

# Prostate-specific membrane antigen expression in regeneration and repair

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Prostate-specific membrane antigen is a type II transmembrane glycoprotein, expressed in benign and neoplastic prostatic tissue as well as endothelial cells of neovasculature from a variety of tumors. The expression of prostate-specific membrane antigen in nonneoplastic neovasculature has not been well studied. Therefore, we studied nonneoplastic reparative and regenerative human tissues, as well as preneoplastic tissue, to determine the presence of prostate-specific membrane antigen-expressing neovasculature. Formalin-fixed paraffin-embedded tissue from keloids, granulation tissue from heart valves and pleura, proliferative and secretory endometrium, and Barrett's mucosa with and without dysplasia were stained for the expression of prostate-specific membrane antigen (3E6). Vessels of proliferative, mid-secretory, and late secretory endometrium were consistently strongly positive for prostate-specific membrane antigen expression in all ten cases of each type (100%). Vessels associated with granulation tissue from pleural peels and heart valves were positive in 10 of 12 cases (83%) and 7 of 10 cases (70%), respectively. Keloids had prostate-specific membrane antigen-expressing endothelial cells in 6 of 15 cases (40%). Prostate-specific membrane antigen was not expressed by vessels associated with Barrett's mucosa with low-grade dysplasia (12 foci), high-grade dysplasia (24 foci), or no dysplasia (18 foci). A variety of nonneoplastic neovasculature expresses prostate-specific membrane antigen, including vessels in proliferative endometrium, granulation tissue, and some scars. This is the first study showing that prostate-specific membrane antigen is expressed in neovasculature from physiologic regenerative and reparative conditions. The folate hydrolase activity of prostate-specific membrane antigen may facilitate vasculogenesis and angiogenesis by increasing local availability of folic acid. These findings will enhance our overall understanding of blood vessel development and will enable us to better understand the effects of anti-prostate-specific membrane antigen therapies, which are already being explored in clinical trials.

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Prostate-specific membrane antigen (PSMA) is a 100 kDa type II transmembrane glycoprotein with folate hydrolase<sup>1</sup> and neurocarboxypeptidase activity.<sup>2</sup> Originally, PSMA expression was identified in prostate cancer cells<sup>3</sup> and was subsequently shown to be consistently expressed in prostatic carcinoma as well as benign prostatic tissue.<sup>4</sup> Normal epithelium of benign tissue has also been shown to express PSMA, including epithelium of the duodenum, kidney, endometrium, and breast.<sup>5–7</sup>

PSMA has also been shown to be expressed in endothelial cells of neovasculature from a variety of tumors, including breast and renal cell carcinoma.<sup>8–10</sup> The role of PSMA in this setting has not yet been elucidated. Although PSMA was not expressed in choroidal neovascular membranes associated with vision loss and macular degeneration in one study,<sup>11</sup> PSMA expression has not been described in other tissues with nonneoplastic neovasculature. It is possible that the folate hydrolase activity of PSMA is important in facilitating vasculogenesis in these tissues, as well as in neoplastic neovasculature, by increasing the availability of folate, which can increase the levels of the proangiogenic molecule nitric oxide (NO) by regenerating the endothelial nitric oxide synthase (eNOS) cofactor, tetrahydrobiopterin (BH<sub>4</sub>).<sup>12</sup>

In this study, we investigate the expression of PSMA in neovasculature associated with nonneoplastic reparative and regenerative human tissues.

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Our novel findings of PSMA expression in these tissues will enhance our understanding of vasculogenesis in various disease states, as well as add to our understanding of the potential impacts of anti-PSMA therapies, some of which are already in clinical trials.<sup>13,14</sup>

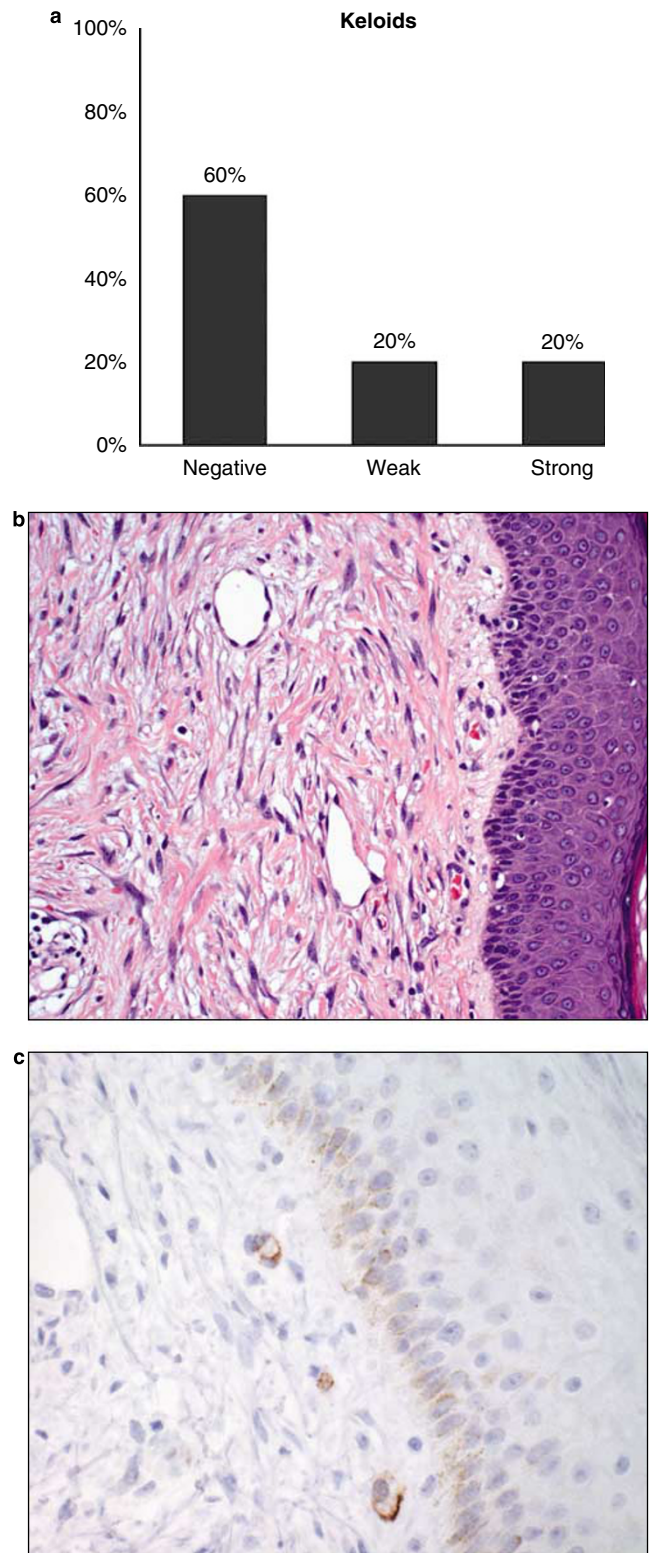
## Materials and methods

Using a protocol approved by the Institutional Review Board at the University of Chicago Medical Center, archival formalin-fixed paraffin-embedded tissue was obtained from keloids (15 cases), granulation tissue from heart valves (10 cases) and pleura (12 cases), proliferative endometrium (10 cases), and secretory endometrium (10 cases mid-secretory and 10 cases late secretory), as well as Barrett's mucosa with low- or high-grade dysplasia (12 foci and 24 foci, respectively) or no dysplasia (18 foci). Dysplastic foci in Barrett's mucosa were determined by three pathologists (IOG, AEN, and JH) who agreed on the diagnosis.

For immunohistochemical studies, 4  $\mu$ m sections were deparaffinized by two xylene rinses followed by two rinses with 100% ethanol. Antigen retrieval was performed by heating the slides in a microwave in a Coplin jar filled with EDTA buffer (pH 9.0). After rinsing briefly in 2  $\times$  Tris-buffered saline (TBS) containing 0.01% Triton at pH 8 (hereafter referred to as TBS rinses), slides were incubated for 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Slides were then incubated with 0.3% bovine serum albumin in 1  $\times$  TBS for 30 min at room temperature to reduce nonspecific background staining and then subjected to two TBS rinses. Slides were incubated for 1 h at room temperature with mouse anti-PSMA monoclonal antibody (clone 3E6; DAKO, Carpinteria, CA, USA; diluted 1:50). Slides were rinsed in TBS and incubated for 30 min with goat anti-mouse IgG conjugated to a horseradish peroxidase-labeled polymer (Envision + System; DAKO). This incubation was followed by TBS rinses, visualization with diaminobenzidine chromogen (DAKO), and then counterstained with hematoxylin. Prostate carcinoma tissue sections served as a positive control. Appropriate negative controls for the immunostaining were prepared by omitting the primary antibody step and substituting it with non-immune mouse serum. Slides were analyzed by light microscopy by two pathologists (IOG and HAA). PSMA expression in vessels was assessed, and any reactivity in vessels was considered positive. Staining intensity was scored on a scale of 0–2, where 0 indicated no staining, 1 indicated weak staining, and 2 indicated strong staining.

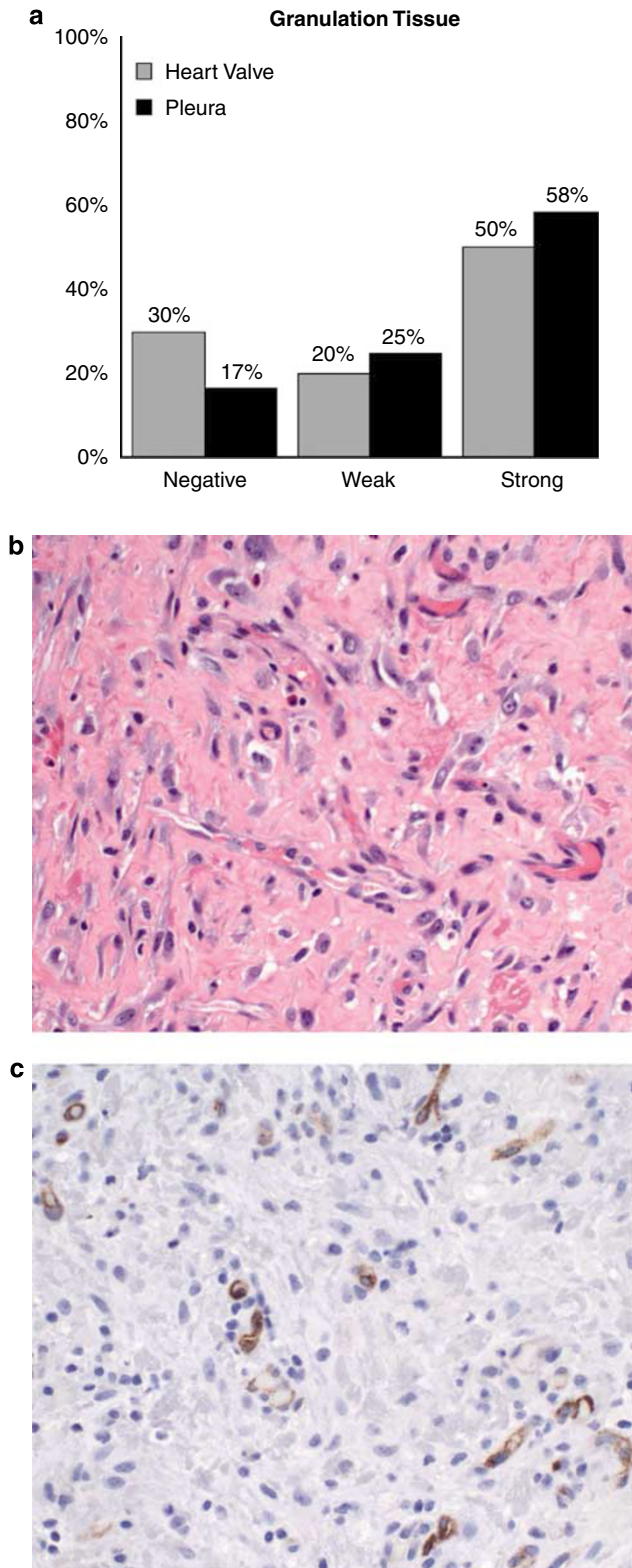
## Results

PSMA was expressed in the endothelial cells in 6 of 15 (40%) cases of keloids (Figure 1a–c). This



**Figure 1** PSMA expression in neovasculature associated with scar tissue (a); H&E (b) and PSMA immunohistochemistry (c).

expression was focal and limited to the papillary dermal capillaries. Half of the positive cases had weak expression, and half had strong expression. Endothelial cells associated with granulation tissue



**Figure 2** PSMA is expressed in granulation tissue neovasculature (a); pleural granulation tissue H&E (b) and PSMA immunohistochemistry (c).

in heart valves and pleura expressed PSMA in 7 of 10 (70%) and 10 of 12 (83%) cases, respectively (Figure 2a–c). Expression was limited to capillaries

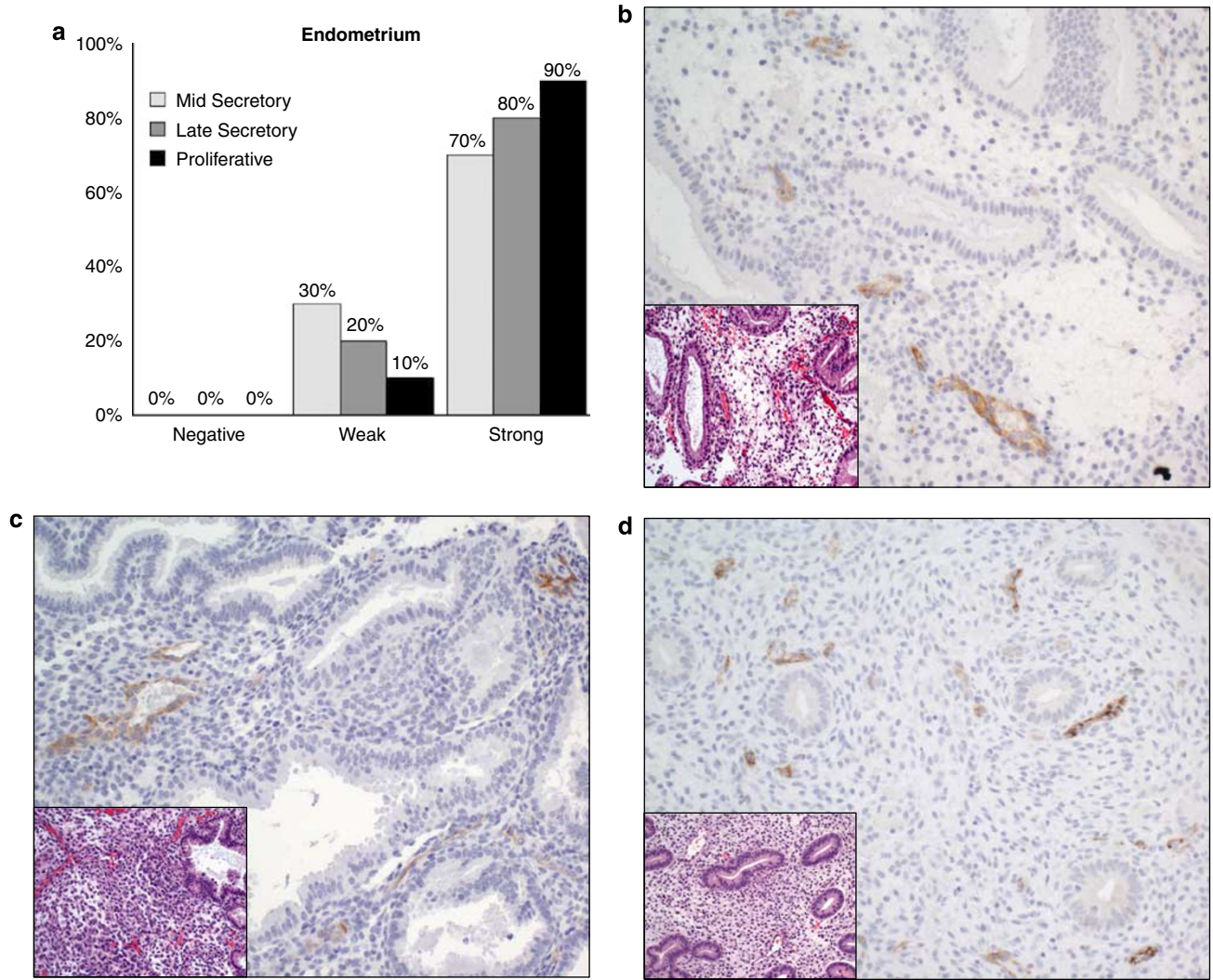
and was patchy in distribution. About 30% of the positive cases had weak expression, and 70% had strong expression. All cases of mid-secretory, late secretory, and proliferative endometrium had endothelial cell PSMA expression (10 of 10 or 100% cases of each type), most having strong immunoreactivity (7 of 10 or 70%, 8 of 10 or 80%, and 9 of 10 or 90% of cases, respectively; Figure 3a–d). PSMA expression was present in most small vessels in a diffuse pattern. Of note, one case of mid-secretory endometrium and two cases of proliferative endometrium had focal weak epithelial glandular expression of PSMA. PSMA was not expressed by vessels associated with Barrett’s mucosa with or without dysplasia, including away from the lesion (Table 1).

## Discussion

This is the first description of PSMA expression in neovasculature associated with nonneoplastic regenerative and reparative tissues. We found PSMA to be expressed by endothelial cells in keloids, granulation tissue from heart valves and pleura, and different phases of cycling endometrium. PSMA was not expressed by endothelium associated with Barrett’s mucosa, even in the presence of associated dysplasia. In this study, the strongest and most consistent expression of PSMA in endothelial cells of neovasculature was in the endometrium, especially in the proliferative phase. The vasculature of the cycling endometrium undergoes predictable periodical changes of proliferation and regression. It expresses the proangiogenic molecules of the vascular endothelial growth factor (VEGF) family,<sup>15</sup> and, interestingly, it has recently been shown that bone marrow-derived endothelial progenitor cells (EPCs) can contribute to the formation of endometrial neovasculature.<sup>16</sup> Although both vasculogenesis and angiogenesis have been extensively studied in wound healing and granulation tissue,<sup>17</sup> the exact mechanisms and pathways of neovasculature development are still unclear. Our novel finding has a variety of implications for our understanding of vasculogenesis and angiogenesis in physiologic regenerative and reparative conditions, and demonstrates that endothelial PSMA expression can occur in the absence of tumor-derived angiogenic factors.

### PSMA Expression in Tumor Neovasculature

Although its expression was once thought to be exclusive to prostate epithelium, PSMA has since been shown to be expressed in neovasculature associated with a variety of tumors. Silver *et al*<sup>6</sup> were the first to describe PSMA expression in capillary endothelium within and around renal cell carcinoma, transitional cell carcinoma of the bladder, and colonic adenocarcinoma. Subsequently, tumor neovasculature in non-small cell lung carci-

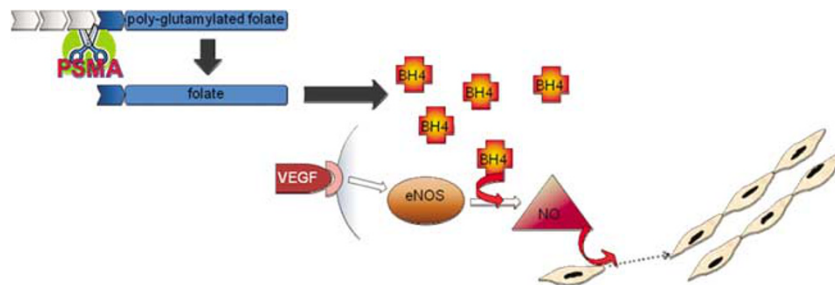


**Figure 3** PSMA is strongly expressed in endometrial neovasculature (a); PSMA immunohistochemistry with H&E inset of mid-secretory (b), late secretory (c), and proliferative (d) endometrium.

**Table 1** PSMA-expressing vessels in regeneration, repair, and dysplasia

	<i>Proliferative endometrium</i>	<i>Secretory endometrium</i>	<i>Pleural peel GT</i>	<i>Heart valve GT</i>	<i>Keloids</i>	<i>BM</i>	<i>BM-LGD</i>	<i>BM-HGD</i>
% of vessels with PSMA	100	100	83	70	40	0	0	0

GT: granulation tissue; BM: Barrett's mucosa; LGD: low-grade dysplasia; HGD: high-grade dysplasia; PSMA: prostate-specific membrane antigen.



**Figure 4** PSMA may facilitate vasculogenesis in reparative and regenerative tissues by increasing the availability of folate, which regenerates the eNOS cofactor BH<sub>4</sub>, permitting NO production and recruitment of endothelial progenitor cells.

noma, breast carcinoma, prostate carcinoma, neuroendocrine carcinoma, pancreatic ductal carcinoma, glioblastoma multiforme, melanoma, soft tissue sarcoma, and testicular embryonal carcinoma was found to have endothelial PSMA expression in most cases.<sup>8,9</sup> Further studies by Chang *et al*<sup>18</sup> demonstrated PSMA mRNA in tumor endothelial cells, confirming that endothelial cells produce PSMA. Interestingly, no expression of PSMA was found in neoplastic endothelial cells in a number of vascular tumors.<sup>18</sup> Because capillaries in adjacent normal epithelium did not express PSMA,<sup>6,8,9,18</sup> it was hypothesized that PSMA expression in tumor-associated neovasculature was dependent on tumor-associated angiogenic factors.<sup>11</sup>

### Vasculogenesis and the Role of eNOS and NO

Our current understanding of vasculogenesis has developed rapidly over the past few years, and the characterization of EPCs, which migrate from the bone marrow in response to vasculogenic signaling molecules, is the subject of several excellent review articles.<sup>17,19–24</sup> The role of VEGF in angiogenesis is well known, and its role in vasculogenesis has recently been elucidated (reviewed in Duda *et al*<sup>25</sup>). Angiogenic growth factors, including VEGF, signal through their receptors to activate eNOS, which catalyzes the breakdown of L-arginine to L-citrulline and NO (Figure 4). The importance of both eNOS and NO in EPC mobilization and vasculogenesis has recently been characterized in the setting of diabetic wound healing<sup>21,23</sup> and in asthma vascular remodeling.<sup>26</sup> Inhibitors of eNOS decrease VEGF-induced angiogenesis.<sup>27,28</sup> In addition, ischemia-induced angiogenesis is reduced in eNOS knockout mice,<sup>29</sup> and in rat models, oral folate can rescue this process in a NO-dependent manner.<sup>30</sup>

eNOS production of NO depends on the cofactor BH<sub>4</sub>.<sup>31</sup> The active form of folic acid, 5-methyltetrahydrofolate, restores BH<sub>4</sub> to allow participation in eNOS reactions.<sup>12</sup> When BH<sub>4</sub> is not available, eNOS generates superoxide radicals,<sup>31</sup> which impair endothelial proliferation.<sup>32,33</sup> Folic acid and its metabolites are also superoxide radical scavengers.<sup>34,35</sup>

### The Role of PSMA in Vasculogenesis

Cellular folate gradients across cell membranes are maintained by the  $\gamma$ -glutamylated form, polyglutamyl folate, which accumulates intracellularly, and must be deconjugated by pteroylpoly- $\gamma$ -glutamate hydrolase, also called folate hydrolase, into a monoglutamylated form to participate in metabolic reactions.<sup>36</sup> PSMA has folate hydrolase activity.<sup>1</sup> Therefore, our findings of PSMA expression in endothelial cells associated with neovasculature may indicate that it is functioning to increase folic acid levels at the site of neovasculature formation,

so that folic acid can exert its proangiogenic effects (Figure 4). In support of this, PSMA has been reported to coimmunoprecipitate with the caveolae marker, caveolin 1,<sup>37</sup> which also coimmunoprecipitates with eNOS,<sup>38,39</sup> suggesting the *in vivo* proximity of PSMA and eNOS.

Explanations for the role of PSMA in angiogenesis have been studied in the context of its expression in tumor-associated neovasculature. Conway *et al*<sup>40</sup> found that PSMA participates in an autoregulatory loop involving  $\beta$ 1-integrin and p21-activated kinase 1 (PAK1) which is necessary for angiogenesis and endothelial cell invasion. Our current hypothesis complements these findings in proposing an alternative role for PSMA in neovasculature development, and specifically in vasculogenesis. It is apparent that PSMA does not play a role in all types or stages of neovasculature formation, because we did not find it to be expressed in Barrett's mucosa, with or without dysplasia, conditions known to have new vessel formation.<sup>41</sup>

### Implications of Current Anti-PSMA Therapies

The strong expression of PSMA in neoplastic prostatic epithelium has led rapidly to clinical trials investigating the therapeutic use of anti-PSMA antibodies. J591 is a humanized monoclonal antibody to PSMA, which is currently being evaluated in clinical trials as a treatment modality for metastatic prostate cancer therapy.<sup>13,14,42</sup> Both immunotherapy and targeted radiotherapy with J591 are being evaluated, and have thus far shown limited toxicity and no adverse immunologic effects.<sup>13,14</sup> These trials were undertaken when endothelial PSMA expression was thought to be limited to neoplastic neovasculature. Our current finding of PSMA expression in nonneoplastic neovasculature, and its likely role in vasculogenesis and angiogenesis associated with reparative and regenerative conditions, is important to consider when determining the enrollment criteria for clinical trials using anti-PSMA therapies, such as regarding patients with cardiovascular disease, diabetes, and asthma.

### Summary

In summary, we found PSMA expression in endothelial cells of physiologic regenerative and reparative neovasculature, which has not been described previously. This is also the first study to show endothelial PSMA expression outside of a tumor and in the absence of tumor-derived angiogenic factors. We hypothesize that in regenerative and reparative tissues, the folate hydrolase activity of PSMA increases the local availability of folic acid, thereby increasing the amount of BH<sub>4</sub> available allowing for the VEGF-mediated production of

NOS by eNOS, resulting in vasculogenesis and angiogenesis.

## Disclosure/conflict of interest

The authors do not have any conflict of interest in the material presented.

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