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Propranolol Targets Contractility of Infantile Hemangioma-derived Pericytes

D. Lee¹, E. Boscolo^{1,2}, J.T. Durham³, J.B. Mulliken^{2,4}, I.M. Herman³, and J. Bischoff^{1,2}

¹Vascular Biology Program and Department of Surgery, Boston Children's Hospital, Boston, MA

²Department of Surgery, Harvard Medical School, Boston, MA

³Graduate Program in Cellular and Molecular Physiology, Sackler School of Graduate Biomedical Sciences and Department of Integrative Physiology and Pathology, School of Medicine, Tufts University, Boston, MA

⁴Department of Plastic and Oral Surgery, Boston Children's Hospital Boston, MA

Abstract

Propranolol, a β -adrenergic receptor (AR) antagonist, was discovered serendipitously to be an effective treatment for endangering infantile hemangioma (IH). Dramatic fading of cutaneous color is often seen a short time after initiating propranolol therapy, with accelerated regression of IH blood vessels discerned after weeks to months. Here we focus on hemangioma-derived pericytes (HemPericytes) isolated from proliferating and involuting phase tumors to assess a possible role for these cells in the apparent propranolol-induced vasoconstriction. HemPericytes express high levels of β_2 AR mRNA, compared to positive control bladder smooth muscle cells. In addition, β_2 AR mRNA levels were relatively high in IH specimens (n=15) compared to β_1 AR, β_3 AR and α_{1b} AR. HemPericytes were assayed for contractility on a deformable silicone substrate: propranolol (10 μ M) restored basal contractile levels in HemPericytes that were relaxed with the AR agonist epinephrine. siRNA knockdown β_2 AR blunted this response. Normal human retinal and placental pericytes were not affected by epinephrine or propranolol in this assay. Propranolol (10 μ M) inhibited proliferation of HemPericytes *in vitro*, as well as normal pericytes, indicating a non-selective effect in this assay. HemPericytes and HemEC were co-implanted subcutaneously in nude mice to form blood vessels, and at day 7 after injection, mice were randomized into vehicle and propranolol treated groups. Contrast-enhanced micro-ultrasonography of the implants after 7 days of treatment showed significantly decreased vascular volume in propranolol-treated animals, but no reduction in vehicle-treated animals. These findings suggest that the mechanism of propranolol's effect on proliferating IH involves increased pericytic contractility.

Introduction

Infantile hemangioma (IH) is a benign vascular tumor that occurs in 4-5% of infants ¹. The tumor follows a unique life-cycle of rapid growth, called the proliferating phase, followed

Corresponding Author: Joyce Bischoff, PhD, Boston Children's Hospital, 300 Longwood Ave, Boston, MA 02115, Phone: 617-919-2192, Fax: 617-730-0231, joyce.bischoff@childrens.harvard.edu.

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by a slow spontaneous involuting phase. This sequence of abnormal vascular growth followed by regression has long fascinated researchers in the field of angiogenesis as a potential wellspring for clues to the regulation of human vascular development and post-natal angiogenesis. For most children, IH does not pose a serious threat and therapy is unnecessary; however, in some instances, IH can enlarge dramatically, threaten vital organs and cause permanent disfigurement. Corticosteroid therapy had been the mainstay treatment until the discovery that oral propranolol accelerates the regression of IH ². Within a relatively short period of time, propranolol has replaced corticosteroid as the first-line therapy for problematic IH. Knowledge of the cellular and molecular mechanisms by which propranolol exerts its effect might lead to further improvements to shorten the duration of treatment, prevent the rebound growth that occurs in some cases ³ and help understand why some IH do not respond to propranolol therapy ⁴.

Storch and Hoeger reviewed three broad mechanisms by which propranolol might act on IH ⁵. First, propranolol might antagonize β -AR-mediated vasodilation, leading to vasoconstriction of IH vessels, which would explain the rapid reduction in volume and color seen with propranolol therapy ⁶. Second, propranolol might block VEGF-mediated angiogenesis, since β -AR signaling has been shown to increase VEGF-A expression ⁷. Third, propranolol might increase apoptosis, as β -AR agonists have been shown to inhibit apoptosis in many different cell types⁵. This would be consistent with the accelerated onset of involution observed in IH patients on propranolol therapy.

In support of these mechanisms, β -ARs have been detected in IH tumor specimens ^{8,9,10} and hemangioma-derived endothelial cells (HemEC) and hemangioma-derived stem cells (HemSC) ¹⁰⁻¹². These reports, combined with data in Figure 1, document the presence of the critical receptor targets for propranolol in IH, establishing a basis for functional studies.

Two groups showed that high concentrations of propranolol (100-300 μ M) reduced VEGF-A levels in HemEC^{13,14}. Chim et. al. also found that propranolol reduced hypoxia inducible factor-1 α and matrix metalloproteinase-2 ¹³. Ji and colleagues reported that propranolol decreased proliferation, induced cleaved caspase-9 and caspase-3 and increased pro-apoptotic proteins p53 and Bax in HemEC ⁹. Wong and coworkers confirmed the increased apoptosis ¹⁴, but other investigators found no increases in apoptosis of HemEC treated with propranolol ¹⁵.

In a later study, Ji and colleagues showed that a β_2 -selective antagonist ICI 118551 (10 μ M) was more effective than the β_1 -selective antagonist metoprolol (10 μ M) in blocking β -AR agonist- stimulated HemEC proliferation ¹¹, suggesting an important role for β_2 AR in this context. Of note, many reports on IH-derived cells studied effects of propranolol in the absence of added agonists. Thus, propranolol may be active as an inverse agonist, that is, inhibiting basal levels of β -AR signaling, as has been shown in other settings ^{16,17}.

We previously showed that HemPericytes from proliferating and involuting phase IH have a reduced contractile activity *in vitro* and are pro-angiogenic compared to normal human pericytes ¹⁸. Herein, we analyzed: 1) expression of β -ARs in HemPericytes, 2) contractile activity of HemPericytes in response to propranolol in the presence/absence of the β -AR

agonist epinephrine, 3) proliferation of HemPericytes in presence of propranolol and 4) effect of a one week propranolol treatment on blood vessels assembled from HemPericytes and HemEC in mice. Our findings indicate that propranolol reverses epinephrine-induced relaxation of HemPericytes *in vitro* and reduces the vascular volume of HemPericyte/HemEC-derived vessels *in vivo*. The propranolol-mediated increased contractility of hemangioma pericytes could explain the apparent vasoconstriction of IH vessels after the initiation of propranolol therapy.

Materials and Methods

In vitro culture of HemPericytes, HemEC and HemSC

Specimens of IH were obtained under a protocol approved by the Committee on Clinical Investigation, Boston Children's Hospital. The clinical diagnosis was confirmed by histopathology. Hem-Pericytes, HemEC and HemSC were isolated as described¹⁸⁻²⁰. Hem-Pericytes were cultured on non-coated tissue culture plates in DMEM 10% fetal bovine serum (FBS), 1× glutamine-penicillin-streptomycin (GPS)¹⁸. HemEC and HemSC were cultured on fibronectin-coated (1 µg/cm²) plates in endothelial basal medium (EBM-2, Lonza CC-3156) supplemented with 20% FBS, EGM-2 SingleQuots without hydrocortisone (Lonza CC-4176) and 1× GPS²⁰.

Non-hemangioma cell culture

Human dermal microvascular endothelial cells (HDMEC) were isolated from neonatal foreskin²¹ and cultured as described for HemEC and HemSC. Human placental and retinal pericytes were purchased from Promocell (C-12981) and Cell Systems (ACBRI 183), respectively, and grown as described for HemPericytes. Smooth muscle cells (SMC) from bladder and bronchus were purchased from ScienCell and cultured in SMC media from ScienCell (Cat. 1101).

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from cells and tissues was extracted using RNeasy Micro kit (Qiagen) and TRIzol (Invitrogen), respectively. cDNAs were synthesized with Quanta qScript cDNA Synthesis kit (VWR Scientific). Primers for qRT-PCR are shown in Table 1. Four housekeeping genes, GAPDH, β-actin, HGPRT, and ribosomal protein S9 were evaluated for consistency among samples: GAPDH was selected for normalizing IH tissue RNA and β-actin was selected for normalizing RNA isolated from IH-derived cells. Reactions were prepared with SYBR Green master mix (Roche) and performed on Applied Biosystems' StepOnePlus Real-Time PCR system.

Contractility assay

A deformable silicone-coated substratum was prepared as described^{22,23}. Polydimethylsiloxane from Sigma-Aldrich (DMPS12M-100G, viscosity 12,500 cST) was pipetted onto marked, round glass coverslips (Fisher Scientific 12-545-81) and spread for one hour at 40 °C, then thermally cross-linked and coated with electrical charge for 13 seconds using a plasma etcher (SPI Plasma-Prep II 11005). The coverslips were coated with 0.1 mg/mL of rat type I collagen (BD Biosciences) and placed in 24-well plates. Plates were

UV-irradiated for five minutes and 4000 pericytes in DMEM 2% FBS, $1 \times$ GPS were pipetted into each well. This assay tested HemPericytes from three different proliferating phase IH (146, 154 and 156), three different involuting phase IH (I-69, I-79 and I-82) and normal human pericytes from retina and placenta. After 48 hours, images were taken under bright field at $10\times$ (Nikon Eclipse TS100) to visualize and quantify “wrinkled” contractile cells at time=0. Contractility at time = 0 was set to 100%.

In a blinded fashion, cells were treated with vehicle (n = 6 wells), propranolol (10 μ M) (n = 6 wells), vehicle + epinephrine (5.5 μ M) (n = 6 wells) or propranolol + epinephrine (n = 6 wells). Propranolol was from Sandoz (NDC 0781-3777-95, 1mg/ml, pH 4), epinephrine was from Hospira (NDC 0409-7241-01, 1 mg/mL) and the vehicle was prepared by the Boston Children's Hospital Pharmacy to match the solution in which propranolol was supplied. One hour after addition of reagents, wrinkled cells were located and images taken. Contractility was quantitated in all four conditions using the formula: $C = N \times L$, N = # of wrinkles and L = length in pixels from ImageJ ²⁴.

siRNA silencing of β_2 AR in HemPericytes

Non-targeting siRNA and β_2 -AR siRNAs were purchased from Dharmacon (D-001810-03-05 and L-005426-01-0005). Non-targeting siRNA has no homology to any known human gene. β_2 -AR siRNAs were a pool of 5 siRNAs, designed to maximize knockdown. HemPericytes from proliferating phase IH (146, 154 and 156) and involuting phase IH (I-69, I-79 and I-82) were grown in DMEM 10% FBS without antibiotics until they reached 60-90% confluence on 12-well plates. Cells were transfected with 10 nM of siRNA using Lipofectamine RNAiMAX (Life Technologies), prepared in reduced-serum media (Gibco OPTI-MEM). The cells were trypsinized 24 hours after transfection and prepared for contractility assays or re-plated on 12-well plates to assess extent of β_2 -AR of knockdown by qRT-PCR. The contractility assays and qRT-PCR were performed 48 hours after transfection.

Proliferation Assays

Cells were plated at 5000 cells/cm² in 48-well plates in growth medium (n=4/condition). Cells were treated twice daily with PBS or propranolol (10 μ M) from Day 1 to 5. Cells were counted using an automated cell counter (Millipore Scepter 2.0); all particles whose diameters ranged 10-25 μ M were included. HemSC isolated from three different IH (94, 97, 133), HemEC isolated from three different IH (150, 158, I-69), and HemPericytes from three different IH (146, 154, 156) were assayed.

In vivo study and micro-ultrasonography analysis

HemECs (158) and HemPericytes (154), 1.25×10^6 of each cell type, were suspended in 250 μ l of Matrigel and injected subcutaneously on the backs of 20 6-week old male athymic nude/nude mice²⁰. Two additional mice were injected with 250 μ l of Matrigel without cells and served as negative controls. At day 7, micro-ultrasonography was used to assess perfused vascular volume^{25,26}. Mice with HemPericyte/HemEC implants with contrast values above those of negative controls were treated with vehicle or propranolol at 5 mg/kg, twice a day for 7 days, by i.p. injection.

Vascular volume was determined by contrast-enhanced micro-ultrasonography using a VisualSonic Vevo 2100 as described^{25,26}. Tail veins of mice were cannulated using modified, winged infusion set (Allegro Medical 549230); non-targeted contrast agent (VisualSonics VS-11913) was injected to quantify vascular volume in the implants. Twenty minutes passed for contrast agent to be cleared from circulation and the above steps were repeated. The two measurements were averaged to determine contrast values.

Microvascular density (MVD) and immunostaining for human CD31+ vessels

Twenty images from mid-Matrigel H & E sections of all animals in vehicle and propranolol groups were taken randomly at 40 $\times$ (Zeiss Axiophot II, equipped with AxioCam MRc5 and supplemented with AxioVision Rel. 4.8 software). Luminal structures containing at least one RBC were counted as one vessel; the average from 20 images was expressed as vessels/mm². Formalin-fixed, paraffin-embedded mid-Matrigel sections were processed and stained with mouse anti-human CD31 (1:40, Dako M0823) or mouse IgG (1:40, Dako X0931) as described¹⁸.

Statistical analyses

Contractility assays were analyzed by two-tailed, paired t-tests. Cellular proliferation assays and histological analyses were analyzed by two-tailed, unpaired t-tests. Contrast values of mice injected with HemECs 158 and Hem-Pericytes 154 were analyzed by two-tailed, paired t-tests. All data analyses were performed using GraphPad Prism Version 5.04 and differences were considered significant when $p < 0.05$.

Results

β_1 -AR and β_2 -AR mRNA in hemangioma-derived cells and hemangioma tissue

β_1 -AR and β_2 -AR mRNA transcript levels were quantified in HemEC, HemPericytes and HemSC isolated from different IH specimens. β_1 -AR was detected at low levels in HemEC but was undetectable in HemPericytes and HemSC (Figure 1A). β_2 -AR was detected in HemEC and HemPericytes at levels above or comparable to bladder smooth muscle cells, the positive control. HemSC expressed low but detectable levels of β_2 -AR (Figure 1B). We extended this analysis to IH specimens from 15 patients at ages ranging from 3 months to 10 years. β_2 -AR was prominently expressed in most IHs, with the exception of 2: one from a 15 month old and one from a 21 month old child (Figure 1D). These two specimens were shown to have expected levels of the endothelial marker CD31 and the pericyte/smooth muscle marker α -smooth muscle actin (data not shown). β_1 -AR, β_3 -AR and the α_{1b} -AR were detected at low and variable levels in IH specimens (Figure 1C, E, F). In summary, mRNA transcript levels for these four AR were variable among the 15 IH specimens tested.

Propranolol reverses epinephrine-induced relaxation of HemPericytes

Pericytes are contractile and regulate capillary blood flow. *In vitro*, calf serum stimulates pericytic contractility while β -AR agonists and cAMP analogs cause pericytic relaxation^{14,27}. We previously reported that HemPericytes exhibit reduced contractile activity *in vitro* compared to normal human retinal and placental pericytes¹⁸. Therefore, we examined contractility of HemPericytes and normal pericytes in response to propranolol and

a β -AR agonist epinephrine. Pericytes were plated on type I collagen-coated silicone membranes and allowed to “wrinkle” the silicone substratum, indicating contractility (Figure 2A). Wrinkled pericytes were quantified at 48 hours, $t=0$, and this value was set at 100%. Propranolol and/or epinephrine were added as indicated for one hour and wrinkled cells were re-quantified. Propranolol was tested at 10 μ M based on prior studies in which 10 μ M propranolol was sufficient to completely block norepinephrine-induced dilation of human coronary arterial segments²⁸. The percent change in contractility compared to $t=0$ in HemPericytes from three different proliferating phase IH, three different involuting phase IH, and normal human pericytes is shown in Figure 2B. Epinephrine-induced relaxation of proliferating HemPericytes was prevented by propranolol (35.4 ± 42.1 versus 89.6 ± 6.9 contractile cells; $p=0.018$). Epinephrine-induced relaxation of involuting HemPericytes was also prevented by propranolol (49.3 ± 26.3 versus 73.3 ± 25.2 ; $p=0.021$). Normal human pericytes from retina and placenta did not relax in response to epinephrine and there was no detectable effect of propranolol (89.2 ± 11 versus 98.6 ± 7.2 ; $p=0.247$) (Figure 2B). Supplemental Figure 2 shows individual contractility data for each pericyte population - hemangioma-derived and normal. Supplemental Figure 1 shows β_2 -AR mRNA levels, determined by qRT-PCR, for each of the pericytes analyzed in the contractility assay.

We used siRNA silencing to reduce β_2 -AR expression in all HemPericytes (Figure 2C), which resulted in a loss of epinephrine-induced relaxation, and thus a loss of the propranolol-mediated reversal of this relaxation (Figure 2C). This data strongly implicates β_2 -AR to be involved in relaxation and propranolol-mediated contractility of HemPericytes in this in vitro assay. The extent to which β_2 -AR mRNA levels were reduced by siRNA is shown by qRT-PCR (Supplemental Figure 2).

Propranolol inhibits proliferation of HemPericytes and normal pericytes

Proliferation of HemPericytes 154, in the presence of 10 μ M propranolol added twice daily over 4 days, was inhibited by 41% compared to the control (Figure 3A). Propranolol reduced the proliferation of placental and retinal pericytes by 48% and 59%, respectively. For comparison, we also assayed the proliferation of HemSC-133, HemEC-158 and HDMEC. Propranolol did not affect the proliferation of HemSC or HDMEC, although it reduced proliferation of HemEC-158 by 37%. The results of proliferation assays of hemangioma-derived cells from three different IH tumors were combined: HemPericytes ($n=3$), HemEC ($n=3$) and HemSC ($n=3$) (Figure 3C). This confirmed the statistically significant inhibitory effect of propranolol on HemPericyte and HemEC proliferation. The results confirm previous studies on the inhibitory effect of propranolol on HemEC^{12,14} and show that proliferation of HemPericytes is also inhibited by propranolol, which may contribute to the anti-angiogenic effect on IH.

Propranolol reduces vascular volume in blood vessels formed in vivo by implanting HemPericytes and HemEC

We previously showed that HemPericytes co-implanted with normal human EC or HemEC formed perfused vascular networks in immune-deficient mice¹⁸. Here, we co-implanted HemPericytes + HemEC and allowed vascular networks to form until Day 7, at which time vascular volume in the implants was measured using contrast-enhanced micro-

ultrasonography. The mice were divided into two groups and treated twice daily for 7 days with either vehicle or propranolol by i.p. injection. Vascular volume was re-assessed on Day 14. Heart rates were 390 ± 52 for the vehicle treated group and 413 ± 5 for the propranolol treated group. Contrast values for individual animals and mean contrast values at Day 7 and Day 14 are shown in Figure 4. Mice treated with propranolol showed a significant reduction in mean contrast value at Day 14, whereas mice treated with vehicle did not. It is unlikely that the propranolol effect was due to changes in blood pressure because in similar experiments in which HemSC were implanted as a single cell type, we did not see a statistically significant reduction in vascular volume in propranolol-treated mice (data not shown). Propranolol had no significant effect on the number of vessels in the implants at Day 14, as determined by counting the number of red blood cell-filled vessels (Figure 4C). To verify that red blood cell-filled vessels were composed of human endothelial cells, sections were stained with an anti-human CD31 mAb (Figure 4D). In this assay, propranolol did not reduce the number of vessels but reduced the perfused vascular volume, consistent with the phenomenon of increased vasoconstriction.

Discussion

Hemangioma-derived endothelial cells (HemEC) and stem cells (HemSC) have been shown to be variably affected by propranolol in *in vitro* assays for proliferation, differentiation and apoptosis. In contrast, relatively little is known about pericytes in IH and how these cells might respond to propranolol. We show that HemPericytes express β_2 AR mRNA and that propranolol reverses epinephrine-induced relaxation of HemPericytes. A limitation of our study is that we did not analyze β_2 AR at the protein level. *In vivo*, propranolol treatment for 7 days reduced perfused vascular volume of blood vessel networks formed from HemPericytes and HemEC. These findings indicate that propranolol increases the contractile activity of HemPericytes, which may contribute to vasoconstriction and reduced blood flow in IH.

HemPericytes isolated from different proliferating phase IH specimens were found to exhibit reduced contractility and increased proliferation compared to normal human pericytes from human retina or placenta¹⁸. Propranolol reduced proliferation of HemPericytes and HemEC, indicating that the agent may also have an anti-angiogenic effect *in vivo*. Propranolol did not inhibit proliferation of fast-growing vasculogenic HemSC that are able to differentiate into pericytes and endothelial cells, and form IH-like vessels in immune-deficient mice^{20,29}.

Considered together with published reports, our data indicate propranolol can target endothelial cells and pericytes in IH, exerting overlapping and distinctive effects on each. More studies are needed to determine how these *in vitro* studies correlate with clinical response to propranolol therapy. In rare instances, some children with IH fail to respond to propranolol therapy. Perhaps low or undetectable levels of β_1 and β_2 -AR, as seen in some IH specimens (Figure 1C,D), explain the ineffective propranolol therapy in these uncommon cases. Analyses of β -AR expression in IH specimens from patients that do not respond to propranolol would be needed to pursue this hypothesis. Another un-explained feature of propranolol therapy is the clinical regrowth of IH that occurs in approximately 20% of cases when the drug is discontinued³. This observation suggests that propranolol does not

produce irreversible effects, either anti-proliferative or anti-vasculogenic, on the cells that drive hemangioma-genesis. Further studies are needed to decipher the clinical features of the rebound phenomenon, combined with *in vitro* and *in vivo* experiments, in order to investigate these concepts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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What is already known about this topic?

Propranolol is an effective treatment for problematic infantile hemangioma.

Propranolol, an antagonist of β -adrenergic receptors, reduces proliferation and increases apoptosis of hemangioma endothelial cells in vitro.

What does this study add?

Propranolol increased contractility of hemangioma pericytes but had no effect on normal pericytes.

Propranolol significantly reduced the vascular volume of blood vessels assembled from hemangioma pericytes and endothelial cells.

Hemangioma pericytes are a likely target of propranolol.

Figure 1. β -AR mRNA in hemangioma-derived cells and hemangioma tissue

Panels **A-B**, mRNA levels in HemEC (n=7), HemPericytes (n=3) and HemSC (n=8) quantified by qRT-PCR. **A**, β_1 -AR mRNA in human cardiac tissue, normalized to GAPDH, set = 1.0. Relative levels in cells, normalized to GAPDH, are shown. **B**, β_2 -AR mRNA in human bladder SMC, normalized to GAPDH set = 1.0. Relative levels in cells, normalized to GAPDH, are shown. Panels **C-F**, IH specimens removed from patients at different ages (shown in months or years) analyzed for **C**, β_1 -AR, **D**, β_2 -AR, **E**, β_3 -AR, and **F**, α_{1b} AR mRNA levels by qRT-PCR. β_3 -AR mRNA in human foreskin, normalized to β -actin, set =

1.0. α_{1b} AR mRNA in bronchial SMC, normalized to β -actin, set = 1.0. Relative levels in tissue, normalized to β -actin, are shown.

Figure 2. Propranolol effects on HemPericyte and normal pericyte contractility

A, Diagram of contractility assay with phase image of HemPericyte wrinkling the silicone substratum. **B**, Contractility of proliferating phase HemPericytes (white bars), involuting phase HemPericytes (grey bars) and normal human pericytes (dark grey bars) at 48 hours after plating was quantified and set to 100%. Percent change in contractility was determined after cells were treated for one hour with vehicle, propranolol, vehicle + epinephrine or propranolol + epinephrine. **C**, Contractility assays performed on HemPericytes (black bars)

with corresponding siRNA β_2 -AR knockdown (grey bars). Values are mean $\pm$ standard deviation. Data analyzed by two-tailed, paired t-tests.

Figure 3. Propranolol effects on proliferation of HemPericytes, normal pericytes, HemEC and HemSC

A, Representative proliferation assay with HemPericyte 154, placental pericytes and retinal pericytes and in **B**, HemSC-133, HemEC-158 and normal human endothelial cells (HDMEC). Cells plated at 5000 cells/cm² in growth media in 48 well dishes (n=4/condition). Cells allowed to adhere for 24 hours (Day 1), and then treated twice daily with PBS (white bars) or propranolol (10μM) (gray bars) until Day 5. Cells counted on Day 1 and Day 5. Values are mean ± standard deviation. Data analyzed by two-tailed, unpaired t-tests.

C, Proliferation at Day 5 in PBS set to 100% (white bars) versus propranolol (grey bars) of HemPericytes 146, 154, 156; HemEC 150, 158, I-69 and HemSC 94, 97, 133.

Figure 4. Propranolol effects on vascular volume in HemPericyte/HemEC Matrigel implants in immune-deficient mice

Immune-deficient nude mice (n=20) implanted sub-cutaneously with HemPericytes and HemEC suspended in Matrigel. At Day 7, mice treated twice daily with vehicle or propranolol by i.p. injection for 7 days. Vascular volume determined by contrast-enhanced micro-ultrasonography. **A**, Contrast values for individual mice. **B**, Mean contrast values at Day 7 and Day 14; data analyzed by two-tailed, paired t-tests. **C**, Vessel density at Day 14 and **D**, Control IgG and anti-human CD31 immunostaining of sections from implants from vehicle-treated and propranolol-treated mice at Day 14.

Table 1
Sequences of primers used for qRT-PCR

Gene	Forward	Reverse
α_{1b} -AR	5'-GATCCATTCCAAGAACTTTCAC-3'	5'-CAGAACACCACCTTGAACAC-3'
β_1 -AR	5'-TCTTTTGTGTGTGCGTGTGA-3'	5'-ATGCTTCTCCCTTCCCCTAA-3'
β_2 -AR	5'-CACCAACTACTTCATCACTTCAC-3'	5'-GACACAATCCACACCATCAG-3'
β_3 -AR	5'-TGAAATCCAGTTGCCATTGA-3'	5'-CACCATGTAAGGCACCACTG-3'
β -actin	5'-TGAAGTGTGACGTGGACATC-3'	5'-GGAGGAGCAATGATCTTGAT-3'
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GATGCAGGGATGATGTTC-3'
HPRT1	5'- TGGACAGGACTGAACGTCTTG-3'	5'- CCAGCAGGTCAGCAAAGAATTGA-3'
S9	5'-GATTACATCCTGGGCCTGAA-3'	5'-ATGAAGGACGGGATGTTTAC-3'