

Von Willebrand Disease: From In Vivo to In Vitro Disease Models

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Abstract

Von Willebrand factor (VWF) plays an essential role in primary hemostasis and is exclusively synthesized and stored in endothelial cells and megakaryocytes. Upon vascular injury, VWF is released into the circulation where this multimeric protein is required for platelet adhesion. Defects of VWF lead to the most common inherited bleeding disorder von Willebrand disease (VWD). Three different types of VWD exist, presenting with varying degrees of bleeding tendencies.

The pathophysiology of VWD can be investigated by examining the synthesis, storage and secretion in VWF producing cells. These cells can either be primary VWF producing cells or transfected heterologous cell models. For many years transfected heterologous cells have been used successfully to elucidate many aspects of VWF synthesis. However, those cells do not fully reflect the characteristics of primary cells. Obtaining primary endothelial cells or megakaryocytes with a VWD phenotype, requires invasive procedures, such as vessel collection or a bone marrow biopsy. A more recent and promising development is the isolation of endothelial colony forming cells (ECFCs) from peripheral blood as a true-to-nature cell model. Alternatively, various animal models are available but limiting, therefore, new approaches are needed to study VWD and other bleeding disorders. A potential versatile source of endothelial cells and megakaryocytes could be induced pluripotent stem cells (iPSCs).

This review gives an overview of models that are available to study VWD and VWF and will discuss novel approaches that can be considered to improve the understanding of the structural and functional mechanisms underlying this disease.

Introduction

Von Willebrand factor

Von Willebrand factor (VWF) is a large multimeric protein that plays an essential role in primary hemostasis. It is released into the circulation upon vascular injury where it binds to collagen to mediate platelet adhesion and aggregation. It also serves as a carrier for coagulation factor VIII and has various roles in processes such as inflammation and angiogenesis.¹

VWF is produced in endothelial cells and megakaryocytes and is stored in Weibel-Palade bodies (WPBs) of endothelial cells and α -granules of megakaryocytes (and platelets).^{2,3} Endothelial cells secrete VWF constitutively in addition to regulated secretion after storage, whereas α -granules only release VWF following platelet

activation. VWF is synthesized in the endoplasmic reticulum as a pre-protein (preproVWF) consisting of several structural domains and when dimerization occurs, the protein will undergo posttranslational modifications.⁴ Moving through the Golgi system, the propeptide is cleaved and multimers will form, before being either secreted constitutively as low molecular weight multimers (LMWMs) or packed as high molecular weight multimers (HMWMs) in the α -granules in megakaryocytes or in a tubular conformation into the WPBs of endothelial cells.⁵ Platelet-secreted VWF constitutes 20% of the total VWF protein and is enriched in VWF HMWMs.^{6,7}

When WPBs fuse with the endothelial membrane, the tubulated VWF multimers uncoil, and are released as long strings into the circulation. These ultralarge VWF multimers are proteolyzed by the enzyme ADAMTS13 into smaller subunits and circulate as coiled inactive VWF units, which are activated by vascular damage. The exposure of subendothelial collagen acts a binding site for VWF, where it unfolds in adhesive strings, exposing their binding site for glycoprotein Ib α (GPIb α), leading to the adhesion, activation and subsequent aggregation of platelets.

Von Willebrand disease

Defects in VWF lead to the bleeding disorder von Willebrand disease (VWD), characterized by mucosa-associated bleeding and bleeding after trauma or surgery. There are several (sub)types of VWD that can be classified on the basis of phenotypic characteristics, caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of VWF.⁸ The severe quantitative VWF deficiency as seen in type 3 VWD is usually caused by genetic defects in the VWF gene leading to homozygous or

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compound heterozygous *VWF* null alleles. Some patients with type 1 VWD (mild quantitative *VWF* deficiency) may have heterozygous *VWF* null alleles, but usually these patients carry heterozygous missense mutations. The functional *VWF* defects in type 2 VWD are mainly caused by *VWF* missense mutations (reviewed in¹).

Research over the years has gathered a vast amount of knowledge about the pathophysiology of VWD and *VWF*, using a variety of disease models. Here, we will discuss the various systems available (Table 1) and that have been developed over the years to study VWD, both in vitro and in vivo. However, to further advance the understanding of VWD, new innovative models and approaches are needed. We will describe those new developments and touch on some applications and future directions (Fig. 1).

Cell models

Endothelial cells and megakaryocytes

Since *VWF* is being synthesized exclusively by either endothelial cells or megakaryocytes, these primary cells would be the most suitable and effective in vitro system to study VWD. However, due to elements such as location and culture issues, the use of these cells appears to be difficult.

Endothelial cells

Human umbilical vein endothelial cells (HUVECs) are frequently used to study *VWF* synthesis and secretion in the healthy situation. HUVECs from healthy individuals are readily available, as umbilical veins are resected after childbirth, and

Table 1

Summary of von Willebrand disease models

Model	Advantages	Disadvantages
Cell models	Low maintenance and costs High throughput screening assays (ie, drugs) Primary cell type Endogenous <i>VWF</i> production and storage (WPBs)	In vitro environment/system Skewed/misleading outcomes Invasive collection procedure Complicated culture circumstances ● Senescence and dedifferentiation (ECs) Lack of cells with VWD phenotype (HUVECs) Invasive collection procedure Complicated culture circumstances ● Limited life span, constant supply needed Transfection/overexpression, aberrant effects (Endogenous) WPBs absent Low isolation success rate Variability in endothelial cell characteristics
● Endothelial cells		
● Megakaryocytes/platelets	Primary cell type/components Endogenous <i>VWF</i> production and storage (α -granules)	
● Heterologous cell systems	Studying specific <i>VWF</i> variants and VWD (sub)types	
● ECFCs	Primary cell type/Endogenous cells Endogenous <i>VWF</i> production and storage (WPBs) Non-invasive collection procedure Simple isolation and culture method	
● Endothelial cell differentiation from iPSCs	VWD patient-specific cell model VWD-in-a-dish Reprogramming from most cell sources Renewable/unlimited proliferation Differentiation potential Therapeutic potential (patient-specific) Gene (editing) and/or cell therapy	Extensive process - chromosomal abnormalities Pluripotent aspect - oncogenic Retroviral gene delivery Labor intensive
3D Tissues/Organoids	Closer to physiological conditions Microvasculature Used for functional assays	In vitro environment/system Skewed/misleading outcomes Labor intensive
Animal models	Physiological environment/system	Labor intensive Expensive/expertise (technical staff) Differences between species/human (anatomic, genomic and physiological) Ethical aspects of animal welfare Expensive housing and maintenance No/low levels of <i>VWF</i> in normal platelets (dog) VWD type 1 (heterozygous) pig asymptomatic VWD type 3 pig not completely deficient in <i>VWF</i> ● Presumably posttranslational defects/instability of mRNA transcripts, no gross <i>VWF</i> gene deletions/rearrangements Factor VIII levels differences (both healthy and VWD) Limitations in sample volume collection (ie, blood, plasma) RIIS/J model low levels of <i>VWF</i> caused by rapid clearance from circulation due to mutation in <i>GALGT2</i> , not an accurate VWD model VWD type 3 no major bleeding problems
● Pig/dog	Naturally occurring VWD (mostly types 1 and 3) Prolonged bleeding times Size comparable to human body Similar anatomic and physiological characteristics to humans	
● Mouse	Naturally occurring VWD (RIIS/J) Genetically engineered VWD models ● knock-out model, VWD type 3 (and 1) ● knock-in models, VWD type 2 Resembling low <i>VWF</i> and factor VIII levels in VWD Known genetic background/profile	

VWD = von Willebrand disease, HUVECs = human umbilical vascular endothelial cells, *VWF* = von Willebrand factor, ECFCs = endothelial colony forming cells, EC = endothelial cell, iPSCs = induced pluripotent stem cells

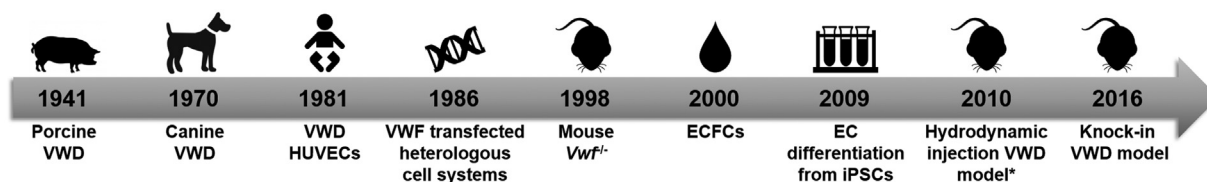


Figure 1. The development of models to study von Willebrand disease (VWD). VWD research has progressed by the discovery and generation of several models, both in vivo and in vitro. VWD is naturally occurring in other mammals, such as pig and dog, but has also been genetically engineered in mouse models. Several cell models have been developed with specific qualities. The combination of all these models and the data generated contributes to the understanding of VWD. **Vwf* knock-out mice have been used to generate this VWD model. VWD = von Willebrand disease, HUVECs = human umbilical vascular endothelial cells, VWF = von Willebrand factor, ECFCs = endothelial colony forming cells, EC = endothelial cell, iPSCs = induced pluripotent stem cells.

require simple isolation techniques.² Besides the use of these normal HUVECs for VWF focused research,^{9–11} there have been studies performed on HUVECs of newborns with inherited VWD. This has been successful for several types of VWD, looking at different aspects, such as cell morphology, VWF production, storage, secretion and activity levels, multimerization patterns, factor VIII production, and platelet binding.^{12–16}

Even though HUVECs with a VWD phenotype have contributed to our knowledge of this bleeding disorder, instances where these disease-specific cells are available are rare, especially when one is interested in studying a specific VWF defect. Therefore, patient-derived (adult) endothelial cells would be a valuable source to study all VWD (sub)types, however, due to their anatomical location, there is the need for invasive procedures to access these cells.¹⁷ Vascular cell isolation can be done by limb vein stripping, but only when patients undergo surgeries such as coronary artery bypass grafting, thus limiting the availability of tissues with a VWD phenotype.¹⁸ Furthermore, in vitro cultures primary endothelial cells readily dedifferentiate and go into senescence, which is a major drawback of these cells.¹⁹

Megakaryocytes and platelets

Like primary endothelial cells, invasive procedures (bone marrow puncture) are needed to collect megakaryocytes, the precursor cells that form and release platelets. Megakaryocytes use similar VWF processing steps to endothelial cells, whilst storing VWF in the α -granules.³ These vesicles appear to be heterogeneous in cargo and, in contrast to WPBs, not all α -granules contain VWF.²⁰

There have been few studies using megakaryocytes, either isolated from bone marrow²¹ or differentiated from circulating hematopoietic stem (CD34⁺) cells in peripheral blood, from VWD patients. A study in 2010 compared the production of platelets from differentiated megakaryocytes and isolated megakaryocytes from a patient with VWD type 2. They showed the regulatory role of VWF-GPIIb interactions in megakaryocytopoiesis and that this was aberrant leading to abnormal platelet structure and production, typical of type 2B VWD.²²

However, in-depth knowledge about the production, storage and secretion of VWF in megakaryocytes and platelets in regards to VWD is restricted. Only a few papers have been published recently and most of these reports are on platelets, reviewed in 2010, with the majority of references from before 1990.²³ The limited research concerning VWF in megakaryocytes and platelets is most likely linked to the difficulties the collection and isolation bring. Megakaryocytes represent a population of less than 0.05% of nucleated cells in the bone marrow, and therefore hampered by location as well as scarcity.²⁴ This can be

overcome by generating megakaryocytes from CD34⁺ blood progenitor cells from either peripheral or cord blood. However, these hematopoietic stem cells circulate in low numbers, which means these stem cells need to be expanded in vitro first before being efficiently differentiated into megakaryocytes, and subsequently platelets.

Furthermore, when able to isolate or generate megakaryocytes and/or platelets, large scale production might be problematic. This approach requires continuous supply of donor material due to limited expansion potential. Because there is no optimal expansion protocol for in vitro megakaryocyte and platelet production, this is currently not a suitable source to generate a reproducible, high throughput cell model for VWD.

Heterologous cell systems

In view of the limited accessibility and challenges of primary cells expressing VWF, in vitro transfection experiments in heterologous cell systems have become an attractive alternative to study the pathophysiology of VWD. Because not all cell types transfected with VWF act similarly regarding the biosynthetic pathway of VWF, this can be applied to study specific mechanisms of VWF mutations.²⁵ These systems are also an effective approach for co-transfections of alleles, making it possible to look at the effect of heterozygous variants in the VWF gene known to be causative of VWD.

Cell types used in these experiments can be divided into different groups, based on the processing and storage pathways of VWF (Table 2). Certain cells, such as COS (monkey kidney tissue) and CHO (Chinese hamster ovary epithelium) cell lines, do produce VWF upon transfection, but lack a regulated secretory pathway and are therefore hindered to store VWF. These lines have shown to be suitable for experiments specifically looking at VWF synthesis, multimerization and constitutive basal secretion.²⁶ Particularly, COS-1 and COS-7 lines have been instrumental in elucidation of mechanisms of VWF multimerization; when transfected transiently with VWF, it was revealed that in addition to the propeptide, both the D' and D3 domains are required for multimer assembly.^{27,28}

More specific, COS cells transfected with VWF harboring cysteine mutations in the D3 domain (p.C1157F or p.C1234W), displayed (and released) only the LMWMs and showed intracellular retention of these mutated forms of VWF in pre-Golgi compartments.²⁹ Co-transfections with wild-type and mutant VWF alleles in COS cells showed that both cysteine substitutions reduced the release of wild-type VWF in a dose dependent manner and these cells failed to form HMWMs. Therefore, even though these cells do not store VWF in vesicles, they are still a valuable tool to study the conformation of the

Table 2
Characteristics of heterologous VWF expression in non-endothelial cell lines

Cell line	Origin	Endogenous vesicles	Organelles containing transfected VWF	WPB-like structure	VWF organized in tubules	Reference
CHO	Chinese hamster ovary	No	No	N/A	N/A	26
COS	Monkey kidney	No	No	N/A	N/A	27
3T3	Mouse fibroblast	No	No	N/A	N/A	99
HEK293	Human embryonic kidney	No	Yes	Round and rod-shaped	Yes ^a	34
CV-1	Monkey kidney	No	Yes	Round and rod-shaped	Yes ^a	25
MDCK-II	Canine kidney	No	Yes	Round shaped	No	100
AT20	Mouse pituitary corticotrope tumour	Yes ^b	Yes	Round and rod-shaped	Yes	38
RIN-5F	Rat pancreas/islet cell tumour	Yes ^c	Yes	Round and rod-shaped	Yes ^a	38

VWD = von Willebrand disease, HUVECs = human umbilical vascular endothelial cells, VWF = von Willebrand factor, ECFCs = endothelial colony forming cells, EC = endothelial cell, iPSCs = induced pluripotent stem cells.

^a Confirmed by electron microscopy.

^b Vesicles contain solely VWF.

^c Vesicles contain both endogenous proteins and VWF; N/A = not applicable.

VWF molecules required for a normal transport pathway, maturation and constitutive secretion.

Interestingly, in some of these cell lines, heterologous VWF expression can induce the formation of de novo WPB-like storage vehicles. This group of cells can give insights in the storage and regulated secretion of VWF and this has been shown for cell lines such as HEK293 (human embryonic kidney cells) (Fig. 2) and CV-1 (monkey kidney cells).²⁵ Especially HEK293 cells have been used extensively in VWF research and are still a frequent and preferred cell line of choice. For example, this approach has led to the discovery that the non-covalent interaction between the propeptide (D1-D2 domains) and the D'-D3-A1 domains of VWF is essential for tubulation and storage of VWF into WPBs.^{30,31} These cells have also been used to look at specific known VWD causing mutations, by transfecting HEK293 cells with modulated VWF constructs.³²⁻³⁴ Our group has recently applied transfected HEK293 cells to investigate a potential small interfering RNA (siRNA) based therapeutic approach for VWD.³⁵ Here we applied siRNAs targeting common heterozygous single nucleotide variants (SNVs) to distinguish alleles harboring heterozygous VWF mutations to inhibit the production of mutated alleles. This allele-specific knockdown of the mutant allele resulted in an improved multimerization pattern, which is aberrant in VWD type 2A.

Finally, there are the cell lines that comprise a regulated secretory pathway, containing endogenous secretory granules, where the exogenous VWF is stored. AtT20 (mouse pituitary tumor) and RIN-5F (rat pancreas tumor) lines belong to this group of cells. RIN-5F cells store the transfected VWF in their vesicles containing other (endogenous) proteins, whereas AtT20 cells produce granules containing merely VWF.^{36,37} A study with AtT20 cells showed that the VWF propolypeptide is necessary for the formation of the VWF-containing granules in the AtT20 cells, contributing to the knowledge about the generation of WPBs.³⁸

Staining with VWF antibodies in these cell systems show that, even though containing VWF, most of these storage vesicles, both endogenous and exogenous, are roundly shaped. However, in combination with electron microscopy, there are rod-shaped structures detected with tubulated VWF. These resemble endothelial WPBs and are known as pseudo-WPBs.^{25,38}

Altogether, these heterologous cell systems have provided an enormous amount of knowledge on the synthesis, storage and secretion of VWF and thus the pathophysiology of VWD. Regardless of the value, these cell models have obvious limitations since only few non-endothelial cell lines can target

recombinant VWF to WPB-like organelles, restricting the applications.³⁹ This needs to be taken into consideration, as WPBs are an important aspect in the processing of VWF. Moreover, WPBs are actually dependent on the tubular assembly of VWF, which drives the formation of these storage vesicles. Because these transfection experiments show that not all cells are able to form WPBs after VWF expression, it is suggested that these cell types may lack the necessary chaperone or adaptor proteins.⁴⁰ Another obvious drawback is the possible aberrant effects overexpression of recombinant VWF with viral promoters will have upon transfection in (non-endothelial) cells.

Furthermore, VWD patients are mostly heterozygous for VWF mutations, which can be modeled in cell systems by co-transfections. It is not a guarantee that co-transfections with wild-type and mutant alleles will lead to an even ratio in expression between the alleles. However, acknowledging the shortcomings, these heterologous cell systems, especially HEK293 cells, have and continue to contribute tremendously towards VWF and VWD research.

Endothelial colony forming cells (ECFCs)

VWF transfection in HEK293 cells has been one of the most recognized model to study VWD for many years. However, in 2000 Hebbels group identified endothelial cells that can be isolated and cultured from peripheral blood, with acceptable expansion capacity in vitro.⁴¹ These cells, by consensus recently named endothelial colony forming cells (ECFCs), but also previously referred to as blood outgrowth endothelial cells (BOECs) or late outgrowth endothelial cells,⁴² represent a population of endothelial cells with a progenitor status, harboring clonal proliferation. They contain the distinct properties and features of endothelial cells such as a cobblestone morphology, expression of endothelial cell surface antigens, and the presence of endogenous WPBs. Likewise, these organelles in ECFCs store VWF, which is released after stimulation, indistinguishable from other endothelial cells⁴³ (Fig. 3). These endothelial-like characteristics, in combination with the simple venepuncture to collect, make ECFCs ideal to study VWF and VWD (and other bleeding/vascular disorders) together with possible therapeutic assessment. We and others have described the use of ECFCs as a feasible cell model to study the effect of VWF mutations on the synthesis, storage, secretion, and string formation of VWF, but also on abnormal cell proliferation, migration, and increased Ang-2 secretion.⁴⁴⁻⁵¹

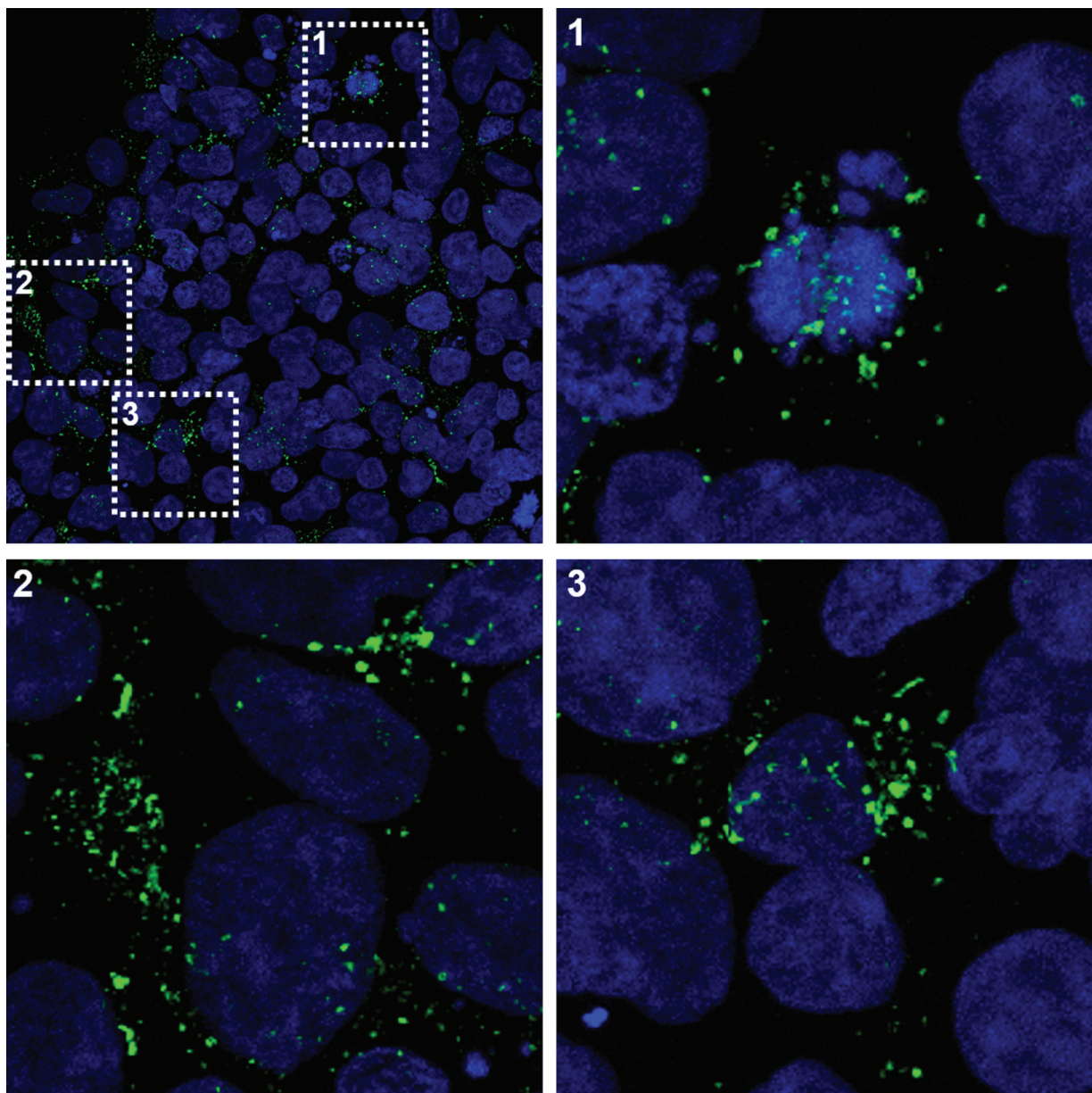


Figure 2. Heterologous cell system transfection with VWF. Upon transfection, human embryonic kidney (HEK293) cells form VWF storage vesicles, so-called pseudo-WPBs that resemble WPBs in endothelial cells. WPB-like structures in the HEK293 cells vary from round to elongated. Confocal images of fixed HEK293 cells, stained for VWF (green) and nuclei (blue, DAPI). Panels 1, 2, and 3 show the zoomed in sections, which are outlined in the enlarged image.

VWF and angiogenesis

VWF has been described as a negative regulator of angiogenesis, and ECFCs have been applied to investigate the pathogenesis of VWD and angiodysplasia.⁵¹ Starke et al found in HUVECs that knock-down of VWF expression leads to an increase in angiogenesis, which was also shown with ECFCs isolated from patients with VWD, showing enhanced angiogenesis.⁵¹ Our group applied ECFCs to investigate the potential pathogenic effect of specific VWF mutations on angiogenesis.⁴⁶ ECFCs, isolated from a type 3 and type 2B patient, displayed increased migratory velocity, and in the majority of VWD ECFCs (8 out of 10) directional migration was impaired. This shows that ECFCs directly isolated from VWD patients displayed the disease phenotype and serve as a good source to study VWF and VWD.^{45,49,51}

Recently, Selvam et al used ECFCs derived from healthy donors to investigate and determine the normal range of angiogenesis. Subsequently, this was compared to angiogenesis levels in ECFCs from VWD patients (all types and subtypes).⁴⁹ ECFCs were assessed for VWF and Ang-2 gene expression, secretion and storage and characterized for cellular proliferation, matrix protein adhesion, migration, and tube formation. Overall, the results indicated that there is great variability in the angiogenic properties of both control and VWD ECFCs.

Challenges and opportunities of ECFCs

Even though VWD ECFCs reflect the pathogenic effects of VWF mutations, significant heterogeneity was observed among individual VWD phenotypes. For ECFCs to be a more robust cell model for use in disease studies, the extent of variability of

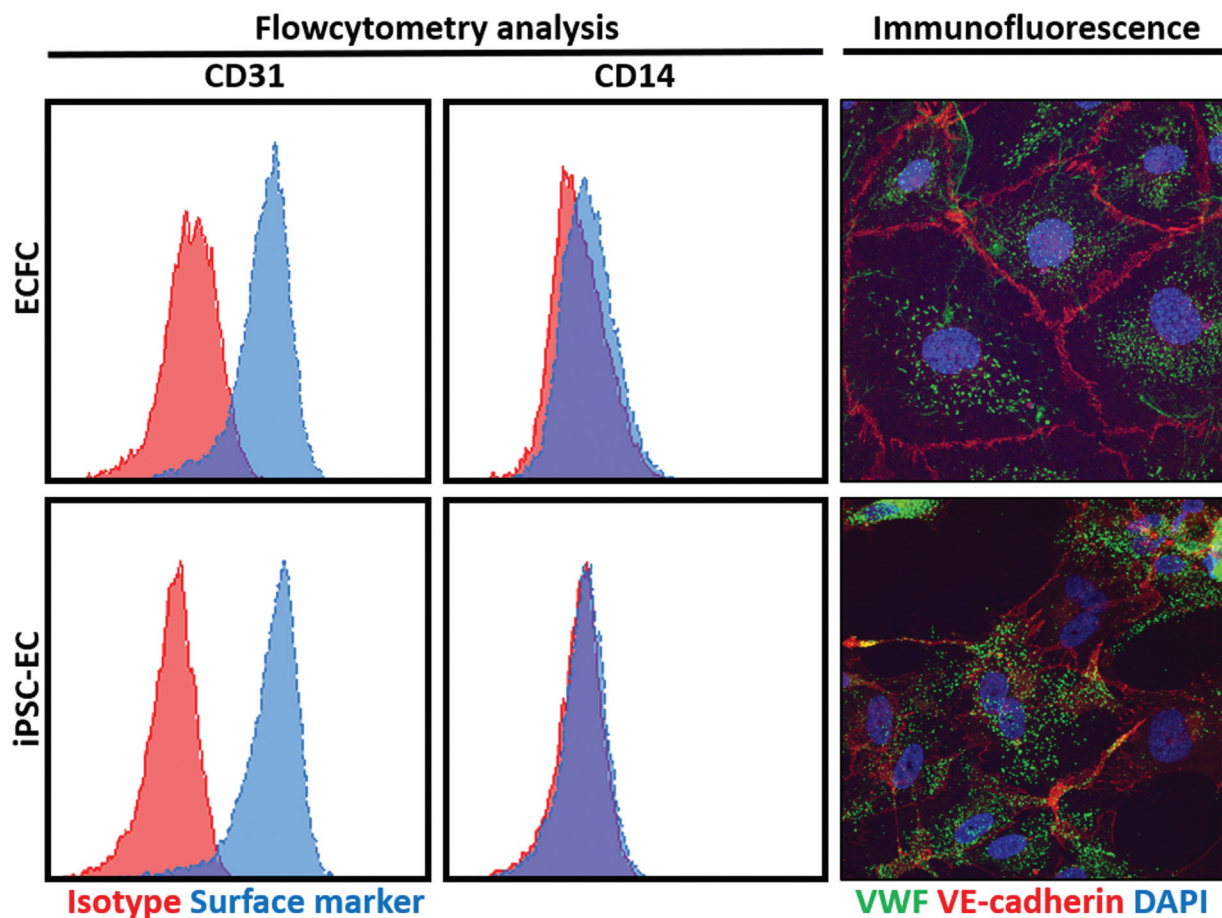


Figure 3. Endothelial cell (surface) marker expression for ECFCs and endothelial cells differentiated from iPSCs (EC-iPSCs). When performing flowcytometry analysis, both endothelial cell sources (ECFCs, top panels; iPSC-ECs, bottom panels) appear positive for endothelial cell surface marker (CD31, platelet endothelial cell adhesion molecule 1 (PECAM-1)) and negative for the monocyte marker (CD14). Cells stained positive for endothelial proteins VWF (green stain) and VE-cadherin (red stain) in immunofluorescent staining, blue stain for nuclei (DAPI). Note that the endothelial cell differentiation protocol includes a CD31⁺ isolation step.

cellular phenotype needs to be understood in more detail. We recently studied a cohort of separate ECFC clones derived from six healthy donors and observed large variations between ECFCs. Not only from different donors, but also amongst clones from individual donors. This variability both in morphology and proliferative potential is being investigated and the origin and age of the ECFC might be involved. However, this should be taken into account when using ECFCs as a cell model.⁵²

There is also the need for standardization of protocols to be able to compare findings across laboratories.⁵³ ECFC isolation and culture methods vary between research groups, and this might affect the cells proliferative capacity and possibly phenotype.⁵⁰ An additional challenge is the rather low success rate of ECFC isolations from peripheral blood (between 40% and 60%). An explanation for these low scores could be the general scarcity of ECFCs in peripheral blood. Kolbe et al showed that ECFCs represent a small fraction of mononuclear cells and their numbers vary considerably between donors⁵⁴ which might also be influenced by pathological conditions, affecting the number of ECFCs circulating in the blood.⁵⁵

In conclusion, there are some challenges and unknowns involved in the isolation and usage of ECFCs such as success rate, low numbers of circulating ECFCs, and whether this is person specific or disease related. Nevertheless, by the use of these cells in

in vitro studies and disease modeling, ECFCs show potential in applications such as drug screening, bioengineering approaches, as well as cell and gene therapies.⁵⁶ ECFCs have made it possible to profile the synthesis and storage of VWF in endothelial cells from individual VWD patients^{44,45} and these results could be of use in the choice of patient-specific therapeutic approaches. This is not only applicable to VWD but also relevant for other diseases that have been studied using ECFCs, such as sickle cell anemia, myeloproliferative neoplasms, hereditary hemorrhagic telangiectasia, and venous thromboembolic disease (reviewed in⁵⁷). In addition to these forward developments, ECFCs have also shown to be a source to reprogram into iPSCs.⁵⁸

Induced pluripotent stem cells (iPSCs)

Reprogramming and differentiation of somatic cells can overcome the lack of disease-specific cells and tissues in disorders like VWD. The generation of iPSCs from human fibroblasts was first achieved in 2007,⁵⁹ and several different cell types, such as peripheral blood mononuclear cells (PBMCs) and keratinocytes, have since been reprogrammed.^{60,61} Nowadays, other cell types, with simple non-invasive collection procedures, have also been reprogrammed, such as cells from hair follicles, urine, and dental pulp from milk teeth.^{62,63} Generated iPSCs are capable of self-

renewal and have the potential to differentiate into almost any cell type, and can thereby create a disease in a dish model. This is especially relevant for cells of the internal organs for which biopsies are not routinely available, such as megakaryocytes and endothelial cells. Acquiring these cells through patient-specific iPSC differentiation can enable better insights into VWD and other bleeding disorders, in combination with additional aims such as (high-throughput) drug screening, development and cell therapy.

ECFCs as reprogramming source

ECFCs have been used as a reprogramming source, with various reprogramming efficiency and results depending on the method to deliver the reprogramming factors^{58,62,64} compared to fibroblast or PBMCs. Regardless, these ECFC-derived iPSC colonies (ECFC-iPSCs) express embryonic stem cell markers and show differentiation potential in all three germ layers in vitro, similar to other somatic cell sources. Furthermore, it has been shown that reprogrammed ECFCs show slightly lower rates of acquired chromosomal abnormalities (compared to the parental cells) in contrast to the other somatic cells tested.⁵⁸ However, to obtain the required cell number, ECFCs need a longer culture period than most other somatic cells such as fibroblast, which could induce genetic changes.

Unfortunately, limited studies have been published where reprogrammed ECFCs have been used in differentiation assays. Orlova et al differentiated ECFC-iPSCs into endothelial cells and showed that these cells expressed similar levels of endothelial-specific surface antigens and perform similar in functional assays compared to endothelial cells from either fibroblast-iPSCs and/or differentiated from human embryonic stem cells.⁶⁵

Furthermore, there are reports that endothelial cell-derived iPSCs have a higher tendency to differentiate to endothelial cells, compared to fibroblast-iPSCs.⁶⁶ This suggests that ECFCs as an iPSC source might be beneficial when differentiation into the endothelial lineage is required, with regards to epigenetic memory and has been reported for a variety of cell types, which predispose iPSCs to favor differentiation towards their cell of origin (reviewed in⁶⁷). This is an important trait when considering the use of iPSCs in disease modeling, drug screening and future (autologous) cell transplantation therapies.

The fact that iPSCs are self-proliferative, in contrast to ECFCs which will go into senescence and/or lose the endothelial morphology after multiple passages, ECFCs as a somatic source could therefore have an advantage and preference when differentiating into endothelial cells for further applications and assays.

Differentiation of iPSCs

There are several protocols and approaches to differentiate human iPSCs from different somatic cell sources into endothelial cells, such as three-dimensional embryoid bodies or co-cultures with stromal cells to induce endothelial cell lineage differentiation.⁶⁸ However, most groups use feeder-free monolayer differentiation on matrix coated culture plates, such as Matrigel or fibronectin, using specific culture mediums with sequentially added recombinant growth factors.^{69,70} The majority of this protocol can be divided into a mesoderm and endothelial differentiation phase. Two populations are generated after the first differentiation round, based on CD31 positive and negative populations. The CD31⁺ cells are further differentiated into endothelial cells, while the CD31⁻ group can be differentiated into

pericytes for co-culture purposes. This will generate substantial numbers of both cell types that can be derived in only 2 to 3 weeks. These cells show the typical endothelial cell-like morphology and express endothelial markers such as vascular endothelial (VE-) cadherin and VWF, and have been used in functional studies (Fig. 3).⁷¹

Regardless, there are some concerns about the efficiency and maturity of iPSC-derived endothelial cells using these protocols. Most endothelial differentiation protocols that have been developed to date generate low endothelial cell yields. Like primary endothelial cells in vitro, these iPSC-derived endothelial cells have restricted proliferative potential and either undergo senescence or endothelium-to-mesenchymal transition after multiple passages. Because these are differentiated in vitro, they are not exposed to impacts from the (tissue) specific environment, such as blood flow and pressure that play roles in endothelial cell differentiation in vivo. This might explain the intermediate (heterogenous) phenotype seen in the population of differentiated endothelial cells; neither committed fully to an arterial or venous fate.⁷² Evidence has also emerged that endothelial cells reprogrammed from iPSCs possess slightly different gene expression and epigenetic patterns compared to primary endothelial cells.⁷³ However, research into the maturation of these differentiated endothelial cells into more specific types of endothelium (arterial, venous, lymphatic) through the manipulation of culture media is ongoing. To mimic these in vivo environments more closely, micro-fluidic 3D systems, like organ-on-a-chip, have been developed.

To generate platelets, pluripotent cells need to be differentiated into megakaryocytes first and this can be done through CD34⁺ progenitor cells derived from sources such as peripheral or umbilical blood, bone marrow, or iPSCs.⁷⁴⁻⁷⁶ Most of these sources have limitations for large scale regeneration of megakaryocytes and platelets and require a continuous supply of donors. However, iPSCs show capacity to serve as a renewable and unlimited cell source that can be expanded in culture and differentiated into megakaryocytes and platelets. The attractiveness of this system is that the same patient-specific iPSCs batch can be used to differentiate into a variety of cell types of interest. With regards to VWD, besides endothelial cell differentiation, these iPSCs can also be used to generate megakaryocytes and subsequently platelets.

Recently, megakaryocytes have been differentiated from CD34⁺ cells from patients with Roifman syndrome, which is a rare congenital disorder characterized by growth retardation, cognitive delay and in some patients thrombocytopenia.⁷⁷ The patient-specific CD34⁺ cells showed defects in megakaryocyte differentiation, with inadequate generation of proplatelets which is a characteristic of this syndrome. This indicates that a differentiation approach from iPSCs into megakaryocytes can be used for disease modeling.

By reprogramming the same somatic cell source and using identical protocols, the differentiation of cells into endothelial cells, might reduce the variation seen in ECFCs and therefore possibly lead to a model for vascular disease.

Potential drawbacks of iPSCs

Even though human iPSCs have emerged as a promising candidate for vascular regeneration medicine,^{70,78} there are still some issues concerning the use of pluripotent cells as a source for cell therapies. Things like low reprogramming efficiency, genetic instability, in vivo functionality and the risk of teratoma

formation from undifferentiated iPSCs are serious matters that are currently under investigation and need to be resolved before further application into the clinic. Research is developing rapidly, to improve the differentiation efficiency of endothelial cells and megakaryocytes from iPSCs by new signaling pathways and novel culture conditions. A deeper understanding of the development of the endothelial cell lineage is required for differentiated cells to become a robust model for vascular diseases and the potential to the safe use of these cells as a patient-specific cell therapy in future.

Animal models

For some research questions cell models are not applicable and therefore animal models are required. Naturally, occurring VWD has been described amongst several animal species, especially mammals, such as dogs and pigs.^{79–82} These animal models have contributed heavily to the understanding of various aspects of VWF and VWD that cannot be addressed through in vitro approaches.

Porcine VWD

VWD in an animal was first reported in 1941, when a bleeding disorder was described in swine by Hogan et al,⁸⁰ making this the oldest known animal model of a human bleeding disorder. Later on, it was revealed that the disease was transmitted as an autosomal recessive trait and identified as a VWD type 3 model.⁸³ The pig is a good model of hemorrhagic disorders since its clotting and platelet characteristics resemble those of humans.⁸⁴ However, there are some important differences when comparing this to human VWD. Homozygous pigs are not totally deficient in VWF, and low but significant amounts of VWF antigen can be detected both in platelets and in endothelial cells from the pulmonary artery and inferior vena cava.⁸⁵ Another restriction of this model is its size and housing cost of the animals and nowadays, research has been restricted with this animal model due to new and better alternatives.

Canine VWD

High costs also apply to the canine model of VWD. The first dog reported with this bleeding disorder was in 1970 in a German Shepherd family⁸⁶ and over the years many other dog breeds have been identified with all three subtypes of VWD. However, canine VWD is a very heterogeneous group of diseases with different subtypes and modes of inheritance⁸⁷ and limited research has been done using dogs as a VWD model in contrast to the porcine model. In 2006, De Meyer et al transduced ECFCs, isolated from type 3 VWD dogs, with VWF, showing that gene therapy of type 3 VWD is feasible.⁸⁸

Murine VWD

For murine VWD, both naturally and genetically engineered VWF knockout mouse models are available.^{89–91} The naturally occurring RIIIS/J mouse, is characterized by typical VWD features such as a prolonged bleeding time and low levels of plasma factor VIII and VWF, however, presenting with a normal VWF multimer distribution.⁹¹ Genetic linkage did not show a correlation between VWF antigen level and genotype, suggesting that VWD in this mouse strain is caused by a defect at a novel genetic locus, distinct from the murine VWF gene.⁹²

By breeding the genetically engineered VWF knockout mouse, a model has been designed that mimics VWF deficiency (type 1 and 3). The generated VWF knockout mouse has been used to generate type 2B VWD models. This has been a great advantage in VWD research as naturally occurring VWD type 2 in animals is very rare. VWD type 2B in humans is characterized by gain-of-function mutations leading to increased affinity for its platelet-receptor, GPIIb α . This phenotype has first been replicated in mice by hydrodynamic injection of mutant VWF constructs into the *Vwf*^{f/-} mice.^{93,94} Even though mimicking VWD type 2B, showing enhanced platelet binding but normal multimerization, the mutant VWF proteins with this approach are (exclusively) expressed by the hepatocytes and therefore only present in the plasma, and not in endothelial cells or platelets. More recently, 2 groups have successfully engineered a VWD type 2B knock-in model in mice.^{95,96} Adam et al introduced the VWD causing mutation p.V1316M into the murine *Vwf* locus and these mice display human VWD-type 2B-like characteristics, such as macrothrombocytopenia, deficiency of HMWVWF, reduction of active VWF levels, circulating platelet-aggregates and a severe bleeding tendency.⁹⁵

Mouse models have advantages such as characterized genetic backgrounds, optimized genome editing approaches, and low cost and maintenance of housing, making this an attractive model to study disease. However, many disorders lack a suitable animal model or are not feasible to use for these experiments. Additionally, there are differences between various species and care needs to be taken when extrapolating results obtained in these studies to humans. Nevertheless, animal models will most likely remain an important model and a requirement in many aspects of disease and drug modeling for the coming years.

Future prospects

Altogether, the collection of these disease models, both in vitro and in vivo, has led to a major part of the knowledge on VWF and the mechanisms underlying VWD (Fig. 1). The generation and differentiation of iPSCs, along with the availability of the human genome and genome editing tools has transformed disease research immensely, leading to the development of new strategies to treat or study vascular diseases.⁹⁷ This approach has already been applied in combination with human iPSCs to correct defective genotypes in vitro for several diseases and conditions, such as hemophilia A.⁹⁸ Studies like these are good examples of combining patient-specific vascular regenerative medicine (in vitro) approaches and animal models. These developments, in combination with three dimensional models, such as organ-on-a-chip and drug screens, could be at the basis to generate novel (cell) therapies for VWD.

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