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# High Density Lipoproteins Rescue Diabetes-Impaired Angiogenesis via Scavenger Receptor Class B Type I

HDL rescues diabetes-impaired angiogenesis

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## **Abstract**

Disordered neovascularization and impaired wound healing are important contributors to diabetic vascular complications. We recently showed that high-density lipoproteins (HDL) enhance ischemia-mediated neovascularization and mounting evidence suggests HDL have anti-diabetic properties. We therefore hypothesized that HDL rescue diabetes-impaired neovascularization. Streptozotocin-induced diabetic mice had reduced blood flow recovery and neovessel formation in a hindlimb ischemia model compared to non-diabetic mice. Reconstituted HDL (rHDL) infusions in diabetic mice restored blood flow recovery and capillary density to non-diabetic levels. Topical rHDL application rescued diabetes-impaired wound closure, wound angiogenesis and capillary density. *In vitro*, rHDL increased key mediators involved in HIF-1 $\alpha$  stabilization including the PI3K/Akt pathway, Siah1 and Siah2, and suppressed the prolyl hydroxylases PHD2 and PHD3. rHDL rescued high glucose-induced impairment of tubulogenesis and VEGFA protein production, a finding associated with enhanced phosphorylation of pro-angiogenic mediators VEGF receptor 2 (VEGFR2) and eNOS. Siah1/2 siRNA knockdown confirmed the importance of HIF-1 $\alpha$  stability in mediating rHDL action. Lentiviral shRNA knockdown of scavenger receptor-BI (SR-BI) *in vitro* and SR-BI<sup>-/-</sup> diabetic mice *in vivo*, attenuated rHDL rescue of diabetes-impaired angiogenesis, indicating a key role for SR-BI. These findings provide a greater understanding of the vascular biological effects of HDL, with potential therapeutic implications for diabetic vascular complications.

## **Introduction**

The vascular complications of diabetes mellitus are characterized by disordered angiogenesis and impairment of ischemia-induced neovascularization. Sufferers of diabetes have reduced coronary collateral formation following vascular occlusion (1), impaired wound healing, and increased rates of amputation (2). Despite advances in the treatment of athero-occlusive disease and the demonstration that intensive blood glucose control attenuates some vascular complications, many diabetic patients with vasculopathy remain refractory to current treatment approaches.

Diabetes-impaired ischemia-induced neovascularization is associated with decreased hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) stability (3; 4), reduced vascular endothelial growth factor (specifically VEGFA) production and signaling via VEGF receptor 2 (VEGFR2) (5), and inhibition of endothelial nitric oxide synthase (eNOS) activity (6). However, the key triggers for these events are incompletely understood, as are potential therapies to minimize these abnormalities.

To date, pre-clinical studies have consistently demonstrated that high-density lipoproteins (HDL) and its main protein constituent apolipoprotein (apo)A-I, exert anti-atherogenic effects (7). Despite this, randomized trials of HDL-raising therapies have not demonstrated clinical benefit. However, long-term mortality follow-up of the Helsinki Heart study found that the incidence of coronary heart disease was significantly reduced when HDL levels were raised by only 5-10% (8), indicating that the biology of HDL is yet to be fully elucidated and perhaps aggressive attempts to raise HDL have other effects at the cellular and molecular level. Increasing evidence demonstrates that HDL exerts endothelial protective and anti-diabetic effects. Infusions of rHDL reduce plasma glucose levels, restore impaired

endothelial function (9) and promote endothelial progenitor cell mobilization (10) in Type 2 diabetic patients. HDL interacts with the cholesterol transporters ABCA1 and ABCG1, and scavenger receptor class B type I (SR-BI). While ABCA1 and ABCG1 are predominantly involved in HDL-mediated cholesterol efflux, SR-BI is known to mediate the vasculo-protective effects of HDL including increasing re-endothelialization and endothelial cell migration (11; 12). Despite a significant amount of work, the vascular biological effects of HDL remain incompletely understood and further efforts to translate HDL into a potential therapeutic agent require a fuller understanding of its properties.

We therefore sought to investigate the effect of rHDL on diabetes-impaired angiogenesis in two murine models of diabetic vascular complications; and to elucidate the mechanisms of action. We report that rHDL rescues diabetes-related impairment of ischemia-driven angiogenesis and wound healing. This occurs via the receptor SR-BI and by HIF-1 $\alpha$  stabilization, enhanced VEGFA/VEGFR2 production and signaling, and increased eNOS activity. These findings may have implications for therapeutic modulation of diabetic vascular complications.

## **Research Design and Methods**

### ***Preparation of discoidal reconstituted HDL***

Apolipoprotein A-I (apoA-I) was isolated from plasma obtained from a pool of multiple healthy donors (>5) by ultracentrifugation and anion-exchange chromatography, as described previously (13). Discoidal reconstituted HDL (rHDL) was prepared by complexing apoA-I with 1-palmitoyl-2-linoleoyl-phosphatidylcholine.

### ***Animal studies***

All experimental procedures were conducted with approval from the Sydney Local Health District Animal Welfare Committee. Male 8-week-old C57Bl/6J, SR-BI<sup>-/-</sup> and wildtype (WT) littermates were rendered diabetic two weeks prior to surgery by a bolus *i.p.* injection of streptozotocin (165 µg/g).

### ***Murine hindlimb ischemia model***

The hindlimb ischemia model was conducted as described previously (14). The left femoral artery and vein were ligated and excised from the hindlimb of mice (n=8–12/group). A sham procedure was performed on the opposite hindlimb. Mice received *i.v.* injections of PBS (vehicle control) or rHDL (200 µg/mouse) via the tail vein every second day following surgery. Hindlimb blood reperfusion was determined by laser Doppler perfusion imaging prior to and immediately following surgery, then at Days 2–3, 7, and 10 post-surgery.

### ***Murine wound healing model***

The wound healing model was conducted as previously described (15). Two full-thickness excisions were created on the dorsum and a silicone splint secured around the wound. For each mouse, one wound received rHDL (50 µg/wound/day) and the other PBS topically

applied directly on the wound. A transparent occlusive dressing (Opsite™) was applied. Digital images and wound area were measured daily. Wound blood perfusion was determined using laser Doppler.

### ***Plasma lipid and glucose concentrations***

Total, HDL and LDL cholesterol concentrations on mouse plasma were determined enzymatically (Roche Diagnostics). HDL cholesterol concentrations were determined following polyethylene glycol precipitation of apolipoprotein B containing lipoproteins. Glucose concentrations were measured using a glucometer (AccuCheck Performa).

### ***Immunocytochemistry***

Fresh frozen 5 µm sections of gastrocnemius muscle from ischemic and non-ischemic hindlimbs were stained to detect the number of new capillaries (CD31<sup>+</sup>, Dako) per myocyte (laminin, abcam). 5 µm sections were taken from the mid-point of paraffin-embedded wound tissues and assessed for CD31<sup>+</sup> neovessels (abcam).

### ***Cell culture***

Human coronary artery endothelial cells (HCAECs, Cell Applications) were cultured in MesoEndo media and used at passages 4–5. Cells were seeded at  $8 \times 10^4$  cells/well and cultured for 8 hours, then treated for 18 hours with rHDL (20 µM, final apoA-I concentration) or PBS. Cells were replaced with fresh DMEM media in glucose conditions for 48 hours. For high glucose conditions, media was supplemented with D-glucose to a final concentration of 25 mM. For the measurement of phosphorylated proteins, cells were stimulated with 10 ng/mL rhVEGF protein (R&D Systems). Each experiment was performed three times independently and in triplicate.

### ***Siah1/2 knockdown in HCAECs***

HCAECs were transfected for 6 hours with 60 nM siRNA for Siah1, Siah2 or control scrambled (Santa Cruz Biotechnology, Inc.) then treated with rHDL (20  $\mu$ M) or PBS for 18 hours and exposed to high glucose (25 mM, 48 hours).

### ***SR-BI knockdown in HCAECs***

Lentiviruses containing either shRNA for SR-BI (shSR-BI) or the empty vector (shControl) were generated in 293T17 cells as described previously (16; 17). Viral titres were quantified using Lenti-X<sup>TM</sup> qRT-PCR Titration Kit (Clontech).

HCAECs were seeded and grown overnight to 50% confluency, then exposed for 24 hours to  $1 \times 10^7$  lentiviral particles/mL containing either shSR-BI or shControl. Transduced cells were seeded at  $8 \times 10^4$  cells/well. Cells were treated with rHDL (20  $\mu$ M) or PBS for 18 hours then exposed to high glucose (25 mM, 48 hours).

### ***RNA expression***

Quantitative real-time PCR was performed for (1) murine *Glut1* (F:5'-TCAACACGGCCTTCACTG-3'; R:5'-CACGATGCTCAGATAGGACATC-3'), *Pparg1a* (F:5'-TGGAGTGACATAGAGTGTGCTG-3'; R:5'-TGTTTCGCAGGCTCATTGTTG-3'), *Hif-2 $\alpha$*  (F:5'-AGGTCTGCAAAGGACTTCGG-3'; R:5'-CAAGTGTGAACTGCTGGTGC-3'), *Phd1*, (F:5'-TAAGGTGCATGGCGGCCTGC-3'; R:5'-TGGCTGCTGCCCCGTTCCCTTG-3'), *Pdk4* (F:5'-CACGTACTCCACTGCTCCAA-3'; R:5'-AGCGTCTGTCCCATAACCTG-3'), *Scarb1* (F:5'-CTGAGCACGTTCTACACGCA-3'; R:5'-GGCCTGAATGGCCTCCTTAT-3'), *Siah1a* (F:5'-GACTGCTACAGCATTACCCACT-3'; R:5'-GTTGGATGCAGTTGTGCCG-3'), *Siah2* (F:5'-CTAACGCCCAGCATCAGGAA-3';



R:5'-GAACAGCCCGTGGTAGCATA-3'), *Hif-1 $\alpha$*  (F:5'-TCCCTTGCTCTTTGTGGTTGGGT-3'; R:5'-AACGTAAGCGCTGACCCAGG-3'), *Vegfa* (F:5'-GGCTGCTGTAACGATGAAG-3'; R:5'-CTCTCTATGTGCTGGCTTTG-3'), *Vegfr2* (F:5'-GCCCAGACTGTGTCCCGCAG-3'; R:5'-AGCGCAAGACCGGGGAGAGC-3') and *36B4* (F:5'-CAACGGCAGCATTATAACCC-3'; R:5'-CCCATTGATGATGGAGTGTGG-3') in murine hindlimbs and wound tissue, and (2) human *SIAH1*, *SIAH2* and  $\beta$ 2-microglobulin (*B2M*) in cultured HCAECs using primers designed previously (16). Relative changes in gene expression were normalized using the  $\Delta\Delta$ CT method to murine *36B4* or human *B2M*.

### ***Protein expression***

Whole cell and nuclear protein extracts were subjected to Western blot analysis and probed with antibodies for PI3K (p85) (abcam), phosphorylated Akt (Ser473), total Akt (Cell Signaling Technology), PHD2, PHD3, HIF-1 $\alpha$  (Novus Biologicals), VEGFA (abcam), VEGFB, VEGFC, VEGFD (R&D Systems), phosphorylated VEGFR2 (Tyr1175), total VEGFR2, phosphorylated eNOS (Ser1177), total eNOS (Cell Signaling Technology) and SR-BI (Novus Biologicals). Even protein loading was confirmed by  $\alpha$ -tubulin (abcam) for whole cell lysates or lamin B1 (abcam) for nuclear fractions. Secreted and cytoplasmic VEGFR1 expression were measured in the media and cytoplasmic fractions by ELISA (R&D Systems).

### ***Matrigel tubulogenesis assay***

Pre-treated HCAECs were seeded at  $8 \times 10^3$  cells/well on polymerized growth-factor-reduced Matrigel and incubated for 4 hours. Tubules were photographed at 40X magnification under light microscopy and total number tubules formed determined using Image J.

### ***Statistical analyses***

Data are expressed as mean $\pm$ SEM. Differences between treatment groups were calculated using a one-way ANOVA (Bonferroni's comparison test *post hoc*) or Student's t-test. A two-way ANOVA (Bonferroni's comparison test *post hoc*) was performed when comparing data at multiple timepoints. Significance was set at a two-sided  $P < 0.05$ .

## **Results**

### ***rHDL rescues diabetes-impaired angiogenesis in vivo.***

We first studied the effects of rHDL in two models of diabetes-impaired vascular complications including: (1) ischemia-mediated neovascularization and (2) wound healing and angiogenesis. In the hindlimb ischemia model, femoral artery ligation reduced blood flow equally in all mice at Day 0 (Figure 1A). In the non-diabetic mice, rHDL infusions promoted blood flow recovery compared with PBS-infused mice, reaching significance at Day 7 ( $P<0.05$ ). Diabetes severely impaired blood flow recovery in PBS-infused mice. However, this was rescued by rHDL infusions ( $P<0.01$ ). Consistent with this, rHDL increased capillary density in the gastrocnemius muscle of ischemic hindlimbs in both the non-diabetic ( $P<0.01$ ) and diabetic mice ( $P<0.001$ ) (Figure 1B). The impact of diabetes on wound healing and angiogenesis was more striking. In non-diabetic mice, topical rHDL application increased wound closure compared to PBS-treated wounds at Day 10 (Figure 1C). As expected, diabetic mice exhibited delayed wound closure. Topical rHDL rescued diabetes-related impairment in wound closure to the level of non-diabetic controls. Furthermore, in non-diabetic and diabetic mice, blood perfusion was elevated in rHDL-treated wounds in the important early stages of wound recovery (Days 2–6) (Figure 1D). Diabetic mice had reduced wound capillary density in PBS-treated wounds (Figure 1E). However, topical rHDL enhanced capillary density in diabetic wounds ( $P<0.05$ ). In both models, we found that diabetes suppressed *Vegfa* expression, which was rescued by rHDL treatment (Supplemental Figures 1A-1B). Taken together, we show that rHDL rescues diabetes-impaired angiogenesis. These effects were independent of changes in glucose and lipid levels (Supplemental Tables 1 and 2). Furthermore, rHDL had no effect on markers of glucose metabolism, including the glucose transporter *Glut1*, the metabolic regulator *Ppargcla* and three genes involved in

metabolic cellular programming: *Hif-2 $\alpha$* , *Phd1* and *Pdk4* (Supplemental Figure 2). This indicates that rHDL does not mediate its effects on angiogenesis via changes in glucose metabolism. Finally, we found there were no differences in *Scarb1* (SR-BI) levels between diabetic and non-diabetic animals in both studies (Supplemental Figure 3A). However, rHDL significantly augmented hindlimb *Scarb1* expression in diabetic mice.

***rHDL rescues high glucose-impaired HIF-1 $\alpha$  stabilization in vitro.***

We then studied the effects of rHDL on key angiogenic pathways *in vitro* in high glucose conditions. Decreased HIF-1 $\alpha$  stability, impaired VEGFA production and eNOS inhibition are strongly implicated in the pathogenesis of diabetes-impaired angiogenesis (3-6). HIF-1 $\alpha$  is post-translationally modulated, beginning with the PI3K/Akt signaling pathway, which induces the E3 ubiquitin ligases Siah1 and Siah2 (18). Under high glucose conditions, rHDL increased PI3K protein expression (Figure 2A,  $P<0.05$ ) and induced an increase in phosphorylated Akt (pAkt) (Figure 2B,  $P<0.001$ ). rHDL increased *SIAH1* and *SIAH2* mRNA levels, irrespective of glucose conditions (Figures 2C–2D,  $P<0.001$ ). The Siahs suppress prolyl hydroxylases (PHD2 and PHD3) that ubiquitinate and target HIF-1 $\alpha$  for degradation. Exposure to high glucose increased PHD2 and PHD3 protein levels (Figures 2E–2F). However, rHDL suppressed both PHD2 ( $P<0.01$ ) and PHD3 ( $P<0.05$ ) levels. Consistent with the decreases in PHDs, rHDL rescued high glucose-induced reductions in both total and nuclear HIF-1 $\alpha$  protein levels (Figures 2G–2H,  $P<0.05$ ). These results show that rHDL rescues high glucose-impaired HIF-1 $\alpha$  stabilization *in vitro*.

***rHDL rescues high glucose-impaired VEGFA/VEGFR2 production/activation in vitro.***

While the VEGFA/VEGFR2 signaling axis has been strongly implicated in diabetic vascular complications (5), other members of the VEGF ligand-receptor family have also been

implicated in diabetes (19-23). We determined the effects of rHDL on the VEGF ligand-receptor family. rHDL prevented high glucose-induced inhibition of VEGFA and VEGFB protein (Figures 3A–3B,  $P<0.05$ ), but did not change VEGFC or VEGFD levels (Figures 3C–3D). While high glucose did not impact on VEGFR1 expression, rHDL significantly decreased both soluble and cytoplasmic VEGFR1 protein irrespective of glucose conditions (Figures 3E–3F). rHDL promoted VEGFR2 phosphorylation (pVEGFR2) (Figure 3G) and augmented eNOS phosphorylation in high glucose (Figure 3H). Finally, consistent with increases in HIF-1 $\alpha$  stabilization, VEGFA/VEGFR2 signaling and eNOS activation, rHDL augmented endothelial cell tubule formation (Figure 3I,  $P<0.05$ ).

***Post-translational HIF-1 $\alpha$  modulators Siah1 and Siah2 mediate rHDL rescue of high glucose-impaired angiogenesis in vitro.***

To elucidate the importance of Siah1 and Siah2 in rHDL-induced HIF-1 $\alpha$  stabilization and VEGFA augmentation, a siRNA knockdown approach was used. In the scrambled siRNA (siScr) cells, rHDL augmented HIF-1 $\alpha$ , VEGFA, pVEGFR2 and tubulogenesis (Figures 4A–4D) in high glucose. However, knockdown of Siah1 and Siah2 abrogated several steps in the angiogenic pathway including rHDL-induced increases in HIF-1 $\alpha$ , VEGFA, pVEGFR2 and tubulogenesis, highlighting an important role for Siah1 and Siah2 in mediating the pro-angiogenic effects of rHDL in high glucose.

***SR-BI mediates rHDL-induced rescue of high glucose-impaired angiogenesis in vitro.***

In HCAECs, high glucose induced a 50% decrease in SR-BI protein (Supplemental Figure 3B,  $P<0.05$ ), consistent with previous studies (24-26). Incubation with rHDL prevented high glucose-induced SR-BI inhibition ( $P<0.05$ ). The role of SR-BI in mediating the pro-angiogenic effects of rHDL in high glucose conditions was next assessed *in vitro* using a

lentiviral SR-BI shRNA (shSR-BI). In the lentiviral control shRNA (shControl) cells, rHDL augmented pAkt, however this was abrogated in shSR-BI cells (Figure 5A). rHDL also failed to augment HIF-1 $\alpha$  expression in shSR-BI cells (Figure 5B). Whilst rHDL increased VEGFA protein in shControl cells, this induction was abrogated by shSR-BI (Figure 5C). Finally, rHDL-induced tubule formation was attenuated in shSR-BI cells (Figure 5D).

***SR-BI mediates rHDL rescue of diabetes-impaired ischemia-induced neovascularization in vivo.***

We then investigated the role of SR-BI in mediating the effects of rHDL on ischemia-mediated neovascularization *in vivo* using SR-BI<sup>-/-</sup> mice. In non-diabetic wildtype mice, rHDL augmented hindlimb blood perfusion, which was attenuated in non-diabetic SR-BI<sup>-/-</sup> mice (Supplemental Figure 4A). In diabetic wildtype littermates, rHDL infusions promoted blood flow recovery, capillary density and augmented mRNA levels of key angiogenic mediators including *Siah1a*, *Siah2*, *Hif-1 $\alpha$*  and *Vegfa* (Figures 6A-6F,  $P < 0.05$ ). However, the pro-angiogenic ability of rHDL was completely abrogated in SR-BI<sup>-/-</sup> diabetic mice, suggesting that rHDL augments ischemia-induced angiogenesis in diabetes via SR-BI.

***SR-BI mediates rHDL rescue of diabetes-impaired wound closure/angiogenesis.***

In non-diabetic wildtype mice, rHDL promoted the rate of wound closure, which did not occur in non-diabetic SR-BI<sup>-/-</sup> mice (Supplemental Figure 4B). In diabetic wildtype littermates, rHDL promoted wound closure and wound angiogenesis and induced *Siah1a*, *Siah2*, *Hif-1 $\alpha$* , *Vegfa* and *Vegfr2* mRNA levels (Figures 7A-7H,  $P < 0.05$ ). The ability of rHDL to promote wound healing and angiogenesis was attenuated in diabetic SR-BI<sup>-/-</sup> mice. Taken together with the *in vitro* studies reported in Figure 5 and the *in vivo* hindlimb ischemia

studies described in Figure 6, this highlights an important role for SR-BI in the induction of angiogenesis by rHDL in diabetes.

## **Discussion**

We report for the first time that rHDL rescues diabetes-impaired angiogenesis by enhancement of ischemia-mediated neovascularization and acceleration of wound closure and wound angiogenesis. *In vitro* studies indicate that these effects may, at least in part, be via enhanced post-translational HIF-1 $\alpha$  modulation and nuclear translocation, increased VEGFA/VEGFR2 production and signaling, and augmented eNOS activity. Siah siRNA knockdown *in vitro* confirmed the importance of post-translational HIF-1 $\alpha$  modulation in mediating the pro-angiogenic effects of rHDL in high glucose. Furthermore, *in vitro* studies using lentiviral shSR-BI knockdown and *in vivo* studies with SR-BI<sup>-/-</sup> mice indicate that these effects of rHDL are mediated by the receptor SR-BI. In summary, we have demonstrated a key role for rHDL in the attenuation of diabetes-related impairment of angiogenesis with implications for the therapeutic modulation of diabetic vascular complications.

Collateral vessel network development is an important response to tissue ischemia following vascular occlusion (27). Similarly the extent of neovascularization in the early stages following wounding is a key determinant of wound closure rate (28). Diabetes is associated with poor outcomes following vascular occlusion and impaired coronary collateral development (29) and patients with peripheral vascular disease manifest increased peripheral limb ulceration and amputation, with cutaneous wounds more prone to amputation (2; 30). Consistent with this, our *in vivo* studies found that diabetes caused impairment of ischemia-induced neovascularization in hindlimbs and in wound closure/angiogenesis, with a more striking impact on wound healing. These are two distinctly different models therefore the timing for capturing changes may vary. Compared to the hindlimb ischemia model which is primarily driven by ischemia-mediated angiogenesis, wound healing is more complex and not solely dependent on angiogenesis but involves other cellular processes including



epithelialization and cellular proliferation. Additionally, a chronic inflammatory state as is commonly seen in diabetic patients, significantly impairs wound healing and can lead to severe unfavorable outcomes such as amputation. HDL also exhibits anti-inflammatory effects (31; 32) and may assist in the more robust impact of HDL. Regardless, rHDL promoted neovascularization in both angiogenic models, rescuing both ischemia-mediated neovascularization and wound healing and angiogenesis, potentially highlighting a new role for HDL in attenuating vascular complications associated with diabetes-impaired neovascularization.

Our *in vitro* studies found that high glucose suppressed post-translational HIF-1 $\alpha$  modulation (via augmentation of PHDs), VEGFA/VEGFR2 production and signaling, and tubulogenesis. HIF-1 $\alpha$  is the pivotal transcription factor involved in ischemia-mediated neovascularization and is governed by a complex orchestration of post-translational regulation. Previous studies have reported that hyperglycemia inhibits hypoxia-induced HIF-1 $\alpha$  stabilization and suggest that mechanisms involving proline hydroxylation are important (33). The current study, however, is the first to directly show that high glucose decreases HIF-1 $\alpha$  stability via post-translational effects including an increase in prolyl hydroxylase (PHD2 and PHD3) expression and a decrease in both total and nuclear HIF-1 $\alpha$  protein. More importantly, rHDL rescued HIF-1 $\alpha$  stabilization at each step in its post-translational regulation by: 1) activating the PI3K/Akt signaling pathway, which 2) triggered an increase in the expression of the E3 ubiquitin ligases Siah1 that 3) suppressed PHD expression and ultimately 4) rescuing hyperglycemia-induced reductions in HIF-1 $\alpha$ . These observations complement a recent study that found rHDL increased post-translational HIF-1 $\alpha$  stabilization but this study was not conducted in high glucose conditions (17). Nuclear localization studies found rHDL augmented nuclear HIF-1 $\alpha$  protein levels, demonstrating strong evidence of the impact of

rHDL and the Siah/PHD axis on promoting nuclear HIF-1 $\alpha$  translocation. Furthermore, siRNA knockdown of Siah1 confirmed the importance of post-translational HIF-1 $\alpha$  modulation in the effects of rHDL in high glucose, as silencing of the Siah1 abrogated key steps in the angiogenic pathway *in vitro*.

As expected, high glucose suppressed VEGFA protein levels *in vitro*. rHDL treatment prevented this decrease, most likely via the stabilization of HIF-1 $\alpha$ , the critical transcription factor mediating VEGFA expression. Our *in vitro* analysis of the VEGF ligand-receptor family found that rHDL augmented VEGFB but had no effect on VEGFC or VEGFD. Both VEGFA and VEGFB bind to VEGFR1, which is also implicated in diabetes-impaired neovascularization (19; 34). However, we found that rHDL decreased VEGFR1 expression, suggesting that VEGFR1 is not mediating rHDL-induced rescue of angiogenesis in high glucose. VEGFR2 is the receptor that regulates the pro-angiogenic effects of VEGFA. Following binding of VEGFA to VEGFR2, the receptor dimerizes and causes the activation of receptor-kinase activity leading to the phosphorylation of the receptor. Phosphorylated VEGFR2 induces the activation of an array of angiogenic signaling pathways (35). We found that rHDL increased the phosphorylation (activation) of VEGFR2 in high glucose. This is particularly important as hyperglycemia reduces VEGFA sensitivity via suppression of VEGFR2 activation (5). Previous studies have found rHDL augments hypoxia-induced VEGFR2 total protein levels (14), but this is the first study to show that in high glucose, rHDL promotes both VEGFA protein expression and signaling via VEGFR2 phosphorylation/activation. The present study also found that rHDL promoted eNOS phosphorylation in high glucose, which is likely to be due to the increase in VEGFA/VEGFR2 and the subsequent increase in Akt phosphorylation (downstream of VEGFR2). Elevated eNOS phosphorylation leads to nitric oxide release, promoting

angiogenic functions including endothelial cell migration, proliferation and vessel growth (36; 37). Consistent with this, other studies have found that rHDL increases eNOS activity *in vitro* and *in vivo* (38; 39). Finally, using a functional Matrigel assay for endothelial tubule formation, we found that rHDL augmented high glucose-impaired tubule formation, suggesting that the effects of rHDL on the key angiogenic proteins is translated into critical cellular processes involved in angiogenic functions.

It is becoming increasingly recognized that HDL exhibits anti-diabetic effects (9; 40). We now show that rHDL rescues diabetes-mediated impairment of hindlimb and wound angiogenesis. This is supported by previous work showing that apoA-I/rHDL augment ischemia-driven angiogenesis and promote angiogenesis-related functions including migration and re-endothelialization (14; 17; 41). However, these studies were not done in the clinically relevant setting of hyperglycemia, one of the key contributors to diabetes-impaired vascular complications. In the current study, the effects of rHDL were independent of changes in glucose and lipid levels. Our *in vivo* gene analysis of both hindlimb and wound tissues found that rHDL had no effect on the expression of the glucose transporter GLUT1 and the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), a powerful regulator in metabolism that is involved in HIF-independent regulation of VEGF and angiogenesis in diabetes (42). Hypoxia tolerance and HIF-1 $\alpha$  stabilization are central to a hypometabolic state characterized by reduced oxygen consumption, such as that seen in diabetes-impaired neovascularization. At a cellular level, metabolic cellular reprogramming involves increased efficiency of energy-producing pathways, via increased anaerobic glycolysis activity, and decreased energy-consuming processes and is mediated via hypoxia-inducible factor-2 $\alpha$  (HIF-2 $\alpha$ ), prolyl hydroxylase 1 (PHD1) and pyruvate dehydrogenase lipoyl kinase isozyme 4 (PDK4) (43). rHDL did not

have any effect on gene expression of any of these markers. Taken together, this confirms that the action of rHDL on diabetes-impaired angiogenesis is not due to glucose stress dependent effects but via a number of mechanisms previously found to contribute to diabetes-impaired neovascularization including: 1) promotion of post-translational HIF-1 $\alpha$  stabilization; 2) VEGFA/VEGFR2 production and signaling; and 3) eNOS activation.

The scavenger receptor SR-BI has been implicated in mediating a number of the endothelial protective effects of HDL such as migration, tubulogenesis and re-endothelialization (11; 12; 17; 44; 45). This is the first study to date that has provided a direct link between SR-BI and the pro-angiogenic effects of HDL in diabetes. We found that diabetic animals had slightly lower levels of *Scarb1* mRNA although this did not reach significance while SR-BI protein levels were significantly reduced under high glucose conditions *in vitro*. Currently, there is no consensus on what happens to SR-BI expression in diabetic patients with one study reporting elevated SR-BI mRNA levels in diabetic patients (46) while two studies reported no differences (47; 48). Data from *in vitro* studies are more robust. Exposure to high glucose is shown to reduce SR-BI levels across several cell types including intestinal Caco-2/15 cells (24), hepatic HepG2 cells (25) and human monocyte-derived macrophages (26). More importantly, rHDL increased SR-BI expression *in vivo* and *in vitro*. Our *in vitro* and *in vivo* data show that SR-BI is critical in mediating the ability of rHDL to rescue diabetes-impaired neovascularization. rHDL was unable to restore high glucose-impaired angiogenesis in shSR-BI cells *in vitro* or rescue diabetes-impaired hindlimb and wound angiogenesis/closure in SR-BI<sup>-/-</sup> mice *in vivo*. Furthermore, compared to wildtype littermates of both the hindlimb ischemia and wound healing models, we found that the ability of rHDL to augment *Siah1a*, *Siah2*, *Hif-1 $\alpha$* , *Vegfa* and *Vegfr2* mRNA levels were attenuated in SR-BI<sup>-/-</sup> mice. Interestingly, SR-BI is the preferred cholesterol acceptor for spherical HDL particles rather

than discoidal rHDL (49). However, it would be expected that following incubation *in vitro*, systemic injection *in vivo*, or topical application to wounds, rHDL would rapidly acquire lipid, forming a spherical particle that is able to interact with SR-BI (50). In support of our findings, a recent study found that SR-BI acts as a cholesterol sensor triggering intracellular signaling and is important for the actions of HDL on endothelial cells (45). SR-BI was also important in mediating ischemia-mediated neovascularization and wound healing/angiogenesis in non-diabetic mice. The role of SR-BI in angiogenesis is further supported by reports that show important signaling pathways including PI3K/Akt are downstream of SR-BI and are associated with angiogenesis-related functions such as migration and proliferation (12).

Despite the vast number of reports demonstrating the therapeutic benefits of HDL on the cardiovascular system, to date there is no translated use of HDL-targeted treatments. There is however, increasing evidence for the anti-diabetic effects of HDL which may present an alternative translation pathway. We found that rHDL rescues both high glucose-related impairment of tubulogenesis *in vitro* and diabetes-impaired neovascularization *in vivo*. The mechanisms for these effects are via increased post-translational HIF-1 $\alpha$  stabilization; VEGFA/VEGFR2 production/activation and signaling; and eNOS activation (Figure 8). Furthermore, SR-BI is important in mediating rHDL-induced rescue of diabetes-impaired angiogenesis. The current study provides a greater understanding into the vascular biological effects of HDL in the context of diabetic vascular complications. This may ultimately facilitate the translation of HDL, not only for cardiovascular disease but also diseases associated with impaired angiogenesis and the vascular complications of diabetes.

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## **Duality of Interest**

No potential conflicts of interest relevant to this article were reported.

## **Author Contributions**

J.T.M.T., H.C.G.P., M.K.C.N., and C.A.B. developed the study design, designed the experiments and interpreted the data. J.T.M.T., H.C.G.P., L.L.D., L.Z.V., A.R., T.T., L.L., Z.E.C., S.C.G.Y., S.R., Y.T.L., and C.A.B. performed the experiments. J.T.M.T., H.C.G.P., and C.A.B. wrote the manuscript. L.L.D., S.R., D.S.C., and M.K.C.N. reviewed and edited the manuscript. C.A.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## **Prior Presentation**

Parts of this study were presented in abstract form at the 75<sup>th</sup> Scientific Sessions for the American Diabetes Association, 5 – 9 June 2015.

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### **Figure Legends:**

**Figure 1: rHDL rescues diabetes-impaired angiogenesis *in vivo*.** *Ischemia-mediated Neovascularization:* Femoral artery ligation was performed on non-diabetic and diabetic C57Bl/6J mice (n=11/group). Mice received *i.v.* injections of rHDL (200 µg/mouse) or PBS (vehicle) on alternate days following ligation until sacrifice. (A) Blood flow perfusion was determined using Laser Doppler; images show high (red) to low (blue) blood flow at Day 10. Laser Doppler Perfusion Index (LDPI) was determined based on the ratio of ischemic (ISC):non-ischemic (NON) hindlimb. White circles, non-diabetic PBS-infused mice; grey triangles, non-diabetic rHDL-infused mice; black circles, diabetic PBS-infused mice; and blue squares, diabetic rHDL-infused mice. (B) Capillaries were identified using immunocytochemistry for CD31<sup>+</sup>, and quantified as number of vessels per myocyte. Photomicrographs represent ischemic gastrocnemius muscle stained for capillaries (CD31<sup>+</sup>, stained red, denoted by arrows) and myocytes (laminin, stained blue). *Wound Healing and Angiogenesis:* Two full thickness wounds were created on non-diabetic and diabetic C57Bl/6J mice (n=11/group). Mice received daily topical applications of rHDL (50 µg/wound) or PBS (vehicle). (C) Wound area was calculated from the average of three daily diameter measurements along the x, y and z-axes. Wound closure is expressed as a percentage of initial wound area at Day 0. White circles, non-diabetic PBS-treated wound; grey triangles, non-diabetic rHDL-treated wound; black circles, diabetic PBS-treated wound; and red squares, diabetic rHDL-treated wound. (D) rHDL:PBS wound blood flow perfusion ratio was determined using laser Doppler imaging; images represent high (red) to low (blue) blood flow at Day 10 in non-diabetic (grey triangles) and diabetic (red squares) mice. (E) Capillaries were identified in wound sections using immunohistochemistry for CD31<sup>+</sup>. Photomicrographs represent wounds stained for CD31<sup>+</sup> (stained brown, denoted by arrows).

Scale bars represent 200  $\mu\text{m}$ . Results are expressed as mean $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. non-diabetic PBS mice; # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.001 vs. diabetic PBS mice.

**Figure 2: rHDL rescues high glucose-impaired HIF-1 $\alpha$  stabilization *in vitro*.** HCAECs were treated with rHDL (20  $\mu\text{M}$ , white bars) or PBS (vehicle, black bars) for 18 h prior to 48 h glucose exposure (5–25 mM), then utilized for RNA or protein analysis. (A) PI3K, (B) phosphorylated Akt (pAkt) relative to total Akt (Akt<sub>T</sub>) protein levels, (C) *SIAH1* and (D) *SIAH2* mRNA levels, normalized to *B2M*; (E) PHD2, (F) PHD3, (G) total HIF-1 $\alpha$  and (H) nuclear HIF-1 $\alpha$  protein levels. Black line separates noncontiguous lanes from the same gel. Results are expressed as mean $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

**Figure 3: rHDL rescues high glucose-impaired VEGFA/VEGFR2 production/activation *in vitro*.** HCAECs were treated with rHDL (20  $\mu\text{M}$ , white bars) or PBS (vehicle, black bars) for 18 h prior to 48 h glucose exposure (5–25 mM), then utilized for protein analysis or Matrigel tubulogenesis assay. (A) VEGFA, (B) VEGFB, (C) VEGFC, (D) VEGFD, (E) secreted VEGFR1, (F) cytoplasmic VEGFR1, (G) phosphorylated VEGFR2 (pVEGFR2) relative to total VEGFR2 (VEGFR2<sub>T</sub>) and (H) phosphorylated eNOS (peNOS) relative to total eNOS (eNOS<sub>T</sub>) protein levels. Black line separates noncontiguous lanes from the same gel. (I) Representative images of tubule formation photographed at 40X under light microscopy. Tubule branches were counted using ImageJ. Results are expressed as mean $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

**Figure 4: Post-translational HIF-1 $\alpha$  modulators Siahs mediate rHDL rescue of high glucose-impaired angiogenesis *in vitro*.** HCAECs were transfected with scrambled (siScr), Siah1 (siSiah1), or Siah2 (siSiah2) siRNA for 6 h then incubated with rHDL (20  $\mu\text{M}$ , white

bars) or PBS (vehicle, black bars) for 18 h prior to 48 h in high glucose (25 mM) and utilized for protein analysis or Matrigel tubulogenesis assay. (A) HIF-1 $\alpha$ , (B) VEGFA, and (C) phosphorylated VEGFR2 (pVEGFR2) relative to total VEGFR2 (VEGFR2<sub>T</sub>) protein levels. (D) Representative images of tubule formation photographed at 40X under light microscopy. Tubule branches were counted using ImageJ. Results are expressed as mean $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.01 vs. relative PBS controls.

**Figure 5: SR-BI mediates rHDL-induced rescue of high glucose-impaired angiogenesis *in vitro*.** HCAECs were transduced with lentivirus ( $1 \times 10^7$  viral particles) expressing shSR-BI-containing vector (shSR-BI) or the empty vector (shControl). Transduced cells were treated with rHDL (20  $\mu$ M, white bars) or PBS (vehicle, black bars) for 18 h prior to 48 h in high glucose (25 mM) then used for protein analysis or Matrigel tubulogenesis assay. (A) Phosphorylated Akt (pAkt) relative to total Akt (Akt<sub>T</sub>), (B) HIF-1 $\alpha$  and (C) VEGFA protein levels. (D) Representative images of tubule formation photographed at 40X under light microscopy. Tubule branches were counted using ImageJ. Results are expressed as mean $\pm$ SEM. \* $P$ <0.05 vs. relative PBS controls.

**Figure 6: SR-BI mediates rHDL rescue of diabetes-impaired ischemia-induced neovascularization *in vivo*.** Femoral artery ligation was performed on diabetic wildtype (WT) and SR-BI<sup>-/-</sup> mice (n=9-10/group). Mice received *i.v.* injections of rHDL (200  $\mu$ g/mouse) or PBS (vehicle) on alternate days following ligation. (A) Laser Doppler Perfusion Index (LDPI) was determined based on the ischemic (ISC):non-ischemic (NON) hindlimb ratio. White circles, diabetic WT PBS-infused mice; grey triangles, diabetic WT rHDL-infused mice; black circles, diabetic SR-BI<sup>-/-</sup> PBS-infused mice; blue squares, diabetic SR-BI<sup>-/-</sup> rHDL-infused mice. (B) Capillaries were identified using immunocytochemistry for CD31<sup>+</sup>,

and quantified as number of vessels per myocyte. Photomicrographs represent ischemic gastrocnemius muscle stained for capillaries (CD31<sup>+</sup>, stained red, denoted by arrows) and myocytes (blue). Scale bars represent 200  $\mu$ m. (C) *Siah1a*, (D) *Siah2*, (E) *Hif-1 $\alpha$* , (F) *Vegfa* and (G) *Vegfr2* mRNA levels, expressed as a ratio of ISC:NON, normalized to *36B4*. Results are expressed as mean $\pm$ SEM. \**P*<0.05.

**Figure 7: SR-BI mediates rHDL rescue of diabetes-impaired wound closure/angiogenesis.** Two full thickness wounds were created on diabetic wildtype (WT) and SR-BI<sup>-/-</sup> mice (n=7-8/group). Mice received daily topical applications of rHDL (50  $\mu$ g/wound) or PBS (vehicle). (A) Wound area was calculated from the average of three daily diameter measurements along the x, y and z-axes. Wound closure is expressed as a percentage of initial wound area at Day 0: white circles, diabetic WT PBS-treated wound; grey triangles, diabetic WT rHDL-treated wound; black circles, diabetic SR-BI<sup>-/-</sup> PBS-treated wound; red squares, diabetic SR-BI<sup>-/-</sup> rHDL-treated wound. (B) Blood flow perfusion was determined using laser Doppler imaging; images represent high (red) to low (blue) blood flow at Day 10 in diabetic wildtype (grey triangles) and SR-BI<sup>-/-</sup> (red squares) mice. (C) Capillaries were identified using immunohistochemistry for CD31<sup>+</sup> (stained brown, denoted by arrows) and expressed relative to wound area. Scale bars represent 200  $\mu$ m. (D) *Siah1a*, (E) *Siah2*, (F) *Hif-1 $\alpha$* , (G) *Vegfa* and (H) *Vegfr2* mRNA levels, normalized to *36B4*. Results are expressed as mean $\pm$ SEM. \**P*<0.05.

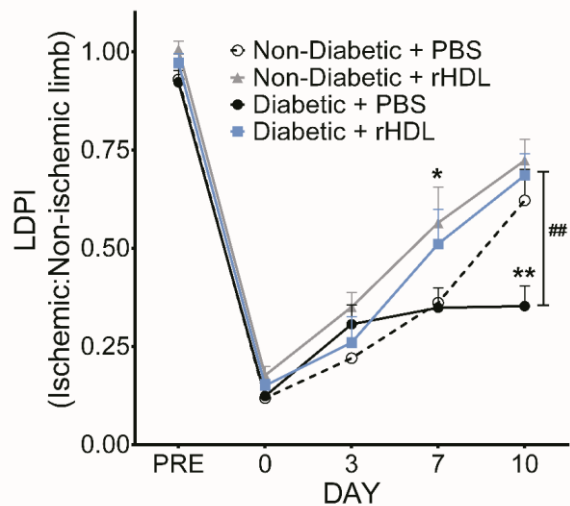
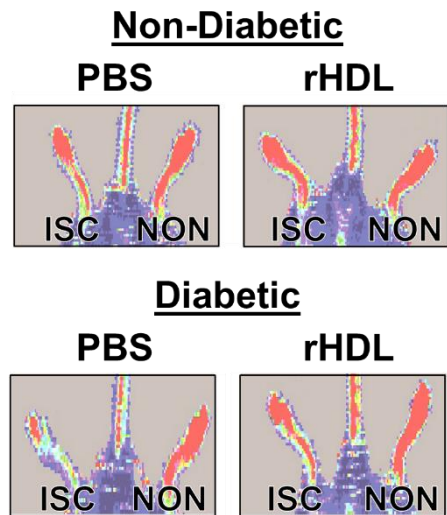
**Figure 8: Proposed mechanism of action of rHDL rescue of diabetes-impaired angiogenesis.** Under high glucose conditions, rHDL activates the PI3K/Akt pathway, inducing the expression of the E3 ubiquitin ligases, Siah1 and Siah2. Increases in Siahs result in the inhibition of two members of the prolyl hydroxylase domain protein family, PHD2 and

PHD3. Suppression of PHD2/3 prevents HIF-1 $\alpha$  from degradation, allowing it to translocate to the nucleus and bind to the hypoxia response element (HRE), activating transcription of pro-angiogenic mediators including VEGFA. VEGFA is released into the circulation where it binds and phosphorylates VEGFR2 further augmenting angiogenesis via the PI3K/Akt pathway and eNOS phosphorylation. These effects of rHDL are mediated via SR-BI.  $\uparrow$  and  $\downarrow$  denotes the effects of rHDL.

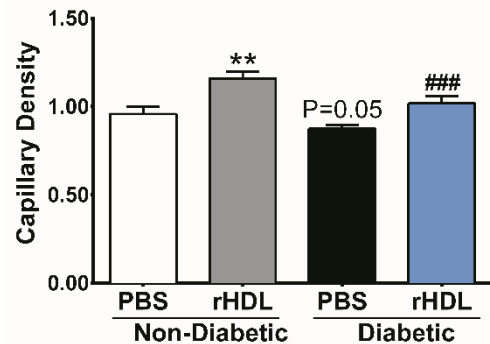
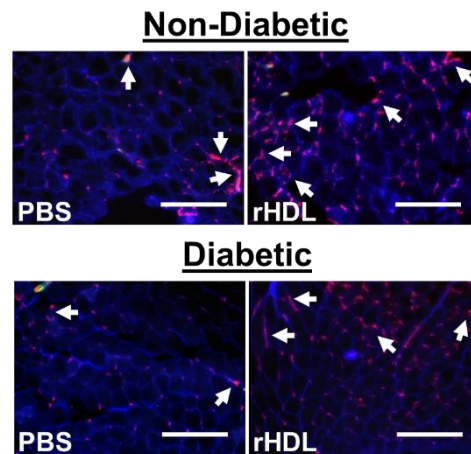


# Figure 1

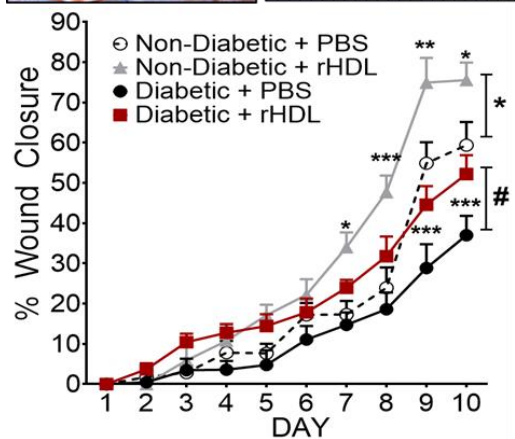
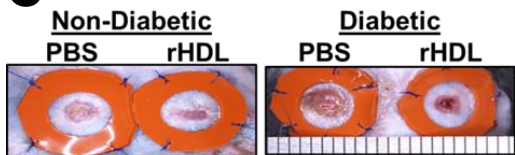
**A**



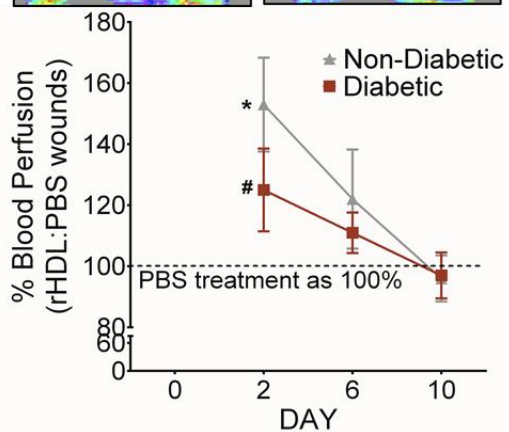
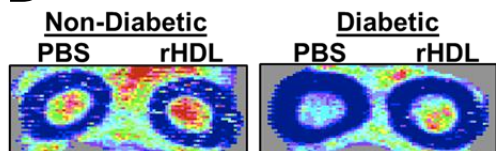
**B**



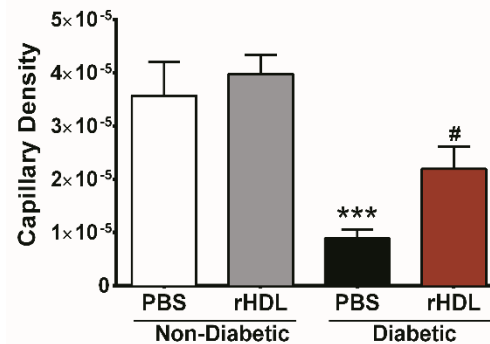
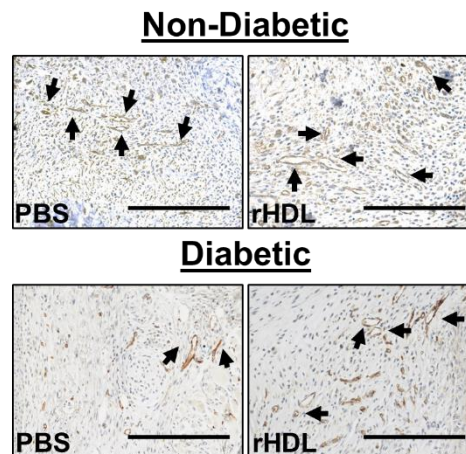
**C**



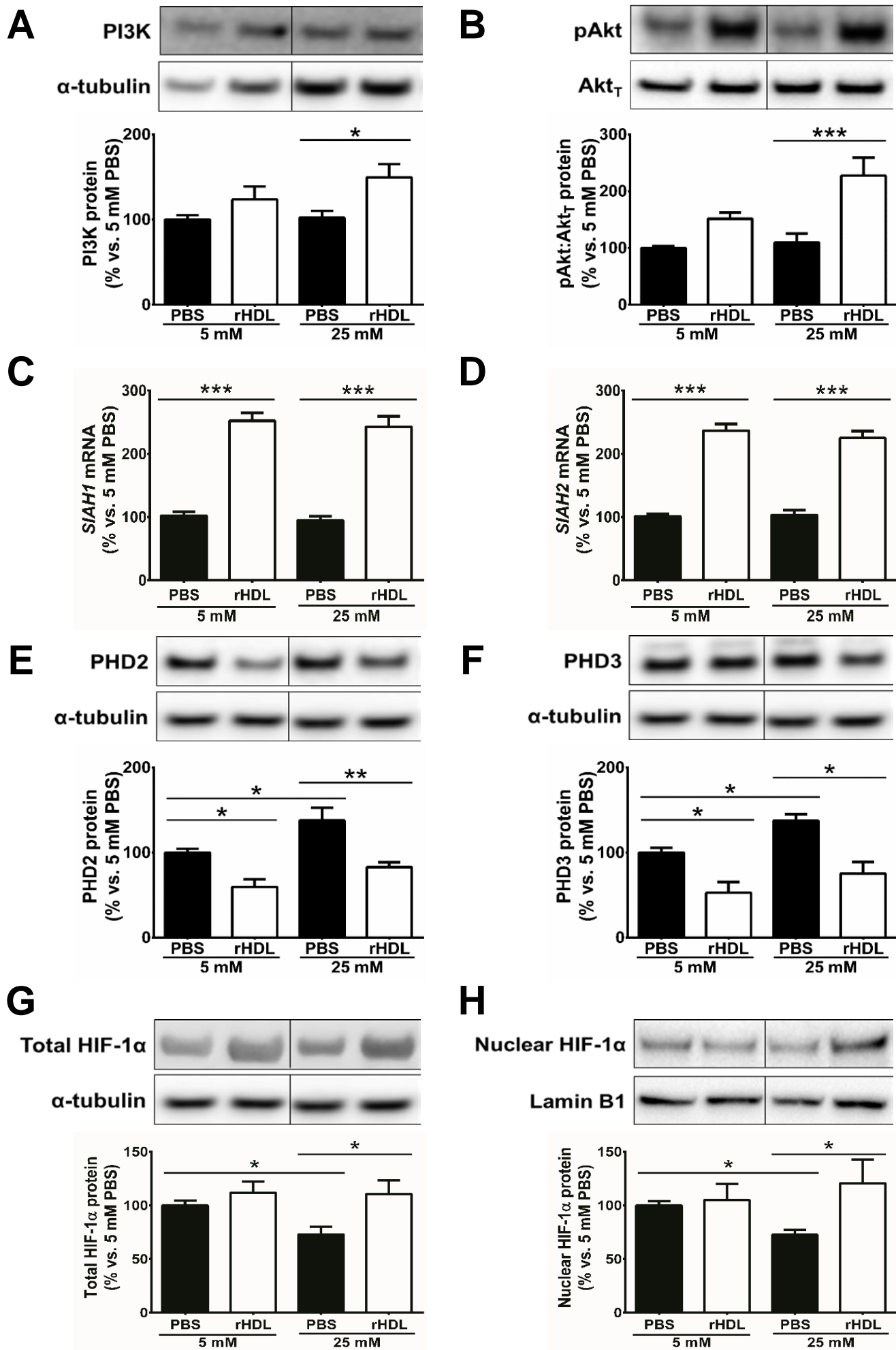
**D**



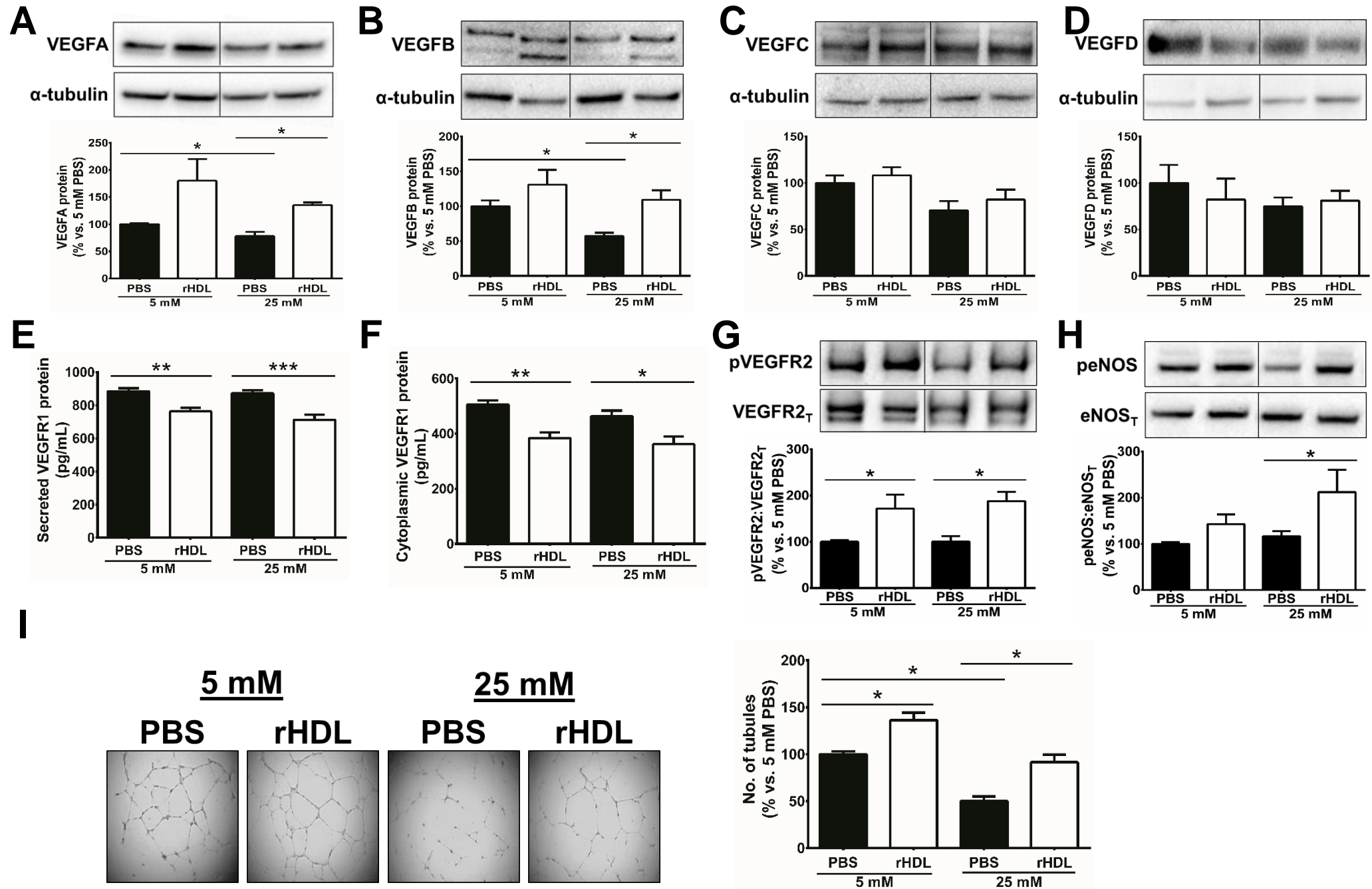
**E**



# Figure 2

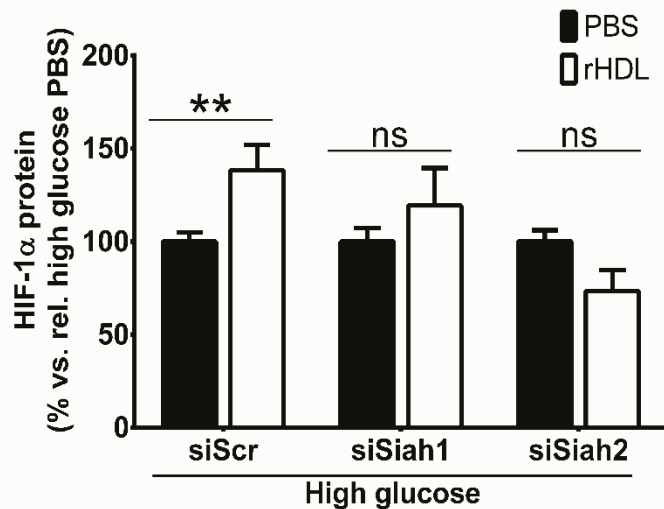


# Figure 3

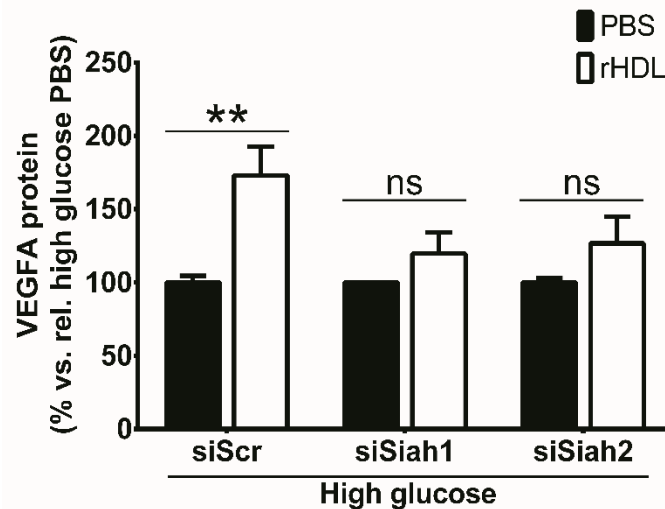


# Figure 4

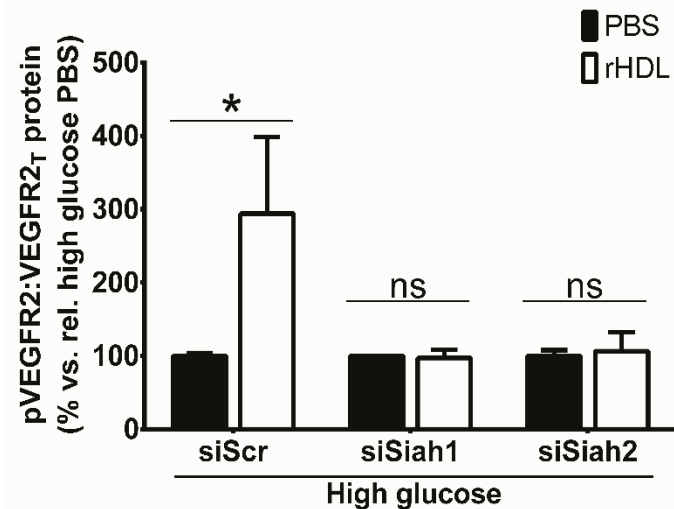
## A



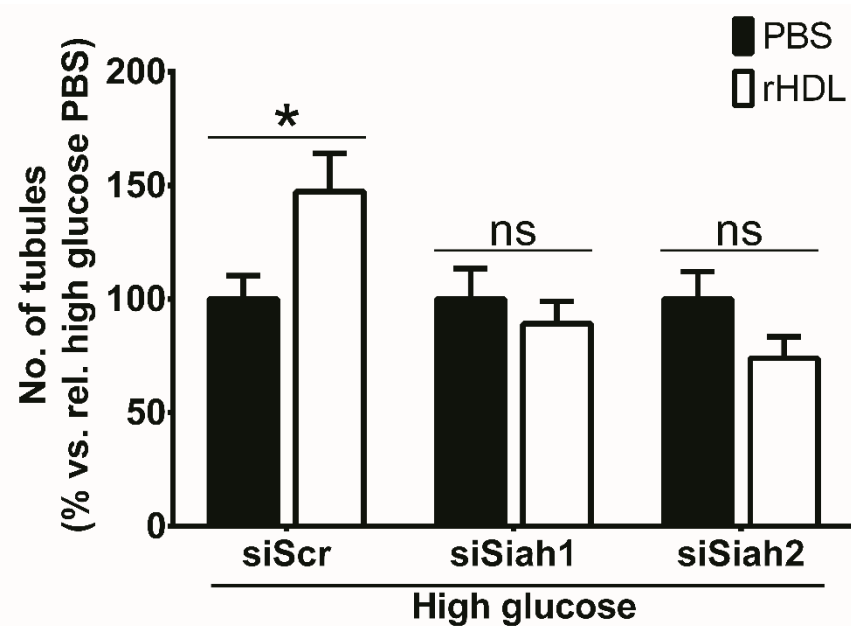
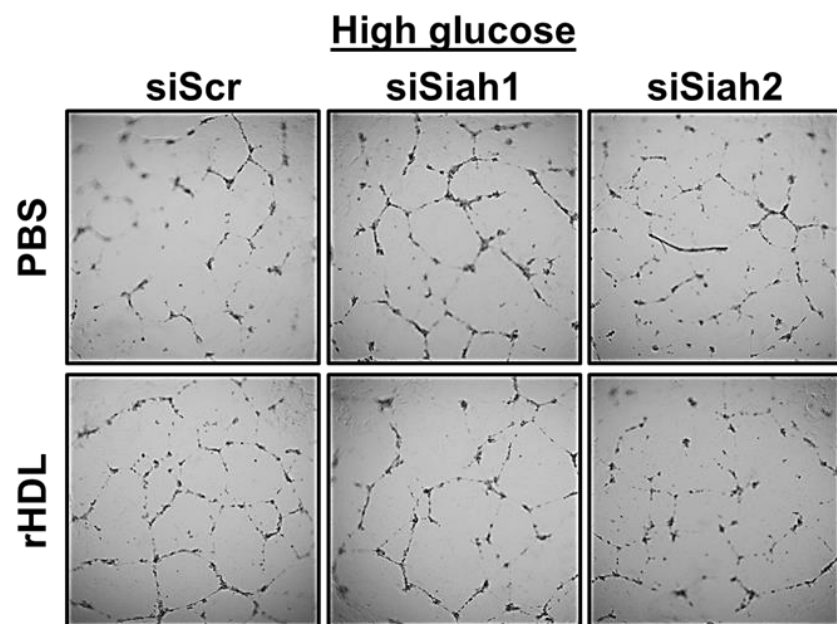
## B



## C

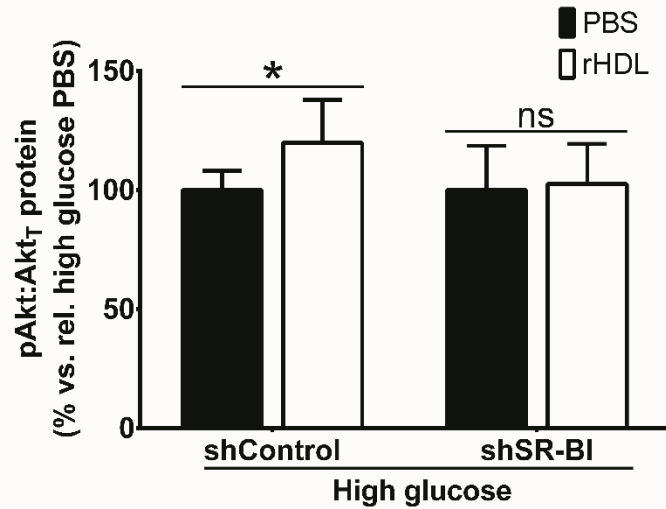


## D

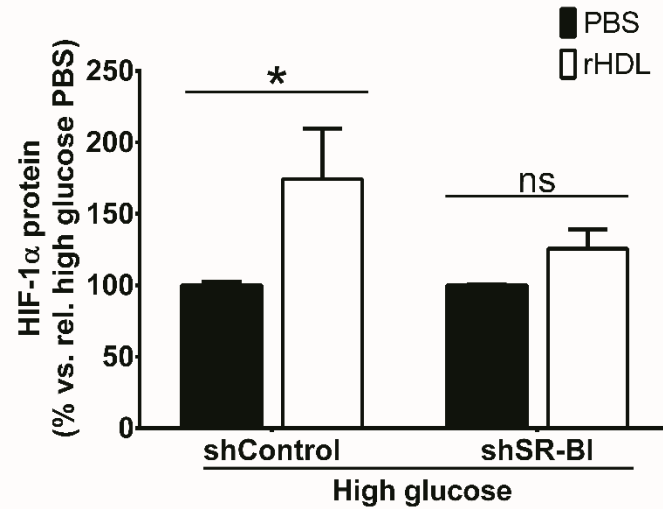


# Figure 5

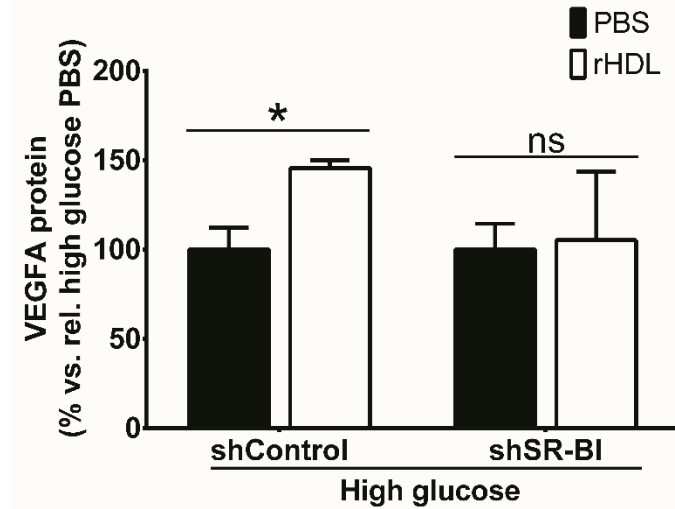
## A



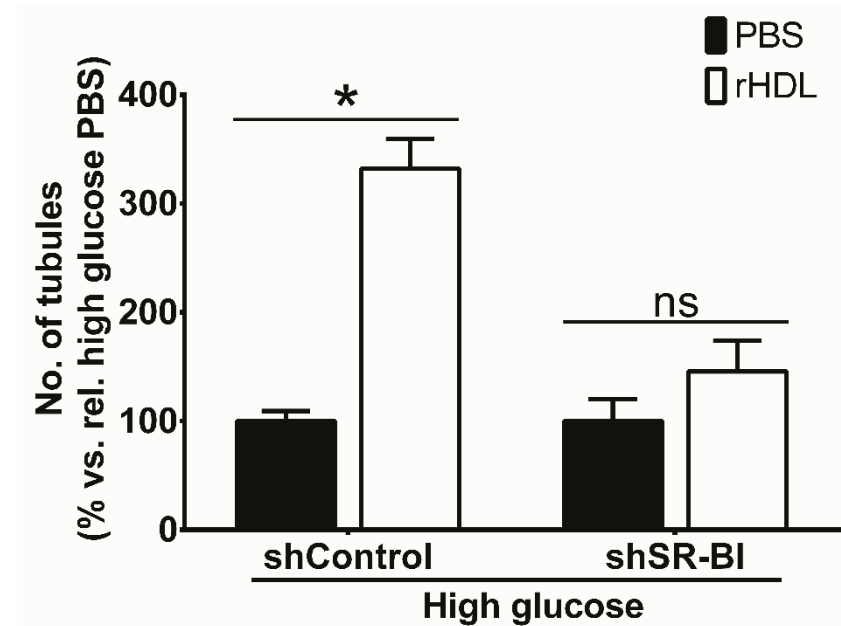
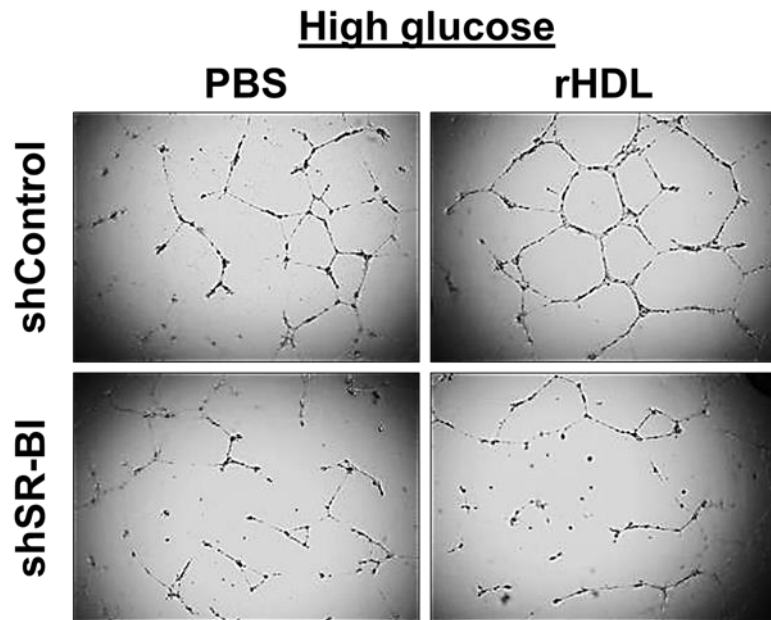
## B



## C

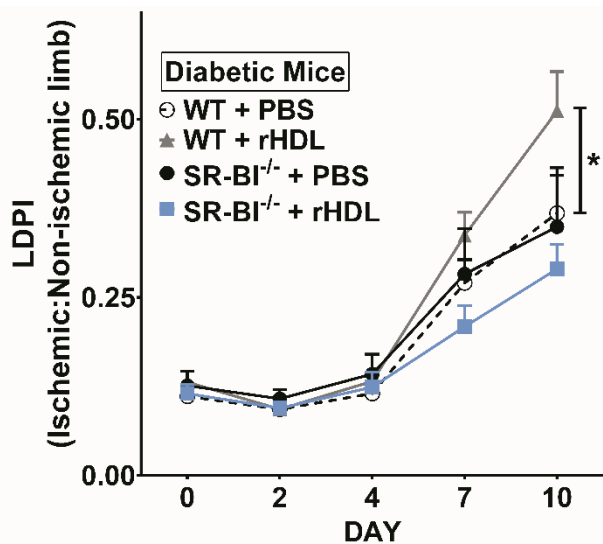
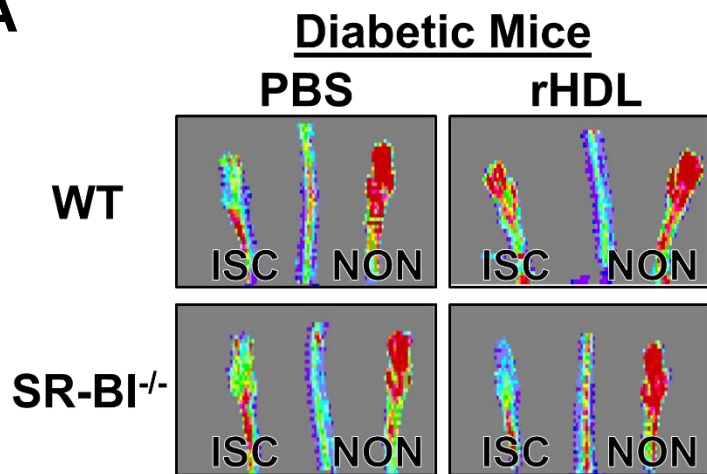


## D

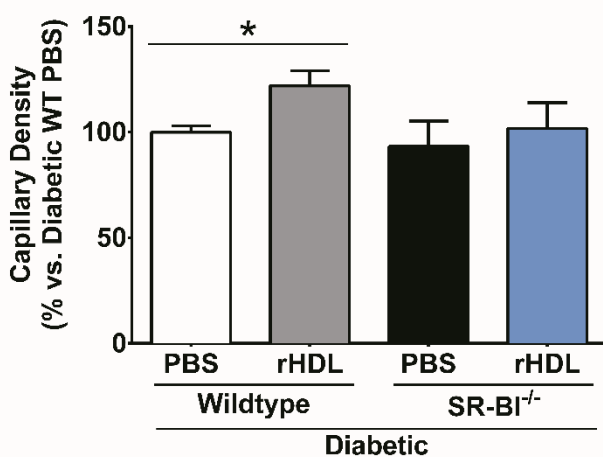
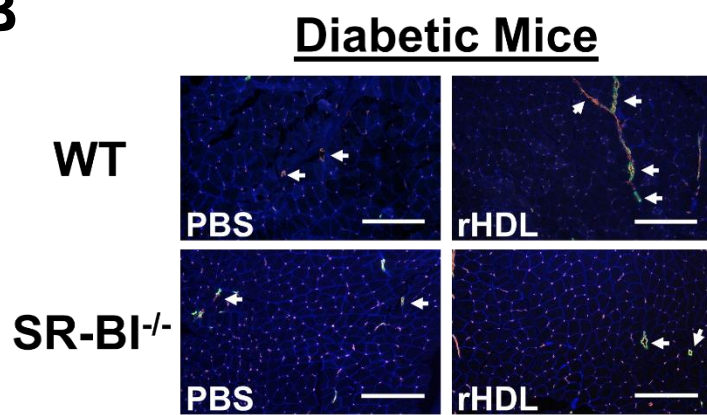


# Figure 6

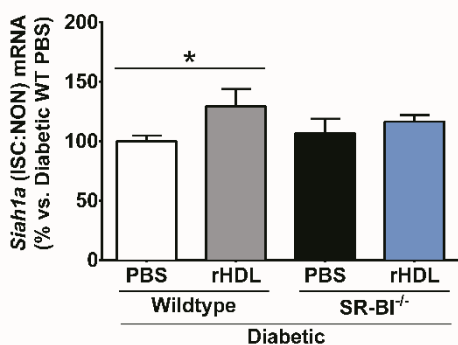
**A**



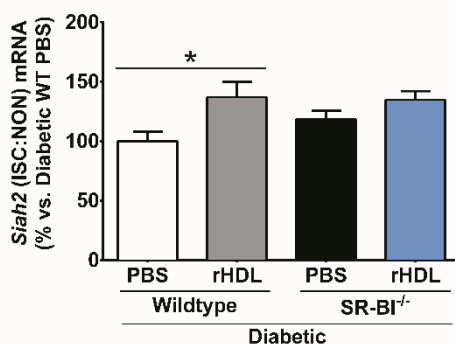
**B**



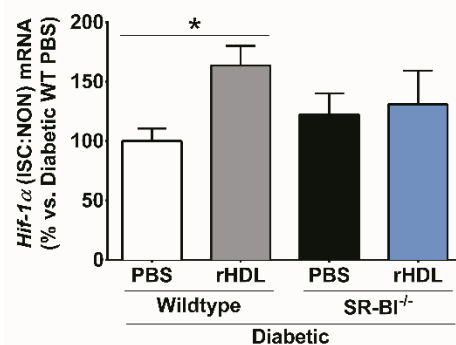
**C**



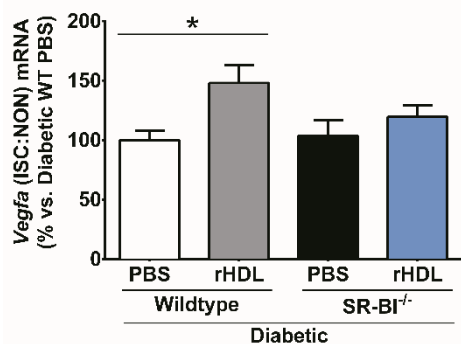
**D**



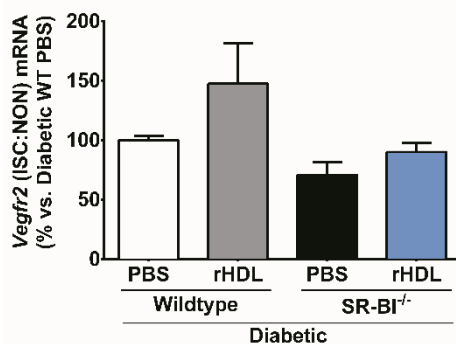
**E**



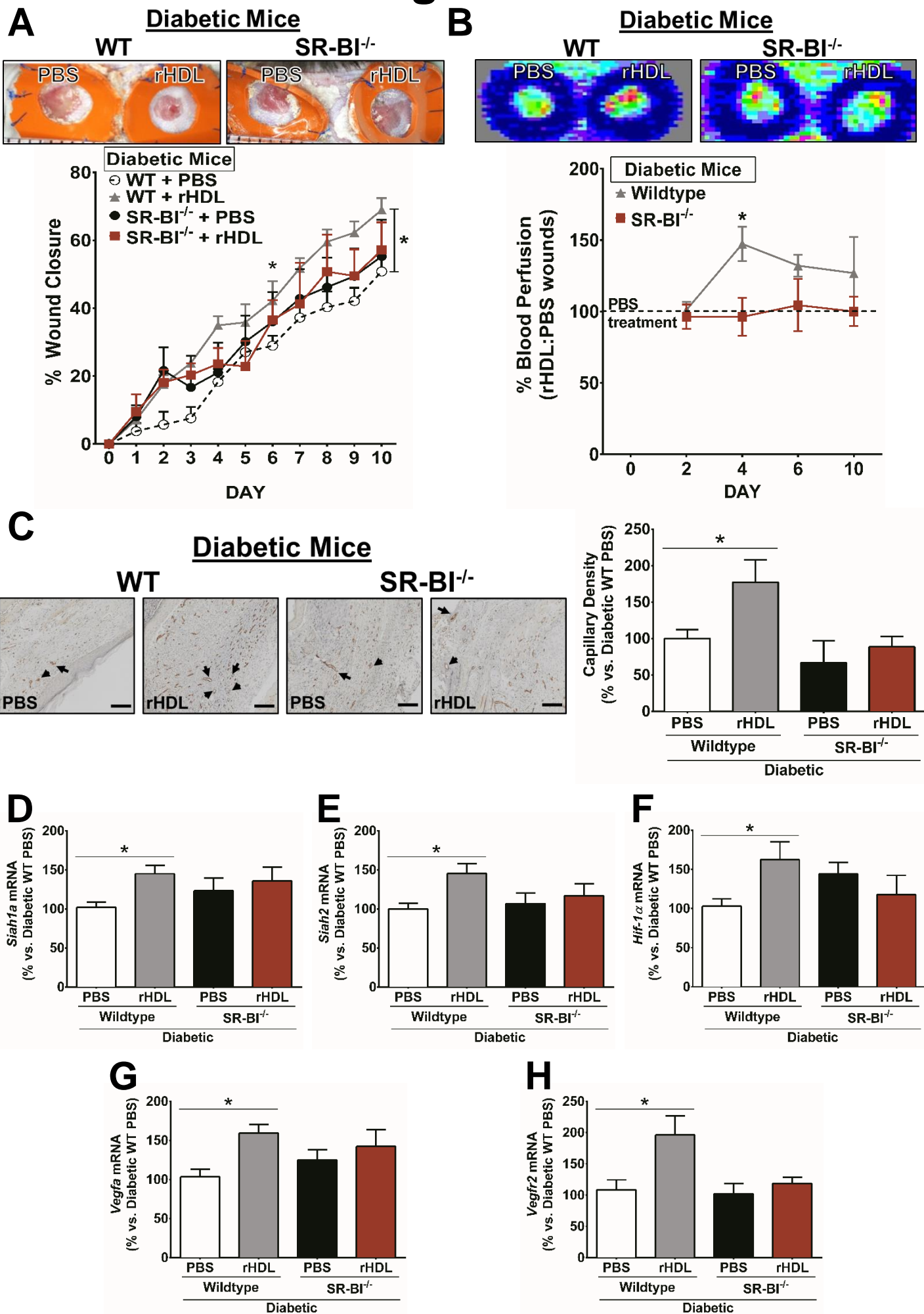
**F**



**G**



# Figure 7



# Figure 8

