Canonical Wnt Signaling Promotes Neovascularization Through Determination of Endothelial Progenitor Cell Fate via Metabolic Profile Regulation

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ABSTRACT

Endothelial progenitor cells (EPCs) contribute to blood vessel formation. Canonical Wnt signaling plays an important role in physiological and pathological angiogenesis and EPC fate regulation. However, the mechanism for Wnt signaling to regulate EPC fate in neovascularization (NV) has not been clearly defined. Here, we showed that very low-density lipoprotein receptor knockout ($Vldlr^{-/-}$) mice, a model of ocular NV induced by Wnt signaling overactivation, have increased EPC numbers in the bone marrow, blood, and retina, as well as an elevated mitochondrial membrane potential indicating higher mitochondrial function of EPCs in the circulation. Isolated EPCs from $Vldlr^{-/-}$ mice showed overactivated Wnt signaling, correlating with increased mitochondrial function, mass, and DNA copy numbers, compared with WT EPCs. Our results also demonstrated that Wnt signaling upregulated mitochondrial biogenesis and function, while inhibiting glycolysis in EPCs, which further decreased EPC stemness and promoted EPCs to a more active state toward differentiation, which may contribute to pathologic vascular formation. Fenofibric acid, an active metabolite of fenofibrate, inhibited Wnt signaling and mitochondrial function in EPCs and decreased EPC numbers in $Vldlr^{-/-}$ mice. It also decreased mitochondrial biogenesis and reactive oxygen species production in $Vldlr^{-/-}$ EPCs, which may be responsible for its therapeutic effect on diabetic retinopathy. These findings demonstrated that Wnt signaling regulates EPC fate through metabolism, suggesting potential application of the EPC metabolic profile as predictor and therapeutic target for neovascular diseases. STEM CELLS 2019;37:1331–1343

SIGNIFICANCE STATEMENT

Dysregulated blood vessel formation is an important pathological feature in multiple diseases such as neovascular eye diseases and cancer. Endothelial progenitor cells (EPCs) are generated in the bone marrow, transported to target tissues through the blood, and differentiate into endothelial cells to form new vessels. This study found that EPC metabolic profile is a direct determinant of EPC fate. The Wnt signaling pathway regulates EPC metabolism, EPC fate, and subsequently, new vessel formation. These findings reveal a new function of Wnt signaling in regulating mitochondrial function in EPCs. This study also suggests a potential significance of EPC metabolic profile as a diagnostic marker and therapeutic target for diseases caused by abnormal blood vessel formation.

INTRODUCTION

The Wnt signaling pathway plays a key role in the regulation of angiogenesis and vasculogenesis in both physiological and pathological conditions [1, 2]. Abnormal blood vessel growth stimulated by different stressors, such as hypoxia, hypoglycemia, mechanical stress, inflammation, and genetic mutations, is closely related to cancer, diabetes, cardiovascular diseases [3, 4], and ocular neovascularization (NV) [5, 6]. The ocular

system is widely accepted and used as a valuable model for NV in nonocular tissues and tumors [7].

Since circulating EPCs were first isolated by Asahara et al. [8], numerous studies suggested that a small population of bone marrow (BM)-derived progenitor cells, play a key role in vascular repair and NV at sites of injury or ischemia. It is believed that the circulating EPCs may home to sites of NV, activate local endothelial cells (ECs; angiogenesis), or differentiate into ECs in situ (vasculogenesis), playing a role in vessel

formation [9–11]. Recently, the "true" EPCs, as enumerated by improved flow cytometry strategy by different groups, are defined as endothelial colony-forming cells, which are able to integrate directly into the developing vessels and form tube-like structure in vitro [12]. On the other hand, the circulating angiogenic cells (also termed early EPCs) regulate angiogenesis via paracrine and autocrine mechanisms.

Dysregulation of the EPC fate, including cell proliferation (self-renewal) and differentiation, is related to the occurrence and severity of microvascular and macrovascular disorders, suggesting a close mechanistic link between EPC dysfunction and abnormal vascular pathology in diabetes [13], cancer [14], and ocular diseases [15–17]. EPC number changes have been reported to associate with cardiovascular disease and to be a promising biomarker for diagnosis and therapeutic evaluation [18]. However, until now the number of EPCs has not been well-accepted as a biomarker for vascular diseases. Hence, researchers are still searching for a more reliable EPC-related biomarker for the development and progression of neovascular disorders.

The function and fate of stem cells, as well as progenitor cells, are closely programmed by their metabolic profile including mitochondrial oxidation and glycolysis in variable physiologic and pathologic conditions [19–21]. The canonical Wnt signaling pathway is one of the main regulators of most types of tissue-specific stem cells in adult mammals [22]. Dysregulation of Wnt signaling is an important cause of impaired mitochondrial biogenesis and reactive oxygen species (ROS) generation, leading to exacerbated pathologic outcomes in tumor development and abnormal NV [23–26]. Several studies have reported that the Wnt signaling pathway regulates NV in the development of ocular diseases [27–32]. In 2013, our group reported that the aberrant activation of the Wnt pathway played an important role in hypoxia-induced EPC release using the oxygen-induced retinopathy mouse model [33]. However, the relationship between the Wnt pathway and EPC fate and metabolism remains elusive.

Very low-density lipoprotein receptor (VLDLR) is a receptor for very low-density lipoprotein and a member of the low-density lipoprotein receptor family. Previously, we have shown that *Vldlr* knockout (KO) results in intraretinal and subretinal NV through activation of Wnt signaling in the retina [32, 34]. However, the impacts of *Vldlr* KO on EPC number and metabolism have not been studied.

In the present study, using the *Vldlr* gene knockout (*Vldlr*$^{-/-}$, VKO) mouse model and isolated EPCs, our study has demonstrated that Wnt signaling regulated the EPC metabolic profile, including mitochondrial oxidation and glycolysis, through which Wnt signaling determined EPC generation, release, homing, and differentiation.

MATERIALS AND METHODS

Animals

Vldlr$^{-/-}$ mice in the C57BL/6J background were originally purchased from Jackson Laboratories (Bar Harbor, ME). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center. Fenofibric acid (FA; AK Scientific, Union, CA) was prepared in dimethyl sulfoxide (DMSO). Animals were intraperitoneally

injected daily with FA (25 μg/g body weight) or vehicle (DMSO) from postnatal day 14 (P14) to P28.

The Wnt signaling reporter mice, transgenic *Axin2*^{lacZ}/*Vldlr*$^{-/-}$ mice, were generated at the University of Oklahoma Health Sciences Center's animal facility by crossing *Axin2*^{lacZ} mice with *Vldlr*$^{-/-}$ mice and littermate *Axin2*^{lacZ}/*Vldlr*$^{+/+}$ mice were used as control mice [29]. Both male and female mice were used in this study.

Primary EPCs Isolation and Culture

EPCs were isolated and cultured as described previously [35]. To obtain mouse primary EPCs, whole BM cells were collected from the femurs and tibias of mice (postnatal day P28) in the morning and one from each group in turn to diminish circadian variation, then suspended in phosphate-buffered saline (PBS) and overlaid onto an equal volume of Histopaque1083 (Sigma, St. Louis, MO), followed by centrifugation for 30 minutes at 500*g*. Mononuclear cells (MNCs) were collected from the interface between the Histopaque and supernatant, and then transferred to a sterile centrifuge tube. The cells were washed three times, plated on dishes precoated with collagen I (1 μg/ml) and cultured in EGM-2MV medium (EGM-2 with EGM-2 bullet kit; Lonza, Basel, Swiss) containing 10% fetal calf serum. After 3 days in culture, unattached cells were removed by a single wash with PBS; the attached cells were cultured in fresh medium for further experiments. To activate Wnt signaling in vitro, primary EPC (P1) were infected with an adenovirus expressing a constitutively active mutant of β-catenin, Ad-S37A, at MOI 50, 72 hours to activate Wnt signaling; to inhibit Wnt signaling, EPCs were treated with an Wnt inhibitor ICG001 (10 μM, 24 hours) and FA (20 μM, 72 hours).

Fluorescence-Activated Cells Sorter Analysis

For quantification of EPCs in the BM, circulation, and retina, fluorescence-activated cells sorter analysis (FACS) was used as described [36]. Samples were transferred into 12 mm × 75 mm polystyrene round-bottom tubes and incubated with CD45-FITC (no. 103108; Biolegend, San Diego, CA), CD133-PE (no. 141204; Biolegend), CD34-BV421 (no. 119321; Biolegend), and VEGFR2-APC (no. 136406; Biolegend) antibodies for 1 hour in the dark. The red blood cells were lysed by adding 2 ml of red blood cell lysis buffer (BD Biosciences, Faranklin Lakes, NZ) for 15 minutes. The remaining cells were washed in 2 ml PBS containing 10% fetal bovine serum and pelleted by centrifugation at 300*g* for 5 minutes and finally resuspended in 250 ml paraformaldehyde (4%). The sample tubes were stored at 4°C in the dark until analysis. The samples were measured by Stratedigm S1300Ex (Stratedigm, San Jose, CA) and CD34+/CD45−/VEGFR2+/CD133+ population was analyzed by FlowJo software (Life Science Software Company, Ashland, Oregon).

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Basically, $\Delta\Psi_m$ of EPCs in the circulation of mice was estimated using flow cytometry and fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, no. 551302, BD, Faranklin Lakes, NZ). Samples were collected as previously described in FACS fraction and then incubated with antibodies for CD45-BV605 (no. 103139; Biolegend), CD34-BV421 (no. 119321; Biolegend), and VEGFR2-APC (no. 136406; Biolegend) for 1 hour. The red blood cells were lysed. Samples were stained with 2.5 μM JC-1 37°C for 15 minutes, washed, resuspended with the assay buffer, and $\Delta\Psi_m$ of

CD34+/CD45−/VEGFR2+ populations were analyzed by flow cytometry immediately.

EPC Metabolic Function Analysis

Mitochondrial function was evaluated using Seahorse XFe96 Flux Analyzer (Agilent, Santa Clara, CA) by measuring oxygen consumption rate (OCR). Wild-type (WT) and $Vldlr^{-/-}$ EPCs were seeded in the Seahorse microplate at a density of 2×10^4 cells per well and were subjected to simultaneous measurement of the two major energy producing pathways, the mitochondrial respiration, and glycolysis using Seahorse XFe96 cell Energy Phenotype Test Kit (Agilent). To further define the role of mitochondrial respiration and glycolysis in cell metabolism, compounds oligomycin (1 μM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 6 μM), and a mixture of rotenone and antimycin A (RAA, 2 μM) were serially injected to measure basal OCR, ATP generation, and maximal OCR. Glucose (10 mM) and oligomycin (1 μM), 2-deoxy-glucose (50 mM), were sequentially injected to measure extracellular acidification rate.

Fluorescent Immunocytochemistry Staining

EPCs were immunostained for Tom20 (Abcam, ab78547) and 3-nitrotyrosine (Abcam, ab61392) following manufacturer's protocol. Images of EPC were collected at the same setting in each experiment under an Olympus Fluoview (Version2.1a; Confocal microscope, ×100 objective). The fluorescence intensities of Tom20 (green) and 3-nitortyrosine (red) were obtained using ImageJ software (NIH) and normalized by DAPI (blue) nuclei fluorescence intensity.

Western Blot Analysis

EPCs were lysed in RIPA buffer containing a protease inhibitor cocktail (ThermoFisher, Waltham, MS). Total protein levels were measured using a BCA protein assay kit (ThermoFisher). An equal amount of proteins was loaded and resolved on a 10% SDS-PAGE gel, and transferred onto a nitrocellulose filter after separation in the gel. The filter was blocked in 5% milk in TBST for 1 hour, and was incubated with primary antibodies for peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1-α; Novus, Littleton, CO, NBP1-04676), Mitochondrial transcription factor A (TFAM; ell Signaling Technology, Danvers, MA, 8076S) and β-actin at 4°C overnight. After washing with TBST, the filter was incubated with HRP-conjugated secondary antibodies for 1 hour. Proteins were visualized, examined, and quantified by densitometry using ChemDoc Imaging System and Image Lab software (BioRad, Hercules, CA).

X-Gal Staining

X-gal staining was performed in primary EPCs from *Axin2-lacZ* reporter mice. Briefly, primary EPCs were isolated from $Axin2^{lacZ}/Vldlr^{-/-}$ reporter mice and $Axin2^{lacZ}/Vldlr^{+/+}$ reporter mice. $Axin2^{lacZ}/Vldlr^{-/-}$ EPCs were cultured with FA (20 μM) for 3 days with DMSO as vehicle control. The cells were fixed in 1× fixation buffer for 30 minutes. The cells were incubated at 37°C for 4 hours in the X-gal staining solution. To quantify X-gal-positive cells, X-gal-positive cells were counted under a microscope in ×10 magnification.

Colony Formation Assay

Colony-forming assay was performed as previously described [37]. Isolated MNCs were suspended in culture medium and seeded in 35-mm methylcellulose-based dish (1×10^6 MNCs per dish; no. 27100, Stemcell Technologies, Vancouver, CA). On the

20th day, the number of colony-forming units (CFUs)/dish was counted. A colony of EPCs was defined as a central core of round cells with elongated sprouting cells at the periphery. The colonies were stained by crystal violet solution (0.5% crystal violet in 25% methanol) for 1 hour and quantified with GelCount colony counter (Optronix, Abingdon, OX, U.K.) and GelCount software.

Cell Differentiation Assay

Total RNA was isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and reverse-transcribed to cDNA using the iScript Reverse Transcription Supermix kit (Bio-Rad) at 37°C for 15 minutes. Gene expression of stemness markers, including octamer-binding transcription factor 4 (OCT4), Nanog, SRY-box2 (Sox2), and Thromboxane B3 (Txb3), was analyzed by SYBR Green Master Mix (Bio-Rad). Primers were as follows: sense: 5′-GCATTC AAACTGAGGCACCA-3′ and antisense: 5′-AGCTTCTTTCCCCATCCCA-3′ for Oct4; sense: 5′-AGGGTCTGCTACTGAGATGCTCTG-3′ and antisense: 5′-CAACCACTGGTTTTTCTGCCACCG-3′ for Nanog; sense: 5′-CAGCTGTCATTTGCTGTGGG-3′ and antisense: 5′-AAATGGGAGGGG TGCAAAAG-3′ for Sox2; sense: 5′-CCACCTCCAACAACACGTTCT-3′ and antisense: 5′-TAAGGAAACAGGCTCCCGAA-3′ for Tbx3, Gapdh was used as a housekeeping gene, in order to normalize the expression targets. The polymerase chain reaction (PCR) was performed following the manufacturer's suggestions.

ROS Assay

ROS was measured using 2′,7′-dichlorodihydrofluorescein (DCF) diacetate (H_2DCFDA) method. EPCs were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 μl of growth medium and cultured. The cells were treated with FA (20 μM, 72 hours). The cells were incubated with 10 μM H_2DCFDA for 30 minutes in the dark at 37°C and 5% CO_2. The relative fluorescence intensity representing the intracellular generation of ROS was measured at an emission wavelength of 535 nm and excitation at 485 nm.

Mitochondrial DNA Copy Number Measurement

Total DNA was extracted from primary $Vldlr^{-/-}$ EPCs and WT EPCs using ZR-*Duet* DNA/RNA MiniPrep Plus Kit (Zymo Research, Irvine, CA). The chip-based digital polymerase chain reaction (dPCR) was performed to quantify copies of mitochondrial DNA (mtDNA) as previously described [38]. The results were normalized by the amount of chromosome DNA.

Statistical Analysis

Data were entered and extracted from PRISM 7 (GraphPad Software, La Jolla, CA). All experiments were performed at least three times. Quantitative data were presented as mean ± SEM and analyzed by Student's *t* test when two groups were compared. Data were analyzed by analysis of variance when more than two groups were compared. The association between qualitative variables was evaluated by Spearman correlation. A *p* value of <.05 was considered statistically significant.

RESULTS

EPC Mitochondrial Function Correlated with EPC Numbers and Wnt Signaling in VKO Mice

As shown in our previous study, absence of VLDLR is associated with ocular NV through the activation of Wnt signaling
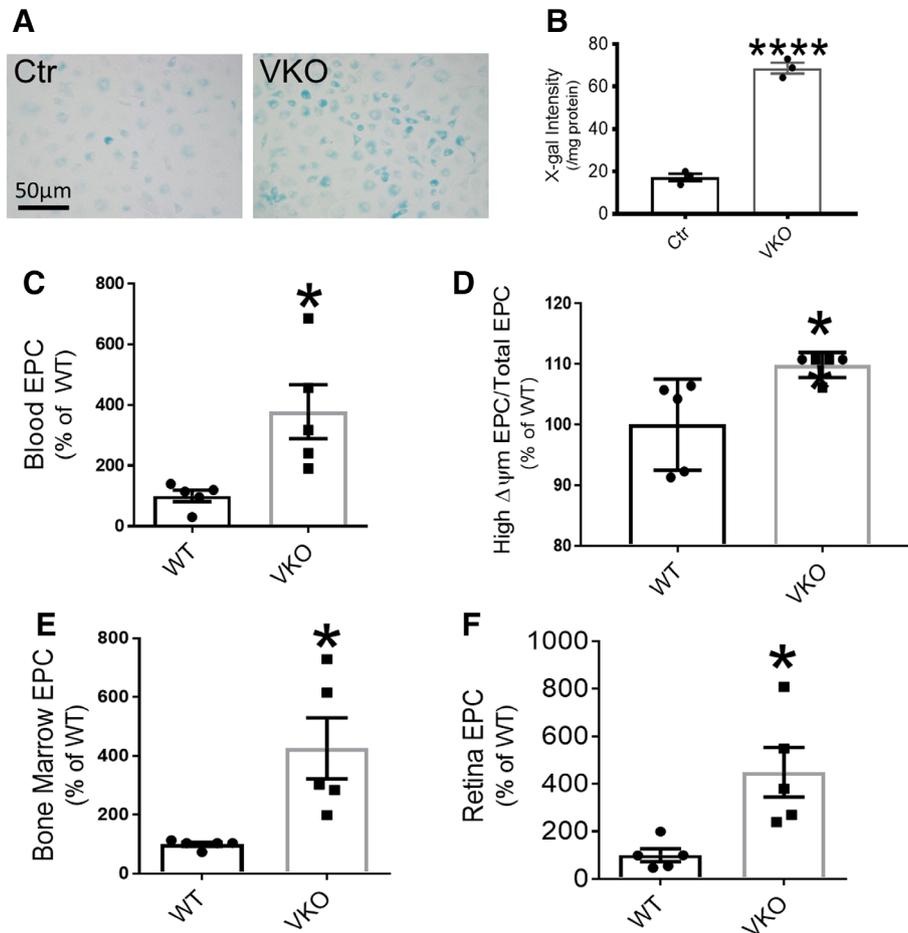
Figure 1. Activation of Wnt signaling increased endothelial progenitor cell (EPC) numbers and mitochondrial function of EPCs in *Vldlr* gene knockout (VKO) mouse. **(A):** X-gal staining of EPCs from the bone marrow of *Axin2^{lacZ}/Vldlr^{+/+}* Wnt reporter mice (Ctr), *Axin2^{lacZ}/Vldlr^{−/−}* Wnt reporter mice (VKO). **(B):** X-gal intensity was quantified and compared; *n* ≥ 3. **(C–F):** EPC population was determined using flow cytometry. (C): EPC population in the blood increased in *Vldlr^{−/−}* (VKO) mice compared with WT (*n* = 5). (D): The population of EPCs with high $\Delta\Psi$m increased in VKO mice (*n* = 5). (E): EPC population in the bone marrow increased in VKO mice (*n* = 5). (F): EPC population increased in the retina of VKO mice (*n* = 5; mean ± SEM; *, *p* < .05; **, *p* < .01; ***, *p* < .001; ****, *p* < .0001).

[32, 34]. Wnt signaling has been shown to regulate EPC release from the BM [33]. To further explore the direct association and mechanism between Wnt signaling and EPC metabolic function/fate determination, we measured Wnt signaling activity in EPCs using Wnt signaling reporter mice, *Axin2-lacZ* transgenic mic*e* [29]. As shown by X-gal staining of EPCs isolated from the BM, Wnt signaling activity was significantly higher in EPCs isolated from *Axin2^{lacZ}/Vldlr^{−/−}* mice (VKO), compared with that in EPCs from *Axin2^{lacZ}/Vldlr^{+/+}* mice (Ctr), indicating Wnt signaling overactivation in *Vldlr^{−/−}* EPCs (Fig. 1A, 1B). Hence, *Vldlr^{−/−}* mice were used as a Wnt activation and pathological ocular NV model in this study. Using flow cytometry, we measured $\Delta\Psi$m of EPCs in the circulation and EPC numbers in the BM, blood, and retina. The results showed that numbers of EPCs in the circulation of *Vldlr^{−/−}* (VKO) mice were threefold higher than that in WT controls (Fig. 1C). Moreover, the percentage of EPCs with high $\Delta\Psi$m in *Vldlr^{−/−}* mice were significantly higher than that in WT mice, demonstrating an increased mitochondrial activity in the circulating EPCs from *Vldlr^{−/−}* mice (Fig. 1D). Consistently, numbers of EPCs in the BM and retina were also increased in *Vldlr^{−/−}* mice (Fig. 1E, 1F). These results suggest that Wnt signaling may play a role in the regulation of the EPC metabolic profile and EPC generation,

release and homing, implying a mechanism by which Wnt signaling promotes NV through upregulation of EPC activity [28, 29, 31–33].

Mitochondrial Respiration Is Dependent on Wnt Signaling Activity In Vivo

To determine the role of Wnt signaling in mitochondrial function of EPCs, the mitochondrial respiration rate of primary EPCs was evaluated by measurement of OCR using Seahorse XFe96 cell Mito Stress Test Kit. The mitochondrial respiration, basal OCR, maximal OCR, and ATP generation were elevated in primary EPCs isolated from the BM of *Vldlr^{−/−}* mice compared with that of WT EPCs (Fig. 2A–2D). Consistently, activation of Wnt signaling using adenovirus expressing a constitutively active β-catenin mutant, *Ad-S37A*, increased mitochondrial function, basal OCR, maximal OCR, and ATP production in WT EPCs (Fig. 2E–2H). On the contrary, inhibition of Wnt signaling in EPCs through a Wnt inhibitor ICG001 treatment for 24 hours significantly reduced the basal OCR, maximal OCR, and ATP generation (Fig. 2I–2L). The activation and inhibition of Wnt signaling by LiCl and *Ad-VLDLR*, respectively (Supporting Information Fig. S1), also demonstrated that mitochondrial function in EPCs was regulated by Wnt signaling.
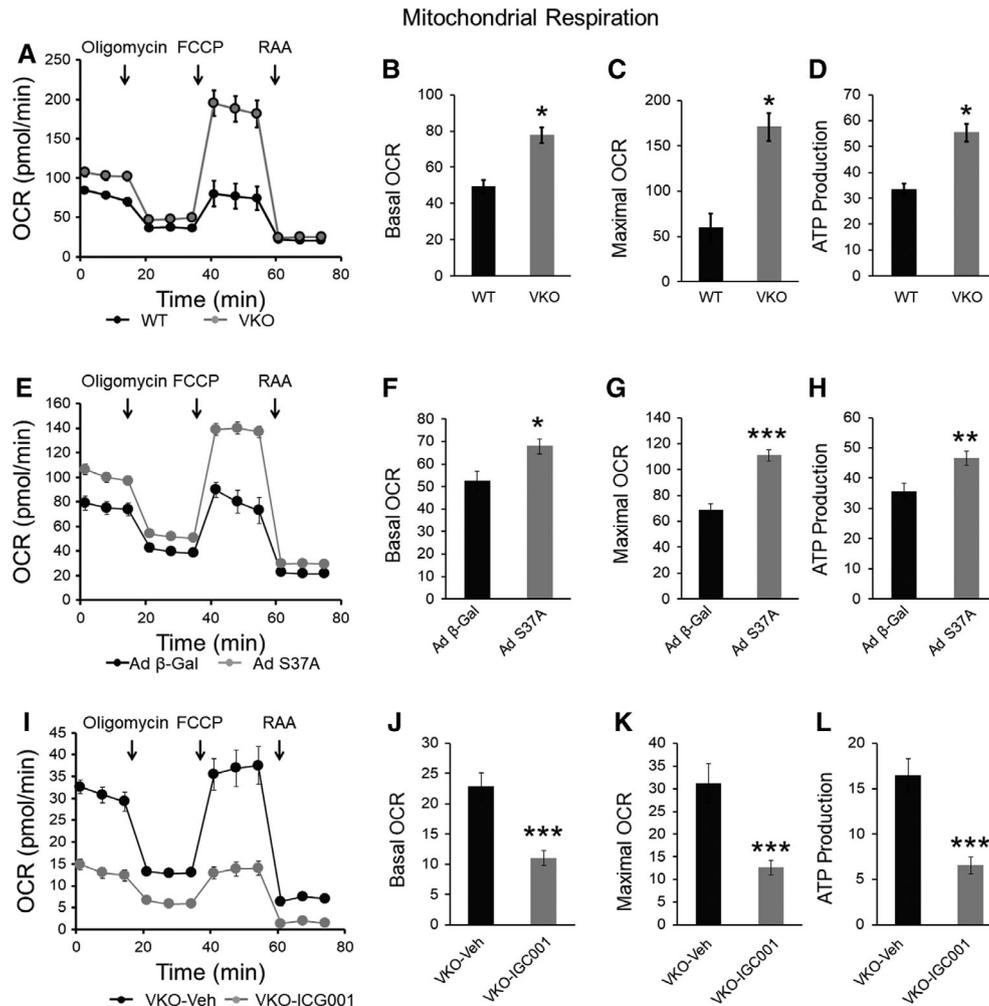
Mitochondrial Respiration

Figure 2. *(Continued on next page)*

Glycolysis of EPC Is Negatively Regulated by Wnt Signaling

Mitochondrial oxidation and glycolysis coordination is known to determine cell fate, especially in progenitor cells. Our results showed that mitochondrial function of EPCs was enhanced by Wnt signaling. Here, we also measured the glycolysis in EPCs using Seahorse XF Glycolysis Stress Test Kit. Interestingly, glycolysis, glycolytic capacity, and glycolytic reserve were decreased in EPCs from $Vldlr^{-/-}$ mice compared with WT EPCs (Fig. 2M–2P). Activation of Wnt signaling in WT EPCs by infection with *Ad-S37A* also resulted in declines of the glycolysis, glycolytic capacity, and glycolytic reserve (Fig. 2Q–2T). Conversely, inhibition of Wnt signaling in $Vldlr^{-/-}$ EPC using ICG001 significantly increased glycolysis, glycolytic capacity, and glycolytic reserve (Fig. 2U–2X). Taken together, these metabolic analyses suggest that Wnt signaling positively regulates mitochondrial oxidation, whereas negatively modulating glycolysis in primary EPC.

Wnt Signaling Facilitates Mitochondrial Biogenesis

To understand the mechanism for the upregulated mitochondrial oxidation in EPCs by Wnt signaling, we further explored whether the effect is through modulation of mitochondrial biogenesis. Immunocytochemistry (ICC) of primary EPCs using an antibody against Tom20, a marker for assessing mitochondrial mass, revealed that the mitochondrial mass in $Vldlr^{-/-}$ EPCs was significantly higher than in WT EPCs (Fig. 3A, 3B). Furthermore, EPCs from $Vldlr^{-/-}$ mice showed increased mitochondrial DNA copy numbers compared with WT EPCs (Fig. 3C). Consistently, Western blotting also showed elevated levels of mitochondrial biogenesis markers peroxisome PGC-1α and TFAM in $Vldlr^{-/-}$ EPCs (Fig. 3D, 3E).

Mitochondria are the major producer of ROS [39], and ROS generation is used as an indicator of mitochondria function. Upregulation of $\Delta\Psi_m$ and ATP generation promotes ROS formation. Here, we measured ROS production with ICC using an antibody for 3-NT, a marker of oxidative stress. The results showed that in EPC from $Vldlr^{-/-}$ mice, 3-NT levels were significantly higher than that in WT EPCs (Fig. 3F, 3G). As shown by DCF fluorescence assay, ROS was overproduced in EPCs from $Vldlr^{-/-}$ mice (Fig. 3H). All of these results suggested that activation of Wnt signaling resulted in the upregulation of mitochondrial biogenesis in EPCs, which may directly promote EPC differentiation.

Activation of the Wnt Signaling Pathway Promotes EPC Differentiation

To determine whether Wnt signaling contributes to the EPC fate determination, we characterized EPCs from $Vldlr^{-/-}$ and WT mice.
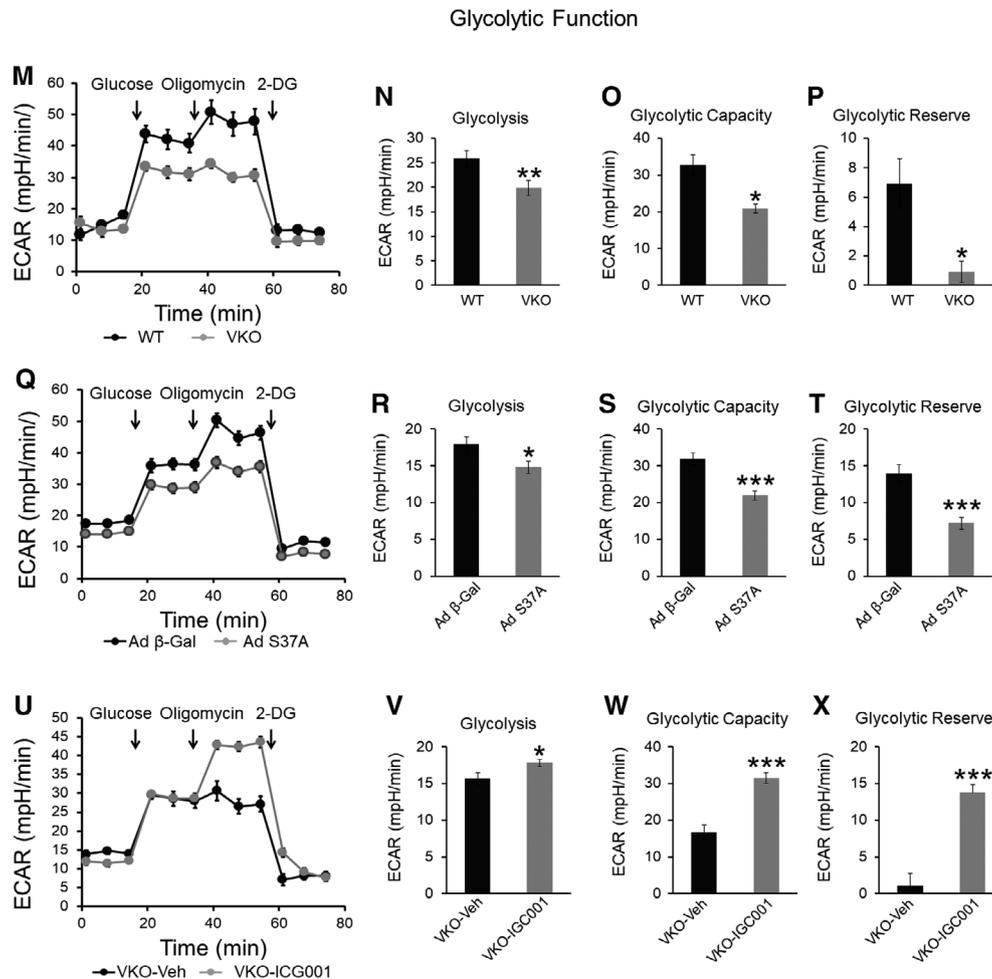
Glycolytic Function

Figure 2. Wnt signaling regulated endothelial progenitor cell (EPC) metabolic profile. **(A–D):** Mitochondrial oxidation was measured using seahorse analysis in isolated EPCs. The effects of injections of reagents (oligomycin, FCCP, RAA, as indicated by arrows) on the average oxygen consumption rate (OCR) trace were measured in the bioenergetic profile in primary EPCs isolated from WT (black) and $Vldlr^{-/-}$ (gray) mice ($Vldlr$ gene knockout [VKO]). **(E–H):** EPCs were isolated from WT mice and infected with control adenovirus (black) and Ad-S37A, expressing a constitutively active mutant of β-catenin (gray). Mitochondrial oxidation was measured using seahorse analysis. **(I–L):** Representative traces of OCR in EPCs from VKO after the treatment with a Wnt inhibitor (ICG001, 10 μM, 24 hours) or with vehicle. (B, F, J) basal OCR, (C, G, K) maximal OCR, and (D, H, L) ATP production were calculated and compared. **(M–P):** Glycolytic profile measured using seahorse glycolysis stress test in isolated EPCs. (M): Effects of injections of reagents (glucose, Oligo, 2-DG, as indicated by arrows) on the average extracellular acidification rate (ECAR) trace were measured in the bioenergetic profile in EPCs isolated from WT and VKO mice. **(Q–T):** Glycolytic profile of EPCs from WT mice infected with Ad-S37A to activate Wnt signaling compared with Ad-β-gal as control virus. (Q): Representative traces for measurement of ECAR of WT EPCs infected with Ad-β-gal (black) and Ad-S37A (gray). **(U–X):** Glycolytic profile of EPCs from VKO mice treated with a Wnt inhibitor ICG001 (10 μM) for 24 hours and vehicle as control. (U): Representative traces measuring ECAR of VKO EPC treated with a Wnt inhibitor ICG001 (gray) or vehicle (black). (N, R, V) glycolysis, (O, S, W) glycolytic capacity, and (P, T, X) glycolytic reserve were calculated and compared. All values are mean $\pm$ SEM; $n \geq 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$.

One of the distinguishing features of EPCs is their colony-forming capacity, which is believed to be a specific functional assay for evaluating undifferentiated stem cells with self-renewal potential [40] and different from cell proliferation assay [41]. The colony formation assay showed that colony numbers as well as colony sizes were decreased in $Vldlr^{-/-}$ EPCs (Fig. 4A–4C). Consistently, mRNA levels of stem cell markers (OCT4, Nanog, Sox2, Txb3) were significantly declined as measured by real-time RT-PCR (Fig. 4D, 4E), indicating that EPCs lost their capability of self-renewal to maintain their stemness under the Wnt overactivation condition. On the

other hand, the proliferation rate, as measured by BrdU incorporation assay, was significantly higher in $Vldlr^{-/-}$ EPCs, compared with that in WT EPCs (Fig. 4F). In order to determine whether Wnt activation promotes EPC differentiation, we used the fluorescence ICC staining of CD31, a marker of mature ECs during the initiation stage of differentiation and found a markedly increased number of cells expressing CD31 from $Vldlr^{-/-}$ mice compared with that from WT mice (Fig. 4G, 4H). These results suggested that the increased BrdU incorporation attributed to the increased cell division of EPCs. As stem cell division including self-renewal and differentiation, increased cell
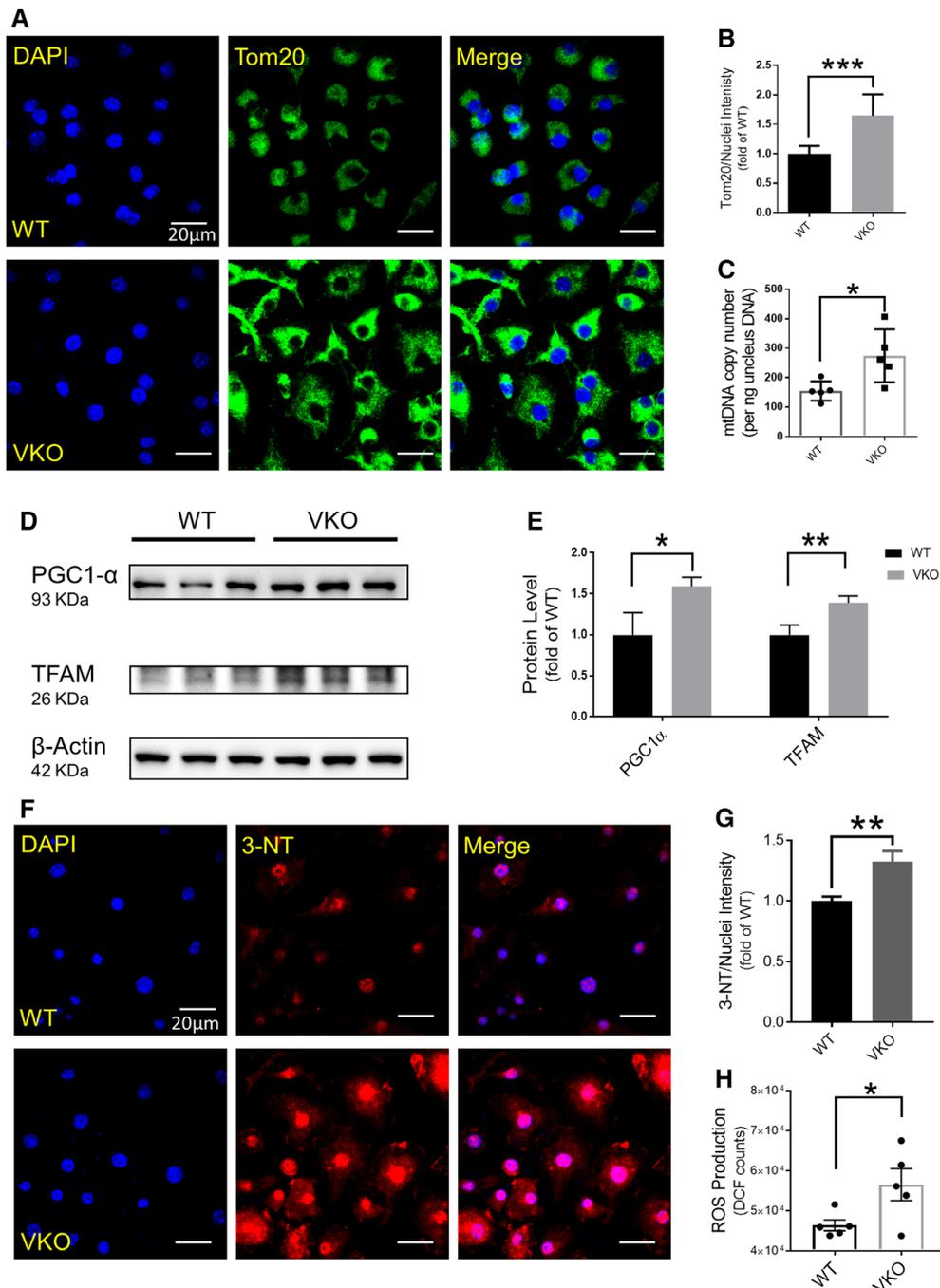
Figure 3. Wnt signaling facilitated mitochondrial biogenesis. **(A):** Representative images of immunostaining of Tom20 (green) of isolated WT and *Vldlr*$^{-/-}$ (*Vldlr* gene knockout [VKO]) endothelial progenitor cells (EPCs) showed the mitochondrial mass, with the nuclei counterstained with DAPI (blue). **(B):** Mitochondrial mass was analyzed by ImageJ, the intensity ratio of mitochondria/nuclei was calculated and compared between WT and VKO EPCs. **(C):** Mitochondrial DNA copy numbers were measured by digital polymerase chain reaction and normalized by chromosome DNA content in isolated WT and VKO EPCs. **(D):** Western blot analysis of PGC-1α and TFAM in primary EPCs isolated from the bone marrow of WT and VKO mice (each lane represent EPCs isolated from 6~7 mice at P28 under same condition), with β-actin as loading control. **(E):** Semiquantification of the Western blots. **(F):** Immunostaining of 3-nitrotyrosine (3-NT) in WT and VKO EPCs to evaluate the reactive oxygen species (ROS) production. **(G):** Quantification of the 3-NT/nuclei intensity ratio by ImageJ. **(H):** Intracellular ROS levels of WT and VKO EPCs were measured using CM-H$_2$DCFDA. All values are mean ± SEM; $n \geq 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$.

division and differentiation markers, whereas decreased self-renewal potential suggested that activation of Wnt signaling not only facilitated the generation and release of EPCs, but also promoted EPC's homing and differentiation in the target tissues, contributing to the new vascular formation. These results implied a profound mechanism by which Wnt signaling regulated EPC migration and differentiation as well as proliferation during pathological angiogenesis [42].
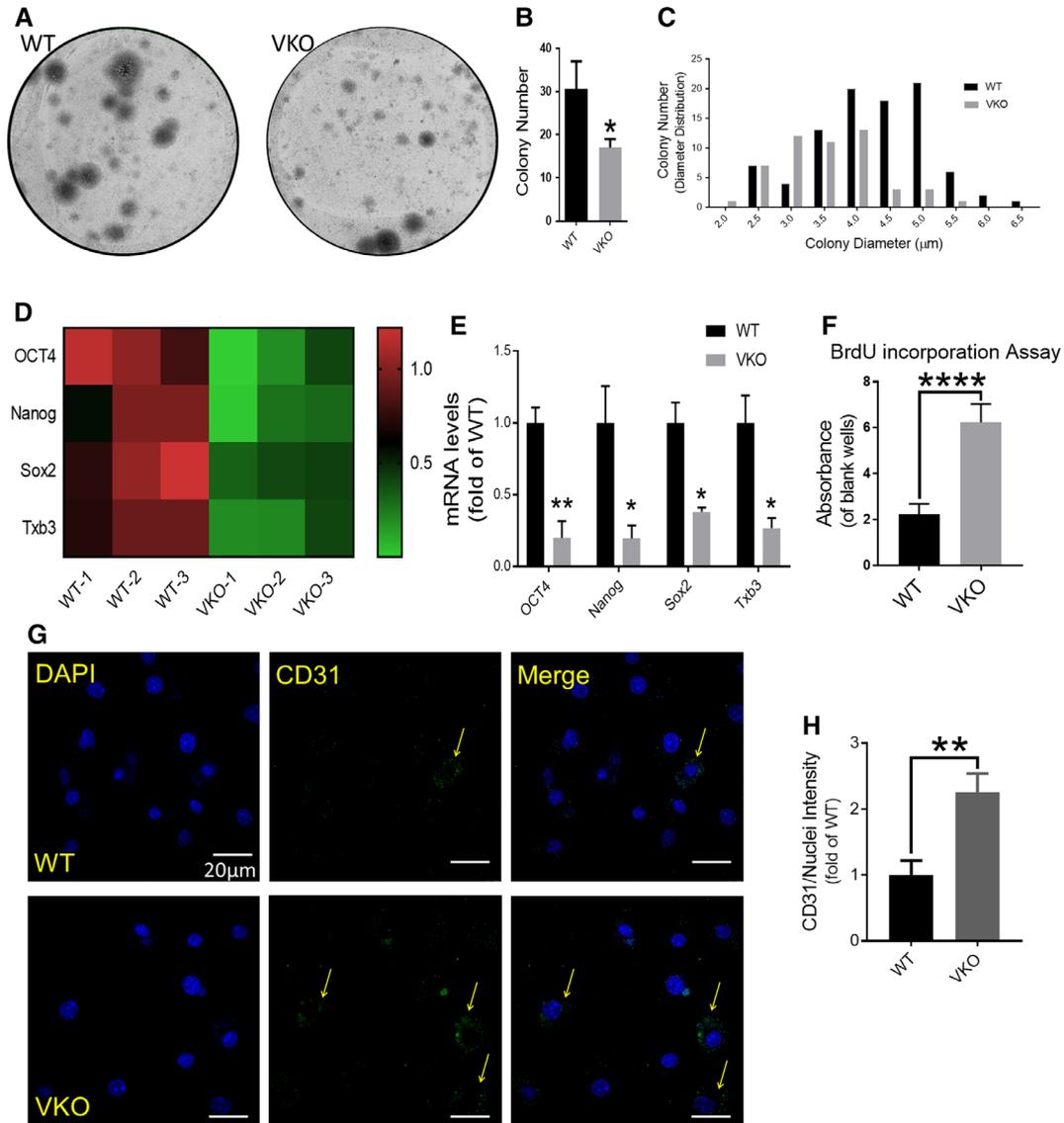
Figure 4. Activation of the Wnt signaling pathway promoted endothelial progenitor cell (EPC) differentiation. **(A):** Representative images of EPC colony formation of primary WT and *Vldlr*⁻/⁻ (*Vldlr* gene knockout [VKO]) EPCs. **(B):** The number of colonies and (C) the size distribution of colonies were analyzed and compared using Gelcount. **(D, E):** mRNA levels of stem cell markers, OCT4, Nanog, Sox2, and Txb3, were measured by qPCR and compared between WT and VKO EPCs. **(F):** The proliferation rates of WT and VKO EPCs were measured by BrdU incorporation assay. **(G):** Representative images of immunostaining of CD31 (green) in WT and VKO EPCs specifying the initiation of differentiation toward endothelial cells. Cells with faint green fluorescence staining were indicated by arrows and **(H)** the CD31/nuclei intensity ratio was quantified by ImageJ (normalized by WT). All values are mean ± SEM; $n \geq 3$; *, $p < .05$; **, $p < .01$; ****, $p < .0001$.

Fenofibrate Acid Downregulates EPC Mitochondrial Biogenesis Through Inhibition of Wnt Signaling

To further confirm that the increased mitochondrial mass and function in *Vldlr*⁻/⁻ EPCs was ascribed to Wnt pathway activation, we treated EPCs from *Vldlr*⁻/⁻ mice with ICG001 for 24 hours and found that ICG001 significantly decreased mitochondrial mass and ROS production, confirming that EPC mitochondrial activity was dependent on Wnt signaling (Fig. 5A, 5B, 5E, 5F). Interestingly, *Vldlr*⁻/⁻ EPCs treated with a peroxisome proliferator-activated receptor-α (PPARα) agonist FA also showed a downregulated ROS production and mitochondrial function (Fig. 5C, 5D, 5G, 5H). As expected, Wnt signaling activity measured using EPCs isolated from Wnt signaling reporter mice, *Axin2*^lacZ/*Vldlr*⁻/⁻ mice, showed FA treatment decreased

Wnt signaling activity in *Vldlr*⁻/⁻ EPCs to levels comparable to that in *Vldlr*⁺/⁺ control EPCs (Fig. 5I, 5J). All of these results suggested that the mitochondrial biogenesis of EPCs were regulated by Wnt signaling.

FA Regulated EPC Fate through Modulation of EPC Metabolic Profile via Regulation of Wnt Signaling

As reported previously, *Vldlr*⁻/⁻ mice develop intraretina and subretina NV [32, 43], and FA attenuated the NV in *Vldlr*⁻/⁻ mice [34]. However, the mechanistic relationship between Wnt signaling and the FA beneficial effect is still elusive. To determine the effect of FA on EPC numbers in the BM, blood, and retina in *Vldlr*⁻/⁻ mice, we treated *Vldlr*⁻/⁻ mice with FA (daily intraperitoneal injections of FA from P14 to P28). EPC numbers
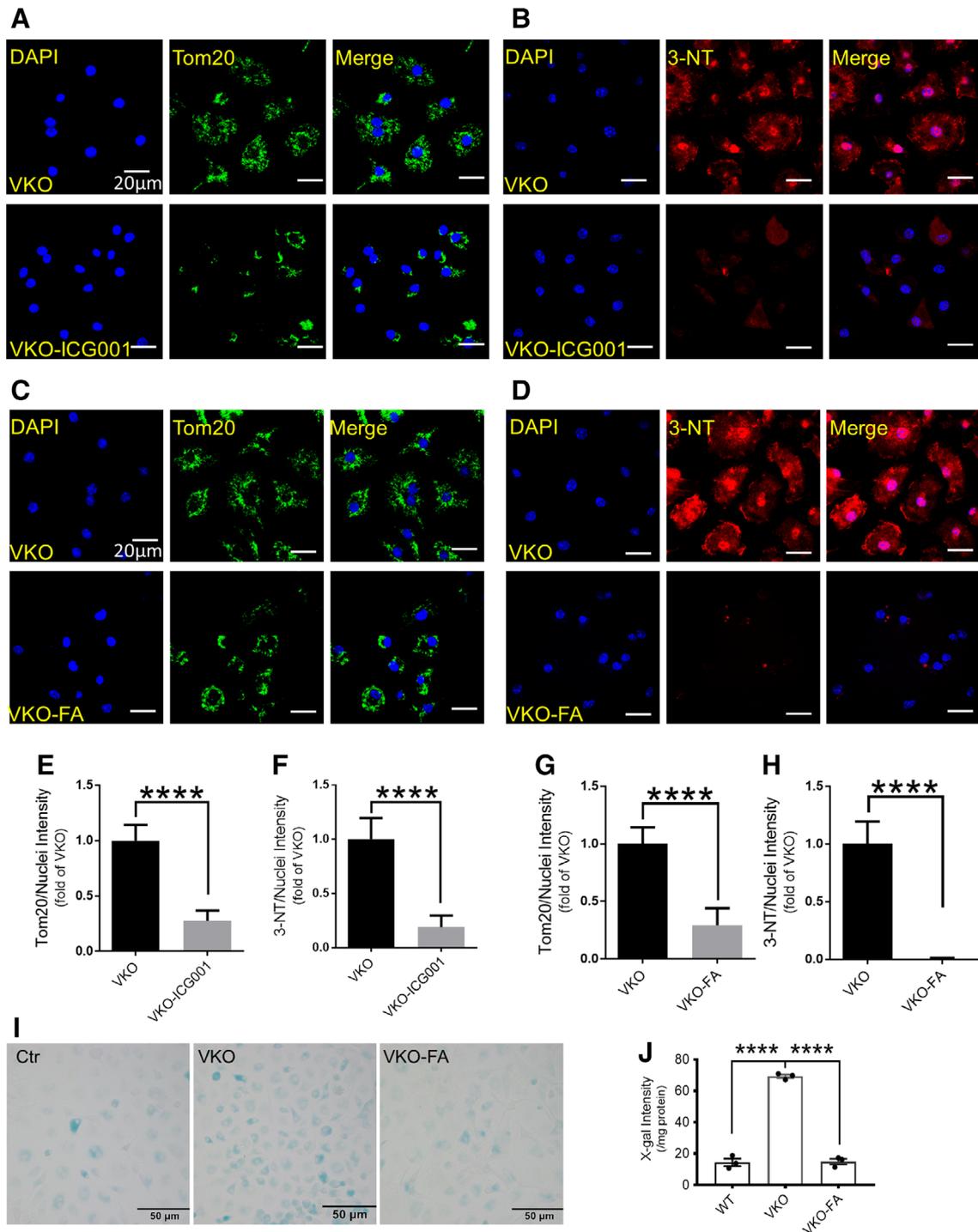
Figure 5. Downregulation of Wnt signaling inhibited endothelial progenitor cell (EPC) mitochondrial activity. **(A, B):** Immunostaining of Tom20 and 3-NT in *Vldlr* gene knockout (VKO) EPCs treated with a Wnt inhibitor (ICG001) or vehicle for 24 hours. **(C, D):** Immunostaining of Tom20 and 3-NT in VKO EPCs treated with fenofibric acid (FA) for 24 hours. **(E–H):** Quantification of the immune-fluorescence (Tom20, green; 3-NT, red) intensity ratio normalized by nuclei staining (blue) using ImageJ in VKO EPCs treated with ICG001 (E, F) and FA (G, H) and vehicle control. **(I):** Representative images of X-gal staining of EPCs from *Axin2^{lacZ}/Vldlr^{+/+}* (Ctr), *Axin2^{lacZ}/Vldlr^{−/−}* Wnt reporter mice treated with vehicle (VKO), or with fenofibric acid (VKO-FA). **(J):** X-gal intensity was quantified and compared. All values are mean ± SEM; $n \geq 3$; ****, $p < .0001$.

were considerably decreased in *Vldlr^{−/−}* mice treated with FA (Fig. 6A–6C). As expected, the ROS production (determined by DCF assay; Fig. 6H) and mitochondrial oxidation in EPC from *Vldlr^{−/−}* mice were also attenuated by FA treatment (Fig. 6D–6G),

indicating a close relationship between EPC mitochondrial function and EPC numbers. As shown by BrdU incorporation assay, FA treatment also reduced EPC proliferation induced by overactivation of Wnt signaling.
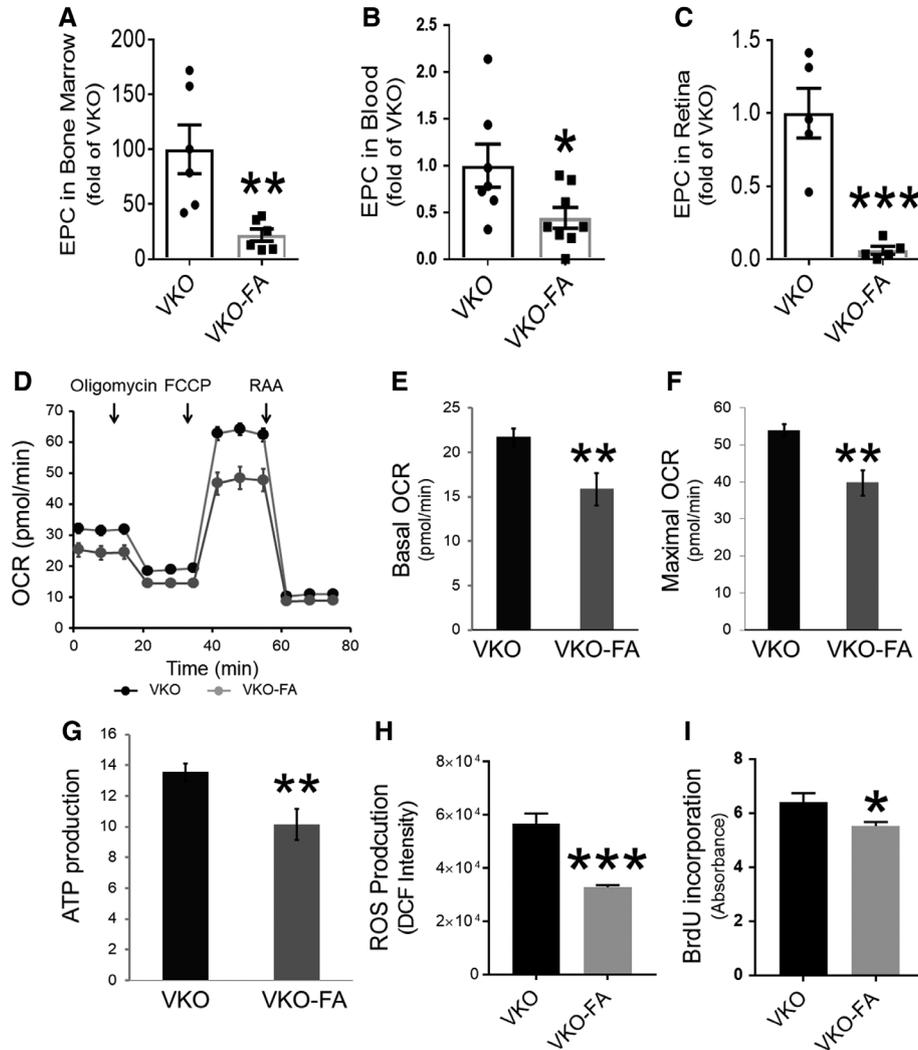
Figure 6. Fenofibric acid (FA) modulated endothelial progenitor cell (EPC) number and EPC mitochondrial function in Wnt activated angiogenesis mouse model. *Vldlr* gene knockout (VKO) mice were treated with FA (25 μg/g body weight, i.p., P14 to P28). **(A–C):** EPC population was determined using flow cytometry. FA treatment decreased EPC population in the bone marrow (A), blood (B), and retina (C) in VKO mice (*n* > 5). **(D):** Representative traces of oxygen consumption rate (OCR) of VKO EPCs treated with FA (20 μM, 72 hours) and vehicle. **(E):** Basal OCR, **(F)** maximal OCR, and **(G)** ATP production were calculated and compared. **(H):** Intracellular ROS levels of VKO EPCs treated with FA and vehicle were measured using CM-H$_2$DCFDA. **(I):** Proliferation rates of VKO EPCs treated with FA and vehicle were measured by BrdU incorporation assay. All values are mean ± SEM; *n* ≥ 5; *, *p* < .05; **, *p* < .01; ***, *p* < .001.

Taken together, these findings revealed that Wnt signaling plays an important role in the determination of EPC generation and homing through regulation of the EPC metabolism pathway.

DISCUSSION

Previous studies have demonstrated that EPCs participate in pathological retinal NV [44]. Our studies have shown that aberrant activation of Wnt signaling promotes retinal NV [32]. However, the role of Wnt signaling in EPC regulation and its mechanism of action has not been defined previously. Here, we provided evidence indicating that Wnt signaling activation promoted EPC generation, release, homing, and differentiation, which contributed to NV at least in *Vldlr*⁻/⁻ mice, a model of pathological ocular NV. Furthermore, we found that this effect of Wnt signaling on EPCs was mediated, at least in

part, through regulation of intrinsic metabolic profile and mitochondrial biogenesis in EPCs. These findings provided new insights into the metabolic regulation of EPC in NV and defined a new function of Wnt signaling, which has potential to contribute to the development of new therapeutic strategies for NV.

Under physiological conditions, new vessel formation is primarily limited to wound healing and female reproductive processes in adults, and, as such, EPCs in the BM are in a relatively quiescence state. Under disease conditions, pathological NV is initiated when genetic or environmental stimuli break the quiescence of EPCs and promote EPC transition toward a more active state [45], and induce EPC generation, release, and homing, resulting in increased EPC numbers in the BM, circulation, and target tissues [9]. Understanding of the molecular mechanism that regulates EPC fate might provide fundamental insights into the pathogenesis of NV in neovascular diseases, including proliferative diabetic retinopathy

(PDR), age-related macular degeneration, and retinopathy of prematurity.

In the present study, in order to investigate the role of Wnt signaling in EPC activation, we used $Vldlr^{-/-}$ mice. As reported by our previous study, VLDLR functions as a negative regulator of Wnt signaling. Wnt signaling is overactivated in the retina of $Vldlr^{-/-}$ mice, resulting in pathological ocular NV [32]. The study demonstrated that Wnt signaling was also activated in EPC isolated from the BM of $Vldlr^{-/-}$ mice. Furthermore, EPC numbers are increased in the BM, blood, and retina in $Vldlr^{-/-}$ mice, suggesting that Wnt signaling promotes EPC generation, release, and homing.

In stem cell self-renewal and differentiation, accumulating evidence has shown that Wnt signaling may actively participate in the pluripotent stem cell fate decision through regulation of cell metabolism [46]. To define the role of Wnt signaling in the regulation of EPC metabolism, we measured mitochondrial respiration and glycolytic rate in isolated EPCs. Our Seahorse analysis demonstrated that mitochondrial function was upregulated in $Vldlr^{-/-}$ EPCs. Although the Wnt pathway in $Vldlr^{-/-}$ mice is overactivated, it is possible that other metabolic perturbations in $Vldlr^{-/-}$ mice could contribute to the neovascular phenotypes. To address this issue, we manipulated the canonical Wnt signaling pathway in WT EPCs with specific activators and inhibitors and then measured the EPC metabolic profile. Consistently, EPC mitochondrial function was upregulated by activation of Wnt signaling using a constitutively active mutant of β-catenin or LiCl, while inhibited by specific Wnt inhibitors, indicating that Wnt signaling directly regulates metabolism in EPC.

The canonical Wnt signaling pathway is one of the important and conserved developmental pathways that control cell fate decision and tissue patterning from early embryonic development all the way to the tissue homeostasis in adult tissues. Despite many significant breakthroughs in Wnt signaling studies in the past decades, some questions remain controversial [23, 47]. Recently, increasing studies have focused on revealing the relationship between the Wnt signaling and cell metabolic profile/energy usage in decisions of cell fate [25].

EPCs, as a type of progenitor cells, have potency to differentiate into EC, which directly contribute to the postnatal vasculogenesis [11]. It has been shown that dysregulation of EPC number and function plays an important pathogenic role in diabetic microvascular complications such as diabetic retinopathy (DR) [16]. However, there are conflicting reports regarding numbers of circulating EPCs in patients with diabetes [48, 49]. This disparity may be ascribed to different EPC markers used in the studies, or just due to different stages of DR studied by different groups, since the EPC number change is a dynamic process through the progression of DR [9]. In nonproliferative diabetic retinopathy, circulation EPC numbers are decreased, whereas in PDR, EPC are increased in the circulation, compared with diabetic patients without DR [49]. In the present study, we provided evidence that EPC mitochondrial function correlated with EPC number and played an intrinsic driving role in the EPC differentiation into ECs during the pathological NV. Flow cytometry measurement of EPC mitochondrial membrane potential ($\Delta\Psi_m$) in the circulation has potential to become a more direct predictor of pathologic NV and to provide an alternative method for prognosis rather than the transient change of EPC numbers.

Although EPC share many properties with adult stem cells, they are described as oligopotent or unipotent and may adopt substantially more precise regulation patterns in their renewal and differentiation. In stem cell proliferation and differentiation, accumulating evidence has shown that Wnt signaling may actively participate in the pluripotent stem cell fate decision through regulation of mitochondrial and cellular metabolism [46]. It has been reported that stem cells heavily rely on glycolysis as a major source of energy. When EPCs differentiate to mature ECs, metabolism profile was changed to mitochondrial respiration [50]. The present study demonstrated that glycolysis in EPCs was decreased by activation of Wnt signaling while increased by a Wnt inhibitor. This finding suggested that Wnt signaling facilitated EPC differentiation toward ECs and the initiation of the process of NV.

Mitochondrial biogenesis and metabolic pattern switches have been suggested as hallmarks of cell differentiation, and stimulating mitochondrial biogenesis is known to promote stem cell differentiation [51]. On the other hand, during stem cell differentiation, in addition to morphological and ultrastructural changes, studies have observed an increase in mitochondrial DNA (mtDNA) content [52, 53] and mitochondrial mass [54]. Our results provided evidence that Wnt signaling activation increased mitochondrial biogenesis in EPC through upregulation of PGC-1α, further supporting the notion that Wnt signaling promotes EPC differentiation.

ROS, a product of mitochondrial oxidation, can promote stem cells to enter the cell cycle [55]. To date, emphasis has been placed on mitochondrial regulation of ROS generation as an inducer of stem cell differentiation [56]. It has been shown that moderately high ROS levels in the progenitor cells sensitize themselves to differentiation, and establish a signaling role for ROS in the regulation of hematopoietic cell fate [57]. In our study, we observed that Wnt signaling activation increased ROS generation in EPCs and provided evidence supporting the role of Wnt signaling in EPC fate determination.

FA, a PPARα agonist, has shown antiangiogenic effects in PDR patients and in several NV animal models including VKO mice [34]. Our previous study reported that FA inhibited the Wnt signaling pathway [30]. The present study indicated that FA significantly decreased mitochondrial biogenesis and ROS production in isolated EPCs in vitro (Figs. 5, 6) and decreased EPC numbers in VKO mice (Fig. 6). These results suggested that directly targeting metabolic pathways of EPC might represent a mechanism responsible for the therapeutic effect of FA on NV.

CONCLUSION

Taken together, our findings suggest that Wnt signaling determines EPC fate through modulation of EPC metabolic profile, contributing to pathological vascular growth. These findings will shed light upon the development of a new strategy for the treatment of the pathological NV. Our results also reveal the potential of EPC metabolic profile as a promising prognosis for pathological vascular formation, especially in ocular diseases.

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AUTHOR CONTRIBUTIONS

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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