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Original Research

Characterization of a Porcine Model for Von Willebrand Disease Type 1 and 3 Regarding Expression of Angiogenic Mediators in the Nonpregnant Female Reproductive Tract

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Von Willebrand disease (VWD), a blood coagulation disorder, is also known to cause angiodysplasia. Hitherto, no animal model has been found with angiodysplasia that can be studied in vivo. In addition, VWD patients tend to have a higher incidence of miscarriages for reasons unknown. Thus, we aimed to examine the influence of von Willebrand factor (VWF) on the female reproductive tract histology and the expression and distribution of angiogenic factors in a porcine model for VWD types 1 and 3. The disease-causing tandem duplication within the *VWF* gene occurred naturally in these pigs, making them a rare and valuable model. Reproductive organs of 6 animals (2 of each mutant genotype and 2 wildtype (WT) animals) were harvested. Genotype plus phenotype were confirmed. Several angiogenic factors were chosen for possible connections to VWF and analyzed alongside VWF by immunohistochemistry and quantitative gene expression studies. VWD type 3 animals showed angiodysplasia in the uterus and shifting of integrin $\alpha_v\beta_3$ from the apical membrane of uterine epithelium to the cytoplasm accompanied by increased vascular endothelial growth factor (*VEGF)* expression. Varying staining patterns for angiopoietin (Ang)-2 were observed among the genotypes. As compared with WT, the ovaries of the VWD type 3 animals showed decreased gene expression of *ANG2* and increased gene expression of *TIE (*tyrosine kinase with immunoglobulin and epidermal growth factor homology domains*) 2*, with some differences in the ANG/TIE-system among the mutant genotypes. In conclusion, severely reduced VWF seems to evoke angiodysplasia in the porcine uterus. Varying distribution and expression of angiogenic factors suggest that this large animal model is promising for investigation of influence of VWF on angiogenesis in larger groups.

Abbreviations: AM, apical membrane; Ang, angiopoietin; BSA, bovine serum albumin; CD31, platelet and endothelial cell adhesion molecule 1; C_T, threshold cycle; ECs, endothelial cells; GE, glandular epithelial cells; HE, hematoxylin-eosin; IHC, immunohistochemistry; NGS, normal goat serum; OE, oviduct epithelial cells; PROCR, protein c receptor; qPCR, quantitative real time PCR; SMA, smooth muscle actin; TEC, Tris/EDTA/Citrate; Tie, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains; UE, uterine epithelial cells; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; VSMC, vascular smooth muscle cells; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; WPBs, Weibel-palade bodies; WT, wildtype

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With a prevalence of 1% to 2%[2] VWD is the most common inherited coagulation disorder. VWD type 1 usually follows an autosomal-dominant inheritance, while the inheritance for the most severe VWD type 3 is autosomal-recessive.[21] Individuals heterozygous for VWD type 3 are in some instances also classified as VWD type 1, as both phenotypes can be indistinguishable.[35] Since VWF plays a main role in hemostasis, an increased bleeding tendency is the primary symptom in VWD patients. This affects the female reproduction system in various ways and may cause symptoms as menorrhagia, spontaneous vaginal bleeding during pregnancy, or excessive bleeding during and after delivery.[11,12] In addition, women affected by VWD show a trend toward higher rates of miscarriages,[13,32] whereas the underlying pathomechanisms are unknown so far.

In contrast to the reproductive tract, the impact of VWD on the gastrointestinal tract has been studied in more detail. 4.5% of VWD type 3 patients develop a phenotype characterized by impaired angiogenesis[8] leading to angiodysplastic lesions in the gastrointestinal tract or the nail fold[14] and angiodysplasia has been proven to be causal for gastrointestinal bleeding in up to 20% of VWD patients.[29] As the reasons for development of angiodysplasia in these patients remain elusive so far, treatment options are still insufficient.[7] Although previous in vitro studies and studies in knockout mice revealed first evidence

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for pathways possibly connecting VWF and angiogenesis, angiodysplasia was described in none of the models.[30,34,40] In those studies, VWF-deficient endothelial cells (ECs) showed enhanced angiogenic properties due to increased VEGFR (vascular endothelial growth factor receptor)-2 signaling. A model of pathways connecting VWF with angiogenesis and vessel maturation via Ang-2, the receptor tyrosine kinase Tie-2, integrin $\alpha_V\beta_3$ and VEGFR-2 was developed,[24] but there might be more angiogenic factors not yet studied in this context (Figure 1). For example, the still not completely elucidated ANG/TIE-system (also including Ang-1 and Tie-1)[12] is essential for angiogenesis, vessel maturation and remodelling[1] and as VEGFR-2 shows a connection to VWF, its strong ligand and major regulator of angiogenesis VEGF,[31] may also be considered to be influenced by VWF. However, a better understanding of the mechanisms leading to the vascular malformations requires a model in which the connections of VWD and angiodysplasia can be studied in vivo in a systemic approach.

Considering these previous findings as well as available data on miscarriages in VWD patients, we hypothesize that impaired angiogenesis might be involved in the fertility of female VWD patients. This might influence (I) ovulation and development of sufficient corpora haemorrhagica as well as corpora lutea, (II) the process of implantation and/or (III) angiogenic processes during placentation leading to impaired nutrition of the fetuses and (IV) the development of a receptive endometrium. The porcine model is more suitable for our purposes than a murine model, as embryos in VWD-affected pigs show an increased intrauterine embryo mortality,[5] while the litter size is normal in VWD-affected mice.[3] The intrauterine mortality, as well as difficulties in breeding of VWD type 3 sows, hampers the provision of large animal numbers for conducting animal studies. Nonetheless, we were able to characterize 2 animals of each genotype in this study to determine if this model is suitable to investigate the influence of VWF on the female reproductive tract in vivo at a sufficient scale in a large animal model. We demonstrate altered blood vessel conformation in the uterus of VWD type 3 animals and varying gene expression and protein distribution of several angiogenic factors among the genotypes.

Materials and Methods

Sampling. Samples were provided by the Thrombosis and Atherosclerosis Unit of the Blood and Vessels Institute, Hôpital Lariboisière, Paris, France. Tissues were harvested from 6 female pigs (*Sus scrofa*) aged between 7 and 15 mo, carrying a natural nonsense mutation of the *VWF* gene.[15] These pigs were part of a colony originating from Mayo Clinic (Rochester, MN, USA by collaboration with EJW Bowie). The colony is a crossbreed of originally Poland China breed crossed with Yorkshire-Hampshire as well as Meishan pigs to downsize the animals. All animal experiments were approved by the French Ethical Committee for Animal Experimentation as well as the French Ministry of Research, Department of Animal Experimentation and Project Authorization (approval number: 0130001) and were performed in accordance with relevant guidelines and regulations. Of the animals examined for this study, 2 were heterozygous for the *VWF* mutation (corresponding to VWD type 1 (V1)), 2 were homozygous (corresponding to VWD type 3 (V3)), and 2 were wildtype (WT) individuals, originating from the same colony and serving as controls. The animals were housed free-ranged in groups and fed with a commercial pig chow adapted to their weight and age. The colony is proven to be free of classic swine fever, Aujeszky disease, and porcine reproductive and respiratory syndrome on a regular basis once a year and France is stated to be free of foot-and-mouth disease. The samples of the 6 animals are referenced by a combination of the respective genotype (WT, V1, V3) and a number indicating the respective pig (1, 2). Breeding of animals V3-1 and WT-1 had been attempted unsuccessfully once. Thus, all pigs were nulliparous. Samples of both uterus horns, both ovaries and both oviducts (ampulla and isthmus) were taken immediately after euthanasia. To minimize the influence of the female cycle, all pigs were euthanized in late estrus, as identified by behavioral assessment of the animals (toleration reflex) to detect if the sows were in heat. All sows were euthanized in late heat, which means shortly after ovulation. Euthanasia was conducted using an overdose of pentobarbital administered intravenously (100 mL/kg) followed by exsanguination. No experiments or manipulations apart from breeding and euthanasia were implemented.

Validation of phenotypes and genotypes. Blood was collected in 3.2% citrate, and plasma was prepared in a standard manner. VWF antigen (VWF:Ag) levels were measured using the STA-Liatest vWF:Ag test kit (Diagnostica Stago S.A.S, Asnières sur Seine, France) adapted for usage in pig. DNA was isolated using the Maxwell16 system and the 16 LEV Blood DNA Kit (Promega GmbH, Mannheim, Germany). The genotype of each pig was determined using quantitative real time PCR (qPCR) and the $\Delta\Delta C_T$ method by comparing the amount of PCR product of the mutant *VWF* gene to 12 VWD type 3 pigs of the same colony previously published by our group.[15]

Tissue processing for histologic examinations. Tissue samples were fixed in neutral buffered formalin, dehydrated, and embedded in paraffin wax. For hematoxylin-eosin (HE), immunohistochemical and immunofluorescent staining sections of 3 to 5 µm thickness were cut and transferred to slides.

HE-staining. HE-staining was conducted according to standard procedures. The histologic evaluation of the sections was done by light microscopy using the Axioskop (Zeiss, Germany) with the camera DP 70 and the corresponding software (Olympus Europa GmbH, Hamburg, Germany).

Immunohistochemistry (IHC). The angiogenic factors Ang-1 and -2, Tie-1 and -2, integrin $\alpha_V\beta_3$ (consisting of the 2 components integrin α_V and integrin β_3), VEGF and VEGFR-2 as well as VWF were analyzed in tissues from the porcine uterus, oviduct, and ovaries, respectively. Sections were treated according to standard procedures (for specifications see Table 1). All antibodies were polyclonal. The incubation with primary antibody diluted in phosphate buffer solution + 1% bovine serum albumin was done overnight at 4 °C in a humidity chamber. DAB (3,3′-diaminobenzidine) was added for visualization. All sections were counterstained with Delafield's hematoxylin. As negative controls, the primary antibody was replaced by pure dilution medium and IgG controls were implemented for all antibodies, respectively. Assessment of the staining intensity and patterns was done semiquantitatively by blinded light microscopic analysis. At least 2 sections per animal and tissue were stained and evaluated by cell types relevant for the different tissues. Staining intensity was scored 0 (= none), 0.5 (= very weak), 1 (= weak), 2 (= moderate) and 3 (= strong) for each cell type. To evaluate uterine and oviduct tissues, randomly chosen areas were assessed on each slide until every cell type included in the analysis (uterine epithelial cells (UE), glandular epithelial cells (GE), oviduct epithelial cells (OE), ECs and vascular smooth muscle cells (VSMC)) had been scored 5 times at different positions on the slides. To evaluate ovary tissues, 2 follicles of each type (primary, secondary, and tertiary follicles) and 2 corpora lutea were randomly chosen (if present) and granulosa

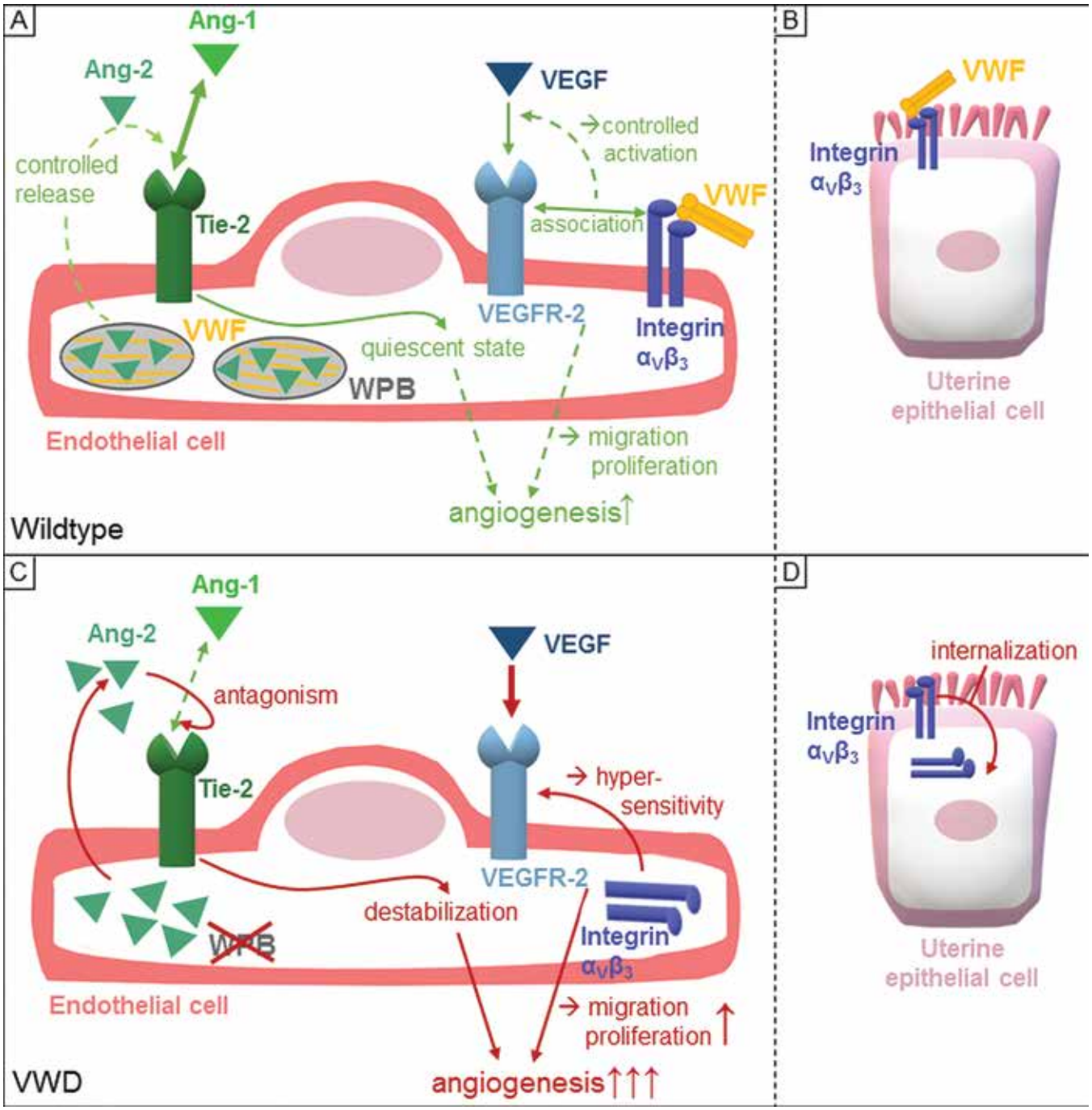

Figure 1. Schematic illustration of hypothesized influence of VWD on angiogenesis. The influence of VWD on angiogenesis is illustrated according to our own results combined with those of Augustin and colleagues,[1] Savant and colleagues,[27] and Randi and Laffan.[25] Green arrows indicate pathways under physiologic conditions; red arrows indicate influence on angiogenesis with missing von Willebrand factor (VWF). (A) Wildtype: In endothelial cells (ECs), VWF binds to integrin $\alpha_V\beta_3$ stabilizing it on the cell surface, facilitating association with vascular endothelial growth factor receptor (VEGFR-2) and thereby controlled proangiogenic action of vascular endothelial growth factor (VEGF). Moreover, VWF is required to build Weibel-Palade bodies (WPB) and regulates their synthesis. Angiopoietin (Ang)-2 is stored within these organelles inside of the ECs and negatively regulates its synthesis. Ang-1/Tie-2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2) signaling keeps ECs in a quiescent state. (B) Wildtype: Integrin $\alpha_V\beta_3$ is also stabilized on the apical cell membrane of uterine epithelial cells. (C) VWD: With missing VWF, integrin $\alpha_V\beta_3$ is internalized into the ECs leading to hypersensitivity of VEGFR-2 for VEGF and subsequently to an increased migration and proliferation of ECs. Ang-2, usually released from WPBs in a controlled manner, is released continuously if WPBs are missing due to the lack of VWF. It subsequently antagonizes Ang-1/Tie-2 signaling leading to destabilization of ECs promoting angiogenesis. (D) VWD: Lack of VWF leads to internalization of integrin $\alpha_V\beta_3$ into the cytoplasm of uterine epithelial cells as well.

and theca cells or luteal cells were scored. In addition, 5 blood vessels were randomly chosen to score their ECs and VSMC. The evaluation was performed dually and the mean of the staining intensity for each cell type of the different genotypes was calculated.

Immunofluorescence. Immunofluorescence was performed for uterine tissue sections of all animals. For immunofluorescent costaining for VWF, smooth muscle actin (SMA) and DAPI, slides were dewaxed according to standard procedures. Antigen retrieval with tris/EDTA/citrate (TEC) buffer and blocking was

Table 1. Protocols and materials used for IHC.

Antibody/ Product code	Manufacturer	Host species	Antigen retrieval	Blocking agent	Dilution	Detection
VWF/ Nr. A0082	DAKO, Santa Clara, USA	Rabbit	Tris/EDTA/ Citrate (TEC) buffer (pH 7.8, 30 min at 96–99 °C)	Normal goat serum (NGS)	1:3000	EnVision+System-HRP detection kit system (DAKO)
Integrin $\alpha_v\beta_3$/ 251672	Abbiotec, San Diego, USA	Rabbit	Citrate buffer (0.1M, pH 6, 20 min at 96–99 °C)	NGS	1:100	EnVision+System-HRP detection kit system (DAKO)
VEGF/ sc-152	Santa Cruz Bio- technology, Dallas, USA	Rabbit	TEC buffer (pH 7.8, 30 min at 96–99 °C)	NGS	1:1000	EnVision+System-HRP detection kit system (DAKO)
VEGFR-2/ ab45010	Abcam, Cambridge, UK	Rabbit	Uterus and oviduct: EDTA buffer (pH 9, 15 min at 96–99 °C); Ovary: citrate buffer (0.1M, pH 6, 20 min at 96–99 °C)	NGS	1:50	EnVision+System-HRP detection kit system (DAKO)
Ang-1/ sc-6319	Santa Cruz Biotechnology	Goat	EDTA buffer (pH 9, 15 min at 96–99 °C)	Bovine serum albumin	1:350	SUPERVision 2 HRP-polymer system (DCS, Hamburg, Germany)
Ang-2/ paa009po01	Cloud-Clone, Katy, USA	Rabbit	Citrate buffer (0.1M, pH 6) microwaved (800W, 15 min)	NGS	1:250	EnVision+System-HRP detection kit system (DAKO)
Tie-1/ ab64477	Abcam	Rabbit	Citrate buffer (0.1M, pH 6, 20 min at 96-99 °C)	NGS	1:250	EnVision+System-HRP detection kit system (DAKO)
Tie-2/ sc-9026	Santa Cruz Biotechnology	Rabbit	Citrate buffer (0.1M, pH 6, 20 min at 96–99 °C)	NGS	1:250	EnVision+System-HRP detection kit system (DAKO)

done as described for IHC. Slides were incubated with primary antibodies against VWF (as used for IHC) and SMA (monoclonal mouse antihuman smooth muscle actin clone 1A4, Dako, Santa Clara, USA, diluted 1:500) overnight. As secondary antibodies goat antirabbit IgG Alexa Fluor 546 and goat antimouse IgG Alexa Fluor 488 (Invitrogen, Waltham) were used, and cell nuclei were visualized with DAPI by fluorescent microscopy using the Axiovert 200M with the according software (Zeiss, Germany).

Gene expression. Expression of genes specified in Table 2 was quantified using qPCR. One representative sample of each reproductive organ comprising all cell types described in the IHC analyses of each pig was placed in RNA*later* RNA-stabilizing solution immediately after harvesting. RNA was isolated using the Maxwell16 system and the Tissue LEV Total RNA Purification Kit (Promega GmbH). Transcription of RNA to cDNA was performed using the GoScript Reverse Transcription System (Promega GmbH). Procedures were performed as suggested in the manufacturer's manual. Specific primers (Table 2) were designed based on the sequence of each chosen gene.[20] For *ITGB3*, different primers were designed or chosen from literature.[4] The expression of all genes analyzed in this study mainly takes place in blood vessel correlated cells, which results in mRNA amounts of these genes influenced by the number of vessels in each sample. Thus, standard housekeeping genes, which are uniformly expressed in all tissue types, were not suitable for normalization of the mean threshold cycle C_T values in this study. Therefore, we chose the endothelial specific genes *PROCR* (protein c receptor) and *CD31* (platelet and endothelial cell adhesion molecule 1) as housekeeping genes. All primers were obtained from Eurofins Genomics (Louisville). For performing qPCR, a LightCycler 2.0 (Roche Pharma AG, Reinach, Switzerland) was used. Results were calculated from the (C_T) for each sample. All assays were performed in duplicates. After ensuring a deviation of both values below 5%, the mean C_T value was calculated.

Data evaluation. The means for both housekeeping genes were used to calculate the relative expression levels by the $\Delta\Delta C_T$ method.[18] The relative changes of gene expression were calculated for each individual animal, using the mean of the WT individuals as calibrator leading to the $\Delta\Delta C_T$ values and the x-fold changes ($2^{-\Delta\Delta^{CT}}$) compared with the WT mean. To compare the gene expression levels of WT animals with VWD animals, the differences between expression levels relative to the WT mean of 100% (or 1-fold expression) of both animals were calculated for each tissue and each gene. Expression levels of VWD animals were compared only if the difference of the x-fold expression between both WT animals was less than 50%. Since effects of components of the ANG/TIE-system notably rely on their ratio to each other,[1] the variation of relative gene expression ratios among the genes of the ANG/TIE-system was determined for each individual. Therefore, the $\Delta\Delta C_T$ was calculated between the respective genes for each individual sample, resulting in the x-fold change. Means and standard errors of the means (SEM) of IHC and qPCR data were generated using SAS Enterprise Guide, Version 7.1 (SAS Institute, Cary).

Results

Characterization of wildtype animals WT-1 and WT-2. *VWF*. The sows were aged 15 (WT-1) and 13 (WT-2) mo at the time of euthanasia and had VWF:Ag Plasma levels of 103% and 112%, respectively. Tissue contained no mutant *VWF* gene product. Immunofluorescent staining of VWF in the wildtype animal showed strong and grained staining patterns (Figure 3 A). Immunohistochemical staining for VWF revealed moderate to strong (ovary) or strong (uterus and oviduct) staining of ECs. In addition, staining of the apical membrane (AM) of UE and OE was found in the WT animals only (data not shown).

Table 2. Primer pairs used for qPCR.

Gene	Primers (5′ → 3′)
VWF	TCCAGAACAACGACCTCACC
	TCACAGAAGCAGGCACAGTC
ITGAV	AGAGCAGCGAGGACTTTGG
	TCAGCGTAATCATCCCCATT
ITGB3[4]	CCACCTTCACCAACATCACCT
	GACAGCAGAGACACCCACAATC
ITGB3 (I)	CCTCAAAAACCCCTGCTATG
	TTCCTCCAGCCAATCTTCTC
ITGB3 (II)	CAACCATTACTCTGCCTCCA
	TTTGCCATAAGCATCCACAA
ITGB3 (III)	AAGAGCCAGAGTGTCCCAAG
	TCCAGATGAGCAGAGTAGCAAG
VEGF	CTTGCCTTGCTGCTCTACCT
	TTCGTGGGGTTTCTGGTCT
VEGFR2	CCAGATGACAGCCAGACAGA
	AGCCTTCAGATGCCACAGAC
ANG1	TAATGGGGGAGGTTGGACT
	TGAATAGGCTCGGTTTCCTT
ANG2	TGAGAGACTGGGAAGGCAAC
	CCTCCTGTGAGCATCTGTGA
TIE1	CATCACCCCAACATCATCAA
	GCAGCATCACTGGCAAAAC
TIE2	TGCCCAGAGAGGTGACATAG
	AAAGTCCCAGACAGACAGCAA
PROCR	GAAACACATCACCACGCATAAC
	CCGTCCACCTACATACAGGAA
CD31	CCCCTTCCAAAAACTTCCTC
	TGCGATGAGTCCTTTCTTCC

Histology. HE-staining of all organs revealed physiologic architecture of tissue and blood vessels (Figure 3 B and 4 A).

Gene expression. In the uterus, the difference of gene expression between both animals was greater than 56% for all genes except *VEGF* and *TIE2,* which showed differences of less than 1.6%. In the oviduct, continuously opposing observations were made for both WT animals. While animal WT-1 showed gene expression levels below the WT mean for all genes, animal WT-2 showed increased levels for all genes, and the difference was 248% or greater except for *TIE2* (19%). In the ovary, the difference in gene expression between both WT animals was 23% or less for *VEGFR2, ANG2,* and *TIE2* (Figure 2 A). qPCR using 3 pairs of primers (*ITGB3* I – III) did not show measurable gene expression in most samples. When an additional fourth pair of primers chosen from literature[4] did not result in measurable results either, we assume these results reflect a nonexistent *ITGB3* gene expression in the respective samples.

Protein expression and distribution. In the uterus, integrin $\alpha_V\beta_3$ was located at the epithelial AM only in WT animals, while there was no or very weak cytoplasmic staining of UE (Figure 3 C). Ang-2 showed no (WT-1) or very weak (WT-2) staining of the epithelial AM and was located in the cytoplasm of UE and GE (Figure 3 D). Ang-1 displayed moderate to strong (UE) and weak (GE) staining intensity of the AM (Figure 3 E). Tie-1 stained very weakly in ECs and VSMCs, but moderately to strongly at the AM of GE. No staining of cytoplasm of UE and GE was seen (Figure 3 F). In the oviduct staining intensity of integrin $\alpha_V\beta_3$ contrasted with the uterus weak (WT-1) or weak to moderate (WT-2) in the cytoplasm of OE and only very weak in the epithelial AM (Figure 4 B). For Ang-2, WT animals showed very weak (WT-1) and weak (WT-2) staining of the cytoplasm of OE only (Figure 4 C). Tie-1 stained very weakly in ECs of both WT animals but not in VSMC. As in GE, the AM was stained, but only with weak to moderate intensity (Figure 4 D). The ovary did not show any trends for both WT animals (data not shown).

Summary. VWF protein was detected clearly in immunofluorescent staining and immunohistochemistry. Histology of blood vessels was normal. WT-1 and WT-2 animals had differences in gene expression of less than 50% and thus their results were compared with the other genotypes for *VEGF* and *TIE2* in the uterus, for *TIE2* in the oviduct, and for *VEGFR2, ANG2,* and *TIE2* in the ovary. Integrin $\alpha_V\beta_3$ was located at the uterine epithelial AM and was very weak at the oviduct epithelial AM. Staining of Ang-2 of the AM was very weak or absent in UE. It was located in the cytoplasm of UE, GE, and OE.

Comparative characterization of VWD type 1 animals V1-1 and V1-2. VWF. The sows were 13 (V1-1) and 7.5 (V1-2) mo old at the time of euthanasia and had VWF:Ag Plasma levels of 20% and 34%, respectively. Uterine tissue contained 43.4% and 40.5% of the mutant *VWF* gene product relative to the previously published reference group [15] and therefore corroborated the identified genotype. The amount of *VWF* mRNA in the reproductive organs ranged from 7.6% to 34.6% (V1-1) and from 4.7% to 78.6% (V1-2) compared with the wildtype mean (Figure 2 B) and in any case below the WT mean. Immunofluorescent staining of VWF in the VWD type 1 animal showed the same staining patterns as observed in the wildtype animal (Figure 3 G). Immunohistochemical staining for VWF revealed moderate (oviduct), moderate to strong (ovary) or strong (uterus) staining of ECs. In contrast to WT animals, no staining of the epithelial AM was found (data not shown).

Histology. As for the WT animals, HE-staining of all organs revealed the physiologic architecture of tissue and blood vessels (Figure 3 H and 4 E).

Gene expression. In the uterus, the *VEGF* expression level ranged below that seen in the WT animals for animal V1-1 but above them for animal V1-2. The reverse trend was found for *TIE2*. In the oviduct, both VWD type 1 animals displayed increased *TIE2* expression compared with the WT animals of 1.6-fold (V1-1) and 1.3-fold (V1-2) compared with the WT mean. In the ovary, there were no obvious differences in expression levels between VWD type 1 and WT animals for *VEGFR2, ANG2,* and *TIE2*.

Protein expression and distribution. In the uterus, in contrast to WT animals integrin $\alpha_V\beta_3$ was not located at the epithelial AM and staining of the cytoplasm of UE was weak (V1-1) and very weak (V1-2) (Figure 3 I). Staining of Ang-2 of epithelial AM in both VWD type 1 animals was moderate (Figure 3 J) and therefore stronger than in WT animals. Ang-1 staining was comparable to the one seen in WT animals and showed moderate to strong staining of the epithelial AM but only weak (V1-1) or no (V1-2) staining of the AM of GE (Figure 3 K). Tie-1 staining intensity was similarly to the one in WT animals. VSMC displayed very weak staining and ECs showed weak (V1-1) or very weak (V1-2) staining. In contrast, the AM of GE stained moderately (V1-1) and moderately to strongly (V1-2). As in WT animals, no staining of the cytoplasm of UE and GE was seen (Figure 3 L). In the oviduct, staining intensity and distribution of integrin $\alpha_V\beta_3$ was comparable to the conditions found in WT animals. The cytoplasm of OE stained weakly to moderately (V1-1) or weakly (V1-2) and the epithelial AM stained weakly (V1-1) and very weakly (V1-2) (Figure 4 F).

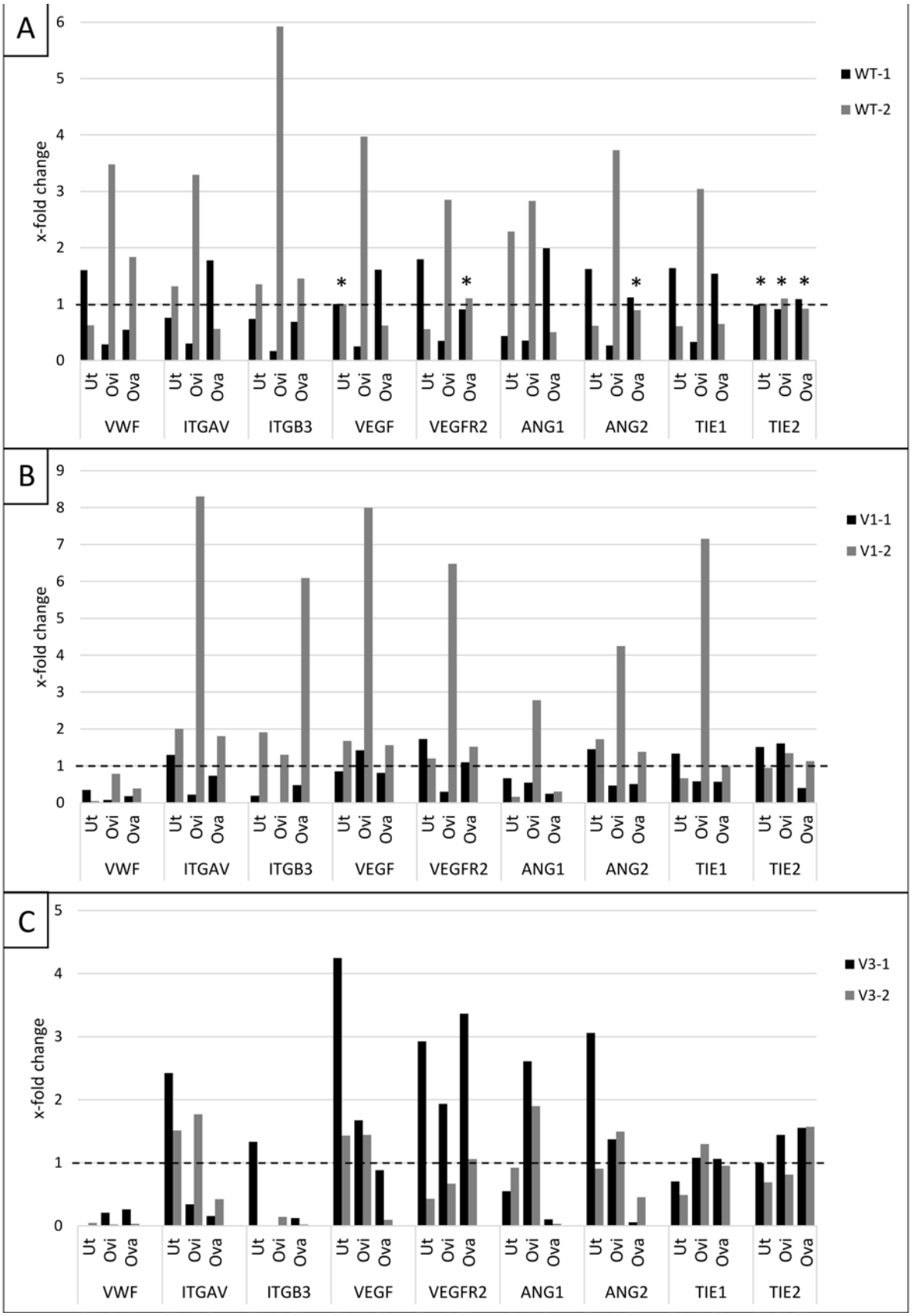

Figure 2. Results of qPCR of wildtype (A), VWD type 1 (B) and VWD type 3 (C) animals for all genes examined. Results are presented as x-fold change compared with the WT mean (indicated by dashed lines). Only if results of wildtype animals, which were used as calibrator, showed < 50% difference between both animals, the corresponding genes and tissues were regarded evaluable for this study. These results are marked by asterisks. Ut = uterus; Ovi = oviduct; Ova = ovary.

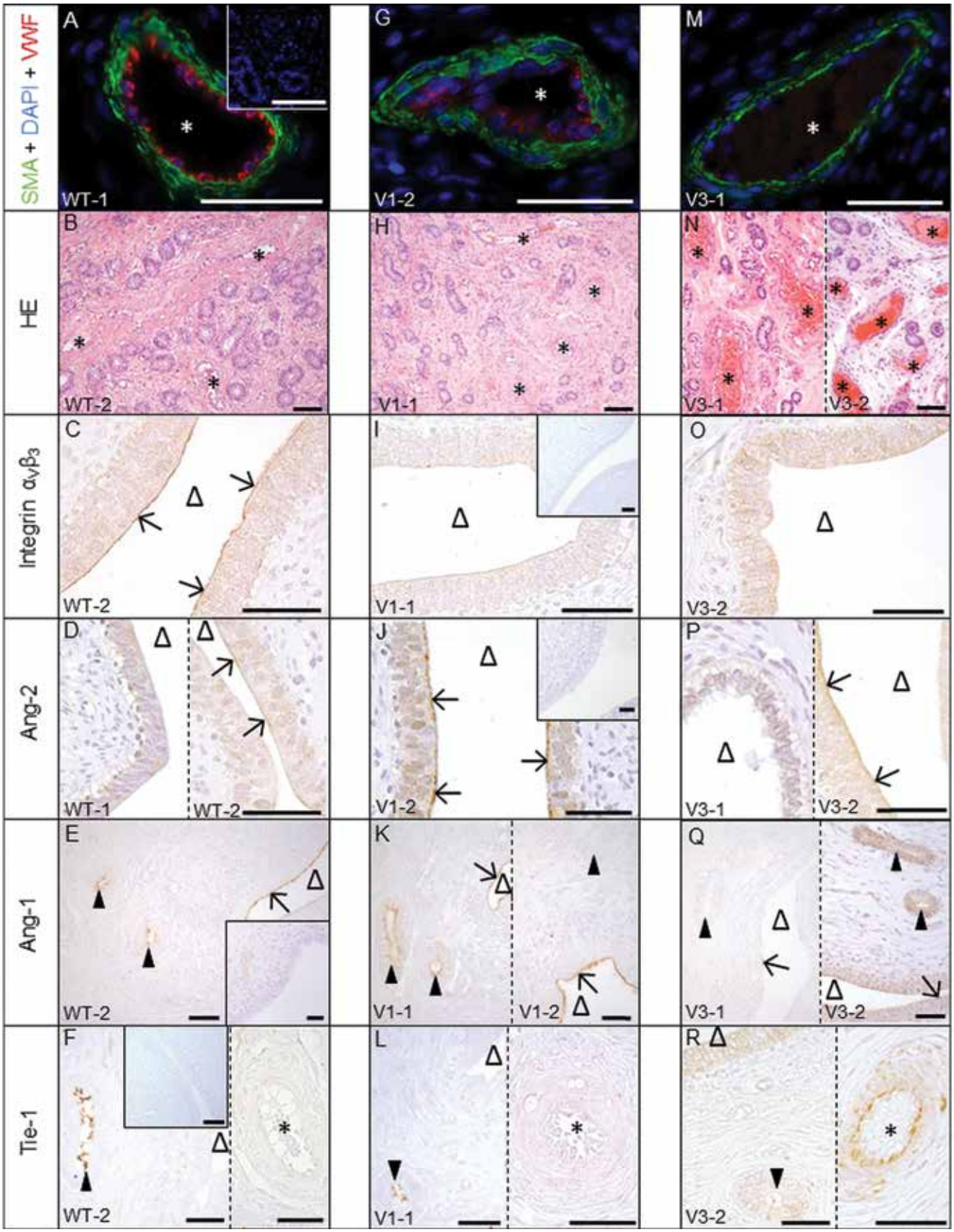

Figure 3. Representative results of histologic examinations of uterine tissue sections. The figure shows representative pictures for WT animals in the left (Athrough F), VWD type 1 animals in the middle (G through L) and VWD type 3 animals in the right column (M through R). *Immunofluorescent costaining of blood vessels:* SMA (green) marks the blood vessel wall and DAPI (blue) marks the cell nuclei. Presence of VWF protein (red) in ECs of WT (A) and VWD type 1 animals (G) is indicated by granular staining, which is rarely seen in ECs of VWD type 3 animals (M). The inset shows the respective negative control. Scale bars = 50 µm; * = blood vessel lumen. *HE-stainings:* blood vessel architecture is physiologic

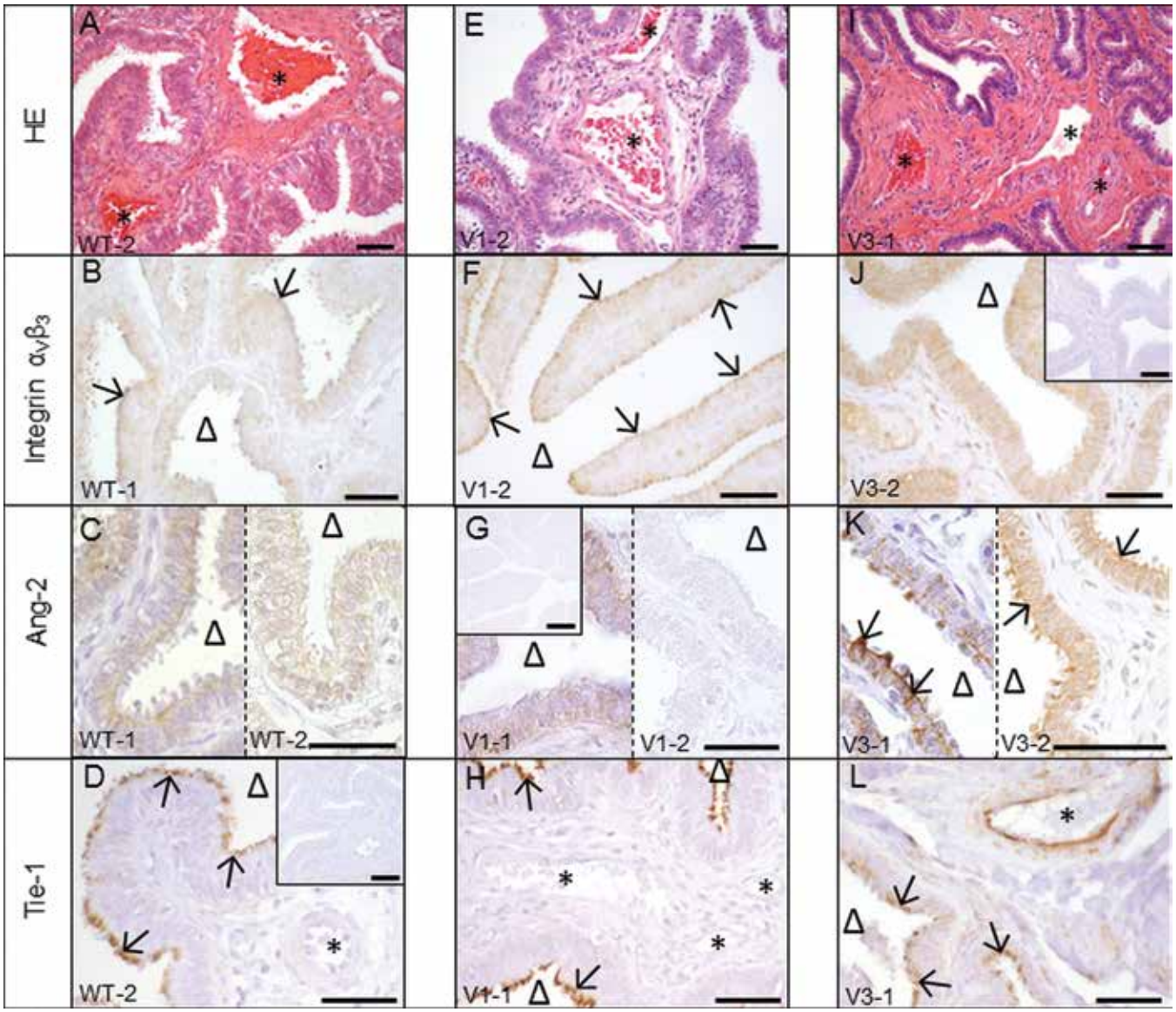

Figure 4. Representative results of histologic examinations of oviduct tissue sections. The figure shows representative pictures for WT animals in the left (A through D), VWD type 1 animals in the middle (E through H) and VWD type 3 animals in the right column (I through L). *HE-stainings:* blood vessel architecture is physiologic in WT (A), VWD type 1 (E) and VWD type 3 animals (I). * = blood vessel lumen. *Integrin* $\alpha_v\beta_3$*:* staining intensity of cytoplasm of oviduct epithelial cells (OE) is comparable in all genotypes, while only WT (B) and VWD type 1 animals (F) show staining of the OE apical membrane and VWD type 3 animals (J) do not. Arrows = staining of OE apical membrane; Δ = oviduct lumen. *Ang-2:* WT (C) and VWD type 1 animals (G) show interindividual variance of staining patterns, while those of VWD type 3 animals (K) are comparable for both animals. *Tie-1:* all genotypes show staining of the OE apical membrane with varying intensity. While WT animals (D) show very weak staining of ECs, no staining is seen in VWD type 1 animals (H) and weak to moderate staining is seen in VWD type 3 animals (L). The insets show the respective isotype controls with no staining. Scale bars = 50 µm.

While animal V1-1 showed weak staining of the cytoplasm of OE as seen in animal WT-2, animal V1-2 did not display any staining for Ang-2(Figure 4 G). Tie-1 did not stain ECs or VSMC but showed strong (V1-1) and moderate to strong (V1-2) staining of the epithelial AM and thus stronger staining than seen in WT animals (Figure 4 H).

Summary. *VWF* expression ranged below WT mean but VWF protein was detected clearly in ECs. Histology of blood vessels

in WT (B) and VWD type 1 animals (H), while both VWD type 3 animals show dilated blood vessels with wider diameter in the uterine lamina propria (N). Scale bar = 100 µm. *Integrin* $\alpha_v\beta_3$*:* staining of the apical membrane of uterine epithelial cells (UE) is seen in WT animals only (C), but not in VWD type 1 (I) and 3 (O) animals. The inset shows the respective isotype control with no staining. Scale bars = 50 µm; ↑ = staining of UE apical membrane; Δ = uterine lumen. *Ang-2:* divergent staining patterns were observed for both WT (D) and both VWD type 3 animals (P), while both VWD type 1 animals (J) showed comparable results with apical staining of UE marked by arrows. The inset shows the respective isotype control with no staining. Scale bars = 50 µm. *Ang-1:* both WT animals show staining of the apical membrane of UE and glandular epithelial cells (GE) (E). Only VWD type 1 animal V1-1 shows the same pattern in GE (K). VWD type 3 animals show divergent staining patterns (Q). The inset shows the respective isotype control with weak staining of fluid within blood vessels only. Scale bars = 50 µm; arrow head = apical membrane of GE. *Tie-1:* WT (F) and VWD type 1 animals (L) show stronger staining for Tie-1 of the GE apical membrane than VWD type 3 animals (R), while these animals show stronger staining of blood vessels. The inset shows the respective isotype control with no staining. Scale bars = 50 µm.

was physiologic. *TIE2* expression was increased in the oviduct. Integrin $\alpha_V\beta_3$ was located in the cytoplasm of UE and very weak at the oviduct epithelial AM. Ang-2 showed moderate staining of the uterine epithelial AM.

Comparative characterization of VWD type 3 animals V3-1 and V3-2. VWF. The animals were 15 (V3-1) and 8.5 (V3-2) mo old at the time of euthanizing and both had VWF:Ag Plasma levels of < 3%. Tissue contained 89.1% and 89.9% of the mutant *VWF* gene product compared with the reference group and therefore corroborated the identified genotype.[15] The amount of *VWF* mRNA in the reproductive organs ranged from 0.8% to 25.9% (V3-1) and from 2.1% to 4.5% (V3-2) compared with the wildtype mean (Figure 2 C), which is partly overlapping with the VWD type 1 animals, but not with WT animals and always below the WT and VWD type 1 mean. Mild profuse to absent immunofluorescent staining was seen in the ECs of the VWD type 3 animal (Figure 3 M). Immunohistochemical staining for VWF in ECs varied between both animals and the different organs. In uterus and oviduct, staining was very weak and weak, in the oviduct weak (V3-1) and weak to moderate (V3-2). As in VWD type 1 animals, no staining of the epithelial AM was found (data not shown).

Histology. In the entire lamina propria and in particular close to the uterine epithelium groups of blood vessels with a noticeable wider diameter were a frequent occurrence. Those vessels were surrounded either by a tunica media comprising several layers of VSMC or by a rather thin tunica media with only a few layers of VSMC (Figure 3 N). No differences in size or number of blood vessels were found in the ovaries (data not shown) or oviduct (Figure 4 I).

Gene expression. In the uterus, *VEGF* expression levels were 4.2- and 1.4-fold higher than those measured in WT animals. In the oviduct, no clear trend was found for *TIE2*. In the ovary, *ANG2* expression of both VWD type 3 animals ranged below that of WT and VWD type 1 animals (0.1- and 0.5-fold compared with WT mean). In contrast, *TIE2* expression ranged above both WT and VWD type 1 animals (both 1.6-fold compared with WT mean). Although WT animals showed a difference of more than 50% in the expression of *ITGB3*, it should be noted that both VWD type 3 pigs showed the lowest *ITGB3* expression (0-fold to 0.14-fold compared with WT mean) for all tissues except from the uterus sample of animal V3-1 (Figure 2 C).

Protein expression and distribution. As in VWD type 1 animals, integrin $\alpha_V\beta_3$ in the uterus was not located at the epithelial AM. Staining of the cytoplasm of UE was moderate and thus stronger than in VWD type 1 animals (Figure 3 O). Staining of Ang-2 of epithelial AM varied in both animals and was either not seen (V3-1) or moderate to strong (V3-2) (Figure 3 P). Ang-1 staining of the epithelial AM varied and was either very weak (V3-1) or moderate to strong (V3-2). Comparable to the conditions in VWD type 1 animals, staining of the AM of GE was very weak (V3-1) or not seen (V3-2) (Figure 3 Q). Tie-1 staining in VSMC was similarly to the one in WT and VWD type 1 animals weak (V3-1) or very weak (V3-2) and staining in ECs was weak (V3-1) but also moderate (V3-2). Very weak (V3-1) or weak (V3-2) cytoplasmic staining of UE and GE was seen in VWD type 3 pigs only (Figure 3 R). In the oviduct, cytoplasm of OE stained weakly to moderately for integrin $\alpha_V\beta_3$ as in WT and VWD type 1 animals, but no staining of the AM was found (Figure 4 J). Only VWD type 3 animals displayed weak to moderate and moderate staining of the cytoplasm of OE for Ang-2 and were the only group showing staining of the AM (Figure 4 K). For Tie-1, ECs stained weakly (V3-1) or weakly to moderately (V3-2). Comparably to the one in VWD type 1 animals, staining of the AM was moderate (V3-1) or moderate to strong (V3-2) (Figure 4 L).

Summary. *VWF* expression ranged below WT and VWD type 1 mean. Staining for VWF protein in ECs was weaker compared with WT and VWD type 1. Altered histology of blood vessels in the uterine lamina propria was found in both animals. *VEGF* expression was increased compared with WT in the uterus. *ANG2* expression was decreased and *TIE2* expression was increased compared with WT and VWD type 1 in the ovary. Integrin $\alpha_V\beta_3$ was not located at the uterine and oviduct AM and showed stronger staining of the cytoplasm of UE compared with WT and VWD type 1. Both animals showed staining of the AM of OE for Ang-2.

ANG/TIE-system ratios. A relatively higher expression of *ANG2* over *ANG1* was shown for all but 3 tissues (uterus of animal WT-2 and ovaries of animals WT-1 and V3-1). A particularly high expression of *ANG2* relatively to *ANG1* (25.1-fold compared with *ANG1*) was found in the ovary of animal V3-2. In contrast, the ovary of animal V3-1 displayed a relative overexpression of *ANG1* over *ANG2* (0.6-fold expression of *ANG2* compared with *ANG1*). Both VWD type 1 animals showed stronger overexpression of *ANG2* compared with *ANG1* (2.2- and 4.9-fold) than both WT animals (0.6- and 1.9-fold). Furthermore, only in the ovary tissues of both VWD type 3 animals, a higher expression of *ANG1* over *TIE2* was seen (expression of *ANG1* compared with *TIE2* 2.0- and 4.5-fold compared with VWD type 1 0.2- and 0.5-fold, and WT 0.1- and 0.2-fold) (Figure 5). No trends were found for other relations within the ANG/TIE-system.

Discussion

This study aimed to characterize a porcine model for VWD type 1 and 3 to further investigate its influence on angiogenic mediators focused on the female reproductive tract. Our results demonstrate an influence of VWD on the female reproductive organs in the investigated model. In a first step, the phenotypes and genotypes of the samples were confirmed to establish a basis for in vivo experiments with respect to VWD and female reproduction. The genetic test by quantification of the mutant *VWF* PCR product[15] matched with the genotype determination by blood VWF:Ag levels and therefore seems to provide reliable results to determine the genotype of the animals. These results were further confirmed by immunofluorescent and immunohistochemical staining for VWF, which was very weak to weak in the VWD type 3 animals. VWD type 1 and wildtype animals differed by the staining of the epithelial AM, which was present only in WT pigs. Concerning the amount of *VWF* gene expression, a clear discrimination was only possible between WT and VWD type 3 animals. Results of VWD type 1 animals overlapped with WT as well as VWD type 3 animal, which is probably caused by different stages of nonsense-mediated mRNA decay of the mutant VWF allele previously shown in this colony.[15] The granular staining pattern of VWF in WT and VWD type 1 animals indicates staining in Weibel-Palade bodies (WPBs)[38] and thus missing granules in immunofluorescent staining indicate a loss of WPBs in that group.

The occurrence of angiodysplastic lesions has most frequently been described in the gastrointestinal tract of VWD patients,[29] but also in the nail fold[14] or the nasal mucosa.[23] However, studies investigating angiodysplastic lesions in different tissue types of animal models for VWD are rare. Enhanced angiogenesis due to *VWF*-knockout was described in mice[34] and the detection of fecal occult blood in mice carrying a *VWF* mutation[3] might indicate the presence of angiodysplastic lesions in their

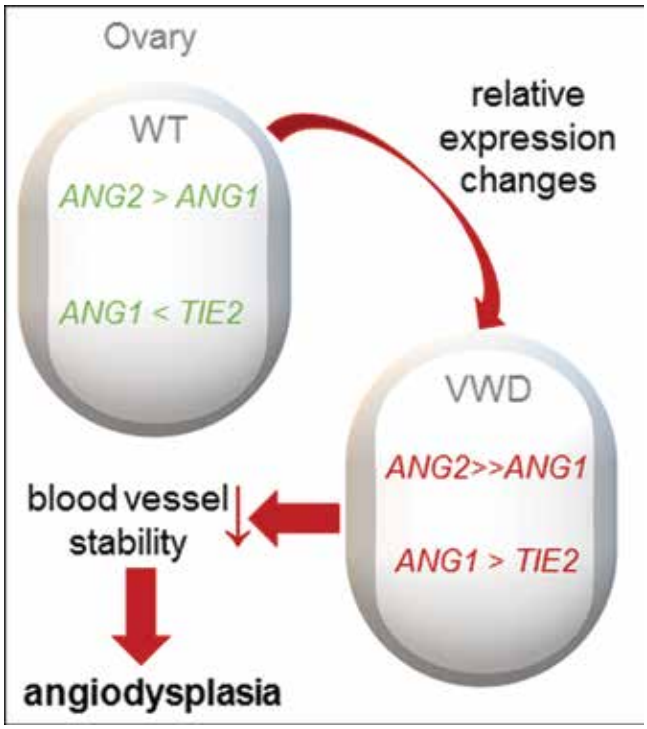

Figure 5. Graphic illustration of *ANG/TIE*-system ratios (qPCR data) observed in the ovary and possible impact on blood vessels. Although gene expression of *ANG2* was reduced in the ovary of VWD type 3 animals, relatively higher expression of *ANG2* over *ANG1* was more pronounced in most VWD animals. Furthermore, a relatively higher expression of *TIE2* over *ANG1* was seen in VWD type 3 animals only. These findings may lead to destabilization of blood vessels and angiodysplasia.

gastrointestinal tract.[19] However, this study is the first to report angiodysplastic lesions of arteries and veins in an animal model for VWD. We identified angiodysplasia in both VWD type 3 pigs examined. Many blood vessels in the lamina propria of both uteri were notably dilated and thin-walled with a wider diameter. Therefore, this animal model allows in vivo investigations of the connection between VWF and angiodysplasia for the first time. In addition to these histologic changes, alterations concerning expression and distribution of investigated angiogenic factors were identified on genetic and protein level.

A shift of integrin $\alpha_V\beta_3$ from the apical cell membrane to the cytoplasm in VWD type 3 animals was mainly seen in the uterus but also in the oviduct. The low integrin $\alpha_V\beta_3$ gene expression in most samples of VWD type 3 pigs compared with the wildtype are in accordance with a previous in vitro study, in which internalization of the integrin $\alpha_V\beta_3$ protein as well as a decreased gene expression was shown in human umbilical vein ECs after *VWF*-knockdown.[34] Integrin $\alpha_V\beta_3$ is compounded of proteins translated from the genes *ITGB3* and *ITGAV*. As reviewed,[39] the different integrin subunits have several functions in physiologic as well as pathologic angiogenesis, also depending on the microenvironment. Therefore, it must be taken into consideration that VWF might influence angiogenesis not only via the β_3-subunit, as suggested by Starke and colleagues,[34] but also via the α_V-subunit. We investigated the expression of *ITGAV*, but WT animals showed a variation of 56%. Thus, we were not able to reveal a clear trend for this gene and the polyclonal antibody used for IHC of integrin $\alpha_V\beta_3$ allows no discrimination between its subunits integrin α_V and integrin β_3. However, our observations made on porcine UE correspond to findings in human umbilical vein ECs,[34] supporting the hypothesis of VWF promoting stabilization of integrin $\alpha_V\beta_3$ on the cell surface (Figure 1). Beyond the influence on angiogenesis, the role of integrin α_V and integrin β_3 in reproductive processes such as implantation of the embryo is noteworthy.[22] While the fundamental role of integrins in establishment of uterine receptivity was described earlier,[16] both integrin-subunits were shown to be expressed in the porcine endometrium at least until day 25 after insemination.[17] This allows the presumption that the described differences among the genotypes concerning integrin $\alpha_V\beta_3$ distribution in the UE may also influence uterine receptivity. Whether the loss of apical staining for VWF in the affected animals in UE and OE might also affect these processes requires further investigation.

VWD type 3 animals showed greater gene expression of *VEGF* in the uterus than did WT animals, but no trends were seen in IHC. It has been proposed that VWF acts on angiogenesis via the VEGF/VEGFR-2-pathway.[25,34] Connecting this pathway to our findings concerning integrin $\alpha_V\beta_3$ mentioned above, mice lacking integrin β_3 show over-sensitivity of VEGFR-2 to VEGF.[26] In contrast, interaction of VEGFR-2 and integrin $\alpha_V\beta_3$ was described to be crucial for full VEGFR-2 activity.[33] These bimodal effects of integrin $\alpha_V\beta_3$ in connection with the influence of VWF on its activity remain speculative so far. Presumably, the internalization of the β_3-subunit found in vitro[34] might mimic the situation of a β_3-knockout and thus leads to VEGFR2-hypersensitivity for VEGF as well. Since VEGF is a potent proangiogenic ligand of VEGFR-2,[32] this influence on sensitivity of the receptor accompanied by higher *VEGF* expression seen in the uteri of VWD type 3 animals may lead to an additive effect resulting in angiodysplastic blood vessels due to excessive VEGF/VEGFR-2-signaling (Figure 1). Furthermore, significantly higher VEGF plasma levels were recently found in human VWD type 3 compared with VWD type 1 and 2 patients.[9] Fragility of capillaries caused by an overexpression of *VEGF* was described earlier.[28] Thus, our results support the hypothesis that a lack of VWF may alter angiogenesis via increased *VEGF* expression and VEGFR-2 signaling.

Another important factor connecting VWF and angiogenesis is Ang-2.[25] In our study, the IHC staining patterns of Ang-2 indicate a modified distribution of this protein. This is in concordance with in vitro findings on varied Ang-2 secretion and storage among genotypes as well as among individuals.[30] This is supposed to be mediated by alterations of the WPBs. VWF is required for maintenance of WPBs, but vice versa size of WPBs has an influence on VWF function[6] and possibly function of other stored proteins. Among the genotype groups in our study, variation of Ang-2 protein distribution was obvious in UE, GE, and OE, but not in ECs. In blood outgrowth ECs of VWD patients, altered intracellular staining of Ang-2 has been described. However, this was not necessarily in accordance with ELISA data.[30] In our VWD type 3 animals, *ANG2* expression in the ovary ranged below WT and VWD type 1 animals. Conversely, *ANG2* expression was increased in most blood outgrowth ECs of VWD patients. However, in these cells, gene expression did not necessarily correlate with basal secretion levels, which were also reduced in some samples.[30] Regarding Ang-1 protein, we found increased or decreased amounts at the uterine epithelial AM in the VWD type 3 animals depending on the individual. Thus, based on our findings, the ANG/TIE-system requires cell type-specific characterization.

For the members of the ANG/TIE-system, clear trends of the gene expression levels were only seen in the ovary. Expression of *ANG2* was lower in the VWD type 3 animals. Ang-2 acts as an antagonist of Ang-1/Tie-2-signaling. Therefore, higher *ANG2* levels associated with angiodysplasia would be expected, as

Ang-2 leads to an activated phenotype of ECs, which allows plasticity of the endothelium.[1] This was already shown in humans with sporadic small bowel angiodysplasia,[10] in connection with reduced VWF in human umbilical vein ECs[34] and blood outgrowth ECs,[30] and also tissue specific in the heart of *VWF*-knockout mice.[40] However, the unexpectedly decreased *ANG2* expression in our VWD type 3 animals was accompanied by simultaneously decreased *ANG1* expression. Thus, *ANG2* expression was in animal V3-2 still relatively higher than *ANG1* expression and this relative difference was even more pronounced than in the other animals. Conversely, animal V3-1 displayed relative overexpression of *ANG1*. The impact of this ratio remains speculative so far, as no angiodysplasia was found in the ovaries so far (Figure 5).

Our study is the first one investigating alterations concerning Tie-1 and -2 due to VWD. The increased expression of *TIE2* seen in the ovaries of VWD type 3 animals indicates the involvement of ANG1-TIE2 signaling. Overexpression of *TIE2* was described earlier in venous malformation[36] and altered dermal angiogenesis.[37] The latter observations were accompanied by increased *ANG1* expression. When reviewing the ratios, we found a relatively higher expression of *ANG1* over *TIE2* in VWD type 3 animals only (Figure 5). Thus, not only increased activation of Tie-2 due to higher secretion of Ang-2 should be considered in terms of connecting VWF and angiogenesis as suggested by Randi,[24] but also the ratios within the ANG/TIE-system. Further evidence for pathways connecting VWF and the Tie-receptors is given by the varying staining patterns observed for Tie-1.

Due to the limited size of our study, a larger number of animals should be examined to validate our findings. However, we were able to show a comprehensive discrimination between the different genotypes due to VWF associated characteristics on genetic as well as protein level. Due to the 3 different genotypes, this model allows not only observations of the almost total lack of VWF, but also moderately reduced VWF levels and in addition, animals in this model can be chosen to minimize variation concerning VWF geno— and phenotype ahead of euthanize. In contrast to VWF expression, the related angiogenic factors examined showed higher variations. Although higher sample sizes will be necessary to verify those first observations, the presence of angiodysplasia as seen in the uteri of VWD type 3 animals can be related to the trends for differences among the genotypes shown. This indicates the influence of VWF on angiogenesis in the female reproductive tract demonstrated here for the first time. The pigs constitute a suitable large animal in vivo model, which enables further investigation of larger groups on the influence of VWF on angiogenesis in general and on female fertility in particular.

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