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## Oxidative stress upregulates Wnt signaling in human retinal microvascular endothelial cells through activation of disheveled

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### Abstract

Abnormal retinal neovascularization associated with various retinopathies can result in irreversible vision loss. Although the mechanisms involved in this occurrence is unclear, increasing evidence suggests that aberrant Wnt signaling participates in the pathogenesis of abnormal neovascularization. Because Wnt signaling upregulation can be induced by oxidative stress through the activation of disheveled (DVL), a key molecule in the Wnt signaling pathway, we investigated whether oxidative stress can activate Wnt signaling and induce angiogenic phenotypes in human retinal microvascular endothelial cells (HRMECs). We found that increased Wnt signaling activity, as well as enhanced angiogenic phenotypes, such as tube formation and cell migration, were detected in the hydrogen peroxide-treated HRMECs. Moreover, these effects were effectively suppressed by a small-molecule Wnt inhibitor targeting the PDZ domain of DVL. Therefore, we propose that targeting abnormal Wnt signaling at the DVL level with a small-molecule inhibitor may represent a novel approach in retinal neovascularization treatment and prevention.

### Keywords

angiogenesis; oxidative stress; pathological neovascularization; Wnt signaling

## 1 | INTRODUCTION

Pathological retinal neovascularization is a hallmark of several retinal diseases including retinopathy of prematurity (ROP) and diabetic retinopathy (DR).<sup>1–4</sup> The inner segment of the retina is vascularized by retinal vessels,<sup>5</sup> where endothelial cells connect and communicate via tight junctions resulting in the formation of the inner blood-retinal barrier.<sup>5,6</sup> This barrier provides a selective mechanism which protects the retina from circulating agents and small molecules, allowing the retina to function in a highly specialized environment.<sup>5</sup> In healthy tissue, retinal blood vessels are maintained in a quiescent state with consideration to growth.<sup>7,8</sup> However, in the event of pathological retinal neovascularization, abnormal angiogenic growth of weakened blood vessels is present at the juncture of the

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CONFLICT OF INTERESTS

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vascularized and avascular retina going into the vitreous. In turn, these weakened blood vessels may lead to blindness as a result of vascular leakage, rupture, scarring, and retinal detachment.<sup>2,9-12</sup>

Although the underlying mechanism of retinal neovascularization is unclear, increasing evidence finds that abnormal Wnt/ $\beta$ -catenin signaling participates in retinal neovascularization.<sup>1,10,12-16</sup> Wnt signaling is essential for the development and differentiation of the retinal vasculature. Moreover, experimental mouse models of oxygen-induced retinopathy (OIR) which depict events that occur during ROP, have shown that abnormal Wnt/ $\beta$ -catenin activity promotes pathological retinal neovascularization in retinopathy.<sup>12,17,18</sup> Vascular endothelial growth factor (VEGF), a potential downstream target gene of the Wnt signaling pathway,<sup>19,20</sup> which controls endothelial cell processes including cell migration and proliferation, is linked to retinal neovascularization.<sup>21,22</sup> Dysregulation of the Wnt signaling pathway has been linked to upregulation of VEGF in endothelial cells.<sup>23-25</sup> Nevertheless, the cause of abnormal Wnt signaling activity is unknown.

Oxidative stress as a result of high concentrations of reactive oxygen species (ROS), also has been linked to pathological retinal neovascularization.<sup>26,27</sup> Oxidative stress is reported to induce key properties of angiogenesis, including cell migration and proliferation, through the stimulation of VEGF in endothelial cells.<sup>28</sup> The main pathogenesis of ROP and DR at the proliferative retinopathy stages is oxidative stress within blood vessel cells.<sup>29-32</sup> We speculate that oxidative stress may be responsible for abnormal Wnt signaling activity in human retinal microvascular endothelial cells, which may be a major contributor to retinal neovascularization. Contrarily, an interrelationship between redox and Wnt signaling has been reported, where redox regulates Wnt signaling at the disheveled (DVL) level.<sup>33-35</sup>

In the current working model of canonical Wnt signaling, cytoplasmic DVL protein is activated via the binding of a secreted Wnt ligand to its transmembrane receptor, Frizzled (FZD).<sup>36-38</sup> Upon activation, FZD interacts directly with the PDZ domain of DVL<sup>39</sup> initiating downstream Wnt signaling.<sup>40-47</sup> However, this interaction between DVL and FZD is also mediated by redox through nucleoredoxin (NXN), a thioredoxin-related redox-regulating protein.<sup>33,34</sup> NXN also binds to the PDZ domain of DVL, blocking Wnt signaling activity by competing with Wnt receptor, FZD. However, oxidative stress can dissociate the interaction between NXN and DVL, enabling DVL to interact with FZD and initiate Wnt signaling.<sup>33,35</sup> Therefore, it is likely that oxidative stress in the retina activates abnormal Wnt signaling at the DVL level. Indeed, while Wnt activity is significantly increased in pathologic neovascularization in the OIR model,<sup>12,48</sup> mutant mice lacking Dvl2, presented significantly reduced levels of neovascularization, suggesting that the DVL proteins play an essential role in the development of retinal neovascularization.<sup>12</sup> Therefore, we reasoned that a Wnt inhibitor targeting the PDZ domain of DVL, may be helpful in developing a novel therapeutic approach for the treatment of retinal neovascularization. However, despite the extensive efforts, the current state-of-the-art DVL-PDZ domain inhibitors are not apposite to be used in the *in vivo* animal model studies.<sup>49</sup> Thus, we decided to use different *in vitro* assays of angiogenesis<sup>50</sup> to assess the idea of targeting DVL with a small-molecule inhibitor of the DVL-PDZ domain.

In this study, using compound 3289–8625, a known inhibitor of the DVL-PDZ domain,<sup>51</sup> together with models of retinal angiogenesis in vitro, we first confirmed that the DVL protein is involved in Wnt-mediated angiogenesis through our analysis of human retinal microvascular endothelial cells (HRMECs) tube formation and cell migration. Then, using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to mimic oxidative stress in the retinal, we showed that H<sub>2</sub>O<sub>2</sub> activated canonical Wnt signaling in HRMECs and further promoted tube formation and enhanced cell migration. Moreover, we found that Wnt inhibitor 3289-8625, effectively suppressed the H<sub>2</sub>O<sub>2</sub>- induced angiogenesis phenotypes in HRMECs, suggesting that selectively targeting the DVL-PDZ domain with small molecules may be a strategy against pathological retinal neovascularization.

## 2 | MATERIALS AND METHODS

### 2.1 | HRMECs culture and Wnt conditional medium preparation

HRMECs were purchased from Angio-Proteomie (Boston, MA). These cells were maintained in Endothelial Cell Medium MV (PromoCell, Heidelberg, Germany) supplemented with Endothelial Cell Supplement Mix (PromoCell, Heidelberg, Germany). Cells from passages 3 to 7 were used for all assays. For production of Wnt and control conditional media, mouse L Wnt-3a cell line stably expressing Wnt-3a (CRL2647; American Type Culture Collection [ATCC], Manassas, VA), and L-cell line (CRL2648; ATCC), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Wnt-3a conditional medium (WCM) and L-cell control conditional medium (LCM) were collected from L Wnt-3a cells and L cells, respectively, after 7 days in culture. Upon assessment, the concentration of LCM and WCM used for all assays was maintained at 10%.

### 2.2 | Compound preparation and HRMECs treatment

Wnt signaling inhibitor 3289–8625 (ENZO Life Sciences, Farmingdale, NY) was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). The working concentration of compound 3289–8625 was 50 μM in 0.05% DMSO, therefore the concentration of the vehicle control and LCM control used contained 0.05% DMSO, unless stated otherwise. HRMECs were treated with conditional medium or 1 μM H<sub>2</sub>O<sub>2</sub> in presence or absence of compound 3289–8625, unless specified differently. The medium was replaced with a growth medium or medium containing compound 3289–8625, following a 30-minute incubation for HRMECs treated with H<sub>2</sub>O<sub>2</sub>, or H<sub>2</sub>O<sub>2</sub> in presence of compound 3289–8625.

### 2.3 | Immunofluorescence assay

HRMECs were treated with either LCM, WCM, WCM in presence of compound 3289-8625, or vehicle, H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub> in presence of compound 3289–8625 for 24 hours. These cells were washed once with PBS (Thermo Fisher Scientific, Waltham, MA) and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at room temperature. Samples were then washed three times for 5 minutes each wash, with PBS. Samples were blocked with PBS containing 5% goat serum and 0.25% Triton-X-100. Primary rabbit anti-human β-catenin (ab32572; Abcam, Cambridge, MA) and phalloidin-TRITC (P5282; Sigma-Aldrich) were added overnight at 4°C in PBS containing 1% goat serum. Samples were washed once

with PBS. Secondary goat anti-rabbit Alexafluor 594 (A-11037; Thermo Fisher Scientific, Waltham, MA) was added to the membranes and incubated for 1 hour at room temperature. These samples were washed three times with PBS for 5 minutes each wash, and then counterstained and mounted with 4',6-diamidino-2-phenylindole (Vector Labs, Burlingame, CA). Samples were visualized using Keyence BZ-X700 fluorescence microscope (Keyence, El Segundo, CA) and five random fields of views were photographed.

#### 2.4 | Quantitative polymerase chain reaction

HRMECs were treated with LCM, WCM, and WCM in presence of compound 3289–8625, or vehicle, H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub> in presence of compound 3289–8625 for 24 hours. Total cellular RNA was extracted from HRMECs using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) as per manufacturer's protocol and the concentration of RNA was analyzed using a NanoDrop (Thermo Fisher Scientific, Waltham, MA). Quantitative polymerase chain reaction (qPCR) was carried out using qScript-XLT 1-Step RT-qPCR kit from Quanta Biosciences (Beverly, MA), with TaqMan primers for human glyceraldehyde 3-phosphate dehydrogenase (Hs02758991\_g1) and human *AXIN2* (Hs00610344\_m1) purchased from Thermo Fisher Scientific (Canoga Park, CA). Manufacturer's protocol was followed for the qPCR and the delta-delta method was used for analysis. Each experimental condition was repeated at least three times.

#### 2.5 | HRMEC tube formation assay

Tube formation assay was performed by using growth factor-reduced Matrigel (Corning Inc, Corning, NY). Matrigel was evenly coated into a six-well plate and HRMECs were seeded at  $1.2 \times 10^5$  cells per well on these Matrigel-coated wells, and subsequently treated with serum-free medium containing either LCM, WCM, and WCM in presence of inhibitor 3289-8625, or vehicle, H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub> in presence of inhibitor 3289–8625. Image acquisition was performed following incubation at 37°C for 6 hours. Acquired images were taken under a phase contrast microscope and were processed and evaluated for the total tube length, branching points, total loops, and area coverage by ImageJ (National Institutes of Health [NIH], Bethesda, MD).

#### 2.6 | HRMEC migration assay

The cell migration assay was performed using the migration and wound-healing system from ibidi (Madison, WI). HRMECs were seeded in a  $\mu$ -35 culture-insert dish at  $3 \times 10^4$  cells per well and incubated overnight. A flat-head forcep was used to carefully remove the culture inserts from culture dish after incubation to create a cell-free gap. Then, HRMECs were treated with serum-free medium containing LCM, WCM, WCM in presence of Wnt inhibitor 3289–8625, or H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> with inhibitor 3289–8625. Images were acquired using a phase contrast microscope after 16 hours. HRMECs migration rates were quantified by analyzing the size of nonmigrated area using the ImageJ (NIH) program.<sup>52</sup> Each experiment was repeated at least three times and there were five random fields of views acquired for each treatment group.

## 2.7 | Luciferase assay

Stable transfected NIH3T3 cell line expressing luciferase under a TCF/LEF promoter was purchased from ENZO Life Sciences Inc (Farmingdale, NY). These 3T3 cells were seeded at  $2 \times 10^5$  cells per well in a 96-well plate (Corning Inc) and incubated overnight. Cells were then treated with either LCM, WCM, and WCM in presence of compound 3289–8625; or vehicle, H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> in presence of 3289–8625, and were cultured for 14 hours. Cell viability and firefly luciferase activity were measured using the ONE-Glo + Tox Luciferase Reporter and Cell Viability Assay kit (Promega, Madison, WI) following manufacturer's protocol. A microplate reader, FilterMax F5 (Molecular Devices, Sunnyvale, CA) was used to measure the cell viability and firefly luciferase activity. All assays were performed in triplicates.

## 3 | RESULTS

### 3.1 | Compound 3289-8625 inhibited Wnt signaling and Wnt-induced angiogenic phenotypes in HRMECs

Compound 3289–8625 binds to the DVL-PDZ domain<sup>51</sup> and blocks Wnt signaling through the inhibition of DVL in different biological systems.<sup>36,53–57</sup> To further validate whether 3289–8625 can also block Wnt signaling in HRMECs, Wnt signaling was activated in HRMECs with Wnt-3a conditional media. *AXIN2* is a known target of Wnt signaling.<sup>58,59</sup> Whereas most Wnt signaling target genes are tissue-specific or developmental stage-specific, the *AXIN2* gene is considered a global transcription target.<sup>60</sup> As expected, we found that *AXIN2* expression was elevated in HRMECs treated with Wnt-3a conditional media and that elevated *AXIN2* expression was reduced when 3289–8625 was added to the Wnt-3a conditional media-treated cells (Figure 1). In addition, immunofluorescence analysis showed that HRMECs treated with WCM had enhanced  $\beta$ -catenin nuclear translocation compared to HRMECs treated with Wnt-3a conditional media in presence of 3289–8625 (Figure 2). Our results indicate that 3289–8625 can effectively block Wnt signaling activity in HRMECs. Wnt signaling regulates retinal neovascularization.<sup>12,61–63</sup> Consistent with a previous report,<sup>62</sup> we found that treating HRMECs with Wnt-3a conditional media enhanced endothelial cell migration and stimulated tube formation (Figures 3,4). However, HRMECs treated with Wnt-3a conditional media in the presence of Wnt inhibitor 3289–8625, had a significantly slower rate of cell migration and regulated tube formation as opposed to cells treated with only Wnt-3a conditional media (Figures 3,4). These data suggest that Wnt signaling is a regulator of HRMECs migration and tube formation. Additionally, inhibiting Wnt signaling can reduce and control cell migration and tube formation in vitro, potentially affecting neovascularization.

### 3.2 | H<sub>2</sub>O<sub>2</sub>-induced Wnt signaling activity in HRMECs

Oxidative stress stimulates Wnt signaling by activating DVL.<sup>33–35</sup> Indeed, using low concentrations of H<sub>2</sub>O<sub>2</sub> to mimic oxidative stress,<sup>33</sup> enhanced levels of canonical Wnt signaling activity were detected in H<sub>2</sub>O<sub>2</sub>-treated NIH3T3 cells, and this increase of Wnt activity occurred in an H<sub>2</sub>O<sub>2</sub> concentration-dependent manner (Figure 5). Moreover, consistent with the notion that oxidative stress activates canonical Wnt signaling at the DVL

level,<sup>35</sup> we found that compound 3289–8625, targeting the PDZ domain of DVL, significantly suppressed canonical Wnt signaling in cells treated with H<sub>2</sub>O<sub>2</sub> (Figure 5).

Oxidative stress has been implicated in the proliferative retinopathy stages of ROP and DR.<sup>26,27</sup> Mouse models of OIR shows that upon removing mice from a high oxygen-concentrated environment and exposing them to normal oxygen levels, H<sub>2</sub>O<sub>2</sub> accumulation induced by the change in environment, is observed in their retina.<sup>30,64,65</sup> There fore, we examined whether H<sub>2</sub>O<sub>2</sub> can also induce Wnt signaling in HRMECs. We found that following 30 minutes of H<sub>2</sub>O<sub>2</sub> treatment, *AXIN2* expression was elevated, and that *AXIN2* elevation could be suppressed by compound 3289–8625 in a dose-dependent manner (Figure 6). Furthermore, immunofluorescence analysis shows that β-catenin levels were upregulated in the nucleus following 30 minutes of H<sub>2</sub>O<sub>2</sub> treatment, and this effect could also be suppressed by adding the compound 3289–8625 to H<sub>2</sub>O<sub>2</sub>- treated HRMECs (Figure 7). These results suggest that H<sub>2</sub>O<sub>2</sub> treatment upregulated the Wnt signaling pathway in HRMECs at the DVL level.

### 3.3 | H<sub>2</sub>O<sub>2</sub>-induced Wnt signaling enhances cell migration and tube formation of HRMECs

Since Wnt ligands, such as Wnt-3a, induce different angiogenic phenotypes in HRMECs,<sup>62</sup> we next evaluated whether H<sub>2</sub>O<sub>2</sub>-activated Wnt signaling can also stimulate these similar angiogenic phenotypes in HRMECs. We found that after 30 minutes of H<sub>2</sub>O<sub>2</sub> treatment, the cell migration rate of HRMECs was enhanced. Moreover, following the addition of 3289-8625 to the cell-culture media, this H<sub>2</sub>O<sub>2</sub>-induced cell migration was reduced (Figure 8). Similarly, we found that H<sub>2</sub>O<sub>2</sub> treatment induced HRMECs tubular network formation by nearly threefold, yet, adding compound 3289-8625 abridged this effect (Figure 9).

## 4 | DISCUSSION

Wnt signaling plays a critical role in the development and differentiation of the retinal vasculature,<sup>13</sup> including its restoration following OIR.<sup>66</sup> Indeed, norrin, a secreted signaling molecule, which is also essential in the retinal vasculature during development, induces Wnt signaling in microvascular endothelial cells, by binding to Wnt receptor, FZD.<sup>66</sup> Furthermore, norrin-induced Wnt signaling increases insulin-like growth factor (IGF-1) expression,<sup>66</sup> a critical factor in the development in the retinal vasculature. Preterm babies with mutations in the norrin/FZD4/LRP5 signaling pathway have decreased levels of IGF-1 that have been associated with the impediment of retinal vascular growth and abnormal angiogenesis.<sup>23,67</sup> Under disease conditions, dysregulation of Wnt signaling has been linked to dysregulation of IGF-1 and has been postulated to be responsible for the ROP pathology.<sup>68</sup>

Mutations in the Wnt signaling pathway have also been linked to several pathologies including defective vascularization of the retina.<sup>48,63,69</sup> Consistent with this notion, aberrant Wnt signaling activity has been shown in a mouse model depicting OIR.<sup>12,48</sup> Furthermore, mutant mice lacking key components of the Wnt signaling pathway have significantly decreased levels of neovascularization,<sup>12</sup> and this effect can be normalized using lithium, a known Wnt activator.<sup>70</sup> In another study, a DR model showed that oxidative stress leads to Wnt signaling activation and is linked to retinal inflammation and neovascularization.<sup>71</sup>

Therefore, targeting specific proteins involved in Wnt signaling may provide insight on understanding and treating retinal neovascularization. Nevertheless, to effectively target abnormal Wnt signaling, a clear view of the mechanism which promotes aberrant Wnt signaling in the retina needs to be further elucidated.

Oxidative stress, as a result of high concentrations of ROS, has been implicated in the pathogenic role of various processes including abnormal retinal neovascularization in ROP and DR.<sup>29,30,72–74</sup> It is known that ROS acts as an intracellular signaling mediator, triggering different signaling pathways including the Wnt signaling pathway. Specifically, it has been reported that oxidative stress can regulate Wnt/ $\beta$ -catenin signaling in a redox-dependent manner.<sup>33,35</sup> In physiological conditions, a thioredoxin-related protein, NXN, is directly bound to the PDZ domain of DVL, through the thiol moiety (-SH) of cysteine, inhibiting the function of DVL in the Wnt/ $\beta$ -catenin signaling cascade. Inhibition of DVL allows  $\beta$ -catenin to form a “destruction complex” consisting of Axin, APC, and GSK-3 $\beta$  resulting in the phosphorylation of  $\beta$ -catenin by a proteasome.<sup>33–35</sup> Treating cells with a low concentration of H<sub>2</sub>O<sub>2</sub> can cause a thiol-disulfide bond to form, leading to the conformational change of NXN. As a result, the inhibitory function of NRX is effected, causing its dissociation from DVL and allowing for DVL to interact with FZD, thus allowing for the upregulation of Wnt signaling and triggering neovascularization.<sup>33</sup> In this present study, we showed that H<sub>2</sub>O<sub>2</sub> enhances vascular-related activities of HRMECs such as tube formation and cell migration. The phenotype induced by H<sub>2</sub>O<sub>2</sub> is similar to that induced by Wnt-3a. However, in the presence of oxidative stress, a small-molecule Wnt inhibitor, targeting the PDZ domain of DVL, can take the place of NXN and modulate Wnt signaling as well as neovascularization. This prevents the accumulation of  $\beta$ -catenin and thus its translocation into the nucleus. As a result, transcription of downstream Wnt signaling genes, including VEGF, does not occur. Furthermore, taking advantage of this small-molecule, we demonstrate that oxidative stress activates Wnt signaling and stimulates the angiogenic phenotypes at the DVL level. Therefore, our findings suggest that ROS-mediated Wnt signaling can be regulated by a small-molecule Wnt inhibitor targeting the PDZ domain of DVL, which in turn can reduce the proangiogenic phenotype of HRMECs. This notion is consistent with the *in vivo* study showing that the mice lacking *Dvl2* have significantly reduced levels of pathological neovascularization.<sup>12</sup>

Although oxidative stress may potentially cause pathological neovascularization, ROS still serves as a line of defense against bacteria and other agents.<sup>31,75</sup> However, under diseased conditions, ROS, such as H<sub>2</sub>O<sub>2</sub>, can trigger angiogenesis by functioning as signaling molecules to regulate cellular signaling,<sup>27</sup> with Wnt signaling being one of the pathways induced by ROS. However, Wnt signaling is important for normal retinal angiogenesis and retinal development, especially in premature infants.<sup>76</sup> Because ROS activates abnormal Wnt signaling at the DVL level, therefore, dynamically easing aberrant ROS-mediated Wnt signaling by selectively targeting the DVL protein with a small-molecule reagent may lead to the development of a novel therapeutic approach for ROP treatment.

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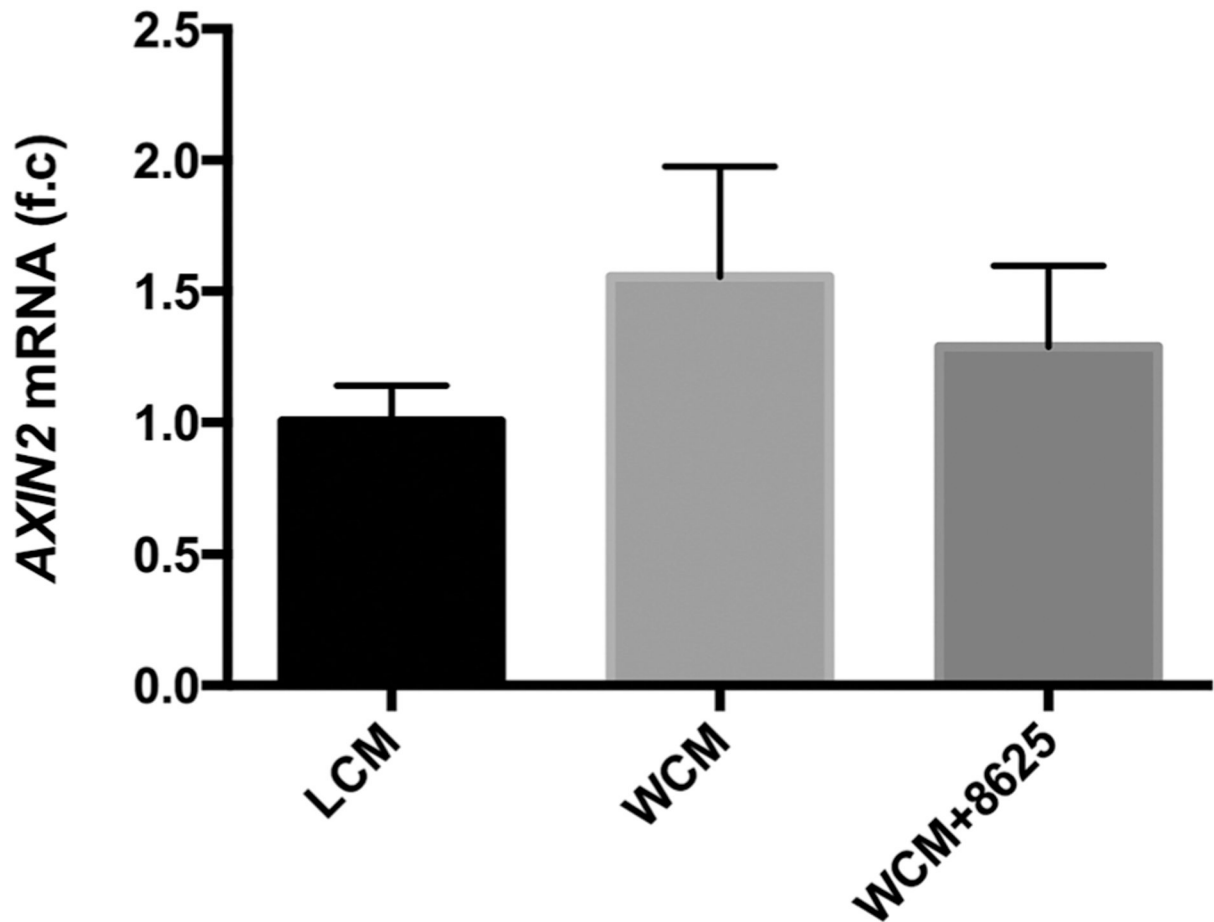
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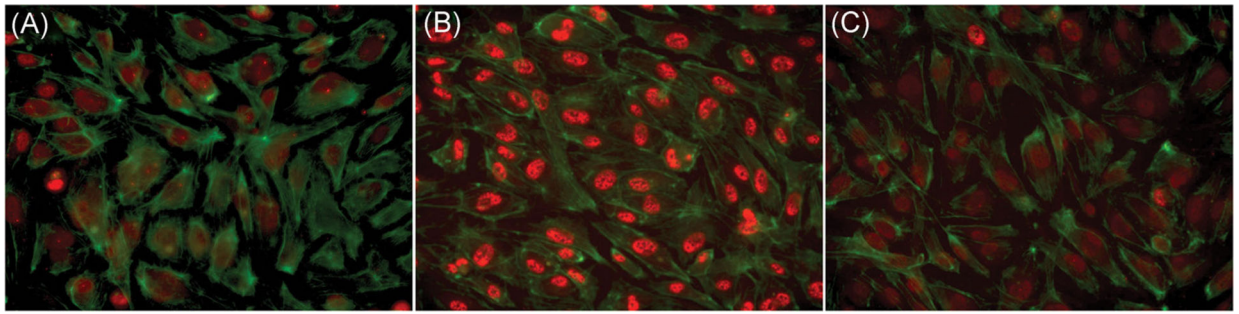
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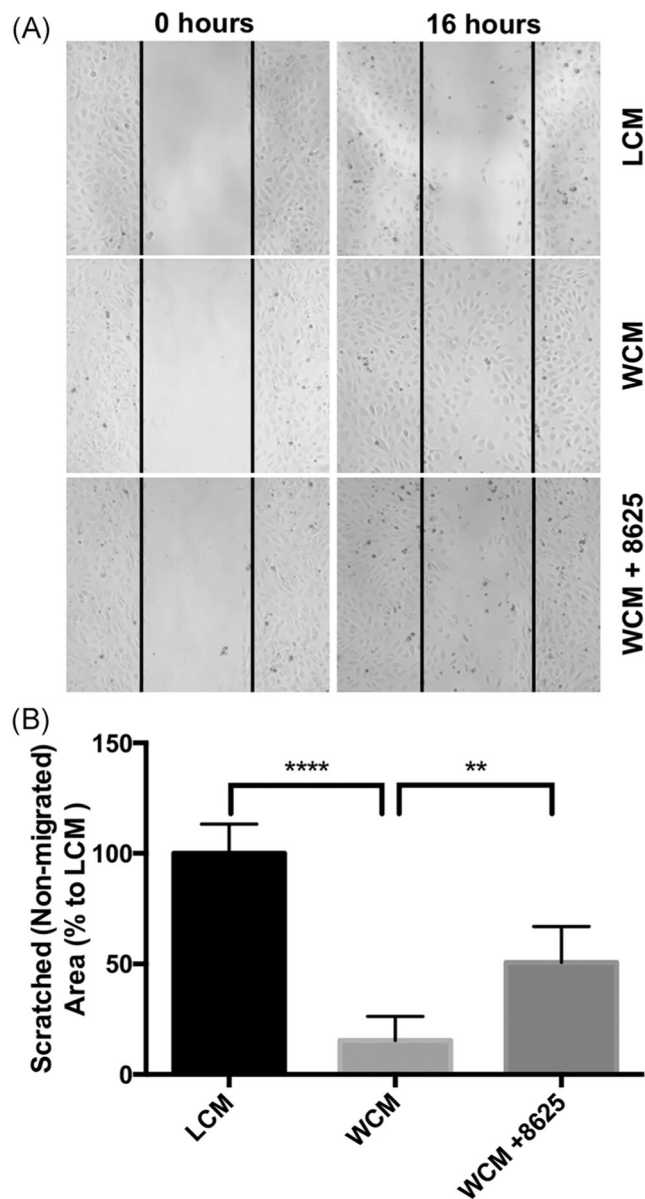


**FIGURE1.**

Compound 3289–8625 regulates Wnt signaling activity in HRMECs. HRMECs were treated with either LCM, WCM, or WCM in the presence of compound 3289–8625 (50  $\mu$ M) for 24 hours. *AXIN2* mRNA expression levels were measured by qPCR. HRMECs treated with compound 3289-8625 presented reduced Wnt signaling levels, indicating that Wnt signaling activity can be regulated by compound 3289–8625 in HRMECs. HRMEC, human retinal microvascular endothelial cell; LCM, L-cell control conditional medium; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; WCM, Wnt-3a conditional medium

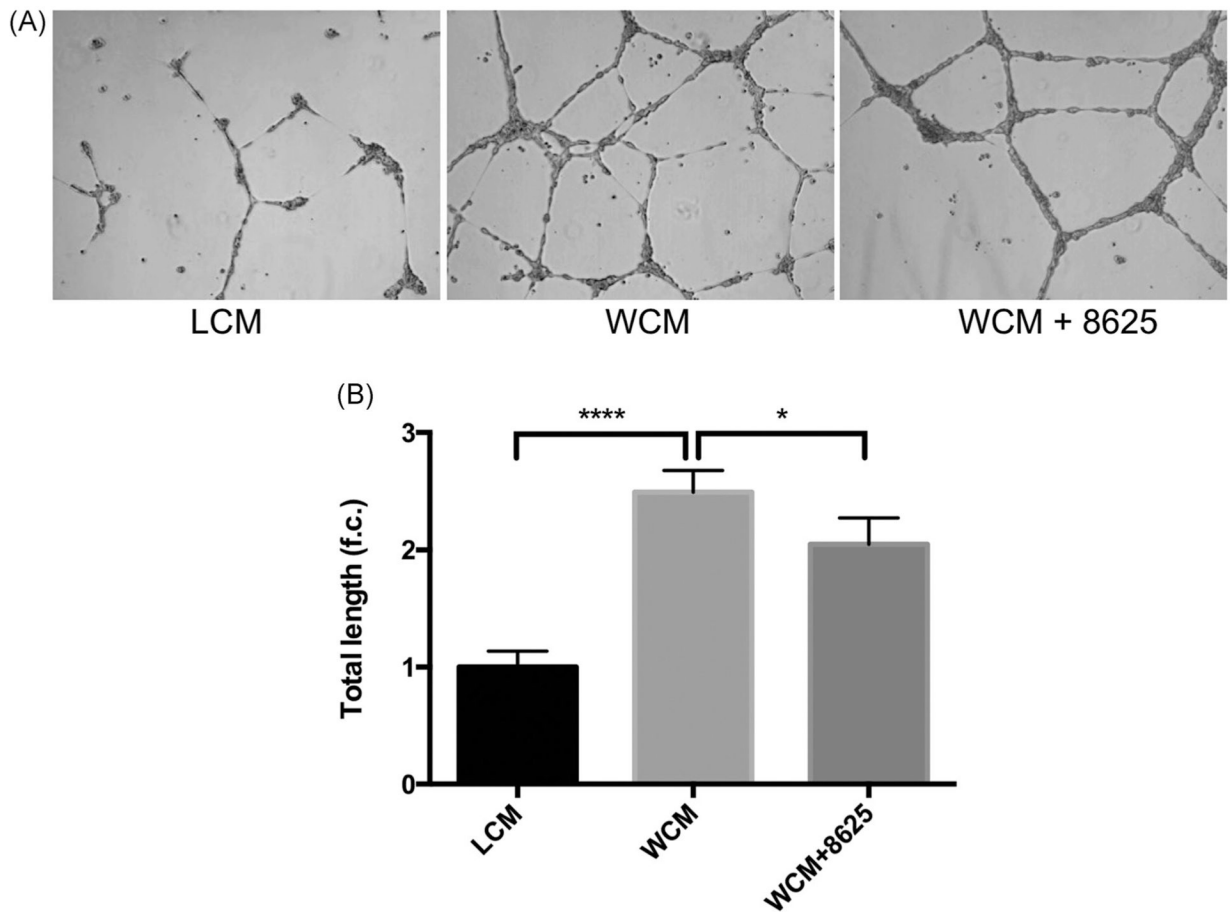
**FIGURE 2.**

Compound 3289–8625 suppressed  $\beta$ -catenin protein level, thus regulating Wnt activity in HRMECs. HRMECs were treated with LCM (A), WCM (B), or WCM in presence of compound 3289-8625 (50  $\mu$ M) (C), for 24 hours. Five random fields were examined on the slide. The green channel of phalloidin staining represents the HRMECs cytoskeleton. Immunofluorescence analysis of total  $\beta$ -catenin protein levels, as indicated by the red channel in HRMECs, shows that WCM enhanced the translocation of  $\beta$ -catenin protein levels into the nucleus. Moreover, compound 3289-8625 can inhibit Wnt signaling activity in HRMECs. HRMEC, human retinal microvascular endothelial cell; LCM, L-cell control conditional medium; WCM, Wnt-3a conditional medium



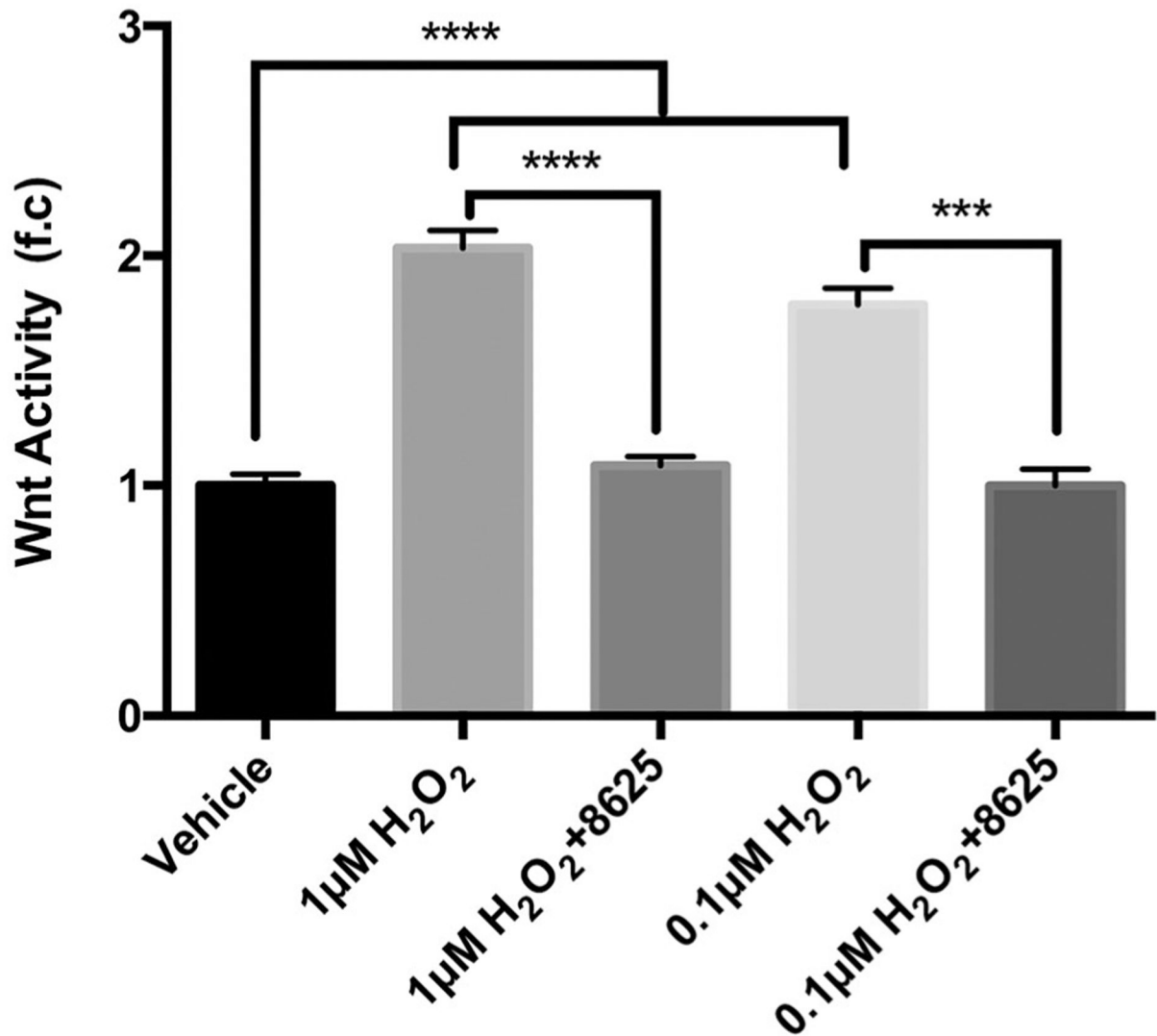
**FIGURE 3.**

Wnt signaling inhibition reduces Wnt-3a- dependent migration in HRMECs. HRMECs were seeded into the  $\mu$ -35 dishes with culture insert. Culture inserts were removed after 24 hours creating a uniformed cell-free surface. Cells were then treated with LCM, WCM, or WCM in the presence of compound 3289–8625 (50  $\mu$ M). Cell images of five random views were taken after 16 hours (A). Image analysis was performed using ImageJ (B). These data show that Wnt-mediated HRMECs migration can be inhibited by compound 3289-8625 (\*\* $P$ 0.01, \*\*\*\* $P$ 0.001 via one-way ANOVA with Dunnett's correction). ANOVA, analysis of variance; HRMEC, human retinal microvascular endothelial cell; LCM, L-cell control conditional medium; WCM, Wnt-3a conditional medium



**FIGURE 4.**

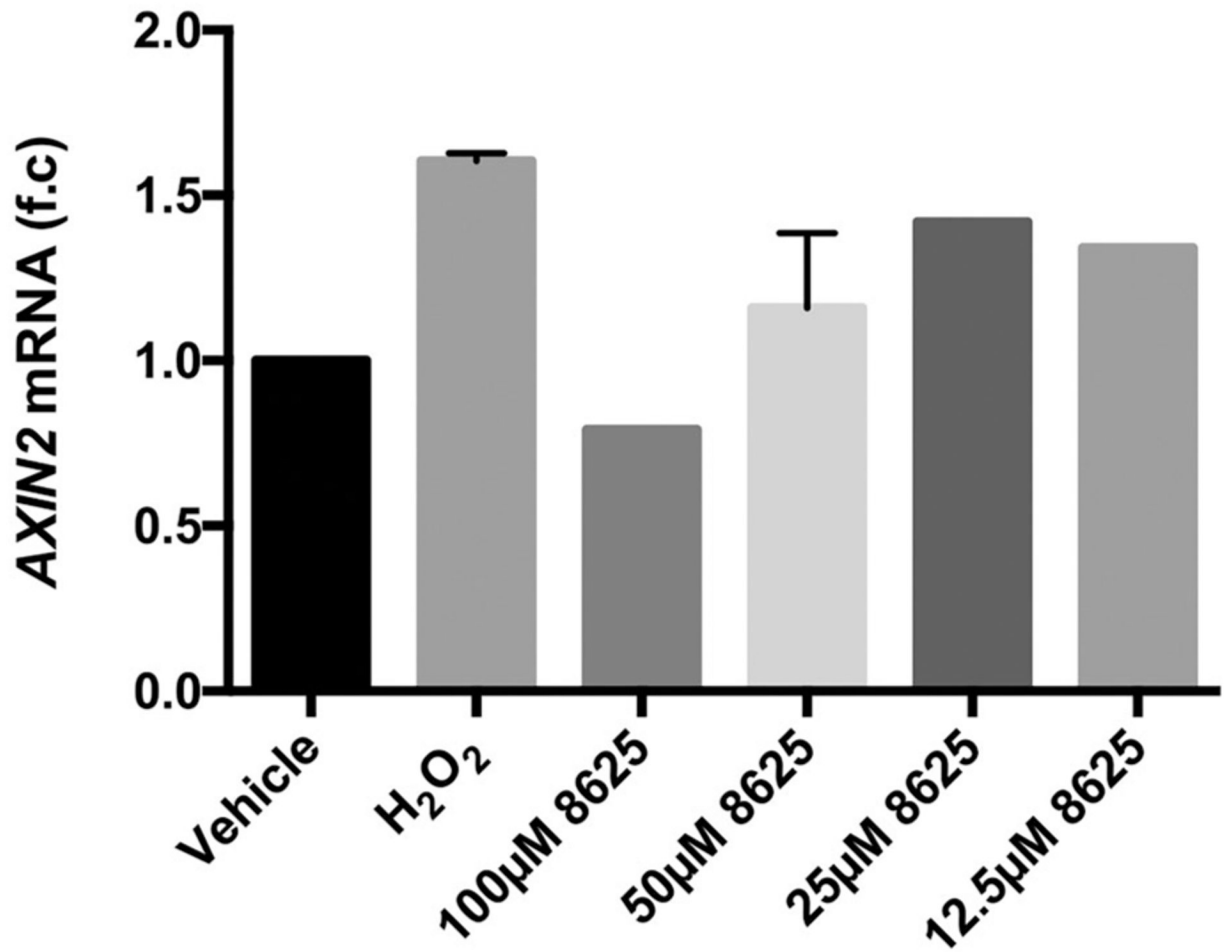
Wnt signaling inhibition reduces Wnt-3a-dependent tube formation in HRMECs. HRMECs were seeded into Matrigel-coated six-well plate. These cells were then treated with LCM, WCM, or WCM in presence of compound 3289-8625 (50  $\mu$ M). Cell images of five random views were taken after 6 hours (A) then analyzed by ImageJ (B). Wnt-induced HRMECs tube formation was inhibited by compound 3289-8625 (\* $P$  < 0.05 and \*\*\*\* $P$  < 0.001 via one-way ANOVA with the Dunnett correction). ANOVA, analysis of variance; HRMEC, human retinal microvascular endothelial cell; LCM, L-cell control conditional medium; WCM, Wnt-3a conditional medium



**FIGURE 5.**

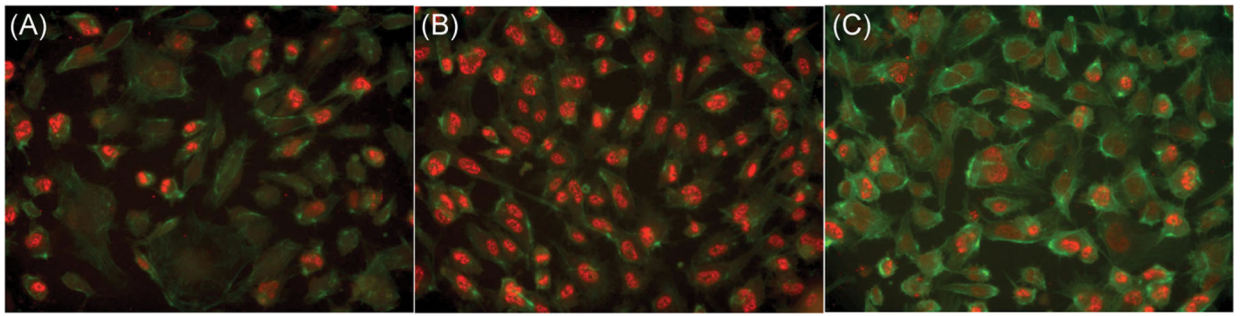
Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induces Wnt activation in the NIH3T3 cell, and compound 3289-8625 suppresses the H<sub>2</sub>O<sub>2</sub>-induced Wnt activation. Stable, transfected 3T3 cells expressing luciferase under control of the Wnt promoter were treated with vehicle, H<sub>2</sub>O<sub>2</sub> (1 or 0.1 µM), or H<sub>2</sub>O<sub>2</sub> (1 or 0.1 µM) presenting with 3289-8625 (50 µM). After 16 hours, Wnt-induced luciferase activities in 3T3 cells were detected (\*\**P* < 0.005 and \*\*\*\**P* < 0.001 via one-way ANOVA with the Dunnett correction). ANOVA, analysis of variance; HRMEC, human retinal microvascular endothelial cell





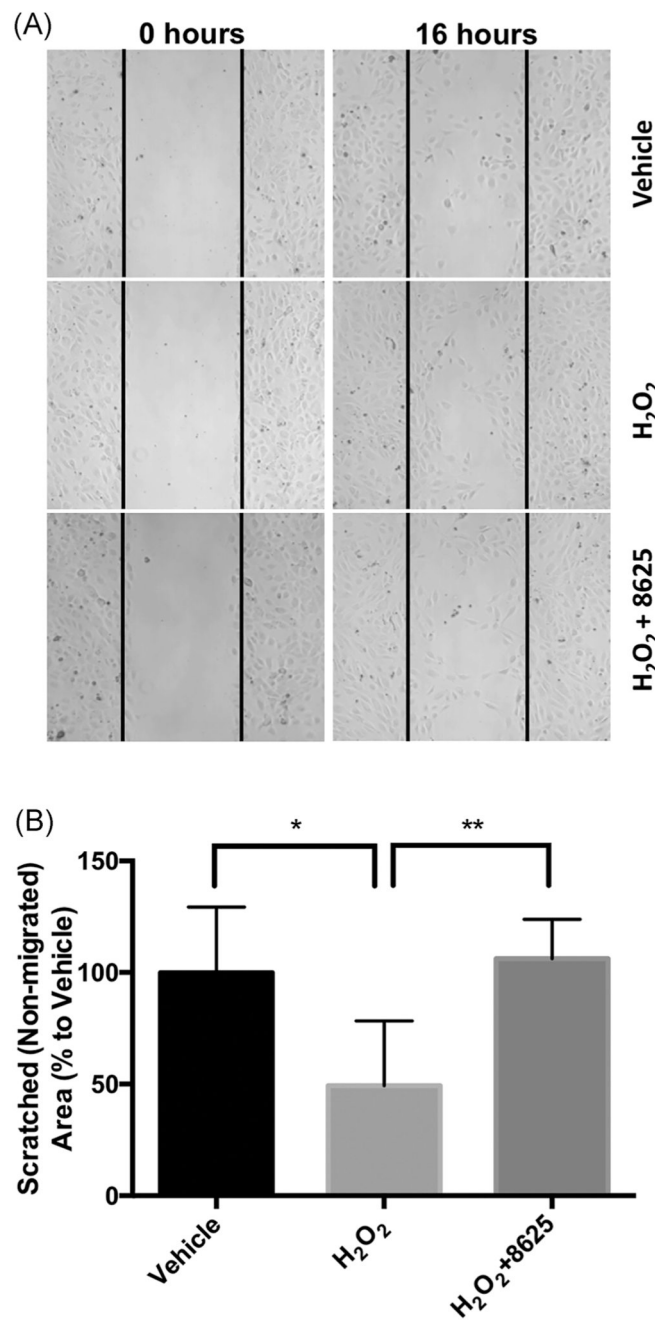
**FIGURE 6.**

Hydrogen peroxide induces Wnt activation in the HRMECs, which can be suppressed by compound 3289–8625. HRMECs were treated with vehicle, H<sub>2</sub>O<sub>2</sub> (1 µM), or H<sub>2</sub>O<sub>2</sub> (1 µM) presenting with compound 3289–8625 at a concentration of 100, 50, 25, or 12.5 µM. The *AXIN2* mRNA expression levels in these cells were measured by qPCR. Compound 3289-8625 can reduce H<sub>2</sub>O<sub>2</sub>-induced Wnt signaling, in a concentration-dependent manner. HRMEC, human retinal microvascular endothelial cell; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction



**FIGURE 7.**

Hydrogen peroxide ( $H_2O_2$ ) increases  $\beta$ -catenin protein level activation in HRMECs and compound 3289–8625 attenuates these effects. HRMECs were treated with (A) vehicle, (B)  $H_2O_2$  (1  $\mu M$ ), and (C)  $H_2O_2$  (1  $\mu M$ ) presenting with compound 3289–8625 (50  $\mu M$ ), for 24 hours. Total  $\beta$ -catenin protein levels were detected by immunofluorescence, as shown by the red channel. The green channel of phalloidin staining represents the HRMECs cytoskeleton. Data suggests  $H_2O_2$  upregulated Wnt activity in the HRMECs. Compound 3289–8625 can inhibit  $H_2O_2$ -induced Wnt activation. HRMEC, human retinal microvascular endothelial cell



**FIGURE 8.**

Inhibiting Wnt suppresses H<sub>2</sub>O<sub>2</sub>-induced HRMECs migration. HRMECs were seeded into the  $\mu$ -35 dishes with culture-insert. Culture inserts were removed after 24 hours, creating a uniformed cell-free surface. Cells were then treated with vehicle, H<sub>2</sub>O<sub>2</sub> (1  $\mu$ M), or H<sub>2</sub>O<sub>2</sub> (1  $\mu$ M) presenting with compound 3289–8625 (50  $\mu$ M). Images of five random views were taken after a 16-hour incubation (A) and then analyzed using ImageJ (B). Our analysis shows that H<sub>2</sub>O<sub>2</sub>-induced Wnt activation mediates HRMECs migration and this phenomenon is reduced with treatment of Wnt modulator, 3289-8625 (\* $P$  < 0.05 and \*\* $P$  <

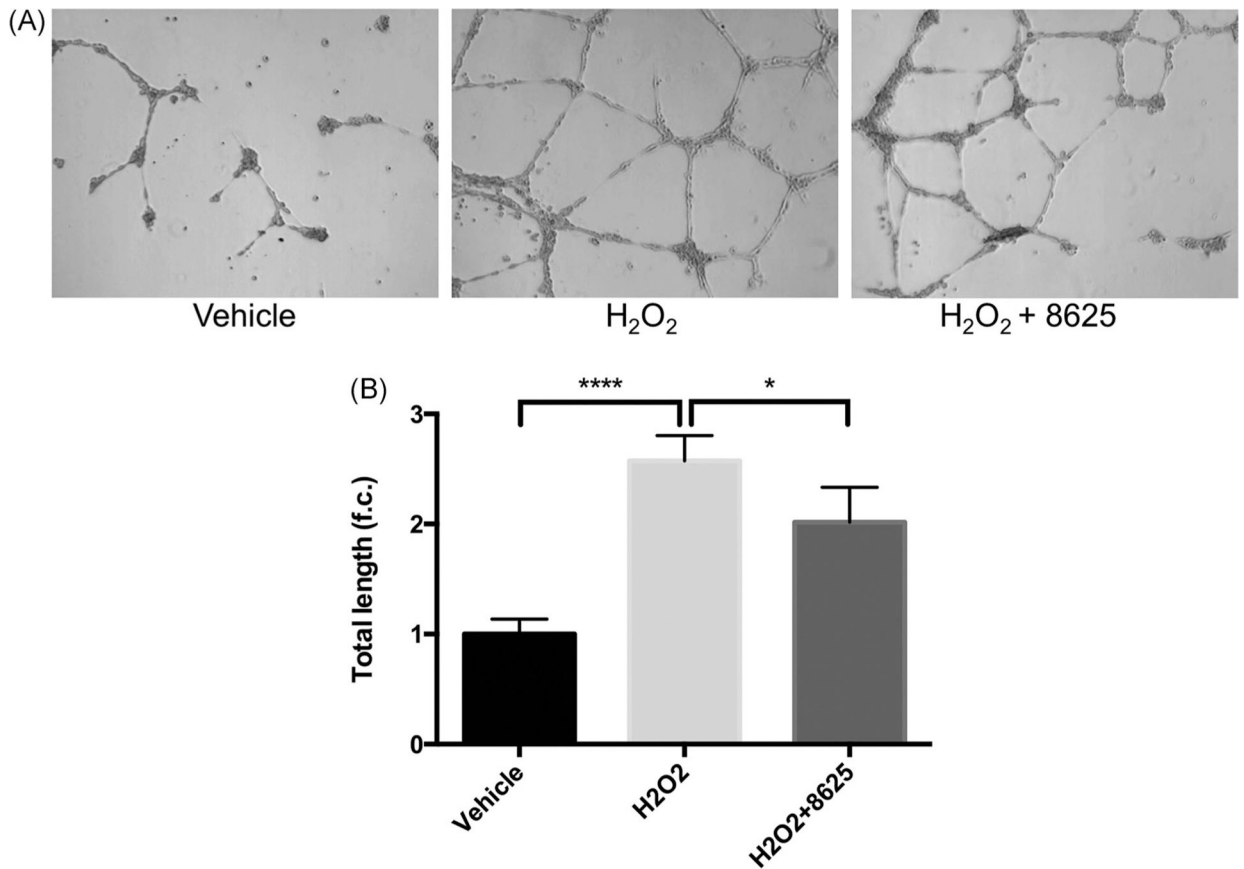
0.01 via one-way ANOVA with the Dunnett correction). ANOVA, analysis of variance;  
HRMEC, human retinal microvascular endothelial cell

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**FIGURE 9.**

Wnt inhibition decreases H<sub>2</sub>O<sub>2</sub>-induced tube formation in HRMECs. HRMECs were seeded into a Matrigel-coated six-well plate. These cells were then treated with vehicle, H<sub>2</sub>O<sub>2</sub> (1 μM), or H<sub>2</sub>O<sub>2</sub> (1 μM) presenting with compound 3289–8625 (50 μM). Cell images of five random views were taken after 6 hours (A) then analyzed by ImageJ (B). Tube formation stimulated by H<sub>2</sub>O<sub>2</sub> induced Wnt signaling can be inhibited by compound 3289-8625 (\**P* < 0.05 and \*\*\*\**P* < 0.001 via one-way ANOVA with the Dunnett correction). ANOVA, analysis of variance; HRMEC, human retinal microvascular endothelial cell