Embryonic Stem Cell-Like Subpopulations in Venous Malformation

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Background: Venous malformation (VM) consists of a network of ectatic anomalous thin-walled venous channels. A role for an activating TIE2 mutation in the development of the dilated luminal vessels in VM, and its proposed involvement of embryonic stem cells (ESCs), led us to investigate the expression of ESC markers in subcutaneous VM (SCVM) and intramuscular VM (IMVM).

Methods: Formalin-fixed paraffin-embedded sections of SCVM from seven patients and IMVM samples from seven patients were analyzed for the expression of Nanog, pSTAT3, OCT4, SOX2, SALL4, and CD44, using 3,3′-diaminobenzidine (DAB) immunohistochemical (IHC) staining. All these samples did not express lymphatic marker D2-40. NanoString mRNA analysis and RT-PCR were performed on snap-frozen samples of SCVM (n = 3) and IMVM (n = 3) from the respective original cohorts of patients included in DAB IHC staining. To confirm co-expression of two proteins, immunofluorescent (IF) IHC staining on two representative samples of IMVM and SCVM samples from the original cohorts of patients included for DAB IHC staining was performed.

Results: DAB IHC staining demonstrated expression of all of the above ESC markers in both SCVM and IMVM samples. IF IHC staining showed that these markers were localized to the endothelium within these lesions and that Nanog, pSTAT3, SOX2, and CD44 were also expressed by cells outside of the endothelium. NanoString mRNA analysis confirmed transcription activation of pSTAT3, OCT4, and CD44. RT-qPCR confirmed transcription activation of Nanog, SOX2, and SALL4.

Conclusion: Our findings support the presence of two ESC-like subpopulations, one within and one outside of the endothelium, of both SCVM and IMVM. Given that the endothelial ESC-like subpopulation expresses the more primitive marker, OCT4, it is exciting to speculate that they give rise to the non-endothelial subpopulation.

Keywords: venous malformation, embryonic, stem cells, markers, pathogenesis

Abbreviations: ACE, angiotensin-converting enzyme; Ang-1, angiopoietin 1; Ang-2, angiopoietin 2; ATIIR1, angiotensin II receptor 1; ATIIR2, angiotensin II receptor 2; DAB, 3,3′-diaminobenzidine; EC, endothelial cell; ESC, embryonic stem cell; H&E, hematoxylin and eosin; IF, immunofluorescent; IHC, immunohistochemical; IMVM, intramuscular venous malformation; PRR, prorenin receptor; RAS, renin–angiotensin system; SCVM, subcutaneous venous malformation; SMC, smooth muscle cell; VM, venous malformation.
INTRODUCTION

Vascular anomalies are classified by the International Society for the Study of Vascular Anomalies classification system into vascular tumors of which infantile hemangioma is the most common, and vascular malformations of which venous malformation (VM) is the most common (1, 2). Vascular malformations may affect arteries, veins, lymphatics, and capillaries singly or in combinations (1).

Venous malformation, which affects 1% of the population (3), is composed of ectatic anomalous venous channels, lined by flat endothelial cells (ECs) (3–5) with absent or deficient smooth muscle cells (SMCs) within the thin vessel walls (3, 5). VM is present at birth, although it may not be noticed until later in life (3–5). It grows proportionately with the child and may suddenly expand in response to hormonal changes, trauma, or incomplete excision (2–4). VM affects different topographic regions and tissues, commonly involving the subcutaneous tissues and less commonly muscle (6). Subcutaneous VM (SCVM) usually presents as a compressible bluish swelling, whereas intramuscular VM (IMVM) often presents later in life with swelling, pain, or loss of function (5). Approximately 40% of VM occur in the head and neck region, 40% in the extremities, and 20% on the trunk (3, 6).

Management of VM is generally unsatisfactory especially for extensive lesions and includes observation, elastic support (7), low-dose aspirin (7), sclerotherapy, such as ethanol sclerotherapy (8, 9), surgery, or a combination of these treatments (10–12).

The pathogenesis of VMs has not been fully elucidated (2, 13), although recent reports have demonstrated a role for mutations of TIE2-L914F (14) and PIK3CA (15) in the biology of these lesions. VMs are mostly sporadic, with 1.2% being familial (16). A mutation in the TIE-2 gene, a receptor for angiopoietin 1 (Ang-1) expressed almost exclusively on ECs, has been identified in familial (17), and in up to 50% of sporadic (18), VM cases. Activating mutations of this tyrosine kinase receptor results in a ligand-independent hyperphosphorylation (14). Vakkula et al. (19) suggest that the TIE2 mutation in ECs may reduce SMC ligand expression causing a local uncoupling between normal SMC recruitment and the proliferation of ECs.

We have recently demonstrated the expression of components of the renin–angiotensin system (RAS): prorenin receptor (PRR), angiotensin-converting enzyme (ACE), angiotensin II receptor 1 (ATII1R), and potentially angiotensin II receptor 2 (ATII2R), in SCVM and IMVM, which suggests a role for the RAS in the biology of VM (20). ATII1R is responsible for the proangiogenic effects of ATII, which may contribute to the increased density of abnormal venous channels within VM (20). The presence of ATII2R may indicate cellular differentiation determination, as proposed by Zambidis et al. (21). Furthermore, ACE is a marker for primitive human pluripotent stem cell-derived hemangioblasts (20). The expression of ACE on the endothelium of VM (20) and the increased expression of stem cell marker c-kit within smaller lesional vessels in blue rubber bleb nevus syndrome (22), a subtype of VM, led us to speculate whether this reflected a primitive phenotype for this endothelium of VM.

A role for TIE2 activation in the formation of increased vessel lumen in vasculature derived from embryonic stem cells (ESCs) (23) parallels the vasculature seen in VMs and led us to hypothesize the expression of a primitive population in endothelium of both SCVM and IMVM.

This study aimed to identify a putative primitive population by their expression of ESC markers, such as Nanog, pSTAT3, OCT4, SOX2, SALL4, and CD44, in SCVM and IMVM.

MATERIALS AND METHODS

Tissue Samples

Previously untreated, SCVM tissue samples from seven patients and IMVM samples from seven patients with a mean age of 22.9 (range, 1.2–54) and 21.1 (range, 8–30) years, respectively, were sourced from the Gillies McIndoe Research Institute Tissue Bank and used in a study approved by the Central Health and Disability Ethics Committee (ref. no. 13/CEN/130). Written informed consent was obtained from the participants.

Histology and Immunohistochemical (IHC) Staining

Hematoxylin and eosin (H&E) staining was performed on 4-μm thick formalin-fixed paraffin-embedded sections of SCVM (n = 7) and IMVM (n = 7) samples to confirm the presence of VM tissues on the slides by an anatomical pathologist (Helen D. Brasch). Negative staining for D2-40 (1:100; cat# M3619, Dako, Glostrup, Denmark) was performed in all cases at the Department of Pathology at Hutt Hospital to exclude lymphatic malformation.

3,3′-Diaminobenzidine (DAB) IHC staining for primary antibodies, such as Nanog (1:100; cat# ab80892, Abcam, Cambridge, UK), pSTAT3 (1:100; cat# 9145, Cell Signaling Technology, Danvers, MA, USA), OCT4 (1:30; cat# MRQ-10, Cell Marque, Santa Cruz, CA, USA), SOX2 (1:200; cat# PA0212, Leica, Newcastle upon Tyne, UK) and ERG (1:200; cat# EP111, Cell Marque, Rocklin, CA, USA), and CD44 (1:1,500; cat# MRQ-11, Cell Marque), was performed on the SCVM and IMVM tissue sections using the Leica Bond Rx auto-stainer (Leica), as previously described (24). Nanog was stained using the ImmPACT NovaRED Peroxidase Substrate Kit (cat# SK-4805, Vector Laboratories, Burlingame, CA, USA) and the ImmPRESS Excel Amplified HRP Polymer Staining Kit (cat# MP-7601, Vector Laboratories). To confirm co-expression of two proteins, immunofluorescent (IF) IHC staining on two representative samples of IMVM and SCVM samples from the original cohorts of patients included for DAB IHC staining was performed with the same primary antibodies at the same concentrations was performed with CD34 (ready-to-use; cat# PA0212, Leica, Newcastle upon Tyne, UK) and ERG (1:200; cat# EP111, Cell Marque), as appropriate endothelial markers. Appropriate secondary antibodies, such as Vectafluor Excel anti-rabbit 594 (ready-to-use; cat# VEDK-1594, Vector Laboratories) and Vectafluor Excel anti-mouse (ready-to-use; cat# VEDK-2488, Vector Laboratories) combinations, were used for IF IHC detection. All antibodies were diluted with Bond TM primary antibody diluent (cat# AR9352, Leica). All IHC experiments were performed as single runs.

Positive human controls tissues used were seminoma for Nanog, SALL4, and OCT4; skin for SOX2; and tonsil for pSTAT3.
and CD44 (25, 26). To determine the specificity of the primary antibodies, appropriate negative controls consisting of combined Flex Negative Control Mouse (ready-to-use; cat# IR750, Dako, Carpinteria, CA, USA) and Flex Negative Control Rabbit (ready-to-use; cat# IR600, Dako) staining was performed on VM tissues.

**Microscopy**

All DAB IHC-stained slides were viewed, and the images were captured using an Olympus BX53 light microscope fitted with an Olympus DP21 digital camera (Tokyo, Japan). IF IHC-stained slides were viewed, and the images were captured using an Olympus FV1200 biological confocal laser-scanning microscope (Tokyo, Japan).

**NanoString mRNA Analysis**

NanoString mRNA analysis was performed on snap-frozen samples of SCVM (n = 3) and IMVM (n = 3) from the respective original cohort of patients included in DAB IHC staining, as previously described (20). Probes for the genes encoding STAT3 (NM_139276.2), OCT4 (NM_002701.4), and CD44 (NM_001001392.1) and the housekeeping gene GAPDH (NM_002046.3) were designed and synthesized by NanoString Technologies (NanoString Technologies, Seattle, WA, USA). NanoString mRNA analysis was performed as a singular run.

NanoString data were analyzed using SPSS (v22, IBM) and validated with nSolver™ software (NanoString Technologies) using standard settings, normalized against the housekeeping gene. Charts were made with Excel.

**RT-qPCR**

Total RNA was isolated from formalin-fixed paraffin-embedded samples of SCVM (n = 3) and IMVM (n = 3) from the original cohorts of patients included for DAB IHC staining, using the RNeasy FFPE Kit (cat# 73504, Qiagen, Hilden, Germany) with DNase digest and the QIAcube system (Qiagen). Total RNA quantity and quality were determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription reactions were performed using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). The expression of stem cell markers was detected using gene-specific TaqMan (Thermo Fisher) primers probes (SOX2: Hs01053049_s1; SALL4: Hs00360675_m1; Nanog: Hs04399610_g1; GAPDH: 4333764T) with the Rotor-Gene Multiplex RT-PCR Kit (cat# 204974, Qiagen). All measurements were performed in duplicate. Gene expression was determined by the Relative Standard Curve Method, using GAPDH as an endogenous control. Graphs were generated with Microsoft Excel, and results are shown as relative expression.

**Statistical Analysis**

The mean levels of mRNA expression for each gene investigated in SCVM vs IMVM were subjected to t-test for quality of means using SPSS v 22, to determine and significant differences.

**RESULTS**

**Histology and DAB IHC Staining**

Venous malformation tissues, characterized by ectatic venous channels in both SCVM (Figure 1A) and IMVM (Figure 1B),
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Figure 1D and IMVM (brown) lesions used in this study did not were identified by H&E staining. The SCVM (Figure 1C, brown) and IMVM (Figure 1D, brown) lesions used in this study did not or minimally expressed D2-40, a lymphatic marker. Nanog (Figures 2A,B, red), pSTAT3 (Figures 2C,D, brown), OCT4 (Figures 2E,F, brown), SOX2 (Figures 2G,H, brown), SALL4 (Figures 2I,J, brown), and CD44 (Figures 2K,L, brown) were expressed on the endothelium of all seven samples of SCVM (Figures 2A,C,E,G,I,K) and seven samples of IMVM (Figures 2B,D,F,H,J,L). Interestingly, cells away from the endothelium also expressed Nanog (Figures 2A,B, red, arrowheads), pSTAT3 (Figures 2C,D, brown, arrowheads), SOX2 (Figures 2G,H, brown, arrowheads), and CD44 (Figures 2K,L, brown, arrowheads) in all samples of SCVM (Figures 2A,C,G,K) and IMVM (Figures 2B,D,H,L).

Positive staining was demonstrated in seminoma for Nanog (Image 1A in Supplementary Material, red), OCT4 (Image 1B in Supplementary Material, brown), and SALL4 (Image 1C in Supplementary Material, brown); skin for SOX2 (Image 1D in Supplementary Material, brown); and tonsil for pSTAT3 (Image 1E in Supplementary Material, brown) and CD44 (Image 1F in Supplementary Material, brown). Negative control SCVM (Image S1G in Supplementary Material, brown) tissue samples demonstrated minimal staining.

IF IHC Staining

Immunofluorescent IHC staining with CD34 (Figures 3A,B, green) and ERG (Figures 3A,B, red) demonstrated CD34+/ERG- (long arrows), CD34+/ERG+ (short arrows), and CD34+/ERG+ (arrowheads) endothelium in SCVM (Figure 3A) and IMVM (Figure 3B) lesions. The CD34+ (Figures 3C,D, green) endothelium expressed Nanog in both SCVM (Figure 3C, red, arrows) and IMVM (Figure 3D, red, arrows) lesions with cells away from endothelium also expressing Nanog (Figures 3C,D, red, arrowheads) within SCVM (Figure 3C) and IMVM (Figure 3D) lesions. The CD34+ (Figures 3E,F, green) endothelium expressed pSTAT3 in both SCVM (Figure 3E, red) and IMVM (Figure 3F, red, arrows) lesions with cells away from the endothelium also expressing pSTAT3 (Figures 3E,F, red, arrowheads), within SCVM (Figure 3E) and IMVM (Figure 3F) lesions. The ERG+ (Figures 3G,H, red) endothelium expressed OCT4 in both the SCVM (Figure 3G, green, arrows) and IMVM (Figure 3H, green, arrows) lesions. The CD34+ (Figures 3I,J, green) endothelium expressed SOX2 in SCVM (Figure 3I, red, arrows) and IMVM (Figure 3J, red, arrows) lesions with cells away from the endothelium also expressing SOX2 (Figures 3I,J, red, arrowheads) within SCVM (Figure 3I) and IMVM (Figure 3J) lesions. The ERG+ (Figures 3K,L, red) endothelium expressed SALL4 in SCVM (Figure 3K, green, arrows) and IMVM (Figure 3L, green, arrows) lesions with no expression of SALL4 on the cells outside of the endothelium. To further characterize the SALL4+ (Figures 3M,N, green) endothelial population, we performed dual staining with SOX2, which confirmed co-expression of SOX2 (Figures 3M,N, red) in both SCVM (Figure 3M) and IMVM (Figure 3N). The ERG+ (Figures 3O,P, red) endothelium expressed CD44 in SCVM (Figure 3O, green, arrows) and IMVM (Figure 3P, green, arrowheads) lesions with cells away from the endothelium also expressing CD44 (Figures 3O,P, green, arrowheads) in SCVM (Figure 3O) and IMVM (Figure 3P) lesions. Dual IF IHC staining showed co-expression of Nanog (Figures 3Q,R, red, arrows) and CD44 (Figures 3Q,R, green, arrows) in cells outside of the
endothelium within SCVM (Figure 3Q) and IMVM (Figure 3R) lesions, inferring the non-endothelial Nanog+ cells and the non-endothelial CD44+ cells are a single population. High powered images of the IF IHC-stained images in Figure 3 are presented in Image 2 in Supplementary Material.

Individual IF IHC staining for each of the aforementioned proteins shown in Figure 3 is presented in Images 3 and 4 in Supplementary Material for SCVM and IMVM, respectively. Negative controls for IF IHC staining for both SCVM and IMVM tissue samples demonstrated minimal staining (Image 5 in Supplementary Material).

**NanoString mRNA Analysis**

NanoString transcriptional profiling of three SCVM and three IMVM samples was normalized against the housekeeping gene GAPDH and averaged confirming the relative abundance of mRNA transcripts for STAT3 and CD44 in all SCVM and IMVM (Figure 4A). Statistical analysis of the mean values revealed no
significant differences between the expression of STAT3 and CD44 between the SCVM and IMVM samples.

**RT-qPCR**
Average expression levels of SOX2, SALL4, and Nanog genes, relative to the housekeeping gene GAPDH, are shown in Figure 4B. There were no significant differences between the mean expression levels of SOX2, SALL4, and Nanog between SCVM and IMVM samples.

**DISCUSSION**
98.8% of VMs arise sporadically. Familial VM that is typically multifocal is inherited in an autosomal dominant manner (2, 3, 5, 14, 19). The activating mutations of the tyrosine kinase receptor, TIE2, in the ECs account for the familial forms and 50% of sporadic (2, 3, 13, 14) VMs. The most common mutation in familial VM is R849W that involves an arginine-to-tryptophan substitution at position 849 in the kinase domain of TIE2 (2, 5, 14, 19). The most common somatic mutation is L914F, which accounts for 77% of patients with mutation-positive VM (14).

The exact mechanism by which mutant TIE2 leads to VMs is unknown (2). The mutations that lead to VM are located in the tyrosine kinase domain, kinase-insert domain, and carboxy terminal tail domains and cause ligand-independent receptor hyperphosphorylation in vitro and increased TIE2 activity (2, 5, 14, 19). The lack of correlation between phosphorylation and strength and severity of patient phenotype suggests a role in qualitative and not just quantitative anomalies in TIE2 signaling (2). The activating TIE2 mutation in ECs may reduce SMC ligand expression causing a local uncoupling between the normal recruitment of SMCs and the proliferation of ECs, resulting in affected vessels containing a disproportionately large number of ECs compared with SMCs (19).
we infer that its expression may be more related to its role in pSTAT3, may be associated with the normal hematopoiesis (28), lesions. Although some of these transcription factors, such as malformations.

first demonstration of the expression of these stem cell markers the scope of this report. However, we believe that this is the pathology warrants further investigation although it is beyond the scope of this study. Interestingly, a recent report demonstrates the use of Y-27632, a Rho pathway inhibitor, for efficient culture of VM ESCs (32), with the use of this cytokine previously reported to be crucial in the culture of ESCs (33).

The core nuclear transcription factors, such as Nanog, pSTAT3, and OCT4, have been used to identify and characterize the ESC population (34). OCT4 works synergistically with SOX2 and Nanog, to regulate various genes required for self-renewal and pluripotency (35, 36). The presence of leukemia inhibitory factor in pSTAT3 leads to its interaction with brachyury to form a loop stimulating the expression of Nanog (36).

Mogler et al. (22) show the presence of c-kit, a stem cell growth factor receptor, in the smaller, but not larger vessels of VM lesions. A potential explanation could be that the larger ectatic vessels are more mature and can no longer maintain a stem cell population, and the smaller vessels act as potential precursors.

Taken together, the novel findings in this report suggest a role of the ESC expression on the TIE2-activating mutation endothelium may possibly predispose to the formation VM phenotype, although this remains the topic of further investigation.

We have recently demonstrated the expression of PRR, a component of the RAS, on the endothelium of both SCVM and IMVM (20). PRR is known to signal through the Wnt/β-catenin pathway (37), and subsequently maintain pluripotency in ESCs (38). The finding of the two ESC-like subpopulations within SCVM and IMVM is novel and suggests that these primitive subpopulations may be a therapeutic target. Work is underway to investigate if these primitive subpopulations expressing the RAS, which can be manipulated by existing medications.

**Study Limitations**

1. Larger studies are needed to confirm the significance of the findings in this study of a relatively small sample size.
2. *In vitro* and *in vivo* functional studies are needed to determine the ESC-like phenotype of these cells and relative role in the patho-etiology of VM.

**ETHICS STATEMENT**

This study was carried out with the approval of the Central Health and Disability Ethics Committee (ref. no. 13/CEN/130) with written informed consent from all subjects in accordance with the Declaration of Helsinki.

**AUTHOR CONTRIBUTIONS**

TI and ST formulated the study hypothesis and designed the study. ET, SS, HB, TI, and ST analyzed the IHC data. TI analyzed the NanoString data. SE and ET performed and analyzed the qRT-PCR results. ET, SS, TI, and ST drafted the manuscript. All authors read and approved the manuscript.
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fmed.2017.00162/full#supplementary-material.

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Conflict of Interest Statement: TI and ST are inventors of the patent application Treatment of Vascular Anomalies (62/287657), 2016. The authors otherwise declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.