Molecules and Cells

Oleanolic Acids Inhibit Vascular Endothelial Growth Factor Receptor 2 Signaling in Endothelial Cells: Implication for Anti-Angiogenic Therapy

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Angiogenesis must be precisely controlled because uncontrolled angiogenesis is involved in aggravation of disease symptoms. Vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR-2) signaling is a key pathway leading to angiogenic responses in vascular endothelial cells (ECs). Therefore, targeting VEGF/VEGFR-2 signaling may be effective at modulating angiogenesis to alleviate various disease symptoms. Oleanolic acid was verified as a VEGFR-2 binding chemical from anticancer herbs with similar binding affinity as a reference drug in the Protein Data Bank (PDB) entry 3CJG of model A coordination. Oleanolic acid effectively inhibited VEGF-induced VEGFR-2 activation and angiogenesis in HUVECs without cytotoxicity. We also verified that oleanolic acid inhibits *in vivo* angiogenesis during the development and the course of the retinopathy of prematurity (ROP) model in the mouse retina. Taken together, our results suggest a potential therapeutic benefit of oleanolic acid for inhibiting angiogenesis in proangiogenic diseases, including retinopathy.

Keywords: angiogenesis, endothelial cells, oleanolic acid, retinopathy of prematurity, VEGFR-2

INTRODUCTION

Angiogenesis, which is the process by which new blood vessels are created from an existing vascular network, must be elaborately controlled because it is important to the progression of various diseases such as inflammation and tumors (Ferrara and Kerbel, 2005; Hanahan and Folkman, 1996). In particular, pathologic angiogenesis that occurs in ocular diseases such as diabetic retinopathy or retinopathy of prematurity (ROP) is an important factor leading to blindness (Aiello, 2005; Gariano and Gardner, 2005). Angiogenesis also plays an essential role in supplying blood flow to various cells during normal eye development. Therefore, understanding the pathophysiology of angiogenesis in the eye and identifying targets to regulate angiogenesis is of great interest.

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Although angiogenesis is complex and requires many steps, the proliferation and migration of vascular ECs is most important in creating new blood vessels. VEGF is an important factor that causes EC proliferation and angiogenesis (Ferrara, 2004; Leung et al., 1989). VEGF is a mitogen that specifically acts on vascular ECs, binding and activating its cognate receptor tyrosine kinase (VEGFR), thereby increasing vascular EC proliferation, growth, and migration. Since VEGF binds to VEGF receptor 2 (VEGFR-2/KDR/Flk-1, herein VEGFR-2) with high affinity, VEGFR-2 induces a major phenotypic change in ECs during angiogenesis (Bernatchez et al., 1999; Gerber et al., 1998; Olsson et al., 2006) and is thus an important target to its inhibition. Thus, developing an inhibitor that targets VEGFR-2 may provide a promising approach for controlling angiogenesis in a variety of diseases.

ROP is a vasoproliferative disease and a major ocular disorder of preterm infants. The pathologies are induced by limiting retinal oxygenation during hypoxia and inducing expression of excessive growth factors, including VEGF, which leads to abnormal blood vessel growth (Hellstrom et al., 2013). Several preventive and therapeutic approaches against ROP have been performed, such as supplemental essential polyunsaturated fatty acids (Connor et al., 2007), vitamin E (Raju et al., 1997), erythropoietin (Chen et al., 2008), and angiopoietin-1 (Lee et al., 2013) treatment; however, therapeutic efficacy has been hindered by a limited understanding of pathologies (Sapieha et al., 2010). Currently, a promising approach against ROP is to use anti-VEGF therapy with VEGF neutralizing antibodies and a recombinant fusion protein (Hellstrom et al., 2013; Micieli et al., 2009; Sapieha et al., 2010).

Recent advances in drug discovery have led to the development of a number of synthetic and natural compounds for a variety of biological assays. However, the conventional process of screening a large number of compounds using biochemical or cellular assays is a time-consuming and costly process and thus a major obstacle to the development of therapeutic agents. A growing body of evidence suggests that virtual screening based on computational methods will allow analysis of the binding capacity of various compounds to defined target proteins and identification of lead compounds with biological activity against target proteins from chemical libraries of compounds (Reddy et al., 2007; Shoichet, 2004). Moreover, there have been many studies of the development of new medicines from traditional herb medicines for, as well as many successful cases, but the traditional assay-based fraction approach is very laborious. Nevertheless, due to the high safety of traditional herbal medicines, the potential for the development of new drugs is promising if new drug candidates are discovered in the traditional herbal medicines. In addition, there is an advantage of predicting the efficacy of the developed drug once drug candidates targeting the FDA-approved drug binding site are identified.

In the present study, we screened for a possible lead compound against VEGFR-2 from the natural compound database (Chen, 2011; Ru et al., 2014). By using the computational approach that docks possible compounds to the binding pocket of VEGFR-2 deduced from the binding of a reference drug (FDA-approved drug lenvatinib (Yamamoto et al., 2014), a multiple tyrosine kinase inhibitor), we predicted the activity of the compounds. We identified the natural product oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) as a substance that binds to VEGFR-2 through virtual screening. Oleanolic acid is a pentacyclic triterpenoid found in a variety of plants (Pollier and Goossens, 2012) that reportedly has several promising pharmacological activities, including hepatoprotective, anti-inflammatory, antioxidant and anti-cancer effects. Although it has been used in some countries as a hepatoprotective drug (Liu, 1995; Reisman et al., 2009) and several studies have reported anti-inflammatory or anti-cancer effects targeting NF-κB (Laszczyk, 2009; Petronelli et al., 2009), the mechanism of action of oleanolic acid has not been fully elucidated.

In the present study, we investigated the effects of the identified oleanolic acid on VEGFR-2 activity in ECs or angiogenesis. We also explored the anti-angiogenic activity of oleanolic acid in an *in vivo* angiogenesis model of retinal development and ROP in mice.

MATERIALS AND METHODS

Preparation of receptors and ligands for virtual screening

We used phytochemicals known to be present in herbs based on the Traditional Chinese Medicine Database @ Taiwan (Chen, 2011) and TCMSP (Ru et al., 2014) as chemical pools of virtual screening. All chemical structure files were downloaded and converted to canonical SMILESs to pdb files by Open Babel 2.3.1 (O'Boyle et al., 2011), after which the pdb files were converted to pdbqt files using MGLTools 1.5.6 (Morris et al., 2009). Structure files of KDR co-complexed with FDA-approved drugs were downloaded from the RCSB PDB (Berman et al., 2000). All non-standard residues in PDB files were removed, after which PDB files were converted to pdbqt files with prepare_receptor4.py command in MGLTools 1.5.6 using options to fix hydrogens and remove water molecules.

Cell culture

HUVECs were purchased from Lonza (USA) and cultured in M199 medium (Millipore-Sigma, USA) containing 20% fetal bovine serum (FBS; HyClone, USA) and antibiotics (Gibco, USA). Cells were cultured at 37℃ in an incubator with a humidified atmosphere of 5% CO_2. Cells between passages 4 and 7 were used for experiments.

Cell viability assay

Cell viability was assessed using a MTT labeling kit (Millipore-Sigma). Briefly, 5×10^3 cells were seeded into 96-well plates (Corning Inc., USA) and then treated with oleanolic acids (Millipore-Sigma) and worenine (Wuhan ChemFaces Biochemical Co., Ltd., China) at the indicated concentrations for 48 h. Cells were subsequently incubated with 100 μl of MTT (5 mg/ml) for 3 h. The blue formazan crystals were then solubilized with acidified isopropanol, after which the formazan levels were determined by measurement of the absorption intensity at 570 nm.

Western blot analysis

Western blot analysis was performed as previously described (Yun et al., 2018). Briefly, cells were treated with the indicated agents for 30 minutes. For oleanolic acid, worenine and VEGF treatment, oleanolic acid (0.1 to 50 μM) or worenine (0.1 to 50 μM) was applied for 2 h before VEGF (20 ng/ml) treatment. The human recombinant VEGF protein was purchased from R&D Systems (USA). Cells were then harvested and processed to SDS-PAGE, after which the blots were incubated with the indicated primary antibodies. The primary antibodies anti-phospho-VEGFR-2, anti-VEGFR-2, anti-phospho-Erk1/2 (CST9106), and anti-Erk (CST9102) were acquired from Cell Signaling Technology (USA). The anti-β-tubulin (sc-9104) and peroxidase-conjugated secondary antibodies (sc-2004, sc-2005) were obtained from Santa Cruz Biotechnology (USA). An ECL Detection kit (Thermo Fisher Scientific, USA) was used to detect protein bands.

Cell migration

Cells were plated on the upper chambers of transwells with 8 μm pores and then inserted into 24 well plates. Next, 6×10^3 cells were seeded into the upper chambers (Corning Inc., USA) for 6 h and starved with media containing 1% FBS for 12 h. Cells were then treated with the indicated agents and allowed to migrate through the pores for another 24 h. For oleanolic acid, VEGF, and FGF treatment, oleanolic acid (10 μM) was applied for 2 h before VEGF (20 ng/ml) of FGF (20 ng/ml) treatment. The membrane in the upper chamber was then separated, washed with PBS and stained with Diff-Quik solution (Sysmex, Japan), after which the membranes were photographed under a microscope (Axioscope, Carl Zeiss, Germany). For quantitative analysis, the number of cells at four sites randomly selected on one membrane was counted and the values of four different membranes were quantified.

Cell proliferation

To evaluate cell proliferation, 7.5×10^4 cells were seeded and incubated overnight, then treated with the indicated agents. For oleanolic acid and VEGF treatment, oleanolic acid (10 μM) was applied for 2 h before VEGF (20 ng/ml) treatment. At 0, 24, 48, and 72 h after the treatments, viable cells were counted using a hemocytometer following trypan blue staining (Millipore-Sigma).

Tube formation

To evaluate tube formation of HUVECs, 300 μl of growth factor-reduced Matrigel (Corning) was coated onto 24-well plates at 37℃ for at least 30 min. Cells were then treated with or without oleanolic acid (10 μM) 2 h before seeding into the Matriegel-coated plates. Cells (2.5×10^4 cells per well) were seeded into the coated plates with or without VEGF (20 ng/ml) and/or oleanolic acid (10 μM), after which they were incubated for another 4 h. Tube length and area were quantified using the Image J software (NIH, USA).

Retinal angiogenesis assay

The litters of C57BL/6 mice were injected with oleanolic acid dissolved in DMSO and sunflower oil mixture intraperitoneally at P4 and P5 at the indicated concentrations, and the retinas were harvested at P6. Animal care and experimental protocols were approved by the Animal Care Committee of Dongguk University (approval number: IACUC-2016-050).

Oxygen induced retinopathy

An oxygen induced retinopathy (OIR) mouse model was developed as in a previous study (Villacampa et al., 2017). Briefly, the litters of C57BL/6 mice were exposed to 85% oxygen from P8 to P11 in a hypoxic/hyperoxic chamber with an O_2 controller (Coy Laboratory Products, USA), then returned to room air for 5 days. Oleanolic acid dissolved in DMSO and sunflower oil mixture was intraperitoneally administrated to the mice at P11 and P15 at the indicated concentrations. All animals were bred in specific pathogen free animal facility and were fed a normal diet (LabDiet, USA) ad libitum with free access to water. All animal care and experimental protocols were approved by the Animal Care Committee of Dongguk University (approval No. IACUC-2016-050).

Immunohistochemistry and morphometric analyses

Retinas were incubated with FITC-conjugated isolectin B4 (IB4, Millipore-Sigma), after which the samples were flat-mounted. All images were obtained using a Nikon Eclipse Ts2 inverted fluorescent microscope (Nikon, Japan) equipped with high-definition color camera (DS-Qi2, Nikon), then analyzed with the NIS Elements Imaging Software (version 4.30; Nikon). To calculate the number of filopodia, stained images were measured along 100 μm vessel length from endothelial tip cells. To determine the filopodia lengths, the images were measured in 5 random 0.05 mm^2 vascular fronts of each retina. The retina vascular densities were determined by analyzing the densities of the IB4-positive areas based on the pixels in five randomly-selected regions of interest. To count the numbers of neo-vascular tufts (NVT) in OIR, vascular tufts with diameters exceeding 50 μm were calculated.

Statistical analysis

Statistical analyses were performed using the Prism software (GraphPad Software Inc., USA) and standard two-tailed Student's *t*-tests assuming unequal variances were used to identify differences between groups. A $P < 0.05$ was considered statistically significant. Quantitative data and figures are presented as the means ± SD.

RESULTS

Oleanolic acid as a potent VEGFR-2 inhibitor

For virtual screening of phytochemicals with VEGFR-2, AutoDock Vina 1.1.2 (Trott and Olson, 2010) with the default options was used to approximate the binding affinity between chemical ligands and VEGFR-2. A Google Clould Engine-based docking platform was constructed to accommodate medium-to-large scale virtual screening, and a total of 400 cores was used to perform docking of 27,760 ligands to 35 VEGFR-2 structures.

Oleanolic acid was found to have similar binding affinity with VEGFR-2 (-10.4 kcal/mol) compared to the reference

drug lenvatinib (-8.0 kcal/mol) in the PDB entry 3CJG of model A coordination (Fig. 1). Oleanoic acid is a phytochemical found in *Achyranthes japonica (Miq.) Nakai, Crataegus pinnatifida Bunge, Foeniculum vulgare Mill., Forsythia viridissima Lindl., Kalopanax pictus (Thunb.) Nakai, Kochia scoparia (L.) Schrad., Ligustrum lucidum W.T.Aiton, Oldenlania diffusa (Willd.) Roxb., Paeonia japonica var. pilosa Nakai, Perilla frutescens var. crispa (Thunb.) H.Deane, Plantago asiatica L., Pogostemon cablin (Blanco) Benth., Polygonum aviculare L., Prunella vulgaris L., Punica granatum L., Rubus coreanus Miq.,* and *Verbena officinalis L.* based on a search of the natural compounds database (Chen, 2011; Ru et al., 2014) (Table 1).

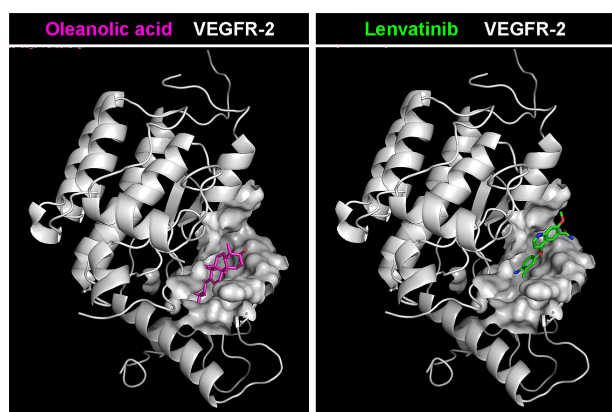

Fig. 1. Scheme for virtual screening of oleanolic acid with VEGFR-2. Oleanolic acid (pink, -10.4 kcal/mol) was bound to VEGFR-2 (grey) with similar binding affinity as the reference drug lenvatinib (green, -8.0 kcal/mol).

Table 1. List of oleanolic acid containing herbs

Scientific name
Achyranthes japonica (Miq.) Nakai
Crataegus pinnatifida Bunge
Foeniculum vulgare Mill.
Forsythia viridissima Lindl.
Kalopanax pictus (Thunb.) Nakai
Kochia scoparia (L.) Schrad.
Ligustrum lucidum W.T.Aiton
Oldenlania diffusa (Willd.) Roxb.
Paeonia japonica var. *pilosa Nakai*
Perilla frutescens var. *crispa* (Thunb.) H.Deane
Plantago asiatica L.
Pogostemon cablin (Blanco) Benth.
Polygonum aviculare L.
Prunella vulgaris L.
Punica granatum L.
Rubus coreanus Miq.
Verbena officinalis L.

Oleanolic acid inhibits VEGF-induced VEGFR2 activation in HUVECs

To determine the cytotoxicity of oleanolic acid in HUVECs, cells were treated with oleanolic acids at different concentrations for 48 h, after which the cell viability was evaluated by MTT assay. Oleanolic acid did not induce cytotoxicity in HUVECs when applied at up to 50 μM (Fig. 2A).

We next examined whether oleanolic acid inhibits VEGFR-2 activity in HUVECs. When cells were treated with VEGF (20 ng/ml) for 30 min with or without pretreatment with varying concentrations of oleanolic acid, oleanolic acids dose-dependently inhibited the VEGF-induced phosphorylation of VEGFR-2 and its downstream signaling protein Erk1/2 (Fig. 2B). At 10 μM, oleanolic acids inhibited approximately 50% and 25% of the phosphorylation of VEGFR-2 and Erk1/2, respectively (Fig. 2C). Thus, we chose 10 μM of oleanolic acid for use in the following *in vitro* experiments. These results indicate that oleanolic acid effectively inhibits VEGF-induced VEGFR-2 activation in HUVECs. Moreover, we identified worenine as another candidate upon virtual screening of phytochemicals with VEGFR-2. Worenine had similar binding affinity with VEGFR-2 (-9.7 kcal/mol) as the reference drug lenvatinib (Supplementary Fig. S1A); however, it could not inhibit phosphorylation of VEGFR-2 and Erk1/2 under non-cytotoxic concentrations in HUVECs (Supplementary Figs. S1B and S1C).

Oleanolic acid inhibits VEGF-induced angiogenesis in HUVECs

To examine whether oleanolic acid inhibited VEGF-induced angiogenesis in HUVECs, we conducted an *in vitro* angiogenesis assay involving cell migration, proliferation, and tube formation. Cells were cultured on the inserts of transwell plates and then treated with VEGF for 24 h. The number of cells that migrated to the bottom of the insert increased in response to VEGF (Figs. 3A and 3B). However, pretreatment with oleanolic acid for two hours inhibited VEGF-induced cell migration to control levels (Figs. 2A and 2B). In the experimental setting for cell proliferation, oleanolic acid also inhibited the proliferation of HUVECs by VEGF to the control level (Fig. 3C). To further analyze the anti-angiogenic effects of oleanolic acid, tube formation of HUVECs on matrigel was evaluated. When cells treated with VEGF, they became elongated and connected to each other to form a web-like structure (Fig. 3D). However, pretreatment with oleanolic acid inhibited the increase of both tube area and length by VEGF (Figs. 3D and 3E). These results indicate that oleanolic acid inhibits angiogenic features of HUVECs induced by VEGF.

Oleanolic acid inhibits developmental angiogenesis in mouse retina *in vivo*.

To investigate whether oleanolic acid inhibits retinal angiogenesis in mice, we administrated oleanolic acid to the peritoneal cavity of postnatal C57BL/6 mice at low (62.5 mg/kg) and high (125 mg/kg) levels at P4 and P5. At 12 h later after the final injection, the retinas were harvested and IB4-stained to measure the number of filopodia and vascular density of blood vessel. The number of filopodia at the front

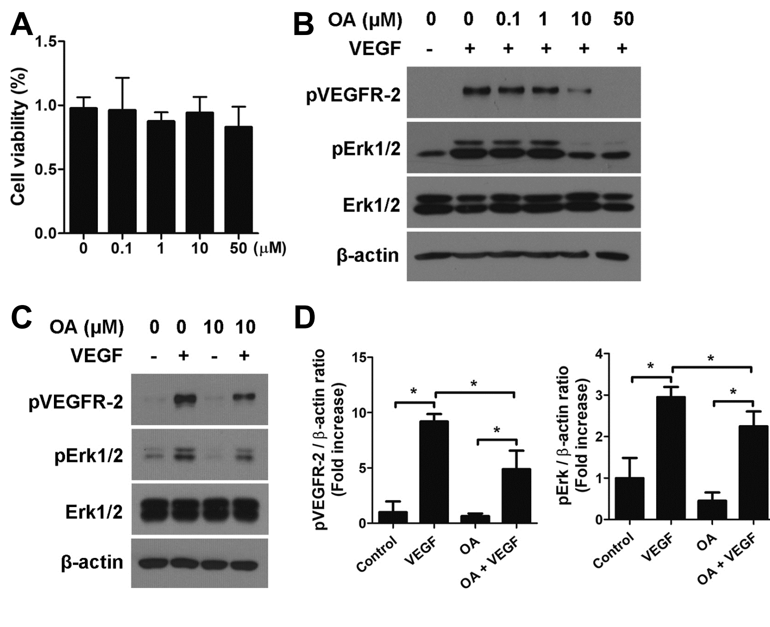

Fig. 2. Effect of oleanolic acid on VEGF-induced VEGFR-2 activity. (A) HUVECs were treated with oleanolic acid at the indicated doses. Cell viability was determined by MTT assay. Note that oleanolic acid at any doses showed no toxicity in HUVECs. The bar graph represents the means ± SD ($n = 4$). (B) VEGF-induced VEGFR-2 phosphorylation (pVEGFR-2) or Erk1/2 phosphorylation (pErk1/2) in HUVECs with or without the pretreatment of oleanolic acid (OA) at the indicated doses was examined by western blot analysis. Erk1/2 and β-actin were used as controls. (C) HUVECs were treated with VEGF (20 ng/ml) and/or oleanolic acid (10 μM). The phosphorylation levels of VEGFR-2 (pVEGFR-2) and Erk1/2 (pErk1/2) were examined by western blot analysis. (D) Quantitative densitometric analysis of western blots in panel (C). The results are fold increases versus the value of the control. The bar graph represents the means ± SD ($n = 3$). * $P < 0.05$ between the two values.

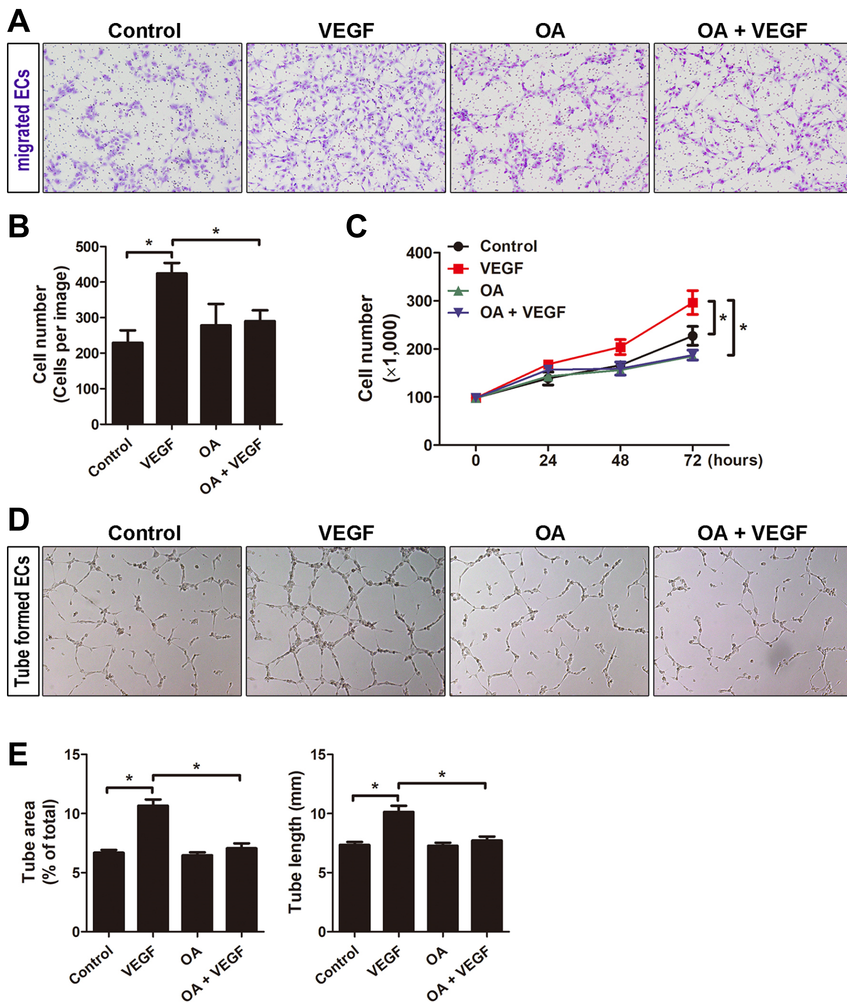

Fig. 3. Effect of oleanolic acid on VEGF-induced changes in angiogenic features of HUVECs. (A) Representative images of cell migration of HUVECs treated with VEGF (20 mg/ml) and/or oleanolic acid (10 μM). The migrated cells were stained with Diff-Quik solution (purple). Original magnification is 100×. (B) Quantitative analysis of cell migration in panel (A). (C) Cell proliferation of HUVECs treated with VEGF (20 ng/ml) and/or oleanolic acid (10 μM) for the indicated times were determined by cell counting. The graph represents the means ± SD ($n = 3$). * $P < 0.05$ between the two values. (D) Representative images of tube formation of HUVECs treated with VEGF (20 ng/ml) and/or oleanolic acid (10 μM). Original magnification is 100×. (E) Quantitative analysis of tube area (% of total area) and tube lengths (mm) in panel (D) was performed. The bar graph represents the means ± SD ($n = 4$). * $P < 0.05$ between the two values.

of retinal blood vessels was significantly smaller in the oleanolic acid treatment group than the control (Figs. 4A and 4B). Consistently, the length of filopodia at the front of the retinal blood vessels was shorter in oleanolic acid-treated mice than in the control (Figs. 4A and 4C). Moreover, oleanolic acid treatment in mice significantly reduced the vascular density of retinas compared to the control (Figs. 4D and 4E). These results indicate that oleanolic acid inhibits angiogenesis in the retina during development.

Oleanolic acid inhibits pathologic angiogenesis in OIR mouse model *in vivo*.

To determine if oleanolic acid inhibits pathologic retinal angiogenesis in mice, we generated an OIR mice model. Neonatal C57BL/6 mice were exposed to 85% oxygen from P8 to P11 in a hyperoxic chamber, then returned to room air until P16. During development of the OIR mouse model, retinal vessels begin to regress by exposure to hyperoxia. On returning to room air, relative hypoxia leads to aberrant neovascular tufts (NVTs) in the avascular retina that abnormally protrudes from the retina surface into the vitreous of the retina (Villacampa et al., 2017). To examine the efficacy of oleanolic acid toward OIR, oleanolic acid was intraperitoneally injected into the mice at low (62.5 mg/kg) and high (125 mg/kg) concentrations at P11 and P15. At one day after final injection, the retinas were harvested and IB4-stained to determine the number of NVT (Fig. 5A). In control mice, we observed many NVTs with diameters exceeding 50 μm, whereas these were significantly smaller in oleanolic acid-treated mice (Figs. 5B-5D). These results indicate that oleanolic acid inhibits pathologic angiogenesis in the retinas of OIR mice.

DISCUSSION

Angiogenesis must be precisely controlled because uncontrolled angiogenesis is involved in aggravation of disease symptoms. VEGF/VEGFR-2 signaling is a key pathway leading to angiogenic responses in vascular ECs. Therefore, targeting VEGF/VEGFR-2 signaling may be effective at modulating angiogenesis to alleviate various disease symptoms (Ellis and Hicklin, 2008). Many studies have shown that drugs that target VEGF/VEGFR-2 signaling are highly effective at inhibiting angiogenesis. For example, bevacizumab, a neutralizing antibody to the VEGF protein, has been approved by the FDA for its efficacy in treatment of metastatic colorectal cancer and is being used to inhibit angiogenesis in a variety of cancers (Ferrara et al., 2004; Hurwitz et al., 2004; Sennino and McDonald, 2012). The inhibitory effect of bevacizumab on angiogenesis and vascular leakage have also been shown to be useful in treatment of ocular diseases such as macular edema and diabetic retinopathy (Arevalo et al., 2009a; 2009b; Avery et al., 2006). In addition, peptides (Bikfalvi, 2004), aptamers (Ng et al., 2006), and soluble decoy receptors (Holash et al., 2002) have been developed as anti-VEGF agents. Despite the advantages of high specificity and low toxicity of those peptide-based drugs, high manufacturing costs and limited routes of administration have

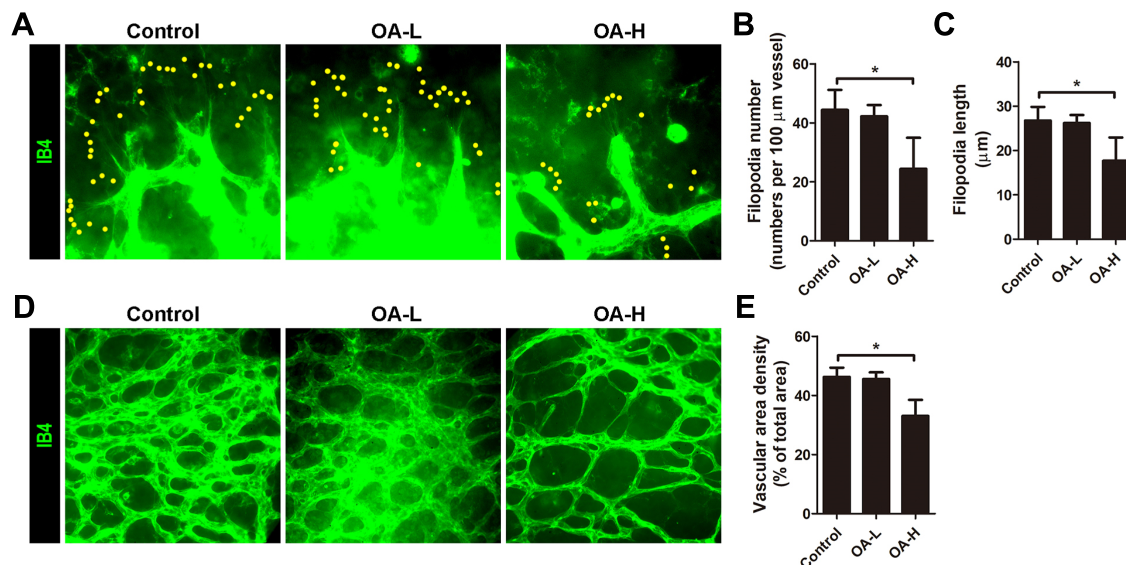

Fig. 4. Effect of oleanolic acid on developmental angiogenesis in mouse retina *in vivo*. (A) Representative images of filopodia formation at two concentrations of oleanolic acid, low (OA-L, 62.5 mg/kg) and high (OA-H, 125 mg/kg). The filopodias were marked with yellow dots. Original magnification is 800×. (B) Quantitative analysis of number of filopodia in panel (A). The graph represents the means ± SD (n = 5). * $P < 0.05$ between the three values. (C) Quantitative analysis of the length of filopodia in panel (A). The graph represents the means ± SD (n = 5). * $P < 0.05$ between the three values. (D) Representative images of retinal vasculature at two concentrations of oleanolic acid, low (OA-L, 62.5 mg/kg) and high (OA-H, 125 mg/kg). Original magnification is 400×. (E) Quantitative analysis of retinal vascular density in panel (D). The graph represents the means ± SD (n = 5). * $P < 0.05$ between the two values. All samples were stained with IB4 (green).

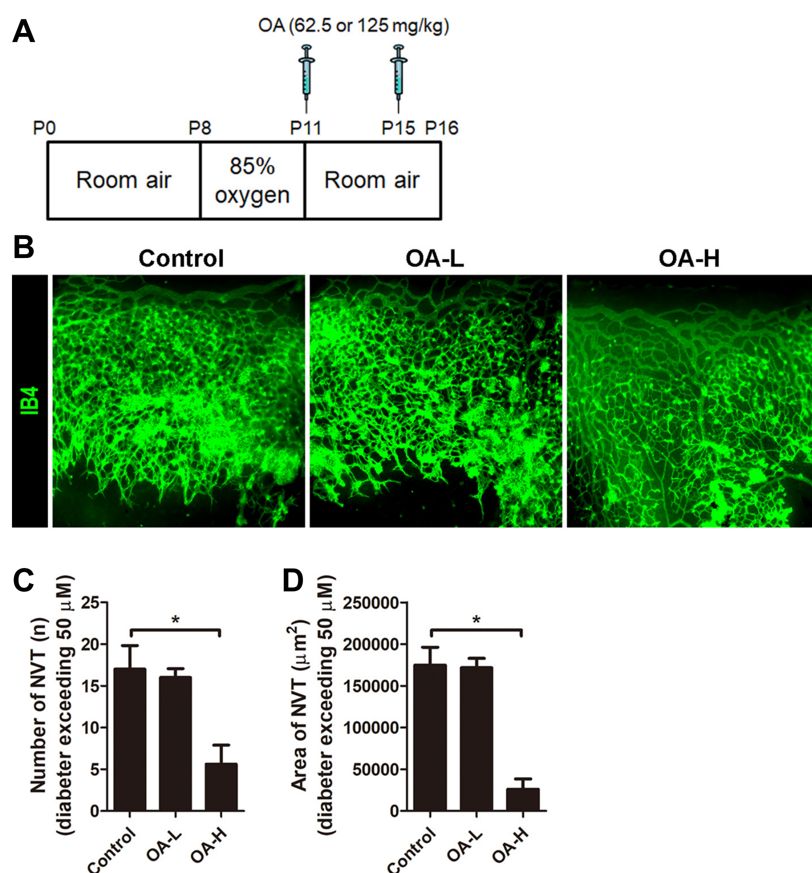

Fig. 5. Effect of oleanolic acid on pathologic angiogenesis in OIR mouse model *in vivo*. (A) Scheme for experimental schedule of OIR. (B) Representative images of vasculature in OIR at two concentrations of oleanolic acid, low (OA-L, 62.5 mg/kg) and high (OA-H, 125 mg/kg). Original magnification is 100×. (C) Quantitative analysis of the number of NVT in panel (B). The graph represents the means ± SD (n = 5). * $P < 0.05$ between the three values. (D) Quantitative analysis of the area of NVT in panel (B). The graph represents the means ± SD (n = 5). * $P < 0.05$ between the two values. All samples were stained with IB4 (green).

limited their use. Given the potential benefits of low molecular weight compounds because of their low cost manufacturing, ease of administration, and long-term chronic administration, we focused on the discovery of small molecule inhibitors for VEGFR-2.

Oleanolic acid is a constituent of various plant leaves used in traditional Chinese medicine to treat liver diseases (Liu, 1995). Although the exact molecular mechanism is unclear, the hepatoprotective effects of oleanolic acid are likely due to the regulation of Nrf2 transcription regulator, which controls the transcription of antioxidant and detoxifying enzymes (Liu et al., 2008; Reisman et al., 2009). Although the mechanism of its effect on ECs is unknown, oleanolic acid was found to inhibit the proliferation of ECs and *in vivo* angiogenesis in chick embryo chorioallantoic membrane (CAM) assays (Sohn et al., 1995). Oleanolic acid also reportedly inhibits tumor necrosis factor-α (TNF-α)-induced upregulation of E-selectin expression in HUVECs, possibly by interfering with NF-kB action (Takada et al., 2010). However, the precise mechanisms of action of oleanolic acid and its molecular targets are currently unknown. In the present study, we identified oleanolic acid as a substance that binds to VEGFR-2 through virtual screening and tested whether it could inhibit VEGFR-2 activity. We demonstrated that oleanolic acid effectively inhibited the activation of VEGFR-2 and its downstream Erk1/2 induced by VEGF in HUVECs (Fig. 1). We also demonstrated that oleanolic acid effectively inhibits angiogenic features induced by VEGF including cell migration, proliferation, and tube formation in HUVECs (Fig. 2). We have used lenvatinib as a reference drug to identify the binding site for VEGR-2 for virtual screening, which raises the question of whether oleanolic acid can also affect other targets of the multiple kinase inhibitor lenvitinib (Yamamoto et al., 2014). We found that oleanolic acid had no significant effect on fibroblast growth factor (FGF)-induced EC migration at concentrations inhibiting VEGFR-2 (Supplementary Fig. S2). These results suggest that oleanolic acid more specifically suppresses VEGFR-2 signaling, even though it binds effectively to the receptor binding site of lenvatinib, a multiple kinase inhibitor. These results also suggest that the specificity of the drug developed through virtual screening needs to be verified and the possibility that the developed drug may have better specificity than the reference drug.

Various eye diseases are associated with functional and morphological changes in the blood vessels in which angiogenesis plays an important role. In particular, angiogenesis is one of the major pathological changes in patients with diabetic retinopathy, age-related macular degeneration, and ROP. Considering the high expression of VEGF in those ocular diseases (Adamis et al., 1994; Sato et al., 2009; Wells et al., 1996) and the high expression of VEGF in the eyes of

patients with angiogenic features relative to those without angiogenic features (Aiello et al., 1994), inhibitors targeting VEGF/VEGFR-2 may have a high therapeutic benefit. ROP is a major ocular complication of preterm infants that progresses via hypoxia induced limitation of retinal oxygenation and excessive VEGF expression, which leads to abnormal vasoproliferation (Hellstrom et al., 2013). Until recently, preventive and therapeutic treatments of ROP have had limited efficacy because the pathologies involved in development of this condition were unknown (Sapieha et al., 2010). However, anti-VEGF therapy is now being used to treat various ocular diseases with positive effects, including in ROP patients (Hellstrom et al., 2013; Micieli et al., 2009; Sapieha et al., 2010).

In the present study, we demonstrated that oleanolic acid inhibits retinal angiogenesis during the development of the mouse retina, which was evidenced by a reduction in filopodia count and vascular area (Fig. 4), and that oleanolic acid further suppresses pathologic retinal angiogenesis in the OIR model, which was evidenced by inhibition of the formation of abnormal vascular tufts (Fig. 5). Moreover, inflammation plays a prominent role in the pathogenesis of ocular diseases, including diabetic retinopathy, age-related macular degeneration, and ROP, while also contributing to the progression of these diseases (Buschini et al., 2011; Gologorsky et al., 2012; Rothova et al., 1996). For example, pro-inflammatory cytokines TNF-α, interleukin (IL)-1β, and IL-6 are known to be elevated in diabetic retinas (Demircan et al., 2006; Krady et al., 2005; Mocan et al., 2006), as well as to cause many functional alterations in retinal vasculature such as leukocyte infiltration and vascular permeability (Tang and Kern, 2011; Yun et al., 2017). In this context, it is interesting that oleanolic acids can inhibit activation of NF-kB, the main signaling pathway of TNF-α, although the mechanism is unclear (Takada et al., 2010). Given that controlling inflammation is as important for the treatment of retinal diseases as controlling angiogenesis, oleanolic acid may have a variety of therapeutic effects, including inhibition of angiogenesis in ocular diseases. However, we showed that oleanolic acid inhibits *in vivo* developmental and pathologic angiogenesis only at a high dose (125 mg/kg). This is presumably because oleanolic acid has a very short half-life (Jeong et al., 2007) and low concentrations of oleanolic acid may not be effective given that oleanolic acids were administered only twice during the experimental procedures (Figs. 4 and 5). Therefore, in order to overcome the pharmacokinetic disadvantages of oleanolic acid and to increase its utility of the treatment, further studies are needed to modify the oleanolic acid formula or alter the route of treatment such as topical administration.

In summary, our study demonstrated the inhibitory effect of oleanolic acid on the activation of VEGFR-2 and VEGF-driven angiogenic features of ECs. We also demonstrated that oleanolic acid inhibits *in vivo* angiogenesis during the development and course of the ROP model in the mouse retina. Together, these results suggest a potential therapeutic benefit of oleanolic acid for inhibiting angiogenesis in ocular diseases.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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