were being degraded in the proteasome, HEK 293T cells were plated in cDMEM media to poly-L-lysine coated 6 well tissue culture plates. Twenty-four hours after plating the cells are switched to Opti-MEM medium supplemented with 100 nM CaCl\(_2\) and transfected with Lipofectamine® 2000 Transfection Reagent (11668-019, Invitrogen). Expression vectors were transfected to mimic the homozygous state. Two hours before media and lysate collection, proteasomal degradation was inhibited using 0.025 mM MG132 (Z-Leu-Leu-Leu-Al) (C2211, Sigma). Forty-eight hours after transfection, cells were washed with PBS and lysed. Intracellular VWF was quantified and compared to uninhibited transfections.

3.4.2 Aberrant Splice Form Characterization

Aberrant splice forms identified in the patient families and produced in transfected HEK293T cells were then characterized for their ability to be synthesized and secreted (VWF:Ag), as well as their activity in terms of platelet binding (VWF:RCo), FVIII binding, and collagen binding ability, as well as multimerization using the methods described in Chapter 2 section 2.4.2: Phenotypic Assays. To ensure alterations in VWF expression levels were caused by the mutant plasmids and not simply the decreased amount of WT in the transfection cocktail a blank pCIneo expression vector not coding for VWF (mock) was also transfected in the homozygous and heterozygous states.
3.4.3 Intracellular Trafficking of Aberrant Splice forms

3.4.3.1 Immunofluorescent Staining & Confocal Microscopy

Biosynthesis and trafficking of the aberrant VWF splice forms was evaluated through immunofluorescent staining of subcellular compartments 48 hours after Lipofectamine® 2000 Transfection Reagent (11668-019, Invitrogen) homozygous and heterozygous transient transfection into HEK293 cells. The primary antibodies used were mouse anti-PDI (endoplasmic reticulum (ER) ab2792, abcam, 1:100), mouse anti-GOLPH4 (cis-Golgi, sc-101054, Santa Cruz, 1:100), goat anti-Rab 27a (pseudo-WPB, sc-74586, Santa Cruz, 1:50), mouse anti-Lamp1 (lysosome, MAB4800, R & D 1:100), and VWF (rabbit anti-human VWF, A0082 DAKO, 1:500). Secondary antibodies include: Alexa Fluor® 488 goat anti-rabbit IgG (A11070, Invitrogen), Alexa Fluor® 568 donkey anti-mouse IgG (A10037, Invitrogen), and rhodamine donkey anti-goat IgG (sc-2094, Santa Cruz). Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.

3.4.3.2 Quantification of Colocalization

Colocalization of the VWF and the organelles was quantified using MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices) to calculate Manders’ Colocalization Coefficient (MCC). Calculation of the MCC measures how much of the VWF staining, co-localizes with the organelle specific stain (Dunn et al. 2011).
3.5 Results

3.5.1 Heterologous Expression System: Human Embryonic Kidney Cells 293 (T) in Culture

Expression vectors reflecting each of the aberrant splice forms found across these three families were transiently transfected into HEK293T cells to determine the functionality of these mutant proteins and whether they would interact with co-produced WT VWF. VWF expression was significantly reduced in all mutant homozygous and heterozygous transfections with the exception of that skipping exon 33 alone (33del) (Figure 3.1). Expression vectors 23del and 23/26del appeared to have a dominant negative effect on the expression of co-transfected WT VWF with expression levels remaining below 35% of WT expression when co-transfected (50:50) with WT VWF expression vectors (Figure 3.1) and intracellular retention of the VWF (Figure 3.2). When transfected in the absence of WT VWF, none of these aberrant splice forms showed a positive VWF release in response to stimulus with PMA; however this stimulated release was achieved in cells transfected with 26del, 23/26del, 33del, or 33-34del alongside WT VWF (Figure 3.1). Normal VWF expression and PMA stimulated secretion was exhibited when WT VWF was co-transfected with the mock expression vector (Figure 3.1).
Figure 3.1: Wildtype (WT) and variant VWF splice form expression from transfected HEK293T cells. Constitutive expression was derived from unstimulated cells and release of VWF from pseudo-WPB was measured after stimulation with PMA. Black significance stars represent a significant difference in constitutive expression of the mutants compared to WT. Green significant bars and stars indicate a significant increase in secretion between constitutive and stimulated HEK293T cells. N=3 (* p<0.05; **p<0.01; ***p<0.001).
Figure 3.2: Intracellular VWF levels from transfected HEK293T cell lysates. Transfections containing 23del, 23/26del, and 33-34del have increased intracellular retention. Homozygously transfected 38del VWF displays less intracellular VWF than WT. The mock control shows no VWF at 100% as expected and the 50% mock is not significantly different than the WT transfection. N=3(* p<0.05; **p<0.01; ***p<0.001).

3.5.1.1 Lack of Proteasomal Degradation of Aberrant Splice Forms

As expression of the aberrant VWF splice forms was decreased in most cases, and intracellular VWF was not always increased (26del and 38del), proteosomal degradation of the splice forms was assessed through inhibition of the proteasome by MG132. Interestingly, no evidence of proteosomal degradation was observed for any of the aberrant splice forms as lysate VWF levels were not seen to increase after inhibition of the proteasome (Figure 3.3).
Figure 3.3: Lack of Degradation of Homozygous Aberrant Splice Forms by the Proteasome. No lysate levels were significantly increased compared to their uninhibited state when treated with the proteosomal inhibitor MG132.

3.5.2 Aberrant Splice form Characterization

As expected, VWF function was generally negatively affected in these aberrant splice forms. Platelet binding (VWF:RCo), FVIII binding, and collagen binding are significantly reduced to negligible levels in 23del, 26del, 23/26del, and 38del aberrant splice forms when produced in the absence of WT VWF (Figure 3.4). When these splicing mutants are co-transfected with WT VWF the functionality of the VWF expressed from the HEK293T cells increases. In the heterozygous state 23del regains marginal FVIII binding and a full capacity to bind collagen (Figure 3.4), 26del regains platelet and collagen binding abilities with a marginal increase in FVIII binding, as well, 23/26del and 38del regain moderate functionality by all three measures (Figure 3.4). Interestingly, the 33del splice form while having abolished the ability to bind platelets and collagen, appears to have an increased affinity for FVIII binding, while perhaps even more intriguingly, the FVIII binding ability is tempered when this mutant is co-expressed with WT VWF (Figure 3.4). The in-frame skipping of exons 33 and 34 enables this aberrant protein to retain moderate platelet and
collagen binding abilities and half the FVIII binding capacity of WT VWF; these functions were only marginally ameliorated with WT co-expression (Figure 3.4).

Multimerization was greatly affected by aberrant splicing of the VWF which can be seen by the lack of multimers in all the homozygous mutant transfections, with the exception of 33-34del which formed multimers exhibiting a slight band shift due to the missing exons aberrantly spliced out of each VWF monomer (Figure 3.5). Slight reparation of the band shift is seen in the heterozygous 33-34del multimers indicating hetero-multimerization of the mutant and WT VWF (Figure 3.5). VWF expressed from heterozygous 23del transfections did not show any restoration of multimerization when co-expressed with
WT VWF (Figure 3.5). The remainder of the mutants (26del, 23/26del, 33del, and 38del) showed no multimerization when transfected alone; however multimerization was restored with WT co-expression (Figure 3.5). Multimerization in the heterozygous 26del and 23/26del transfections, explains the restoration of VWF:RCo and to varying degrees of FVIII and collagen binding in these proteins. It is surprising that without a reparation of multimerization, heterozygous 23del regained some FVIII and collagen binding functionality (Figure 3.4). Exon 26 skipping may interfere with the full range of multimerization when co-transfected with WT VWF, as there appears to be a lesser degree of HMWM in the 50% 26del VWF than in the other mock heterozygous transfections (23/26del, 33del, and 38del) (Figure 3.5). Restoration of secreted multimers was seen from the heterozygous PTC-inducing mutant transfections (33del and 38del) which may indicate that the prematurely truncated splice forms are not interacting with the co-transfected WT allowing a full range of multimers to be secreted from the HEK293T cells (Figure 3.5).
Figure 3.5: Multimers from recombinant WT and mutant VWF splice variants secreted from HEK293T cells. Lane 1: 2A VWD abnormal control; Lane 2: WT-VWF; Lane 3: 100% 23del; Lane 4: 50% 23del; Lane 5: 100% 26del; Lane 6: 50% 26del; Lane 7: 100% 23/26del; Lane 8: 50% 23/26del; Lane 9: WT VWF; Lane 10: 2A VWD abnormal control; Lane 11: NPP normal control; Lane 12: 100% 33 del; Lane 13: 50% 33del; Lane 14: 100% 33-34del; Lane 15: 50% 33-34del; Lane 16: 100% 38del; Lane 17: 50% 38del.
3.5.3 Intracellular Trafficking of Aberrant Splice forms

3.5.3.1 Immunofluorescent Staining and Confocal Microscopy

Transfected HEK293 cells were stained for VWF and organelles involved in VWFs biosynthesis (ER, Golgi, and pseudo-WPBs) and as no proteosomal degradation was observed (section 3.5.1.1), lysosomes were stained to determine where the aberrant splice variants are trafficked and retained in the cell, leading to their decreased expression. In the WT VWF transfections, we saw that the VWF was commonly punctate, however, there was also some diffuse staining (Figure 3.6). Colocalization occurred between the WT VWF and the ER marker PDI, and some colocalization with the cis-Golgi, likely due to the VWF passing through these organelles during normal VWF biosynthesis (Figure 3.6). Again there is a strong colocalization with pseudo-WPB marker Rab27a as the VWF is packaged for regulated release. Little colocalization was observed between the WT VWF and the lysosomes of these transfected HEK293 (Figure 3.6).

Homozygous transfections of the 23del expression vector, showed colocalization between the mutant VWF and the ER and the lysosomes. The staining for the pseudo-WPB marker Rab27a appeared to overlay with the mutant VWF staining to a lesser extent, and as we noted previously, when the mutant VWF was trafficked to pseudo-WPBs these organelles would not release their VWF (Figure 3.7). A similar pattern of colocalization was seen in the heterozygous 23del transfections with prominent yellow colocalization with the ER and the lysosomes (Figure 3.8). Trafficking of homozygous VWF skipping exon 26 (26del) appears to stop in the ER. Colocalization with PDI was observed; however, there does not appear to be significant overlap between the mutant VWF and the Golgi, pseudo-WPBs, or the lysosomes. This may indicate ER retention of the mutant VWF (Figure 3.9). When co-transfected with WT, the 26del VWF continues to colocalize with the ER, but colocalization with Rab27a may be increased (Figure 3.10). The concurrent skipping of exons 23 and 26 displays colocalization primarily with the ER and pseudo
WPBs; again, little colocalization was seen between the mutant VWF and the Golgi or the lysosomes (Figure 3.11). Similar patterns of colocalization and staining were found when this mutant was transfected in both the homozygous or heterozygous states (Figure 3.12). Homozygous exon 33 skipping (33del) showed a more diffuse staining of the mutant VWF. While diffuse, it appeared to colocalize with the ER, the cis-Golgi, and the lysosomes indicating that while this recombinant splice product can be secreted constitutively, it may also be degraded by the cells (Figure 3.13). When transfected in the heterozygous state, the intracellular VWF appeared to slightly more punctate and had a similar colocalization profile to homozygous transfections (Figure 3.14). Skipping of exons 33 and 34 (33-34del) have a punctate distribution, colocalizing with the VWF biosynthesis pathway including the pseudo-WPBs which indicate this mutant is trafficked to storage organelles in both the homozygous and heterozygous states (Figure 3.15; Figure 3.16) Though based on these expression studies without the presence of WT VWF, the release of this stored VWF cannot be stimulated. Finally, skipping of exon 38 (38del) showed strong colocalization with the ER, where it may be retained (Figure 3.17). Heterozygous 38del transfections showed strong colocalization with the pseudo-WPB markers which may support the concept of a lack of interaction between the WT and 38del VWF, in that the WT VWF can be trafficked to pseudo-WPBs (Figure 3.18).
Figure 3.6: Immunofluorescent Staining of Intracellular Trafficking of WT VWF. Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.7: Intracellular Trafficking of VWF- Homozygous Skipping Exon 23 (23del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.8: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exon 23 Skipping (23del). Organelles are stained in red, and VWF in green with Dapi (blue) staining for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.9: Immunofluorescent Staining of Intracellular Trafficking of VWF- Homozygous Skipping Exon 26 (26del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.10: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exon 26 Skipping (26del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.11: Immunofluorescent Staining of Intracellular Trafficking of VWF-Homozygous Skipping Exons 23 and 26 (23/26del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.12: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exons 23 and 26 Skipping (23/26del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.13: Immunofluorescent Staining of Intracellular Trafficking of VWF-Homozygous Skipping Exon 33 (33del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.14: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exon 33 Skipping (33del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.15: Immunofluorescent Staining of Intracellular Trafficking of VWF- Homozygous Skipping Exons 33 and 34 (33-34del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.16: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exons 33 and 34 Skipping (33-34del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.17: Immunofluorescent Staining of Intracellular Trafficking of VWF-Homozygous Skipping Exon 38 (38del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
Figure 3.18: Immunofluorescent Staining of Intracellular Trafficking of VWF Heterozygous for Exon 38 Skipping (38del). Organelles are stained in red, and VWF in green with Dapi staining (blue) for the nucleus. Scale bar is to 5 µm. Imaging was conducted at room temperature using a Quorum Wave Effects Spinning Disc confocal microscope at 60X magnification and captured with a Hamamatsu Orca high resolution camera and ImageJ software analysis.
3.5.3.2 Quantification of Colocalization

Co-staining of transfected HEK293 cells for the various aberrantly spliced VWF and markers of four organelles through which VWF biosynthesis occurs enabled the evaluation of mutant trafficking to explain the decreased expression of these mutants. Because observation of confocal colocalization can be subjective, the degree of colocalization was quantified using MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices). Aberrant splice products 23/26del and 38del showed significant increases in colocalization with the ER lumen marker PDI (Figure 3.19A). While many of the mutant transfections showed a trend of decreased colocalization with the Golgi body marker Golph4 but this was only significant in the homozygous 23/26del transfection (Figure 3.19B). The skipping of exon 23 (23del) and exons 33-34 (33-34del) showed decreased colocalization with the pseudo-WPB marker Rab27a in both homozygous and heterozygous transfections (Figure 3.19C). Similar decreases in pseudo-WPB localization were observed in the homozygous 33del and 38del transfections (Figure 3.19C). Lysosomal colocalization showed the most variable results. Homozygous and heterozygous 23del and 38del, and heterozygous 26del had significantly increased lysosomal colocalization compared to WT VWF. Colocalization with the lamp1 marker was decreased in heterozygous 23/26del and homozygous 33-34del splicing mutants (Figure 3.19D).
Figure 3.19: Quantification of WT and mutant VWF colocalization with organelle markers. N=40-50 cells per condition (* p<0.05; ** p<0.01).

3.6 Discussion

Elucidation of the molecular pathogenesis of VWD mutations using heterologous HEK293T cell models is able to increase our understanding of VWF biology. Through investigation of synthesis, secretion, and functionality of six aberrant VWF splicing variants causing exon splicing we deduced that the inclusion of exon 23 may be essential for trafficking of VWF through the biosynthesis pathway, and particularly for release of the exon skipped VWF. The location of exon 23 in the D’D3 assembly explains its negative effects on FVIII binding ability and multimerization of VWF. VWF lacking exon 23 would be translated
properly after the exon skipping until the C-terminal end of VWF; therefore, it is possible that the mutant VWF is forming dimers that can associate with WT VWF. This could be impairing multimerization and increasing retention of co-produced VWF in the HEK 293T model as well as in our patients from Chapter 2 who exhibited decreased plasma VWF:Ag and BOEC with increased intracellular retention of VWF. Exon 26 skipping is also found in the functional domains assigned to FVIII binding and multimerization; however, significant intracellular retention was not observed in HEK293T cells transfected with this mutant therefore we can infer that there is something particular about exon 23 that affects trafficking or retention of VWF. When exon 26 was skipped either singly (26del) or in conjunction with exon 23 (23/26del), multimerization was restored in the mock heterozygous transfections. This indicates that in the absence of exon 26 a dominant negative effect is not imposed on WT multimerization, or that these mutants can incorporate into WT multimers, but not form multimers on their own. These multimeric observations coincided with previous studies producing these recombinant VWF mutants in COS-7 cells (James et al. 2004). The double exon skipping of exons 33 & 34 was the only in-frame splicing variant that was able to be expressed, though at a reduced level in this heterologous cell model (p<0.05). This trafficking may not directly reflect biosynthesis of VWF in ECs, as in Chapter 2 we observed minimal VWF expression from patient T151’s BOEC when they were producing this aberrantly spliced VWF. This discrepancy may be based on variable VWF biosynthesis across the two cell types, or the decreased VWF expression in the BOEC may be due to interactions between the exon 33-34 skipping VWF and the VWF produced from their c.3939 G>A allele causing increased VWF retention. In the homozygous state, this in-frame mutant VWF is able to form band-shifted multimers and retain moderate VWF:RCo, FVIII binding, and collagen binding abilities; therefore, if this variant was able to be released from the patient’s ECs it would be immensely beneficial to this Type 3 VWD patient. This in-frame double exon skipping splice form essentially removes the D4N domain of the D4 assembly; this region has been noted as an initial binding site for ADAMTS-13 to VWF in its globular
form, allowing for docked ADAMTS-13 to be poised for VWF proteolysis upon the unraveling of VWF multimers (Zanardelli et al. 2009). It would be interesting as a continuation of this study to assess the ability of these splicing variants to associate with, and be cleaved by ADAMTS-13 and observe if the 33-34del mutant has an extended half-life, or reduced proteolysis due to the lack of the D4N domain.

Trafficking and particularly secretion from the HEK293T cells appeared to be the major contributor to decreased VWF levels across these expression studies, particularly for the in-frame splicing mutants 23del, 26del, 23/26del and 33-34del. Macroautophagy, thought previously to assist solely with protein degradation and recycling, has been implicated in regulating secretory pathways, including recently the secretion of VWF by ECs (Pfeffler 2010, Abrahamsen & Stenmark 2010, Manjithaya & Subramani 2011, Deretic et al. 2012, Torisu et al. 2013). Torisu et al. have observed an association between VWF, WPBs, and autophagosomes (Torisu et al. 2013) where inhibition of macroautophagy causes a decrease in both constitutive and stimulated WPB release in vitro and in vivo (Torisu et al. 2013). Further elucidation of the interaction between WPBs and autophagosomes in VWF secretion is required, however perhaps the mutant VWF splice forms that can traffic to WPBs are interfering with the interaction between WPBs and autophagosomes, reducing or abolishing the ability of WPBs to release the mutant VWF.

It is interesting that the two aberrant transcripts that introduce PTCs displayed different expression and functionality profiles in this heterologous cell model. While it is presumed that the introduction of a PTC will target the aberrant transcript for NMD as a regulatory mechanism to control protein expression (Frischmeyer & Dietz 1999), it has been noted that this is not always the case, and that transcripts with PTCs may serve other functions as truncated proteins (Sittler et al. 1996, Lazaridis et al. 2000). Wang et al. (2011) postulated that disruption of intra-chain disulfide bond formation, and dimerization by VWF mutation prevents the aberrant VWF from passing ER quality control mechanisms which would lead to ER retention and either aggregation or intracellular degradation of the mutant VWF as we observed with 38del. Despite
the quality control mechanisms in the ER, some of the aberrant VWF molecules may leave the ER and continue to be trafficked to WPBs for storage (Wang et al. 2011). The observation of consistent expression of the exon 33 skipping splicing variant within the normal population (N=6), as well as the constitutive expression of 33del VWF in HEK293T cells and its apparent ability to bind FVIII in the absence of WT VWF may reveal that this truncated protein plays a role in circulation as a chaperone to FVIII. Premature truncation of this VWF variant would prevent its multimerization into full HMWM; however, this truncated VWF may comprise a larger proportion of VWF constitutively expressed from ECs, rather than stored in WPBs. Similar retention of functionality was not observed for the exon 38 skipping VWF variant (38del), and while this transcript is likely subject to NMD, if low levels of the transcript escape degradation, it is likely retained briefly in the ER due to its lack of a C-terminal domain (Cocquerel et al. 1998, Pagny et al. 1999) and is then degraded by the cell as we observed an increased colocalization with lysosomes.

While useful, these heterologous HEK293T cell models are not precise replicates of how VWF would be produced in vivo, or by ECs in vitro. Transfection of the aberrant splicing expression vectors can be complicated by over or under expression within a cell, and may not properly reflect the heterozygous state, particularly in immunofluorescent staining confocal microscopy as the degree to which each cell was transfected with WT or mutant VWF is variable. Additionally these heterologous cells are smaller than the ECs which natively produce VWF thus posing challenges in trafficking VWF synthesis. Intracellular compartments such as the ER comprised most of the cell, which may have artificially increased the degree of colocalization with ER markers. HEK293 cells can form distinct pseudo-WPBs when transfected with WT VWF; however, as transient transfection often leads to over expression of recombinant proteins, these pseudo-WPBs can be difficult to distinguish.

Accurate modeling of aberrant splicing outcomes is challenged more than regular mutation expression studies using this model particularly when trying to investigate the outcome of the PTC
containing transcripts. This is because during the process of splicing, with the joining of each exon pair, exon junction complexes are deposited near the site of the junction, and when these complexes are found downstream of a termination codon, the cell recognizes the transcript as aberrant, targeting it for NMD (Frischmeyer & Dietz 1999, Lewis et al. 2003, Wollerton et al. 2004, Isken & Maquat 2008). This is not replicated in the transfected HEK293T cell model because the expression vectors sequence is that of the VWF cDNA, without the inclusion of splicing markers; therefore, these expression systems better model the genomic deletion of the exons in question. Despite the lack of exon junction complexes to target these transcripts for NMD as would likely occur \textit{in vivo}, as discussed, some transcripts can escape NMD degradation or be tolerated at low levels (Baek & Green 2005, Pan et al. 2006, Blencowe 2006); therefore, this model lends some insight as to what would occur in these instances.

3.6.1 Limitations and Further Studies

The characterization of these mutants is limited in part by the use of a heterologous cell model as HEK293(T) cells may process VWF in a slightly different manner than endothelial cells where VWF is natively produced. Additionally, the use of expression vectors reflecting the VWF cDNA lacking the exon(s) skipped in the splicing process may be more representative of genomic deletions rather than splicing errors as these expression vectors remove the influence exon junction complexes would have on PTC recognition and targeting for NMD. Thus, these characterization results would likely be most accurate for the in-frame variants (23del, 26del, 23/26del, 33-34del), rather than those which introduce PTCs (33del and 38del). The use of transient transfections rather than stable transfections may also have influenced our results, particularly the intracellular trafficking, as transient transfection can induce over-expression of VWF where trafficking may not be representative of VWF produced at physiological levels. Over-expression of these transiently transfected VWF vectors may present artificially increased retention in the ER or targeting of the
protein to the lysosomes. As well, when transfecting the HEK293 cells to visualize the mock heterozygous state (50% WT: 50% mutant), it is impossible to control each cell’s uptake of the vectors in these ratios. Therefore, imaging and quantification of colocalization may be skewed toward WT or mutant VWF in any given cell.

If this chapter were to be expanded, it would be interesting to include assessments of the binding and cleavage ability of these aberrant splice variants by ADAMTS-13. This would be especially interesting for the 33-34del variant as this D4N region of VWF is thought to act as a preliminary binding site for ADAMTS-13 (Zanardelli et al. 2009) and therefore it’s removal by aberrant splicing may increase the half-life of this variant in circulation. Additionally it would be interesting to conduct the co-staining experiments including markers for the autophagosomes as these organelles are known to be associated with WPB trafficking and have even been shown to contain VWF themselves (Pfeffer 2010, Manjithaya & Subramani 2011, Deretic et al. 2012, Torisu et al. 2013). Labelling of either the WT VWF expression vector or the aberrant expression vectors with myc-his tags would be ideal for further elucidation of multimerization in the mock heterozygous state. This would allow us to distinguish between heteromultimerization and solely secretion of homomultimerized WT VWF.
CHAPTER 4
Variant VWF Splice Forms in Normal Subjects

4.1 Summary

Alternative splicing is quite predominant across the human genome with approximately 90% of human genes undergoing this diversifying process (Pan et al. 2008, Barash et al. 2010). Patterns of alternative splicing have been known to vary between cell types and with changes in the cellular microenvironment. Intron 1 splicing of the hemostatic protein VWF is known to regulate its expression in endothelial cells; however the splicing process has no bearing on VWF expression in megakaryocytes (Yuan et al. 2013). While the process of VWF splicing has been observed to affect expression of the VWF gene (Yuan et al. 2013) and many mutations to VWF have pathologically affected VWF splicing (James et al. 2004, Gallinaro et al. 2006, James et al. 2007, Plate et al. 2010, Castaman et al. 2010, Corrales et al. 2011, Pagliari et al. 2013), alternative splice variants have yet to be identified for this coagulation factor.

In this chapter we assess the propensity for alternative splicing of VWF across the normal endothelium by investigating VWF mRNA produced by three normal blood outgrowth endothelial cell (BOEC) lines, human umbilical vein endothelial cells (HUVEC), and human microvascular endothelial cells (HMVEC) with and without subjection to biochemical (estrogen, histamine, and DDAVP), and biomechanical (laminar shear stress of 15 dynes/cm²) stimuli. VWF mRNA was evaluated with nine overlapping, broad scanning amplifications of VWF transcripts, in silico analyses were performed to identify which intron/exon boundaries contained the weakest consensus splice sites, and then PCRs were targeted to assess exon skipping at sites of the weakest splice sites. The weakest donor sites were found at exons 14, 27, and 44, while the weakest acceptor sites were associated with exons 1, 9, 18, and 20.
PCRs targeting skipping of these exons showed more amplification across the cell lines and stimulation states than initially expected, indicating that these alternative exon skipping transcripts may be more common than previously thought. HMVEC were the least likely to amplify the targeted exon skipping sequences, showing no amplification of exon 9 skipping when stimulated with plasma or DDAVP, no amplification of exon 14 skipping unless stimulated with estrogen, weaker amplification of exons 20 and 27 skipping compared to the BOEC or HUVEC lines, and only amplified exon 44 skipping transcripts when stimulated with histamine. When amplifying exon 9 skipping four amplicons were produced in some of the samples: 1121bp representing the expected transcript; ~925bp thought to skip exon 14; ~905bp thought to skip exon 15; and the most predominant band amplified at 709bp skipping of exons 14 and 15. The additional skipping of exons 14-15 was found across all samples except HMVEC exposed to plasma or DDAVP and the ECs exposed to shear stress. Amplicons of the expected size for the exon 9 skipping PCRs were found in HUVEC exposed to plasma or DDAVP, HMVEC when unstimulated or exposed to estrogen or histamine, and at least one BOEC line under each of the conditions except for the application of shear stress.

Exon 33 skipping was the most commonly amplified, appearing in all samples regardless of stimulation state. Skipping of exon 44 was the least commonly amplified with only a faint scattering of bands across the samples including: histamine-stimulated HMVEC and BOEC line #2 (B2), estrogen stimulated BOEC line #1 (B1) and HUVEC, B1 when stimulated with media supplemented with DDAVP, HUVEC when stimulated with plasma treated with DDAVP, and HUVEC, B1, and B2 when media was supplemented with plasma not exposed to DDAVP. All of these targeted exon skipping transcripts, with the exception of exon 44 skipping would introduce a premature termination codon (PTC) and target the alternative transcript for nonsense mediated decay (NMD). Identification of these potential alternative VWF transcripts may provide an additional mechanism in which splicing of VWF affects its gene expression. The
presence of these transcripts should be confirmed using RNA-Seq technology on a larger number of
subjects, as well as in comparison to VWF mRNA populations in the platelet transcriptome. It would also be
widely beneficial to look into largescale databases of the human transcriptome, such as the Mammalian
Gene Collection, to determine if other VWF splice variants have been detected but thus far
unacknowledged.

4.2 Introduction

4.2.1 Alternative Splicing: A Natural & Common Process

Alternative splicing is a process found to occur in approximately 90% of human genes (Pan et al.
2008, Barash et al. 2010) to increase protein diversity without expansion of the genome (Black 2003).
There are multiple mechanisms through which alternative splicing can occur, such as exon skipping, intron
inclusion, use of alternate 5’ and 3’ splice sites, or alternate transcription start sites or poly-adenylation sites
(Kim et al. 2007, Sugnet et al. 2004). Exon skipping is the most common type of alternative splicing in
higher eukaryotes and intron retention is the least common, occurring when the splicing machinery does
not recognize weak splice sites flanking small introns (Kim et al. 2007, Sakabe & de Souza 2007). Weaker
5’ splice sites, identified by a less stringent binding affinity for the U1 snRNA, can be a major selective force
in alternative splicing. The U1 snRNA itself is highly conserved, however splice site sequences have
become more variable throughout evolution (Kim et al. 2007, Ast 2004) which can decrease exon
recognition, and promote alternative exon skipping (Stamm et al. 1994, Carmel et al. 2004, Sorek et al.

Generally, alternative splicing or exon skipping that introduces a frameshift and a downstream
premature termination codon (PTC) is selected against, which may explain why the majority of alternative
cassette exons maintain the original reading frame; however, this does not explain why ~30% of
alternatively spliced exons disrupt the reading frame and introduce a PTC (Koren et al. 2007). When PTCs are introduced upstream of exon junction complexes, these transcripts are recognized as aberrant and will commonly be targeted for nonsense mediated decay (NMD) to ensure quality control in gene expression (Lewis et al. 2003, Wollerton et al. 2004, Isken & Maquat 2008). It has been proposed that occurrences of NMD targeting may not be as stringent as previously thought, and some PTC containing transcripts may tolerated in low does (Baek & Green 2005, Pan et al. 2006, Blencowe 2006), or even serve alternate functions to the primary protein. With ~19,000 human genes undergoing alternative splicing, it can be difficult to determine which of the many mRNA isoforms will provide functional downstream consequences (Kalsotra & Cooper 2011). Much of diversity of the transcriptome appears to involve stochastic noise from low-abundance transcripts generated from alternative splicing that is not conserved known as “noisy splicing” (Melamund & Moult 2009, Pickerell et al. 2010).

4.2.2 Splicing Variance across Cellular Environments

As previously mentioned, splicing patterns and proportions of various splice forms can vary depending on the context in which the transcript is being produced (Johnson et al. 1999, Chen et al. 1999, Karpova et al. 2001). Many alternative splicing patterns are also known to be sensitive to the tissue in which they are produced. Interferon regulatory factor-3 (IRF3) produces two alternative splice forms IRF3 and IRF3a, in many tissues through the use of different 5’ exons; in the brain there is a significant predominance of the IRF3a splice form compared to other tissues (Xu et al. 2002). Additionally, the serine/threonine protein kinase WNK1, has been shown to have an alternate splice form produced only in the kidney which uses an alternative 4th exon introducing an in-frame stop codon and an additional in-frame start codon which would produce the remainder of the WNK1 protein in frame however the kinase domain would be disrupted (Xu et al. 2002). Inclusion of the 5th exon in the striated muscle-specific cardiac troponic T (cTNT) gene varies based on developmental stage; in adult skeletal and cardiac muscle tissues the exon
is excluded, whereas it is included during embryonic development of these tissues (Ryan & Cooper 1996). Spliceopathies involving this switch in alternative splice forms have been known to cause myotonic dystrophy (DM) where the conversion to predominantly exon 5 exclusion does not occur and cTNT splicing retains its fetal pattern into adulthood (Osborne & Thornton 2006, Caillet-Boudin et al. 2014). During B cell maturation, the transcriptional regulator CCCTC-binding factor (CTCF) induces pausing of RNA polymerase II (pol II) in exon 5 of CD45 transcripts which leads to changes in exon 5 inclusion once B cells are mature (Irimia & Blencowe 2012, Shukla et al. 2011). Similar pausing or arrest of pol II elongation has been known to occur under situations of cellular stress which has been shown to drastically affect alternative splicing regulation of genes involved in RNA binding and processing, leading to an increase in the propensity of PTC introductions and acting as an immediate mechanism to control gene expression (Irimia & Blencowe 2012, Ip et al. 2011).

Mechanical stimulation has been shown to regulate alternative splicing of many shear stress responsive genes in mechanocytes such as vascular endothelial growth factor (VEGF) which splices differently based on the degree of mechanical stress experienced by the cell (Houck et al. 1991, Seko et al. 1999, Maes et al. 2004, Saygili et al. 2011, Huan & Tang 2013, Murphy & Hynes 2014). Alternative splicing of many other genes such as insulin-like growth factor (IGF-I) (Tang et al. 2004, Goldspink 2005, Goldspink & Goldspink 2005, Dluzniewska et al. 2005, Heinemeier et al. 2007, Matheny et al. 2010), tension-induced/inhibited proteins (TIPs) (Bannister et al. 2000, Jakkaraju et al. 2005), tenascin C (TnC) (Jones et al. 2000, Hsia & Schwarzbauer 2005, Keller et al. 2007), collagen XII (Keller et al. 2007), CD44 (Ponta et al. 2003, Keller et al. 2007), and serum response factor (SRF) (Kemp et al. 2000, Yang et al. 2000) is mechano-responsive (Huan & Tang 2013). These alternative splicing patterns can be moderated by shear stress through alterations in intracellular Ca$^{2+}$ signaling combined with altered phosphorylation signaling
and intracellular depolarization which alter proportions of splicing enhancing and silencing factors as well as influence splice site selection by the spliceosome complex (Huan & Tang 2013).

4.2.3 VWF & the Importance of Splicing of Intron 1

von Willebrand factor (VWF) is an integral hemostatic protein involved in both primary and secondary hemostasis. VWF is biosynthesized in both endothelial cells (ECs) and megakaryocytes and platelets. The highly polymorphic VWF gene extends over 178kb on the short arm of chromosome 12 and contains 52 exons separated by 51 intronic stretches (Mancuso et al. 1989, Gallinaro et al. 1990). The exons range dramatically in length from exon 50's minimal 40 nucleotides, to the expansive 1.3kb exon 28; intron lengths are similarly divergent across the VWF gene, spanning from the 97bp of intron 29, to intron 6 which comprises 19.9kb (Mancuso et al. 1989). Exon 1 of VWF encodes the 5’ untranslated sequence and the promotor region of VWF expression; this is separated from exon 2: the translational start site of VWF by a 1228bp intron (intron 1) (Yuan et al. 2013). When VWF undergoes transcription and splicing, these introns are removed and the remaining exons are spliced together to form an approximately 8.5kb VWF transcript which encodes for this vital coagulation factor (Mancuso et al. 1989).

VWF expression levels are known to vary throughout the vasculature, with veins expressing more VWF than arteries, and venules more expressive than arterioles; even within particular vascular beds, VWF expression has been shown to vary between neighbouring endothelial cells (Senis et al. 1996, Yamamoto et al. 1998, Patel et al. 2008). A recent study by Yuan et al. 2013 suggests, that one factor contributing to variable VWF expression is the splicing of VWF’s first intron (Yuan et al. 2013). Through transient transfection of HUVEC with luciferase-reporter plasmids associated with the VWF promotor region with and without the first intron of VWF, it was noted that splicing of VWF’s first intron enabled the expression of the reporter gene in these assays, whereas in the absence of the first intron of VWF, and subsequently the action of splicing, expression was decreased (Yuan et al. 2013). This enabling of expression was not
specific to only the first intron of VWF, as insertion of the second intron of the β-globin gene was also able to rescue expression from with VWF promotor (Yuan et al. 2013). While this finding indicates that it is the act of splicing that effectively enables expression through the VWF promotor, there are some introns that affect this change (such as VWF intron 1 and beta-globin intron 2) and others which offer no expression (mouse down syndrome critical region 1 intron 2), or partial rescue of expression (hagfish factor X intron 6) through the VWF promotor (Yuan et al. 2013). This dependence on intronic removal by splicing for expression is not a universal effect; however, it has been observed in other genes as well as VWF (Nott et al. 2003, Bracham & Korf 2008, Yuan et al. 2013).

As VWF is produced in both ECs and platelets, this presents the possibility of cell type specific differences in VWF splicing, and the effect of VWF splicing on promotor activity and VWF gene expression. Yuan et al. 2013 also explored this possibility through similar transient transfections into a megakaryocyte cell line and uncovered that splicing of intron 1 is not a requirement, nor does it have an effect on VWF expression in megakaryocytes (Yuan et al. 2013). This untranslated region upstream of exon 2 has been shown to play a role in vascular bed specific expression of VWF, where VWF expression is driven by this promotor region in ECs of the brain, heart and skeletal muscle, but not the aorta, lungs, kidney, spleen, or liver (Minami et al. 2002). Despite the fact VWF splicing has been found to affect VWF expression, alternative splice variants for VWF have yet to be identified in the normal population.

4.3 Objectives

This chapter aims to assess the potential for alternative splicing of endothelial VWF within the normal population and across vascular bed using three normal BOEC lines, HUVEC, and HMVEC. These endothelial cells will be subjected to biochemical and biomechanical stimuli to determine if these factors play a role in regulating alternative splicing of VWF.
4.4 Materials and Methods

4.4.1 Derivation of Normal Endothelial Cell Lines

BOEC were derived from three normal controls following the protocol outlined in Section 2.3.3.1. HUVEC and HMVEC (ATTC) were evaluated in this section to look at the heterogeneity of EC populations and how VWF splicing across different vascular beds may differ from normal BOEC.

4.4.2 Biochemical Stimulation of Cells

4.4.2.1 DDAVP Stimulation

Blood was collected from normal individuals into sodium citrate anticoagulant and half of it was then incubated with 100 ng/mL DDAVP for 90 minutes as seen in Hashemi et al. (1990). DDAVP and non-DDAVP treated blood samples were then centrifuged at 5000xG for 10 minutes to isolate the plasma. Plasma samples were then mixed 50:50 with Opti-MEM reduced serum media (Life Technologies) and applied to ECs grown to 90% confluence on 10cm collagen-coated tissue culture plates for 48 hours. ECs were also treated with 50 ng/mL DDAVP supplemented cEGM2 in the absence of pretreated plasma for 48 hours to assess the effect of DDAVP alone. RNA was extracted from the stimulated cells using the QIAamp RNA Blood Mini Kit (QIAGEN).

4.4.2.2 Estrogen & Histamine Stimulation

ECs were grown to 90% confluence on 10 cm collagen-coated tissue culture plates and then treated with either 2 µM of estrogen or 1nM Histamine for 48 hours before the plates were washed and RNA was extracted from the stimulated cells using the QIAamp RNA Blood Mini Kit (QIAGEN).

4.4.3 Biomechanical Stimulation of Cells

ECs were plated on 0.8 µluer slides (Ibidi) and grown to 90% confluence. They were then subjected to laminar flow with a shear stress of 15 dynes/cm² for 48 hours using an Ibidi pump system.
RNA was then extracted using an RNAqueous®-Micro Kit (Ambion). Shear stress experiments were not completed with HMVEC as sufficient numbers of cells would not stay adherent to the slides under shear to conduct RNA extraction and analysis.

4.4.4 Evaluation of Alternative Splicing

4.4.4.1 General Broad Scanning RT-PCRs

RNA collected from stimulated and unstimulated ECs was reverse transcribed and VWF cDNA was amplified in 9 sections as described in Section 2.4.5. Subsequent cDNA amplifications were visualized on 1% agarose gels by electrophoresis and any bands of unexpected sizes were extracted using the QIAquick Gel Extraction Kit (28704, Qiagen) and sequenced by Sanger sequencing.

4.4.4.2 In Silico Analyses

Alamut 2.0 in silico analysis software was used to assess the strength of each of VWF’s natural splice sites. This program incorporates predictions from five different splice site prediction programs: Splice Site Finder Like, Max Ent Scan, NNSplice, GeneSplicer and Human Splicing Finder. The acceptor (3’) and donor (5’) splice sites for each exon were evaluated by each of these programs and the three acceptor and donor sites most consistently ranking in the weakest 10% were selected for further investigation of potential alternative splicing. Each site needed to be ranked in the weakest 10% by at least 3 of the in silico programs.

4.4.4.3 RT-PCRs Targeting Predicted Weak Splice Sites

Primers were designed to target the predicted exon skipping of the exons with the three weakest acceptor or donor sites, as well as exon 33 skipping which was noted in the normal population during qRT-PCRs in Chapter 2 (Figure 4.1). Primer sequences can be found in Appendix B. PCRs specific to exon skipping transcripts predicted above were performed on VWF cDNA from the stimulated and unstimulated
EC RNA to determine if cellular stimulation or cell type affected alternative splicing of VWF’s weakest splice sites. PCR products were visualized on 1% agarose gels and underwent Sanger sequencing.

**Figure 4.1: Targeted Exon-Skipping PCR Primer Design.** Example showing targeted exon 9 skipping where the sense primer bridges the 3’ end of exon 8 and the 5’ end of exon 10, and the reverse primer is located in exon 17, employing primers from the broad-scanning PCRs

### 4.5 Results

#### 4.5.1 General Broad Scanning RT-PCRs

The RNA from stimulated and unstimulated ECs was reverse transcribed and amplified to visualize the VWF mRNA in nine broad scanning sections, to identify any large scale aberrant banding patterns indicative of alternative VWF splicing. Amplification at the expected band size was observed for each of the nine broad sections of VWF with no extra bands representative of alternative splicing indicating that alternative splicing of VWF, if present is found in low abundance.

#### 4.5.2 In Silico Analyses

As no indicators of alternative splicing were observed in the VWF mRNA from normal BOEC, HUVEC or HMVEC when observed with broad scanning RT-PCRs, *in silico* analyses were performed to identify the splice sites most vulnerable to alternative splicing. The acceptor splice sites that were mostly commonly assigned to the weakest 10% were found at exons 1, 9, 18, and 20 (Table 4.1). The donor sites that were mostly commonly assigned to the weakest 10% were found at exons 14, 27, and 44 (Table 4.1).
While the acceptor site on exon 1 was recognized as a weak consensus site by four of the splicing analysis programs, this technique of targeted exon skipping assessment was not possible for exon 1 due to the lack of constitutively included mRNA sequences upstream of exon 1 to design the primers on, therefore the skipping of exon 1 was excluded from our analyses.

Table 4.1: VWF Exons with the Weakest Acceptor and Donor Sites as Predicted By *In Silico* Assessment Programs.

<table>
<thead>
<tr>
<th>Exons with the Weakest Acceptor Sites</th>
<th>Exons with the Weakest Donor Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXON 1</td>
<td># Weak Assessments</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>EXON 14</td>
<td># Weak Assessments</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td>44</td>
<td>4</td>
</tr>
</tbody>
</table>

**4.5.3 Targeting Predicted Weak Splice Sites**

Variable amplification was observed across the samples and exon skipping transcripts, however surprisingly each of the estimated transcripts was amplified in more than one RNA subset. An example of the amplification of these targeted amplifications is depicted from unstimulated normal BOEC RNA grown in static culture (Figure 4.2). All bands of expected and aberrant sizes amplified through targeted PCRs were sequenced to confirm their identity as VWF transcripts.

PCRs targeted to amplify exon 9 skipping showed amplification of a strong band approximately 400bp shorter (709bp) than expected for a VWF transcript skipping only exon 9 (1121bp), despite optimization of the PCR as seen in Figure 4.2 amplified a band of the expected size. This band was amplified in all of the samples regardless of biochemical stimulation except for the RNA from HMVEC exposed to plasma with and without DDAVP, as well as media supplemented with DDAVP (Table 4.2). This
band was also not amplified in ECs after exposure to shear stress. Upon sequencing, this transcript was found to skip both exons 14 and 15 (Figure 4.3). In addition to this exon 14-15 skipping band few of the samples (Table 4.2) amplified three other products (Figure 4.4): 1121bp the size expected for the exon 9 skipping primer set, another at ~925bp in length expected to be exon 14 skipping, and an approximately 905bp band thought to represent skipping of exon 15. Unfortunately the sequencing of the ~925bp and ~905bp bands were difficult to successfully separate during gel extraction and identification through sequencing was not possible.

Figure 4.2: Amplification of Each Targeted Exon Skipping Transcript in RNA from a Normal Unstimulated BOEC. RT-PCR amplifications were run on a 1% agarose gel at 110volts for 30 minutes. Lane 1) Targeted exon 9 skipping; Lane 2) Targeted exon 14 skipping; Lane 3) Targeted exon 18 skipping; Lane 4) Targeted exon 20 skipping; Lane 5) Targeted exon 27 skipping; Lane 6) Targeted exon 44 skipping; Lane 7) Negative control; Lane 8) Targeted exon 33 skipping.

Figure 4.3: Double Exon 14-15 Skipping. Identified as the 709 bp in the exon 9 skipping amplifications.
Figure 4.4: Quadruple banding observed in some exon 9 skipping PCRs. 1% agarose gel run at 95 volts for 75 minutes. 1121bp expected band size for the exon 9 skipping primers; 925bp possible exon 14 skipping; 905bp possible exon 15 skipping; 709bp exon 14-15 skipping.

When amplifying target sequences specific to exon 14 skipping, all of the samples amplified a band ~500bp larger than expected for the primer pair amplifying the PCR. In addition to this larger band, a band of the expected size for exon 14 skipping was observed in HUVEC cultured with plasma with and without DDAVP supplementation, as well as estrogen supplementation and the application of 15dynes/cm² shear stress (Table 4.2). All three BOEC lines also produced an amplification product of the expected size for exon 14 skipping when left untreated, or stimulated with estrogen, histamine, and plasma with or without DDAVP supplementation (Table 4.2). The third BOEC line was the only line to amplify exon 14 skipping when treated with media supplemented with DDAVP in the absence of plasma. Sanger sequencing of these two PCR products showed quality VWF sequence between the exon 8/10 bridging primer and the reverse primer 500bp downstream for the band of the expected size; unfortunately, upon trying to
sequence the larger than expected band, the sequence was unreadable, containing double peaks throughout the sequence.

Transcripts specific to the skipping of exon 18 amplified across all samples regardless of chemical stimulation, with the exception of HMVEC exposed to plasma with and without DDAVP, as well as media supplemented with DDAVP (Table 4.2). However when subjected to shear stress at 15 dynes/cm² amplification was only seen in normal BOEC line #2 and the HUVEC samples. Sequencing of this amplification product showed the VWF sequence expected from the primer bridging exons 17/19 and a downstream antisense primer at the end of VWF exon 21 (Appendix B).

When targeting skipping of exon 20, we saw amplification in all cell lines under all chemically stimulated and unstimulated conditions; however, amplification in HMVEC samples appeared to be decreased relative to the RNA from the HUVEC and BOEC samples (Table 4.2). When subjected to shear stress amplification of exon 20 skipping transcripts again only occurred in normal BOEC line #2 and HUVEC. Sequencing of this transcript was unintelligible and identification of this transcript as the target sequence can only be based on the accurate size of the amplification product produced.

Targeted amplification of transcripts skipping exon 27 and exon 33 skipping were observed across all cell lines whether subjected to biochemical stimulus, biomechanical stimulus, or no stimulus at all (Table 4.2). Sequencing was as expected for the primer sets employed (Appendix B). Amplification specific to exon 44 skipping was the least commonly amplified of all of these predicted locations (Table 4.2). Faint but definite bands were seen in HUVEC when stimulated with either estrogen or plasma treated with DDAVP. Weaker banding patterns appeared when HUVEC and BOEC line #1 were stimulated by DDAVP in the cellular medium, without the pre-treated plasma. Unfortunately, sequencing of this amplification product was also impossible to derive adequate identification from and therefore, inferences as to its identity were made solely on band size and lack of additional bands being amplified.
Table 4.2: Amplification profiles of exon skipping sequences from targeted PCRs. N=3 It is to be noted that shear stress manipulation was not conducted on HMVEC so in the 15 dynes/cm² shear stress column “all lines” refers to all 3 normal BOEC lines as well as HUVEC. B1= BOEC line #1, B2=BOEC line #2, B3=BOEC line #3, No Amp= target did not amplify across any of the cell lines.

<table>
<thead>
<tr>
<th>Targeted Transcript</th>
<th>No Stimulus</th>
<th>Histamine</th>
<th>Estrogen</th>
<th>Media + DDAVP</th>
<th>Plasma + DDAVP</th>
<th>Plasma no DDAVP</th>
<th>15 dynes/cm² shear stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 9 Skipping</td>
<td>HMVEC, B3</td>
<td>HMVEC, B2</td>
<td>HMVEC, B2</td>
<td>HUVEC, B3</td>
<td>HUVEC, B1, B3</td>
<td>HUVEC, B1, B3</td>
<td>No Amp</td>
</tr>
<tr>
<td>(1121bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 9 Skipping</td>
<td>HMVEC, B3</td>
<td>HMVEC, B2</td>
<td>HMVEC, B2</td>
<td>HUVEC, B3</td>
<td>HUVEC, B1, B3</td>
<td>HUVEC, B1, B2, B3</td>
<td>No Amp</td>
</tr>
<tr>
<td>(925bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 9 Skipping</td>
<td>HMVEC, B3</td>
<td>HMVEC, B2</td>
<td>HMVEC, B2</td>
<td>HUVEC, B3</td>
<td>HUVEC, B1, B3</td>
<td>HUVEC, B1, B2, B3</td>
<td>No Amp</td>
</tr>
<tr>
<td>(905bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 9 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All but HMVEC</td>
<td>All but HMVEC</td>
<td>All but HMVEC</td>
<td>No Amp</td>
</tr>
<tr>
<td>(709bp)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 10 Skipping</td>
<td>B1, B2, B3</td>
<td>B1, B2, B3</td>
<td>HMVEC, B1,B2,B3</td>
<td>B3</td>
<td>HUVEC, B1,B2,B3</td>
<td>HUVEC, B1,B2,B3</td>
<td>HUVEC</td>
</tr>
<tr>
<td>Exon 11 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All but HMVEC</td>
<td>All but HMVEC</td>
<td>All but HMVEC</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 12 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 13 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 14 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 15 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 16 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 17 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 18 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 19 Skipping</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 20 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 21 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 22 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 23 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 24 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 25 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 26 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>B2, HUVEC</td>
</tr>
<tr>
<td>Exon 27 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 28 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 29 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 30 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 31 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 32 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 33 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 34 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 35 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 36 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 37 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 38 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 39 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 40 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 41 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 42 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 43 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
<tr>
<td>Exon 44 Skipping</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines-HMVEC weaker</td>
<td>All Lines</td>
</tr>
</tbody>
</table>
4.6 Discussion

Alternative splicing transcripts of VWF likely represent a small proportion of the total VWF transcriptome. This low abundance of alternatively spliced transcripts, particularly if they were subject to nonsense mediated decay (NMD), would explain why these transcripts were not distinguished by the broad scanning RT-PCRs of the VWF mRNA. Even the exon 33 skipping transcript identified at a proportion 13±0.3% of the VWF mRNA in normal BOEC grown in static culture was not identified by broad scanning RT-PCRs of the normal RNA. It was upon targeted amplification of the exon 33 skipping transcript from patients T151 and T152 that this variant was identified in normal controls. If each of these transcripts predicted by in silico analysis exists in the endothelium, they would most commonly introduce frameshifts and premature termination codons (PTC) into the VWF transcript downstream of the skipped exon (Table 4.3). It makes sense that these transcripts would be produced in small amounts, and possibly as a product of ‘noisy splicing’ (Melamund & Moult 2009) as transcripts experiencing NMD comprise a significant portion of the VWF transcriptome can lead to VWD. Exon 9 skipping induced by the VWF mutation c.1109+2 T>C in intron 9 has been shown to cause Type 1 VWD through NMD of the aberrant transcripts (Castaman et al. 2010). Exon 44 skipping is the exception to this trend where skipping of exon 44 introduces an in-frame change with no additional amino acid substitutions at the site of exon skipping/ the junction of exons 43 and 45 (Table 4.3). It is interesting that the VWF exons with the weakest acceptor and donor sites are primarily those which introduce PTCs into the VWF sequence and therefore would be degraded by NMD and are most likely selected against. If the VWF exons most likely to be skipped are those which would induce NMD of the alternative transcripts, it may be presumed that for VWF, the importance of alternate splicing of VWF is intended to control gene expression by reducing VWF expression through the NMD pathway when these alternate transcripts are produced. It is possible that in silico analyses of VWF canonical splice site strength is not the best predictor of alternative splicing potential.
Table 4.3: Characteristics and downstream outcomes of targeted and observed exon skipping proposed by *in silico* analyses and exon 33 skipping.

<table>
<thead>
<tr>
<th>Exon Skipping</th>
<th>Exon(s) Size (bp)</th>
<th>Frame Effects</th>
<th>Amino Acid (AA) Change</th>
<th>Downstream PTC Introduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 9</td>
<td>112</td>
<td>Frameshift</td>
<td>Glu→Ala</td>
<td>PTC 14AA into Exon 11</td>
</tr>
<tr>
<td>Exon 14</td>
<td>196</td>
<td>Frameshift</td>
<td>Thr→Pro</td>
<td>PTC 11AA into Exon 15</td>
</tr>
<tr>
<td>Exon 15</td>
<td>216</td>
<td>In-Frame</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Exons 14 &amp; 15</td>
<td>412</td>
<td>Frameshift</td>
<td>Thr→Lys</td>
<td>PTC 2AA into Exon 16</td>
</tr>
<tr>
<td>Exon 18</td>
<td>161</td>
<td>Frameshift</td>
<td>Ser→</td>
<td>PTC 4AA into Exon 19</td>
</tr>
<tr>
<td>Exon 20</td>
<td>139</td>
<td>Frameshift</td>
<td>Cys→Trp</td>
<td>PTC 15AA into Exon 21</td>
</tr>
<tr>
<td>Exon 27</td>
<td>136</td>
<td>Frameshift</td>
<td>Gly→Ala</td>
<td>PTC 28AA into exon 28</td>
</tr>
<tr>
<td>Exon 33</td>
<td>44</td>
<td>Frameshift</td>
<td>Gly→Ala</td>
<td>PTC 32AA into Exon 34</td>
</tr>
<tr>
<td>Exon 44</td>
<td>111</td>
<td>In Frame</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The only predicted in-frame alternative transcript, the skipping of exon 44, was the least ubiquitously amplified. It would be interesting to further confirm the existence of this variant on the proteomic level through Western blotting, and continue with further *in vitro* expression studies in HEK293T cells and functional assays to determine potential applications for the VWF splice variants *in vivo*. Exon 44 skipping variant may remove a portion of the GpIIb-IIIa binding domain for assisting platelet association with VWF therefore while being an in-frame truncated splice variant, its functionality may vary from that of wildtype (WT) VWF in circulation.
The amplification of four different splicing products with the RT-PCRs targeted toward exon 9 skipping was a novel finding. The predominance of the 709bp transcript skipping both exons 14 and 15 is of particular interest as it was present in most of the samples regardless of EC stimulation with biochemical and biomechanical factors, with the exception of HMVEC exposed to DDAVP and/or plasma which did not produce any amplicons for this PCR. As this joint skipping introduces a frame shift and PTC only 2AAs into exon 16, it is presently unclear what the physiological significance of this transcript would be. As with the proposed exon 44 skipping product, the in-frame skipping of exon 15 should be probed for in a Western blot, and functional assessment of this protein and its expression should be analyzed as these exons are part of the VWF propeptide which is known to be critical for VWF multimerization, WPB formation, and intracellular trafficking of VWF (Zucker et al. 1979, Verweij et al. 1987, Wise et al. 1988, Wise et al. 1990, Wagner 1990, Mayadas & Wagner 1992, Rehemtulla & Kaufman 1992, Purvis & Sadler 2004, Valentijn et al. 2011).

It is unfortunate that the approximately 1.1kb band observed in the exon 14 skipping could not be sequenced successfully as it may have identified an additional splice product in this area, possible inclusion of intron 14, or the use of a cryptic splice site in that area. Alternatively, this additional band may simply be the product of insufficient PCR optimization. Ideally to identify conclusively positive amplification, these PCRs would have been optimized using positive controls, such as a vector containing the proposed exon skipping sequences; however, this was not the case in this chapter. PCRs were ‘optimized’ by assessing amplification at a wide variety of primer annealing temperatures and performing the amplification at the annealing temperature that produced a single band at the size expected for the primer design. In the instances where the amplification products from these targeted PCRs could not be sequenced clearly the sequence may have been contaminated throughout the gel extraction process, or it may be an indication that the predicted transcripts are less likely to exist in the VWF mRNA population compared to variant
amplifications which were able to be sequenced. Unfortunately, with the primer design bridging the exon that was expected to be skipped, sequencing was unable to capture and illustrate the actual site of exon skipping. The site of exon skipping was disguised at the site of primer binding or the tail end of the sequencing product which tends to confer messy sequence is not the best predictor of alternative splicing potential.

Identification of these potential alternative VWF transcripts may provide an additional mechanism in which splicing of VWF affects its gene expression. The presence of all these amplified transcripts should be confirmed using RNA-Seq technology on a broader number of subjects, as well as in comparison to VWF mRNA populations in the platelet transcriptome to assess cell type-mediated changes in VWF splicing. While this experimental design has some limitations it leaves us with a good starting point to further investigate the occurrence of alternative splicing of VWF in the normal population. This study has raised questions as to whether alternatively spliced VWF transcripts serve a purpose other than regulating VWF gene expression through targeting these PTC-containing alternative transcripts for NMD.

Shear stress exposure at 15 dynes/cm² appears to decrease the degree to which in-frame variant splice forms are produced as both exon 15 and exon 44 skipping are not observed in samples isolated from ECs under shear stress (Table 4.4). When observing the frame-shifting variants the story is less clear. While amplification of exon 9 or exon 14 skipping, and the co-skipping of exons 14-15 are not observed after shear stress exposure for 48 hours, skipping of exon 18 or 20 is merely decreased, and evidence of exon 27 or exon 33 skipping remains strong after exposure to shear (Table 4.4). While it appears that shear stress must be a regulator of endothelial VWF splicing, the functional effects of these changes remain unclear, and may depend on the results of in depth functional proteomic investigation of these splice variants. Lack of proteomic data aside, these observations may suggest a general trend toward shear stress as a down-regulator of alternative splicing, presuming that the in-frame variants will be removed, or
at least reduced, and any remaining PTC-inducing transcripts will be targeted for degradation before translation, unless they produce an otherwise functional protein. This idea is supported by our data from Chapter 2 which showed a similar trend away from production of aberrant in-frame transcripts after the exposure of patient BOEC with pathologic splicing mutations to shear stress at 50 dynes/cm² for 48 hours (Table 4.4).

**Table 4.4: Effects of shear stress on aberrant & alternative VWF transcripts.** Grey shading= transcripts decreased after shear stress exposure. No shading= transcripts maintained or increased after exposure to shear stress.

<table>
<thead>
<tr>
<th>Splicing Transcript</th>
<th>Frame Effects</th>
<th>Effects of Shear Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 23 Skipping</td>
<td>In-Frame</td>
<td>Decreased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 26 Skipping</td>
<td>In-Frame</td>
<td>Decreased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 23/26 Skipping</td>
<td>In-Frame</td>
<td>Decreased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 33 Skipping</td>
<td>Frameshift-PTC</td>
<td>Increased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 33-34 Skipping</td>
<td>In-Frame</td>
<td>Decreased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 38 Skipping</td>
<td>Frameshift-PTC</td>
<td>Increased @ 50dynes/cm²</td>
</tr>
<tr>
<td>Exon 9 Skipping</td>
<td>Frameshift-PTC</td>
<td>Not amplified @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 14 Skipping</td>
<td>Frameshift-PTC</td>
<td>Not amplified @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 15 Skipping</td>
<td>In-Frame</td>
<td>Not amplified @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 14-15 Skipping</td>
<td>Frameshift-PTC</td>
<td>Not amplified @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 18 Skipping</td>
<td>Frameshift-PTC</td>
<td>Decreased @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 20 Skipping</td>
<td>Frameshift-PTC</td>
<td>Decreased @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 27 Skipping</td>
<td>Frameshift-PTC</td>
<td>Strong amplification @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 33 Skipping</td>
<td>Frameshift-PTC</td>
<td>Strong amplification @ 15dynes/cm²</td>
</tr>
<tr>
<td>Exon 44 Skipping</td>
<td>In-Frame</td>
<td>Not amplified @ 15dynes/cm²</td>
</tr>
</tbody>
</table>

While we had hoped this chapter would shed light on the possibility of splicing variability across different vascular beds, the fairly ubiquitous amplification of these targeted exon skipping sequences...
makes it difficult to draw specific conclusions. HMVEC appear to have less propensity toward alternative exon skipping than HUVEC or BOEC when unstimulated or subjected to stimulation by histamine, estrogen or DDAVP manipulations (N=3). HMVEC responses to shear stress were unable to be evaluated as they would not remain adherent on the Ibidi µleur slides throughout the duration of shear stress exposure, and vast quantities of cells appeared to be removed over the course of the 48 hours. The majority of HUVEC splicing products appeared to align with transcripts present in the BOEC populations; however, occasionally even the various BOEC lines would display different patterns in VWF splicing amplification, such as only B3 amplifying exon 14 skipping after incubation in media supplemented with DDAVP, or only B2 and HUVEC amplifying exon 18 and 20 skipping transcripts under application of 15 dynes/cm² shear stress. This intra-BOEC variability may be explained by inter-individual differences affected by concentrations of splicing factors or single nucleotide polymorphisms (SNPs) in their VWF sequence which affect the binding of splicing factors and intron/exon recognition. Use of the splice-junction microarrays has shown to be useful in assessing varying ratios of splicing isoforms across different tissues types and may be useful to further elucidate the transcripts patterns here (Johnson et al. 2003).

Primer design to amplify a specific target does not allow for the discovery of additional alternative splicing transcripts. RNA-Seq can assess alternative splicing in a more neutral manner amplifying 200-300bp overlapping reads across the transcripts to identify any variations in splicing patterns, not biasing the identified targets with specifically designed primer targets. The frequent amplification of all the predicted transcripts seemed unlikely and the first suspicious target in this excessive amplification may be the primer design for the targeted exon skipping PCRs. Primers in this assessment were designed to anneal half in the exon upstream (eg. exon 8) of the exon predicted for skipping (eg. exon 9), and half in the downstream exon (eg. exon 10) (Figure 4.1). Since these primers were 18-20 nucleotides long, it may be possible that only half the primer bridging the proposed exon skipping is actually binding to the VWF mRNA and allowing
for PCR amplification. In retrospect, it may have been better to design the primers in the regions next to the site of proposed exon skipping rather than over the hypothesized exon junction, so that double banding of included and excluded transcripts could be observed; however, to prevent preferential amplification of the more abundant WT splice variant, primers were designed to bridge the proposed site of exon skipping.

While this manner of assessing alternative splicing is not ideal, it was the method feasible for our investigation. This method of broad scanning and targeted PCRs, can only assess alternative splicing transcripts present at high levels, or those targeted directly which introduces a primer bias and decreases the possibility to identify alternative splicing variants not targeted by the chosen primer. Other techniques that may be considered to follow up on these findings are: splice-junction microarrays, Nanostring Technology®, or RNA-Seq. Splice-junction microarrays can be used to target specific exon junctions with oligonucleotide probes, and the fluorescent intensities of the different probes can lend insight as to the ratios of alternative transcripts (Chen et al. 2012; Johnson et al. 2003). Some of the same issues present in our current study exist with the use of splice-junction microarrays and Nanostring technology®, in that the search for alternative transcripts must be targeted to specific sequences, or specific splicing events, making it difficult to assess all potential splicing variants. RNA-Seq is useful in this regard as this powerful technology can produce millions of short sequence reads, and identifying transcripts present even on small scales (Wang et al. 2009, Robertson et al. 2010, Martin & Wang 2011, Chen et al. 2012); however, this enhanced utility comes at a price.
CHAPTER 5
Thesis Discussion

5.1 Summary

von Willebrand factor (VWF), is a complex, multimeric glycoprotein critical for both primary and secondary hemostasis as an adhesive surface to cover exposed subendothelial matrix and aggregate activated platelets at sites of vascular injury, and also as a chaperone for the anti-hemophilic coagulation factor VIII (FVIII). VWF is produced in platelets and endothelial cells (ECs) and splicing of VWF intron 1 is shown to promote VWF gene expression in ECs; however, no such effect was observed in megakaryocytes expressing VWF (Yuan et al. 2013). Approximately 95% of human multi-exon genes are thought to undergo alternative splicing, and interestingly, despite its large size, alternative splicing variants of VWF have not been described in the literature (Pan et al. 2008; Barash et al. 2010). It is thought that 15% of VWF mutations affect splicing of VWF leading to the common bleeding disorder von Willebrand disease (VWD).

In this thesis we describe three such mutations distributed throughout exonic regions, canonical splice sites, and intronic sequences and characterize the aberrant splice products these genetic mutations produce. The various downstream pathogenic effects of these mutations such as: intracellular retention whether in the ER or WPBs, decreased VWF secretion through NMD of aberrant transcripts, and potential dominant negative retention of co-expressed WT VWF are investigated.

Additionally, this thesis has investigated the potential for alternative splicing of VWF through exon skipping at the weakest acceptor and donor sites as assessed through in silico splice site analysis. Interestingly, the majority of VWF’s weakest consensus splice sites were found to surround exons which, if removed from the VWF sequence, would introduce premature termination codons (PTCs) and target these transcripts for nonsense mediated decay (NMD). This may support the idea that VWF splicing is almost
entirely constitutive, and that if produced, the most predicted VWF alternative splicing transcripts would function to control VWF expression by limiting the amount of translatable mRNA transcripts. In the study of alternative splicing, the struggle is two-fold: 1) to find alternative splice forms for a gene of interest, which has been made simpler in recent years with the advent of RNA-Seq technology, and 2) to determine if an alternative transcript is functional on a proteomic level, or simply contributing to the well-recognized phenomena of `splicing noise` (Melamund & Moult 2009).

5.2 VWF Splicing Mutations and VWD

Several large cohort studies have been completed to assess the mutation status of VWD patients, and across cohorts, it has been noted that splicing mutations comprise approximately 15% of VWD cases when mutations could be identified (Cumming et al. 2006; James et al. 2007, Castaman et al. 2007). The majority of these sequences variants reside in the highly conserved canonical splice sites at the intron/exon boundaries; however, these are not the only location that can affect splicing if mutated. As we observed, in addition to canonical sequence changes, exonic and intronic sequence changes can have pathologic effects on VWF splicing as well. In this study, the effects of splicing were evaluated across platelet and blood outgrowth endothelial cell (BOEC) RNA to allow for splicing analysis between cell types, but primarily because the yield and quality of VWF mRNA from platelet RNA extractions was often insufficient for conclusive analysis of aberrant splice products.

Laboratory confirmation of VWD subtypes across the patients included in this study was relatively straight forward following the clinical assays for VWD assessment, as well as more in depth probing into collagen and FVIII binding abilities of patient plasma VWF. Unfortunately, none of the families investigated in this study were local to our study area, and many samples had to be shipped across the country. This increased the margin of error for sample quality and some possible sample degradation was observed in
the plasma multimers from Family 2, patients V69 and V70, which displayed a slight decrease in high molecular weight multimers (HMWM). Similar degradation was observed with cross country shipping of these samples had led to the initial classification of these patients as Type 2A (James et al. 2004). Fortunately, BOEC were able to be isolated from this family and full range multimers could be observed in expressed VWF, confirming their diagnosis as Type 1 VWD which is more consistent when considering the similar levels of VWF:Ag and VWF:RCo observed in these patients. However, it is possible as we observed VWF displaying exon 23 skipping and exon 26 skipping may interfere with multimerization of co-expressed WT VWF, that the individuals in this family present varying phenotypes between Type 1 and Type 2A depending on the proportion of aberrant transcripts they are producing.

While these particular splicing mutations are not predominant in the VWD population, splicing mutations are thought to comprise approximately 10% of pathologic VWF mutations, which may be an underestimation since this mostly refers to mutations in canonical splice sites. Further studies into the downstream consequences of these splicing mutations may be beneficial for patients whose splicing mutations create (moderately) functional VWF which is retained within the cell to the patient’s detriment as is the case observed with patients T151 and T152. If their endothelium was able to release this aberrant exon 33-34 skipping VWF, T151 could likely be treated with DDAVP rather than factor concentrates, and T152 may become asymptomatic. Stimulation of BOEC with IL-11 should be investigated to observe potential WPB release as IL-11 acts through a different mechanism than DDAVP to stimulate WPB exocytosis (Ragni et al. 2008, Ragni et al. 2011, Ragni et al. 2013).

Analysis of the splicing variants of Family 1 and 2 has gleaned insight into VWF splicing and the inter-exon relationships required for normal splicing. In both families 1 & 2, we see double exon skipping where the introduction of a mutation affecting splicing of one exon (exon 26 or 34) has negative effects on the splicing of an upstream exon, exons 23 and 33 respectively. These relationships between exons likely
speaks largely to the effect of trans-acting splicing enhancers and silencers that are disrupted by the removal of the downstream exon (e.g. exon 26 or 34), or are disrupted simply by the genetic mutation itself as exon 23 is found to be skipping on its own when the mutation is found in exon 26. It is known that speed of splicing is variable depending on the size of the intron/exon, and is influenced by changes in RNA structure as the transcript is being transcribed. Speed of transcription and splice site exposure alters the speed and order of splicing. As introns are not necessarily removed in 5' to 3' sequence, the removal of a downstream exon (such as exons 26 or 34), may influence the exclusion of an upstream exon (such as exons 23 or 33). Therefore, RNA structure, mutation location, as well as speed and order or splicing may be affecting the splice variants observed in this thesis.

In using BOEC as an in vitro model of patient endothelium, it was promising to see the patient phenotype reflected their levels of VWF expression, which is consistent across other in vitro studies of VWD (Berber et al. 2009, Starke et al. 2011, Wang et al. 2013, Starke et al. 2013). Cellular response to PMA also corresponded well with patient response to DDAVP. This reflectiveness of the patient condition endears us to the use of BOEC as a preferential model to study not only VWD but other disorders of the endothelium. Studies involving BOEC have also determined these cells retain phenotypes of the endothelium acquired in vivo, such as chronic obstructive pulmonary disease (Paschalaki et al. 2013).

5.3 Characterization of Aberrant VWF Splice Variants

While BOEC are a useful tool in the investigation of VWD, the patient BOEC required to study a particular VWF variant in both the heterozygous and homozygous state are not always available or easily accessible in part, because, the success rate for BOEC isolation is only 80-85% (Martín-Ramírez et al. 2012, Wang et al. 2013). Therefore, the use of heterologous cells systems may be useful despite their
inherent limitations. Heterologous cell systems can provide valuable insight into pathogenic mechanisms of VWF mutations; however, as these cells are not the native source of VWF production, differences in exocytotic machinery, intramolecular chaperones, and post-translational modifications may affect the expression and functionality of the recombinant proteins produced. As these heterologous cell systems model VWF production through transfection of expression vectors, analysis may be convoluted by overexpression of the transfected vectors, or an inaccurate representation of the mimicked heterozygous state. In these studies, the majority of data between cell types was consistent; with slight differences in lysate levels between transfected HEK293T cells and patient BOEC which may be a limitation of the heterologous cell system, or possibly attributed to the extent of aberrant transcript production in the patient BOEC. VWF lacking exons 33-34 showed differences in expression between BOEC and HEK293T expression systems, where the mutant protein could be expressed in the heterologous cell system; however, patient T151 exhibited minimal BOEC VWF expression. Again, this difference may be ascribed to cell model differences, or is possibly complicated by the compound heterozygosity of T151 for the splicing mutation c.5842+1 G>C and the nonsense mutation c.3939 G>A.

Throughout the investigation of these aberrant transcripts it was observed that exon 23 is likely required for multimerization and secretion of VWF. The skipping of exon 23 remains in-frame and exerts a dominant negative effect on the multimerization and secretion of co-expressed VWF. This exon falls within the multimerization domain of VWF and so its inability to multimerize is not surprising; nevertheless it is interesting that it can interfere with the WT VWF monomers preventing their multimerization. When exon 26 is skipped in conjunction with exon 23, we see a restoration of multimerization in the mock heterozygous transfections. Both the 23del and 23/26del splicing variants exhibit significant intracellular retention not seen when exon 26 is skipped alone, indicating that exon 23 is required for trafficking VWF during biosynthesis, regardless of its interaction with WT VWF.
The double exon skipping of exons 33-34 was the only aberrant VWF splice form that was able to form full range HMWM without co-expressed WT VWF. This may be because this D4N region of VWF is not ascribed to an essential functional domain of VWF, and this in-frame change may simply change, and possibly hinder proteolysis of the mutant VWF by ADAMTS-13. Despite this aberrant splice form’s ability to multimerize and preservation of moderate VWF functionality as assessed by VWF:RCo, collagen binding, and FVIII binding, this aberrant protein is retained in the cell. Increased intracellular retention in the homozygous and heterozygous transfections of HEK 293T, a lack of response to PMA in the absence of WT VWF and lack of response to DDAVP in patient T151 all indicate that these exons of VWF are essential for VWF secretion from ECs.

Different expression profiles were shown for the two transcripts identified which would induce frameshifts and downstream PTCs. Where exon 38 exhibited decreased expression through probable intracellular retention in the ER and subsequent degradation, VWF lacking exon 33 appeared to be expressed constitutively at a level similar to WT VWF. Although this may be an artifact of the heterologous expression system lacking the exon junction markers which would denote this transcript as containing a PTC, in combination with this mutant’s retained FVIII binding capacity, and its presence in the normal population, it lends support to the prospect of this splicing variant being identified as an alternative VWF splice variant.

5.4 VWF Splicing in the Normal Population

Prior to this study, alternative splicing of VWF has not been investigated and this protein was assumed to be spliced constitutively with its 52 exons in the normal population in the absence of genetic mutations affecting splicing. *In silico* analysis of VWF canonical splice junctions predicted that the most
likely exon skipping to occur would involve skipping of exons 1, 9, 14, 18, 20, 27 or 44. Interestingly, each of these predicted splicing outcomes with the exception of exon 44 skipping would introduce a PTC, likely targeting these transcripts for NMD. In addition to these predicted splicing variations, skipping of exon 15 and the combined skipping of exons 14-15 were observed throughout the normal endothelial cell (EC) population to varying degrees. While the commonly observed, double exon 14-15 skipping transcript would also introduce a PTC, the exon 15 skipping alone retains the reading frame and this alternative splice product would be more likely to continue through translation. Exon 33 skipping was observed throughout the simulated and unstimulated EC, and quantified as representing 13±0.3% of the VWF population across 6 normal BOEC lines grown in static culture.

The observation of these ubiquitous PTC containing variants such as exon 33 skipping, and skipping of exons 14 & 15, as well as the periodic identification of in-frame splicing variants such as exon 15 or exon 44 skipping, leads to the speculation that VWF may indeed experience alternative splicing along with the other 95% of human multi-exon genes (Pan et al. 2008, Barash et al. 2010). The outcome of these PTC containing transcripts whether predicted or confirmed, is unclear, while the inclination is to assume they are targeted for NMD and therefore likely play a role in mediating gene expression of VWF (Soergel et al. 2000, Isken & Maquat 2008, Pimentel et al. 2014, Carpenter et al. 2014 ). Conversely, the introduced PTCs, may be alternatively selecting 3’ termination sites and some of these variants may be translated to serve other functions within the endothelial cell, or in the circulation. It was observed that despite lacking the capacity for multimerization, truncated VWF lacking exon 33 and terminating protein production in exon 34 of the D4N domain of VWF retains its ability to be expressed and bind FVIII, postulating that this alternative splice product may in fact play a role in FVIII chaperoning in circulation.

Confirmation of these predicted splicing variants on the proteomic level is the clear next step in confirming the identity of the alternative VWF splice variants and determining their function; can they be
secreted or do they remain primarily intracellular? As has been conducted on the exon 33 skipping splice variant, these other predicted splicing variants should be characterized in vitro to shed insight as to their function. Are these observations remnants of ‘noisy splicing’ awaiting degradation through NMD, or will they become alternate splice forms serving physiologic functions yet to be uncovered? This characterization is especially important for the in-frame splicing variants that have been identified as they are the most likely to proceed through protein translation and biosynthesis.

Significant variability was observed across the different EC lines and biochemical and biomechanical stimuli. This may be ascribed to splicing variation across various vascular beds, explaining differences in targeted splicing amplification across BOEC versus HUVEC versus HMVEC. Yet, the regulation runs deeper than simply differing by vascular bed as across BOEC lines a great deal of interpersonal variation in splicing patterns was observed. Application of shear stress at 15 dynes/cm² appeared to be the most constant regulator of alternative splicing of VWF. Most of the targeted alternative splice sites were amplified under static conditions, regardless of biochemical stimulation; however amplification of these alternative splice products was often significantly down-regulated or abolished after 48 hours exposure to shear stress at 15 dynes/cm².

5.5 VWF Splicing and Shear Stress

Within this study we investigated the effect of shear stress on splicing of VWF under two circumstances: (1) application of high shear stress at 50 dynes/cm² and its effect on the distribution of WT and aberrant VWF transcripts across a patient population affected by pathologic splicing mutations, and (2) application of moderate shear stress at 15 dynes/cm² to normal BOEC and HUVEC to assess any changes biomechanical stimulation may have on alternative splicing of VWF. Across these two
applications, some general patterns were observed. The degree to which the aberrant and alternative transcripts were produced across all ECs was highly influenced by the application of shear stress to the cells. This indicates that these variant splice forms may be produced at different levels from cell to cell, and vascular bed to vascular bed depending on the extracellular environment and the possibly the degree of shear stress to which the endothelium is exposed.

Whether derived from a pathogenic splicing mutation (skipping of exon(s) 23, 26, 23/26, or 33-34) or observed in the normal population (skipping of exon 15 or 44), in-frame splicing variants were decreased under shear stress at both 15 and 50 dynes/cm². On the other hand, many of the exon skipping transcripts which introduced a PTC, whether in the patients (exon 33 or 38 skipping), or in the normal population (skipping exon 27, or 33), are maintained or even increased in situations of shear stress. This apparent maintenance or up-regulation of PTC containing transcripts with shear stress may be more profound in transcripts produced from splicing mutations, as the ‘alternative’ amplifications which introduce at PTC with skipping of exon(s) 9, 14, 14-15, 18, or 20, were not observed in ECs exposed to shear stress at 15 dynes/cm².

As we know, shear stress responsive elements (SSREs) are key regulators of endothelial transcriptional regulation, and these observations of shear dependent splicing of VWF may provide support for the influence of SSREs on not only changes in transcription and gene expression profiles but also pre-mRNA splicing. Alternatively, the effect of the SSREs on gene expression may in fact be modulated through alterations in splicing. To further deduce the effects of shear stress on EC VWF splicing, it would be of interest to quantify the various identified aberrant and alternative splicing isoforms in ECs after exposure to different durations of shear stress, different flow patterns, and a range of shear stress applied. This could help elucidate whether shear stress based changes are moderated in an “on” or “off” fashion, or
if they relate more closely to the phenotype of the EC, quiescent with chronic laminar flow, and activated with exposure to acute and/or perturbed flow patterns.

5.6 Significance, Future Directions, and Final Conclusions

The work presented in this thesis has identified the molecular pathogenesis of Types 1 and 3 VWD across three families with different putative splicing mutations, characterizing the splicing outcomes of the exonic mutation c.3538G>A, the canonical splice site mutation c.5842+1G>C, and the intronic branch point variant c.6599-20A>T. The conclusions of these characterization studies have supported the growing data that mutations outside of canonical splice sites can lead to pathologic splicing, likely through the disruption of key splicing enhancers and silencers. Functional assessment of the identified splicing variants observed that exons 23 and 26 are integral for VWF multimerization and that the in-frame skipping of exon 23 has a dominant negative effect on co-expressed WT multimerization, intracellular trafficking and expression. In addition to characterization of these aberrant splice forms, several possible alternative splicing variants were observed throughout several normal BOEC lines, as well as HMVEC and HUVEC, where VWF was previously thought to be an exception to the rule and only be spliced constitutively. Shear stress has also novelty been linked to the splicing of VWF, appearing to down-regulate in-frame transcripts whether aberrant or alternative, and has shown mixed results regarding transcripts introducing PTCs.

Future studies should be carried out to confirm the alternative VWF splice forms predicted and identified throughout this study on using a quantitative transcriptome technology such as RNA-Seq, as well as on the proteomic level to determine whether these transcripts are transcribed, and whether they produce intracellular or secreted proteins. Characterization of alternative splice forms through in vitro expression studies should help to elucidate any potential functions for these variants within the endothelium or in circulation. Once these alternative transcripts have been confirmed, their regulation should be investigated
with more extensive experimentation and RNA-Seq or splice site-specific microarrays to further understand the effect of different shear stress applications and tissue or vascular bed specific splicing patterns.

In conclusion, the work presented in this thesis supports the observation that canonical splice sites are not the only regions where a mutation can negatively affect splicing. As well, this thesis contributes to the knowledge of VWF’s functional domains providing novel insights into the role of exon 23 and the D4N domain in intracellular trafficking of VWF. The novel idea of alternative splicing of VWF and its regulation and role in coagulation and endothelial cell biology is introduced here as a variety of alternative splicing transcripts were found in the normal EC RNA of BOEC, HUVEC, and HMVEC. These observations open new windows for research into the mechanisms shear stress’ influence on splicing, particularly in VWF, as well as uncovering the existence and physiological role of functional alternative VWF splice forms.
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APPENDIX A
Thesis Protocols

A.1 VWF:Ag ELISA
Protocol based upon DAKO recommended conditions. Buffers A, B, and C are stable at 4°C for 2 months. Check for growth before using. Reagent E is stable indefinitely.

**Buffer A: PBS, pH 7.2**
10 mM Na₂HPO₄ 0.71 g Na₂HPO₄ or 5 ml 1M solution
145 mM NaCl 4.238 g NaCl or 14.5 ml 5M solution
500 ml with dH₂O, pH 7.2

**Buffer B: Washing/Dilution Buffer, PBS 0.5 M NaCl, 0.1% Tween 20, pH 7.2**
10 mM Na₂HPO₄ 0.71 g Na₂HPO₄ or 5 ml 1M solution
500 mM NaCl 14.61 g NaCl or 50 ml 5M solution
0.1% Tween 20 0.5 ml Tween 20 (cut off end of tip for easier pipetting)
500 ml with dH₂O, pH 7.2

**10X Buffer B:**
100 mM Na₂HPO₄ 8.52 g Na₂HPO₄
5 M NaCl 175.32 g NaCl

600 ml with dH₂O Microwave 2 minutes to dissolve. pH 6.15 for 1X=7.2. HPO₄ Buffer is not temperature sensitive.

**Buffer C: 0.1 M Citric Acid-Phosphate Buffer, pH 5**
0.0347 M Citric Acid 3.34 g Citric Acid
0.0667M Na₂HPO₄ 4.73 g Na₂HPO₄
500 ml with dH₂O, pH 5.0

**Reagent D: Color Reagent**
7.5 ml Buffer C
5 mg tablet OPD (o-Phenylenediamine) (Dissolve before adding peroxide)
3.1 μl 30% H₂O₂

2X for 1 plate, 3X for 2 plates.
Allow to sit for 5-10 minutes to confirm no yellowing without HRP.

**Reagent E: Stop Solution, 1 M H₂SO₄**
1M H₂SO₄ 5.56 ml 95% H₂SO₄
100 ml with dH₂O
Wrap bottle with aluminum foil. Be aware that diluting acid will generate heat.

**Coating Antibody:** Rabbit Anti-Human von Willebrand Factor, DAKO A0082.
Detecting Antibody: Rabbit Anti-Human von Willebrand Factor HRP, DAKO P0226.
Human Standard Curve Plasma: Human CryoCheck Reference plasma

Day 1: Coating wells with first antibody

1. Dilute coating antibody in Buffer A to 10μg/ml. Rabbit Anti-Human von Willebrand Factor, DAKO A0082, 3.1 μg/μl
   
   33.9 μl/10.5 ml (1)  
   72.6 μl/22.5 ml (2)  
   96.77 μl/30 ml (3)

2. Add 100 μl/well, Immulon 4 HBX plate (Dynex). Apply plate sealer and store 4°C, O/N.

Day 2: Dilutions, Binding, Detecting Antibody and Plate Development

3. Dilute samples with Buffer B on ice, using Biorad Titertube Micro Tubes. Prepare standard curves with reference plasmas (8 points, plus a blank and a control 1:100). Starting dilutions of the standard curve:
   Human 1:20, Mouse 1:5. Human samples 1:40 and 1:80.
4. Wash plate with 250 μL Buffer B, let sit 3 minutes, invert, tap hard until dry. Repeat 2X.
5. Add 100 μL diluted samples/well. Cover plate with plate sealer. Let sit room temperature for at least 2 hours. (4°C O/N will also work if absolutely necessary).
6. Wash plate as above 3 times. (Human samples can be aspirated).
7. Dilute detecting antibody Rabbit Anti-Human von Willebrand Factor HRP, DAKO P0226, 1.1 μg/μl, in Buffer B.
   For human VWF, ~1:8000
   1.77 μl/12 ml (1)  
   3.32 μl/22.5 ml (2)  
   4.43 μl/30 ml (3)
   For mouse VWF, 5 μg/μl, (from B. Montgomery’s lab)
   47.5 μl/10.5 ml (1)  
   102.3 μl/22.5 ml (2)  
   136.4 μl/30 ml (3)
8. Add 100 μl/ well and recover plate. Incubate at least 1-hour room temperature.
9. Prepare Reagent D: dissolve the OPD tablets on Buffer C.
10. Wash plate as above 3 times. In the last wash, add the H₂O₂ to buffer D.
11. Add 100 μl Reagent D/well. Cover plate and incubate 12-30 minutes, until standard curve is apparent.
12. Stop reaction with 100 μl Reagent E.
13. Read results at 492 nm using the plate reader.
A.2 Collagen Binding ELISA

CBA:Buffer
NaCl     3.5g (0.12M)
Imidazole 0.68g (0.02M)
Citric Acid 0.526g (0.005M)
500ml    pH 7.3 (Add about 25 drops of 5N NaOH)

Sodium Carbonate/Bicarbonate Buffer ph9.6
1.465g   NaHCO3
0.795 g  Na2CO3
500ml    pH9.6

Reagent D: Color Reagent
7.5 ml Buffer C
5 mg tablet OPD (o-Phenylenediamine) (Dissolve before adding peroxide)
3.1 μl 30% H2O2

2X for 1 plate, 3X for 2 plates.
Allow to sit for 5-10 minutes to confirm no yellowing without HRP.

Reagent E: Stop Solution, 1 M H2SO4
1M H2SO4________________________5.56 ml 95% H2SO4
100 ml with dH2O
Wrap bottle with aluminum foil. Be aware that diluting acid will generate heat.

Coating: ICN : bovine collagen I (95%)/ III(5%)
Human Standard Curve Plasma: Human CryoCheck Reference plasma

1: Coat plate with 100 ul of collagen : 3% i.e.3mg/ml→20ug/ml in PBS , pH7.4
   ↓ RT o/n    at least 2days
   ↓ wash (x3 with CBA buffer)

2: 3%BSA in CBA buffer—this is a block step, so add 3% bsa to a portion of the CBA and add 100ul per well
   ↓ 1h  37°C

3: 100ul of plasma dilutions (diluted with 3%BSA in CBA buffer)
   Plasma Samples: I did 1:100 and 1:200
   Standards: Used Normal Reference Plasma and did standard curve (8 points, starting with 1:10), as with VWF ELISA
   ↓ 3h    37°C
   ↓ wash (x3 with CBA buffer )

4: Anti VWF Ab HRP 1.1g/L or 1.3g/L (P0226). Diluted 1:500 in CBA buffer.
5: Add 100 μl Reagent D/well. Cover plate and incubate 12-30 minutes, until standard curve is apparent.

6: Stop reaction with 100 μl Reagent E.

7: Read results at 492 nm using the plate reader.

**A.3 FVIII Binding ELISA**

**TBST (10X) Buffer**
Tris 12.1g  
NaCl 29g  
**Tween20 2.5mL**  
dH$_2$O to 500mL pH 8.0

**TBST + 0.35M CaCl$_2$**
50mL TBST + 2.58g CaCl$_2$

**TBST + 10mM CaCl$_2$**
48.6mL TBST + 1.4mL TBST+0.35M CaCl$_2$

**Buffer C**
Citric Acid 3.65g  
NaH$_2$PO$_4$ 4.73g  
dH$_2$O 500mL pH 5

**OPD Solution**
7.5mL Buffer C  
5mg OPD tablet  
3.1 ul H$_2$O$_2$

**Coating Antibody:** Rabbit Anti-Human von Willebrand Factor, DAKO A0082.  
**Detecting Antibody:** anti-human FVIII/HRP (Affinity Biologicals #F8C-EIA-D)  
**Human Standard Curve Plasma:** Human CryoCheck Reference plasma

**Day 1: Coating Plates**
- 100ul Coating Solution/well & incubate overnight at 4°C

**Day 2: Immobilization of VWF & rFVIII**
- Wash 3 times in 250ul of TBST  
- Dilute standards and samples in TBST+3% BSA
- Add 100ul of sample to plate & incubate 2 hours at room temperature
- Wash 3 times in 250ul of TBST
- Incubate with 125ul of TBST+0.35M CaCl$_2$ for 1 hour at 37°C
- Wash 3 times in 250ul of TBST
- Add 100ul of 1.25IU/mL rFVIII diluted in TBST+10mM CaCl$_2$ and incubate for 1 hour at 37°C
- Wash 3 times in 250ul of TBST
- Add 100ul of anti-human FVIII/ HRP diluted in 1:3 TBST+3% BSA & incubate for 1 hour at room temperature
- Add 100ul OPD solution and incubate until standard curve appears
- Stop reaction with 100ul of 1M H$_2$SO$_4$
- Read results at 492nm using the plate reader

A.4 BOEC Isolation

Reagents

- CPT vacutainers (cat no: 362780, BD – Southern Syringe)
- Phosphate buffered saline (PBS), pH 7.2
- C-EGM-2: EBM-2 (Lonza; cat.no. CC-3162) supplemented with the entire growth factor bullet kit, 10% (v/v) FBS (hyclone) and 1% penicillin (10,000U/ml)/ streptomycin (10,000μg/ml)/ amphotericin (25 μg/ml, Invitrogen; cat.no. 15240-062). Store at 4°C for one month.
- Fetal Bovine Serum (FBS, Hyclone; cat.no.SH30070.03)
- Collagen I plates, 6 well- plates (BD Biosciences Discovery Labware; cat. No.356400)
- Rat tail collagen I (BD Biosciences Discovery Labware; cat. No. 354236)

1. Collect 48ml blood into 6 CPT vacutainer tubes. Invert tubes to mix anticoagulant additive with blood. (Do not shake as this can cause hemolysis). Note donor and anything unusual when blood was drawn and mix well.

2. Store tubes upright at room temperature until centrifugation. (blood samples should be centrifuged within 1 hour of blood collection).

3. Centrifuge at room temperature (18-25°C) in a horizontal rotor (swing-out head) for 30 minutes at 1600 RCF. (note: remix the blood sample immediately prior to centrifugation by gently inverting the tubes 8-10 times).

4. Remix the blood sample immediately after the centrifugation by gently inverting the tubes 8-10 times

5. Remove the serum +MNCs layer and add to 8 mls of PBS+10% serum

6. Centrifuge the MNCs at 520g for 10min at room temperature with a high brake.
7. Aspirate and discard the supernatant. Following this initial centrifugation the pellet of cells is often loose. Care should be taken to avoid aspirating cells.

8. Gently tap loose the pelleted cells and resuspend in 10 ml of PBS (+10%FBS). If there are multiple tubes, cell pellets can be serially combined at this point.

9. Repeat steps 5-7 one time.

10. Remove 10μl of cell suspension and mix with 90μl of PBS (1:10 dilution). Remove 30μl of the diluted sample and mix with 30μl trypan blue. Count viable cells on a hematocytometer and calculate the total number of MNCs in the sample.

11. Centrifuge the cell suspension at 520g for 10 min at room temperature with a high brake and aspirate the supernatant.

12. Tap loose the cell pellet and resuspend MNCs in cEGM-2. A seeding density in the range of 3-5 x10^7 MNCs/4mls is ideal for ECFC colony formation.

13. Pipette 4ml (3-5x10^7 MNCs) into each well of 6 well tissue culture collagen plate and place in a 37 C, 5% CO₂ humidifier incubator

Media changes

14. After 24 hours (day 1), slowly remove the media from the well with a pipette. Media is removed at a rate of 1ml per 4-5 sec. Leave some liquid in the well to prevent drying of the plate surface.

15. Slowly add 2ml of cEGM-2 to the well.

16. Slowly remove the 2ml of the media and add 4 ml cEGM-2 to the well. Return the culture plates in the incubator.

Change media daily for the first 7 days by removing and adding slowly the media. From day 8, change the media every other day.
A.5 Calcium Phosphate Mediated Transfections of HEK293T Cells

Reagents: Solutions should be prepared fresh. pH is critical for this assay. Solutions can also be made, aliquoted and stored at -20°C.

**2X HEPES Buffered Saline (HBS) pH 7.15**
50mM HEPES 0.595g
280mM NaCl 0.815g
H₂O 50 ml
pH to 7.15 Filter sterilize 0.22μm

**0.07 M Na₂HPO₄**
0.497g
H₂O 50 ml
Filter sterilize 0.22μm

**2 M CaCl₂**
14.7g
H₂O 50 ml
Filter sterilize 0.22μm

**Coating Plates with Poly L Lysine**
*optional but recommended; improves cell adhesion
Poly L Lysine (10mg/ml) aliquots are in door of RK Roxy freezer.
Dilute 10 mg/ml 1:100 in tissue culture grade water (Sigma P7890)
i.e. 0.5 ml Poly L Lysine in 49.5 ml Sigma Water
Add 1.5 ml/10 cm plate, swirl to coat
Incubate for 1.5 hours, “rocking” every 15 mins
Wash 2x with 5 ml HBSS
*best to do day of but can do the day before, wrap in foil and store in fridge

**Day -1: Plating Cells**
Aspirate off media
Trypsonize cells by washing 1-2X with 10ml HBSS, aspirate off
Add 1-1.5 ml trypsin. Let sit 1 min in incubator.
Add 10 ml DMEM/10%FBS/1XP/S/1XL-Glutamine to stop reaction. Pipette up and down to wash plate, so no clumps.
Pool cells together in conical tube. Spin 1000g for 8 mins. Remove supernatant.
Add 40 ml DMEM/10%FBS/1XP/S/1XL-Glutamine to cells to resuspend (pipette up and down to resuspend).
Place drop of 10 μl of cells to hemocytometer. Follow protocol for counting cells.
Plate 2-4x10⁶ HEK 293T cells/100mm dish in 10ml DMEM/10%FBS/1XP/S/1XL-Glutamine.

**Day 0: Transfection**
Cells should be 50-75% confluent. Batch transfections can also be performed, multiply amounts as appropriate, and add 1 ml mix/10 cm dish.
1.5 ml DNA tubes
Total DNA: 20 μg, use calf thymus to make up the difference
pβgal plasmid: 3.2 μg
pClneo-VWF, pCDNA3.1, etc.: 10 μg
* one negative control plate, 1 pβgal control plate (also WT βgal, mutant βgal etc.)
Add 20 μg DNA to 1.5 ml eppendorf tube
Add 440 μl sterile dd H₂O or 0.1X sterile TE
Add 62.5 μl 2 M CaCl₂ to tube. Flick hard to mix.

5 ml HBS Phosphate tubes
Add to 5 ml eppendorf tubes
500 μl 2X HBS
10 μl 0.07 M Na₂HPO₄

Add DNA mixture dropwise to 5ml tubes, and bubble top to bottom at least 10 times. Do one tube every 1 minute. Let sit 20 mins. Very mild milky appearance will form. Add to plates slowly (P1000), gently swirl to mix. You should see a fine “grains of salt” precipitate on the cells at the highest magnification.

Day 1: Medium Change * No more than 17 hours after transfections
Aspirate off media.
Add 8-10 ml 1X PBS 1mM EGTA to plates. Wait 2 mins, swirling periodically to remove precipitate. Aspirate off PBS. Add 10 mls of OptiMEM with 1X ITS, 1X P/S.

Day X: Medium and cell harvest
72 hours after transfections:
Pipette off media into a 15 ml conical tube, labelled. Spin 500g for 8 mins.
Collect 100 μl from each in 0.6 ml tubes for ELISA.
Pool all wild-type media (including WT βgal), all mutant media (including mutant βgal) into 50 ml conical tubes. If necessary, top WT up with HBSS to make even with mutant.
Filter in Centricon Plus-70 centrifugal filter device. Spin 3400 g, 18°C, 20 mins. Spin more if necessary.
Wash with 25 ml HBSS. Spin 3400 g, 18°C, 20 mins. Spin more if necessary.
Invert with collection cup. Spin 1000 g, 18°C, 2 mins.
Aliquot concentrated protein from collection cup into 0.6 ml tubes (4x10 μl, 100 μl for rest, estimate last tube). Store at -80°C.

In mean time:
Wash plates with 10 ml HBSS. Aspirate off HBSS.
Add 0.625 ml Lysis buffer to plates. Use rubber scraper to scrape off cells to bottom. Pipette into 1.5 ml tube (approximately 1 ml lysate).
Spin 13000 rpm, 3 mins, remove supernatant to fresh tube. Store lysate at -80°C.

β-galactosidase reporter assay using Applied Biosystem Galacto-Light Plust kit
* Buffers need to be at room temp.
Dilute Galacton-Plus 1:100 in Galacto reaction buffer diluent.
100 μl reaction buffer/luminometer tubes.
10 μl cell lysates/tube (or lysis buffer control) every 30 seconds.
Incubate 1 hour (min. 30 mins, must be same for all tubes)
Add 150 μl Accelerator II to the tubes every 30 seconds. IMMEDIATELY read in LB501 luminometer using protocol 3.
## Appendix B: Primer Sequences

### B.1 Genotyping Primers

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