

# Decreased eNOS Protein Expression in Involuting and Propranolol-Treated Hemangiomas

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**Objective:** To examine the location and degree of endothelial nitric oxide synthase (eNOS) protein expression in hemangioma growth, involution, and during propranolol therapy.

**Design:** Cross-sectional study.

**Setting:** University hospital.

**Patients:** Pediatric patients with hemangiomas.

**Interventions:** Fresh human hemangioma specimens at various stages of development were harvested. Effective propranolol therapy had been implemented in some patients. Quantitative assessment and localization of eNOS protein expression was performed on each specimen by Western blot analysis and immunohistochemical analysis, respectively.

**Results:** Hemangiomas in a proliferative phase (group 1: n=4; mean [SD] age, 4.25 [2.06] months), an early involuting phase (group 2: n=6; 12.00 [1.64] months), and

a late involuting phase (group 3: n=6; 23.30 [1.97] months) were harvested. The mean (SD) eNOS protein expression was 0.88 (0.41) in group 1, 0.26 (0.26) in group 2, and 0.15 (0.08) in group 3, respectively. A statistically significant decrease in eNOS protein expression was observed between proliferating and involuting hemangiomas (group 1 vs group 2 and group 3;  $P \leq .01$ ) but not between early and late phases of involution ( $P = .17$ ). In a separate propranolol treatment group (n=7), the eNOS protein level was significantly lower than in age-matched controls (n=7; 0.08 [0.1] vs 0.45 [0.45];  $P = .03$ ). Immunohistochemical analysis demonstrated eNOS to be predominately in endothelial cells lining mature blood vessels.

**Conclusion:** Expression of eNOS protein decreases during the hemangioma lifecycle. Propranolol may suppress hemangioma growth by inhibiting expression of eNOS protein and subsequent production of nitric oxide.

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**H**EMANGIOMAS ARE NONMALIGNANT vascular tumors, composed of vascular endothelial cells, that undergo a unique growth and resolution phase. They are the most common vascular anomaly in infants and children.<sup>1</sup> They affect nearly 2% to 3% of neonates at birth and 10% of all infants by 12 months of age. Hemangioma development comprises essentially 3 phases, namely proliferation, quiescence, and involution. During the proliferative phase hemangiomas grow by rapid endothelial cell division, with the fastest component occurring at 3 to 6 months of life. Hemangiomas subsequently enter a slower, albeit continuous, phase of growth at around 6 to 9 months age. By 12 months of age, most hemangiomas have reached their maximal size and have ceased to grow (qui-

escence). At 12 to 18 months of age, hemangiomas then enter into an involution phase of gradual loss of volume and replacement of endothelial cells with fibrofatty tissue.<sup>2</sup>

Many hemangiomas grow uneventfully and are left untreated to later undergo their natural involution phase. Unfortunately, some patients require early intervention when function and form are compromised by rapid growth. Ulceration, bleeding, compression of vital organs, massive size, and expectant scarring are typical events that prompt urgent therapy for hemangiomas. Sensitive anatomic sites for hemangioma development include the periorbital, pelvis, perineum, lumbosacrum, face, ear, and airway. Traditional treatment protocols have included surgical resection and nonsurgical approaches, such as intralesional in-

jection of corticosteroids, chemotherapeutic agents (vincristine sulfate, interferon alfa), and laser therapy. Recently, Léauté-Labrèze et al,<sup>3</sup> for the first time, reported that propranolol can dramatically inhibit hemangioma growth. Much interest in propranolol therapy for hemangiomas has ensued since this original article. Similarly, our group demonstrated a 97% effective rate in reducing the size and growth of proliferating and involuting hemangiomas with low-dose propranolol.<sup>4,5</sup> However, minor surgical treatments were still necessary in nearly half of these patients.

Many related growth factors and receptors likely contribute to hemangioma proliferation and involution. In cultured hemangioma-derived endothelial cells and hemangioma tissues, fetal liver kinase-1 (Flk-1)/vascular endothelial growth factor receptor 2 (VEGFR-2), VEGFR-1, tunica interna endothelial cell kinase-1 (Tie-1), Tie-2, and angiopoietin-2 are strongly expressed.<sup>6</sup> VEGF expression is reduced in involuting hemangioma.<sup>7</sup> In addition to VEGF, hemangioma cells express high levels of several cellular markers, including proliferating cell nuclear antigen, E-selectin, and basic fibroblast growth factors.<sup>8-10</sup> Insulin-like growth factor-2 (IGF-2) expression is also upregulated in proliferating hemangiomas, indicating a role of IGF-2 in hemangioma growth.<sup>11</sup> While apoptosis, known as programmed cell death, is thought to be critical during the involution of hemangiomas.<sup>12</sup>

Evidence suggests that regulation of apoptosis and production of vascular associated growth factors is controlled by release of nitric oxide, a potent endothelial cell mitogen, vascular permeability factor, and vessel dilator.<sup>13</sup> Its role in angiogenesis, vasculogenesis, and vascular remodeling is well established. Three enzymes produce nitric oxide and include neuronal, inducible, and endothelial nitric oxide synthase. Primary production and release of nitric oxide is performed by endothelial nitric oxide synthase (eNOS). Because of nitric oxide's role in vascular development, we hypothesized that its constitutive production, by eNOS, may help regulate the hemangioma lifecycle and possibly the impact of propranolol therapy. Using fresh human hemangioma specimens from children of various ages, we explored this relationship through quantitative and qualitative measurement of eNOS during proliferation, involution, and propranolol therapy.

## METHODS

### PATIENT TISSUE COLLECTION AND PRESERVATION

This study was approved by the institutional review board of the University of Arkansas for Medical Sciences, Little Rock. Fresh tissue was harvested upon the surgical removal or sampling of hemangioma after written informed consent was obtained from the patient's family. Following excision, the central core of each hemangioma was isolated and processed for experimental analysis. This provided some consistency in tissue sampling and removed any potential char debris from the specimen. Adjacent normal tissues in some patients with hemangiomas were harvested to provide control tissue specimens. Upon harvest, the tissue was immediately divided into 2 sec-

tions with 1 portion stored at  $-80^{\circ}\text{C}$  and the remaining portion fixed in 10% formalin (pH, 7.0) and embedded in paraffin for further analysis. Tissue sections were stained with hematoxylin-eosin for routine histologic examination.

### WESTERN BLOT ANALYSIS

Total proteins were extracted from 10 mg of frozen hemangioma tissue with 200  $\mu\text{L}$  of T-PER tissue protein extraction reagent (Pierce, Rockford, Illinois) added with Mini Protease Inhibitor Cocktail (Roche, Indianapolis, Indiana). Thirty micrograms of the total protein was loaded onto NuPAGE 4–12% Bis-Tri gels (Invitrogen, Carlsbad, California) for electrophoresis, transferred to a nitrocellulose membrane, and probed with either polyclonal rabbit antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz, California) at 1:200 dilution, polyclonal rabbit antibody against CD31 (Santa Cruz Biotechnology) at 1:500 dilution or monoclonal mouse antibody against  $\beta$ -actin (Santa Cruz Biotechnology) at 1:1000 dilution. The blot was incubated with a horseradish peroxidase (HRP)-conjugated either goat anti-rabbit IgG (Invitrogen) or goat anti-mouse IgG (Invitrogen), and the protein was visualized by using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). The eNOS protein expression level was semiquantitatively assessed, compared to  $\beta$ -actin level, by using NIH software Image J (National Institutes of Health).

### IMMUNOHISTOCHEMICAL ANALYSIS

To examine eNOS protein expression and its location, formalin-fixed and paraffin-processed sections (5  $\mu\text{m}$ ) of hemangioma tissues were deparaffinized and rehydrated. Epitope retrieval was performed by steaming sections for 20 minutes in citrate buffer (pH, 6.0). After quenching the endogenous peroxidase activity and blocking the nonspecific binding sites, sections were incubated overnight with polyclonal rabbit antibody against eNOS (Santa Cruz Biotechnology) at 1:500 dilution at  $4^{\circ}\text{C}$ . In the negative control slide, no primary antibody was applied. The mouse brain tissue was used as positive control as suggested by Santa Cruz Biotechnology. After washing with PBS, the antibody staining was visualized by using UltraVision Detection System (Fisher Scientific, Hampton, New Hampshire). The staining results were validated by a blind review performed by a pathologist (C.-Y.F. or A.S.) who has rich experience with vascular anomalies and immunohistochemical analysis.

### STATISTICAL ANALYSIS

Western blotting results were expressed as means (SDs), and the difference between any 2 groups was calculated with *t* test.  $P < .05$  was set as statistical significance.

## RESULTS

All hemangioma tissues were confirmed by separate pathological examination at our institution. Based on the clinical characteristics and patient age of harvested specimens (**Table**), all patients untreated with propranolol were divided into 3 groups, including hemangiomas in a proliferative phase (group 1:  $n=4$ ; mean [SD] age, 4.25 [2.06] months), an early involuting phase (group 2:  $n=6$ ; 12.00 [1.64] months), and a late involuting phase (group 3:  $n=6$ ; 23.30 [1.97] months). In addition, to compare eNOS expression in the patients whose hemangioma were treated with propranolol with those untreated with propranolol,

**Table. Characteristics of Patients With Hemangiomas<sup>a</sup>**

Patient No./Sex/Age, mo	Lesion Location
Patients not treated with propranolol	
1/M/4	Forehead
2/M/7	Right cheek
3/F/2	Head
4/F/4	Posterior scalp
5/M/11	Forehead
6/F/12	Right arm
7/F/12	Forehead
8/F/15	Scalp
9/F/14	Nasal tip
10/M/11	Right postauricular
11/M/20	Scalp
12/F/22	Right lower lip
13/F/24	Right groin/thigh
14/F/25	Right neck
15/F/25	Right intraoral
16/M/24	Upper lip
Patients treated with propranolol	
17/F/5	Nasal bridge
18/F/9	Right cheek
19/M/12	Postauricular scalp
20/M/12	Nasal tip
21/F/13	Right cheek
22/F/14	Nasal tip
23/M/16	Right temple

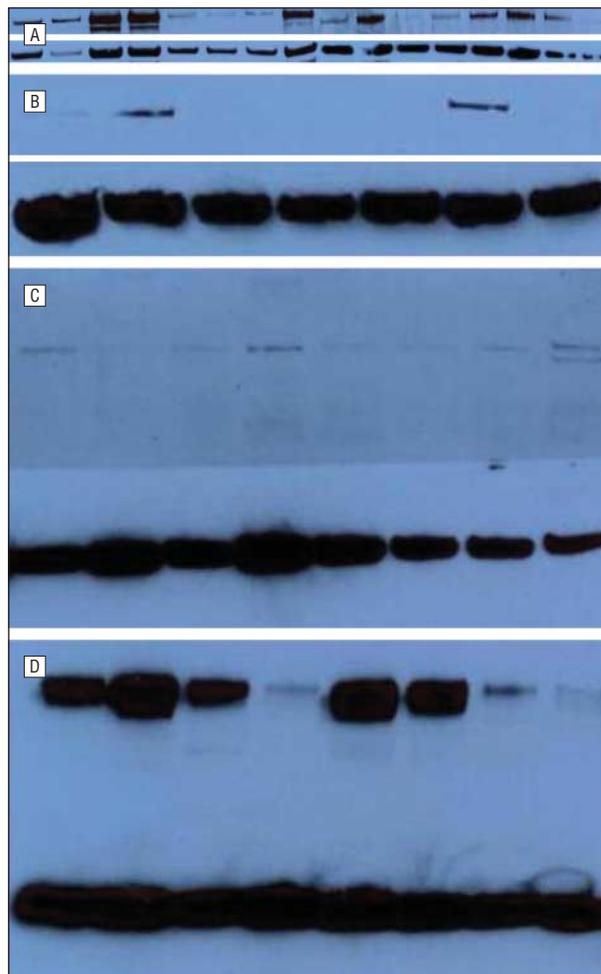
<sup>a</sup>Group 1 includes patients 1 to 4; group 2, patients 5 to 10; and group 3, patients 11 to 16. All lesions were focal.

2 groups were established. One was a control group (n=7; age, 10.9 [4.1] months), in which patients did not receive propranolol treatment, another 1-treatment group (n=7; age, 11.6 [3.6] months), in which patients were treated with propranolol. There is no statistical difference between these 2 groups in terms of the age.

Examination of the patients providing propranolol-treated specimens revealed that surgical removal was performed due to either incomplete response (4 patients) or intolerance of therapy or adverse effects (3 patients). Propranolol therapy was continued at, or near, the time of excision.

Semiquantitative analysis of eNOS protein expression was performed by Western blot analysis. With  $\beta$ -actin being the loading control, the mean (SD) eNOS protein level was 0.88 (0.41) in group 1, 0.26 (0.26) in group 2, and 0.15 (0.08) in group 3, respectively. A statistically significant decrease in eNOS protein expression was observed between proliferating and involuting hemangiomas (for group 1 vs group 2,  $P=.01$ ; for group 3,  $P=.001$ ) but not among early and late phases of involution ( $P=.17$ ) (Figure 1A and Figure 2A). In the propranolol treatment group, eNOS protein level was significantly lower than in the control group (treatment group, 0.08 [0.1]; control group, 0.45 [0.45];  $P=.03$ ) (Figure 1B and Figure 2B).

Normal specimens were harvested adjacent to hemangioma tissue as a representation of normal skin and subcutaneous tissue. Western blot analysis revealed bands so weak that detection was too difficult for comparison with the hemangioma results. In essence, eNOS expression was represented by very weak bands indicating very low levels (Figure 1C). To determine if patterns in endothelial



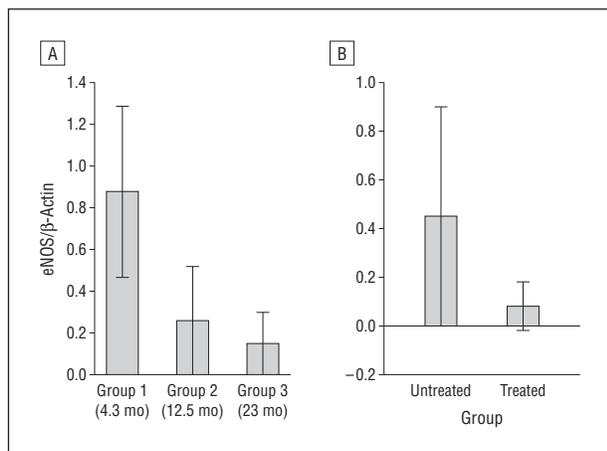
**Figure 1.** Endothelial nitric oxide synthase (eNOS) protein expression was detected by Western blot analysis. A, Patients without treatment of propranolol in different age groups. B, Patients treated with propranolol.  $\beta$ -Actin was used for the loading control as well as the control for semiquantization of eNOS protein level. C, Normal tissues from patients with hemangiomas. D, CD31 protein expression was detected by Western blot analysis.

cell concentration affected eNOS results, CD31, a known endothelial cell marker, was examined by Western blot analysis. CD31 expression level demonstrated considerable variability among the various age groups of hemangiomas excised (Figure 1D). No consistent changes in CD31 expression were correlated with age or treatment.

To examine the location and distribution of eNOS protein, hemangioma tissues were stained using a specific eNOS antibody. Like the positive control that is positively stained for eNOS in the neuron cell cytoplasm, eNOS was expressed mainly in the cytoplasm of clumped endothelial cells and primitive hemangioma vasculature. In some vessels that appeared normal in development, a few endothelial cells also stained positively. Smooth muscle and stromal cells were nonreactive to this antibody (Figure 3).

#### COMMENT

Nitric oxide synthases (NOS) are a family of enzymes that are responsible for production of nitric oxide from



**Figure 2.** The mean ratios of the intensity of the protein band of endothelial nitric oxide synthase to  $\beta$ -actin by Western blot analysis was calculated by Image J Software. The bars indicate standard deviations. A,  $P = .01$  for group 1 vs group 2;  $P = .001$  for group 1 vs group 3;  $P = .17$  for group 2 vs group 3. B,  $P = .03$  for the untreated group vs the treated group.

L-arginine. So far, 3 major isoforms of NOS have been found, including neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS 2), and endothelial NOS (eNOS or NOS3).<sup>14</sup> eNOS is mainly expressed in the endothelial cells playing a key role in both angiogenesis and vasculogenesis. eNOS is activated by multiple factors, which include the laminar shear stress causing phosphorylation of serine residues of eNOS; the increased cytoplasmic calcium level activating calmodulin that phosphorylates serine residue 1179 of eNOS after the stimuli of VEGF, estrogen, bradykinin and so forth; and the metabolic stress that also causes S1179 phosphorylation of eNOS via activated adenosine monophosphate kinase.<sup>13,15-19</sup> The activated eNOS has variable complex functions in both physiologic and pathologic conditions. It produces nitric oxide that plays a critical role in regulating blood vessel tone and blood flow, inhibiting vascular smooth muscle cell proliferation, and modulating the interaction of endothelium with leukocyte.<sup>20</sup> However, the role of eNOS is not limited to the cardiovascular system. In an eNOS knockout mice model, it was found that macrophage eNOS regulates proinflammatory gene expression such as nuclear factor- $\kappa$ B (NF- $\kappa$ B).<sup>21</sup> In addition, as reviewed by Ying et al,<sup>22</sup> eNOS/nitric oxide pathway closely modulate events in tumors, including promoting angiogenesis, antiapoptosis in tumor epithelial cells, stimulating cancer cell cycle progression and proliferation and enhancing tumor cell vascular invasion.

Hemangioma development mainly comprises 2 phases of growth. In the proliferation phase, hemangioma is predominately composed of immature hemangioma endothelial cells that are dividing rapidly. In the involuting phase, the endothelial cells are replaced by mature vascular channels and fibrofatty tissue in a slow but continuous process that can last years.<sup>23</sup> Because many vascular growth factors and receptors likely contribute to hemangioma development, we sought to investigate the role of eNOS, a key regulator of endothelial cell growth and proliferation, in hemangioma proliferation and involution. At the same time, propranolol was fast becoming a popular route for treating problematic hemangiomas

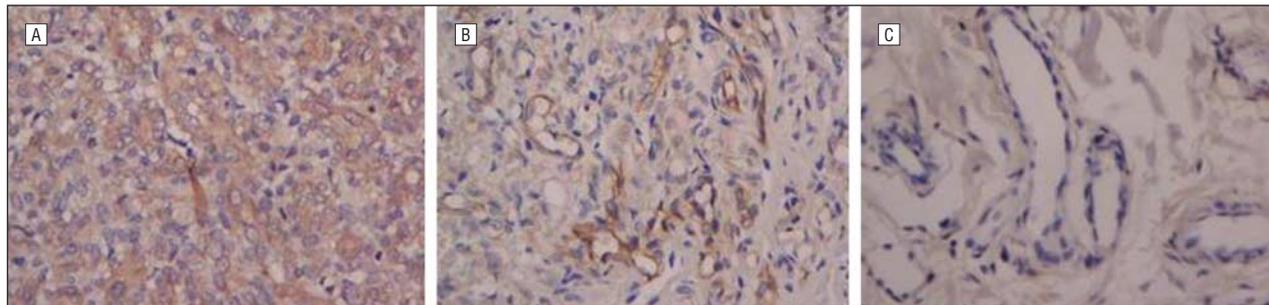
at our institution and many others owing to its profound impact on early growth.<sup>3,5</sup>

In this study, we demonstrate eNOS protein level to be 3.4 times greater in proliferative phase hemangiomas compared with their involuting counterparts. More important, a consistent and gradual reduction in eNOS protein level is seen during later stages of involution. Immunohistochemical staining shows eNOS protein to be predominantly expressed in the cytoplasm of hemangioma endothelial cells. These findings suggest that a high level of eNOS, and subsequent NO production, may be a major contributor to the rapid growth of hemangioma endothelial cells in proliferative phase. Although the exact role of eNOS is unknown, it is possible that eNOS is part of the VEGF pathway regulating hemangioma endothelial cell division. VEGF has been shown to upregulate eNOS messenger RNA and protein expression,<sup>24</sup> while it is also detected at higher levels in proliferative than in involuting hemangiomas. Similarly, VEGF has been shown to promote proliferation of hemangioma vascular endothelial cells in vitro.<sup>25,26</sup>

To examine the possibility that alterations in eNOS expression was related to a change in endothelial cell concentration, we checked expression of CD31, an endothelial cell marker, by Western blot analysis. The goal was to essentially normalize the denominator as the number of endothelial cells within the specimens tested. There was considerable variability of CD31 levels among the hemangioma specimens (Figure 1D) that was not consistent with age or stage of involution. This result suggests that despite changes in the endothelial cell density of the hemangioma architecture, changes are not predictable based on age. This further implies that changes in eNOS expression are not directly related to presumed changes in endothelial cell density at various stages of a hemangioma's lifecycle.

More important, and to the point, even if endothelial cell density decreased with involution or propranolol therapy, this does not necessarily imply a direct impact on the amount of eNOS expression. A high level of eNOS expression could be stimulated in a small number of endothelial cells and vice versa (a low level of expression in high number of cells) depending on the upstream regulators. In essence, alterations in eNOS expression is not necessarily linked to a change in the number of endothelial cells.

Propranolol, a nonselective  $\beta$ -blocker, has been quickly adopted as the newest and most effective therapeutic approach to problematic hemangiomas. Propranolol is now used internationally for treating hemangiomas owing to its remarkable effect in shrinking hemangiomas and a limited adverse effect profile.<sup>3</sup> Although several hypotheses have been proposed regarding propranolol's mechanism in reducing hemangiomas, most have not been directly confirmed by laboratory evidence. We are of the fundamental belief that a key factor in propranolol's effectiveness is hemangioma hemodynamics and cellular proliferation. Both of these mechanism are controlled, somewhat, by the release of nitric oxide. Various levels of this potent cytokine have an impact on regulation of vascular permeability, dilation, and endothelial cell growth.



**Figure 3.** Endothelial nitric oxide synthase (eNOS) detection by immunohistochemical analysis (IHC) demonstrates specific expression in endothelial cells. Reductions in eNOS protein expression are incidentally evident by IHC in sequentially older patients: 7 months (A), 9 months (B), and 15 months (C). Samples were stained with polyclonal rabbit antihuman eNOS antibody (original magnification  $\times 400$ ). Quantification by IHC was not performed.

The present study is the first report, to our knowledge, on the reduction of eNOS protein by propranolol therapy. Similar to involuting specimens, a significant decrease in the expression of eNOS in hemangiomas was discovered in patients treated with propranolol compared with the age-matched controls. Both groups were in the same phase of the hemangioma lifecycle (mean age, 10-11 months) so the effect of age and involution on eNOS expression could be considered negligible. Similarly, specimens examined in this study had a good response to propranolol but required definitive management owing to drug intolerance or tissue residuum. Thus, the eNOS expression determined in these tissues adequately represents levels following the successful impact of propranolol therapy on hemangioma.

Furthermore, the results from this study are similar to those seen in a recent rat model of cirrhosis examining eNOS expression following propranolol therapy. In this model, propranolol decreased eNOS activity by 47% and eNOS expression by 75%.<sup>27</sup> We thereby suspect that the effect of propranolol in hemangiomas may be partially due to the decreased expression and activity of eNOS. A low level of eNOS can be a direct correlate to the reduced NO production leading to vasoconstriction and reduced vascular tone. Physical evidence of this physiologic characteristic is present in treated lesions as they become softer and smaller within few weeks of administration.<sup>3,28</sup> In addition, decreased eNOS can theoretically impair hemangioma endothelial cell proliferation and provide long-term control of hemangioma growth.

Despite these results, the study is limited in providing a clear picture on how reduced eNOS expression controls hemangioma proliferation and stability. Further investigation on the direct role of nitric oxide on hemangioma endothelial division and differentiation is necessary through in vitro and in vivo analysis before broader conclusions can be made. Inhibition of eNOS during such work may further elucidate the role of this ubiquitous enzyme in hemangiomas. Furthermore, we recognize this as pilot work in examining the complex array of cytokines involved in hemangioma regression and the mechanism of propranolol in treating this common birthmark. Numerous factors involved in vascular biology and development undoubtedly contribute to the chemical cascade and cellular response that leads to hemangioma involution. Increasing our sample size and exploring upstream and downstream regulators of

eNOS expression is necessary to support the results from this study.

In conclusion, to our knowledge, this study is the first to suggest a potential role for eNOS expression, and subsequent nitric oxide production, in the life cycle of hemangioma development. Reduced levels of eNOS in involuting and propranolol-treated hemangiomas point to important hemodynamic and endothelial cell processes involved in hemangioma development. This study alludes to the mechanism by which propranolol has an impact on hemangioma dissolution.

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**Author Contributions:** Drs Dai, Hou, Fan, Saad, Suen, and Richter had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Richter. *Acquisition of data:* Dai, Hou, Buckmiller, and Richter. *Analysis and interpretation of data:* Dai, Fan, Saad, Suen, and Richter. *Drafting of the manuscript:* Dai and Richter. *Critical revision of the manuscript for important intellectual content:* Hou, Buckmiller, Fan, Saad, and Suen. *Statistical analysis:* Dai and Richter. *Obtained funding:* Suen. *Administrative, technical, and material support:* Dai, Hou, Suen, and Richter. *Study supervision:* Fan and Saad.

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