Original Research

Norisoboldine, an alkaloid compound isolated from Radix Linderae, inhibits synovial angiogenesis in adjuvant-induced arthritis rats by moderating Notch1 pathway-related endothelial tip cell phenotype

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Abstract

Synovial angiogenesis is well recognized as participating in the pathogenesis of rheumatoid arthritis (RA) and has been regarded as a potential target for RA therapy. Previously, we have shown that norisoboldine (NOR) can protect joints from destruction in mice with collagen II-induced arthritis (CIA). Here, we investigate the effect of NOR on synovial angiogenesis in adjuvant-induced arthritis (AA) rats, and clarify the mechanisms *in vitro*. NOR, administered orally, significantly reduced the number of blood vessels and expression of growth factors in the synovium of AA rats. *In vitro,* it markedly prevented the migration and sprouting of endothelial cells. Notably, the endothelial tip cell phenotype, which is essential for the migration of endothelial cells and subsequent angiogenesis, was significantly inhibited by NOR. This inhibitory effect was attenuated by pretreatment with *N*-{*N*-[2-(3,5-difluorophenyl) acetyl]-(*S*)-alanyl}-(*S*)-phenylglycine tert-butyl ester, a Notch1 inhibitor, suggesting that the action of NOR was related to the Notch1 pathway. A molecular docking study further confirmed that NOR was able to promote Notch1 activation by binding the Notch1 transcription complex. In conclusion, NOR was able to prevent synovial angiogenesis in AA rats, which is a putatively new mechanism responsible for its anti-rheumatoid effect. The anti-angiogenesis action of NOR was likely achieved by moderating the Notch1 pathway-related endothelial tip cell phenotype with a potential action target of the Notch1 transcription complex.

Keywords: norisoboldine, rheumatoid arthritis, angiogenesis, tip cells, Notch1

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease associated with significant function disability and morbidity. The pathogenesis of RA is complex and involves interconnected B- and T-cell autoimmune pathways, cytokine networks and angiogenesis.[1,2] The transformation of synovium increases invasiveness into cartilage and bone, further exacerbating joint damage.[3] Traditional therapeutic options for RA include analgesics, non-steroidal anti-inflammatory drugs, glucocorticoids and biological and small-molecule agents.[3] However, these disease-modifying antirheumatic drugs have substantial limitations, including partial efficacy and poor tolerability.[3,4] Therefore, developing new strategies with better clinical efficacy and safety is urgently needed.

During the development of RA, synovium hyperplasia and consequent pannus formation largely depend on the local blood supply.[2] Thus, preventing the formation of synovial blood vessels should not only reduce the blood supply to starve the pannus, but also limit the vessel-dependent damage outside the joints.[4] Many endogenous and exogenous inhibitors of angiogenesis (i.e. the formation of new capillaries from pre-existing blood vessels) have been found to be able to ease synovial injury in RA. For example, angiostatin could repress synovial inflammation, vascularization and pannus formation in a murine collagen II-induced arthritis (CIA) model.[5] Endostatin could prevent

the progression of arthritis in various animal models via $\alpha v\beta 3$ integrin-dependent mechanisms.[6] Vatalanib, an inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinase, could effectively attenuate knee arthritis in rabbits.[7,8] TNP-470, an angiostatic derivative of fumagillin, was able to prevent arthritis when administered before the onset of the disease by inhibiting the expression of methionine aminopeptidase-2 (an enzyme involved in neovascularization).[9,10]

Norisoboldine (NOR) is the primary isoquinoline alkaloid constituent of Radix Linderae, the dry roots of *Lindera aggregate* traditionally used in Chinese medicine to treat chest and abdominal pain, indigestion, regurgitation, cold hernia and frequent urination. Our previous studies have demonstrated the beneficial effect of NOR on joint destruction in CIA mice. The minimum efficient dosage of NOR was only 10 mg/kg when orally administered from the booster day for 20 consecutive days.[11] However, the precise antiarthritis mechanisms of NOR remain unclear. In the present study, we evaluated the effect of NOR on synovial angiogenesis in adjuvant arthritis (AA) rats and further interpreted the underlying mechanisms with human umbilical vein endothelial cells (HUVECs) *in vitro*.

Materials and methods

Reagents and chemicals

NOR (>98%) was given by Dr Guixin Chou (Figure 1). The structure was identified by ^{1}H- and ^{13}C-nuclear magnetic resonance and confirmed by the literature.[12] The purity was tested by high-performance liquid chromatography (see Supplementary Figures 1–4 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.011416/-/DC1). Medium 199 (M199), endothelial cell growth medium-2 (EGM-2), trypsin, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). New-born calf serum (NBCS) was purchased from PAA Laboratories GmbH (Morningside, QLD, Australia). *N*-{*N*-[2-(3,5-difluorophenyl)acetyl]-(*S*)-alanyl}-(*S*)-phenylglycine tert-butyl ester (DAPT), curcumin, dexamethasone, Tween-20, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), dithiotheitol and phenylmethylsulphonyl fluoride were purchased from Sigma Chemical (St Louis, MO, USA). Notch1 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, enhanced chemiluminescent plus reagent kit and peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from KangChen Bio-tech (Shanghai, China). Cleaved Notch1 antibody was obtained from Bioworld Technology (St Louis Park, MN, USA). Recombinant human $VEGF_{165}$ was obtained from Peprotech (Rocky Hill, NJ, USA). The other chemicals and reagents used were of analytical grade.

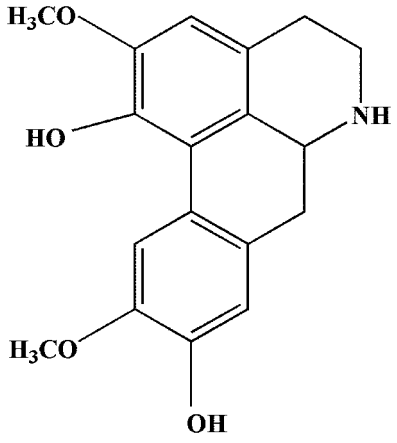

Figure 1 Structure of norisoboldine

Induction of arthritis

Six-week-old male Sprague–Dawley rats that weighed 180–220 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). The procedures and protocols of the study followed our institutional guidelines and were conducted in accordance with the Declaration of Helsinki and the Guideline for the Care and Use of Laboratory Animal in Jiangsu Province, People's Republic of China. The animals were housed in a room with a constant temperature ($23 \pm 2^{\circ}C$) and 12/12 h light/dark cycle and fed a standard diet and water *ad libitum*.

Arthritis was induced by an intradermal injection of 100 μL of 10 mg/mL *Mycobacterium butyricum* paraffin oil suspension into the base of the right hindpaws and tails of the rats. As a control, the same volume of liquid paraffin alone was injected the same way. All of the time points were relative to the day of AA induction, which was designated day 0. From day 14, a secondary response gradually appeared, characterized by the swelling of the left hindpaws and joints. NOR and dexamethasone (a positive control) were suspended in 0.8% sodium carboxymethylcellulose solution and orally administered during the autoimmune phase from day 14 to day 23. One hour after the last administration, the animals were sacrificed by etherization. The left hind knee joints were promptly removed, and the synovium tissues were prepared for the immunohistochemistry assay.

Isolation and treatment of HUVECs

Umbilical cords were obtained from Nanjing MaiGaoQiao Hospital (Nanjing, China). Endothelial cells were isolated from the cords as described previously.[13] The harvested cells were seeded into 25 cm^2 flasks precoated with 0.2% (w/v) gelatin (Promega, Madison, WI, USA). The culture medium consisted of M199 supplemented with 20% (v/v) NBCS, 2 mmol/L L-glutamine, 5 U/mL penicillin G, 5 μg/mL streptomycin sulfate and 7.5 μg/mL endothelial cell growth supplement (Sigma). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed after 24 h and every two days thereafter until confluence was achieved. All of the experiments were performed using HUVECs within passages 3–5. Trypsin (0.25%) in phosphate-buffered saline (PBS) was used for digestion and passage.

Fibrin bead assay

This experiment was conducted as described previously.[14,15] In brief, HUVECs were mixed with Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a concentration of 400 HUVECs per bead in 1 mL EGM-2 (Gibco). Beads with cells were shaken gently every 20 min for four hours at 37°C with 5% CO_2 and then incubated in 5 mL EGM-2 for an additional 12–16 h. Subsequently, the beads were re-suspended at a concentration of 200 cell-coated beads/mL in 2.5 mg/mL fibrinogen (Sigma) and 0.15 units/mL aprotinin (Sigma) and seeded in a 24-well plate (0.5 mL/well), followed by adding 0.625 units of thrombin (Sigma). Human skin fibroblasts were layered on top of the clot at a concentration of 2×10^4 cells/well. Capillary-like sprouts in the fibrin bead assay appeared on days 2–3. Lumen formation began around days 5–6. Over the course of 10–20 days, extensive networks were formed. Different treatments were added on day 3, and five random ×200 fields were photographed for each well on day 14. Tip cells were counted, averaged and compared.

Cell proliferation assay

The proliferation assay of HUVECs was performed using a Cell-Light TM 5-ethynyl-2′-deoxyuridine (Edu) DNA Cell Proliferation Kit (Ruibo Biotech, Guangzhou, China).[16–18] In brief, HUVECs were cultured in 96-well plates at 2×10^4 cells/well with different concentrations of NOR (1, 3, 10 and 30 μmol/L) or curcumin (10 μmol/L) in the presence or absence of VEGF (10 ng/mL) for 24 h. The cells were then incubated with EdU for another eight hours. Five random ×100 fields were photographed for each well, and the EdU-positive cells were counted, averaged and compared.

Cell migration assay

The HUVEC migration assay was performed using a transwell Boyden chamber (Corning Costar, Cambridge, MA, USA) that contained a polycarbonate filter with a pore size of 8 μm. The cells were starved with 2% NBCS medium for two hours and then harvested and re-suspended with medium. Subsequently, 2×10^5 cells were seeded in the upper well of each chamber, and the chambers were inserted into 24-well plates that contained 2% NBCS medium with or without 10 ng/mL VEGF or various concentrations of NOR (1, 3, 10 and 30 μmol/L). After incubation for six hours at 37°C, the non-migrated cells on the upper surface of the membrane were removed by a PBS-soaked cotton swab, and the migrated cells on the bottom surface of the transwell membrane were stained by 5% Cresyl violet for 20 min. The migrated cells for each sample were counted in five randomly selected high-power fields.

Tube formation assay

The formation of vascular-like structures by HUVECs was assessed by a previously described method.[19] Briefly, 200 μL matrigel (4 mg/mL in PBS) was added to 24-well plates. The plates were then incubated at 37°C for 30 min to allow the gel to polymerize. HUVECs (5×10^4 cells/cm^2/well) were plated into the culture plates coated with matrigel and incubated with NOR (1, 3, 10 and 30 μmol/L) or curcumin (10 μmol/L) in the presence or absence of VEGF (10 ng/mL) for 24 h. Five ×100 fields were then randomly selected for each sample, and the tube lengths were measured, averaged and compared with ImagePro Plus (Media Cybernetics Inc., Bethesda, MD, USA).

Rat aortic ring assay

As described previously,[20] the thoracic aortas were obtained from six-week-old Sprague–Dawley rats and immediately transferred to a culture dish with serum-free medium. After gently removing the fibroadipose tissues, the aortas were cut into 1 mm long rings and set in 96-well plates pre-coated with 40 μL growth factor-reduced matrigel. Additional matrigel (40 μL) was added into the aortic rings to allow solidification. Serum-free endothelial growth medium (200 μL) supplemented with 200 μg/mL endothelial cell growth supplement was then added to the wells. The microvessel sproutings of aortic rings were observed in nine days. After treating the aorta rings with NOR (1, 3, 10 and 30 μmol/L) or curcumin (10 μmol/L) in the presence or absence of VEGF (10 ng/mL) for six days, microvessel sprouting was quantified by evaluating migrated cells. Five random ×100 fields were photographed, and migrated cells were counted, averaged and compared.

Reverse transcription polymerase chain reaction analysis

The extraction of RNA and conversion to cDNA were performed. HUVECs in the logarithmic growth phase were placed into six-well flat-bottom tissue culture plates at a density of 2×10^5 cells/mL. The cells were then treated with different concentrations of NOR (1, 3, 10 and 30 μmol/L) or curcumin (10 μmol/L) in the presence or absence of VEGF (10 ng/mL) for three hours. Total RNA (0.5 μg) was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using oligo $(dT)_{18}$ primer and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) at 37°C for 50 min. The primer sequences were chosen as follows: hairy/enhancer-of-split related with YRPW motif (HEY) 1 primers, (sense) 5′ CGA GGT GGA GAA GGA GAG TG 3′ and (antisense) 5 CTG GGT ACC AGC CTT CTC AG 3′; HEY2 primers, (sense) 5′ TTC AAG GCA GCT CGG TAA CT 3′ and (antisense) 5′ GGG CAT TTT ACT TCC CCA AT 3′; neuropilin-2 (Nrp-2) primers, (sense) 5′ GTG GTT CAT CTT GAC CTT GT 3′ and (antisense) 5′ ATT CTT CTT CTG CAA CCT CA 3′; neuronal cell adhesion molecule (Nr-cam) primers, (sense) 5′ AAC ATA TGG GTA GAG AGT ATA TTT 3′ and (antisense) 5′ CTT TGC ATT CCA GTT CAT ATT AA 3′; Myosin-X (MyoX) primers, (sense) 5′ ATC TTC GCC ATC GCC AAC GAG 3′ and (antisense) 5′ GTT GTT GTA CAC GGT CTT CGC 3′; cell division cycle 42 (Cdc42) primers, (sense) 5′ GCC CGT GAC CTG AAG GCT GTC A 3′ and (antisense) 5′ TGC TTT TAG

TAT GAT GCC GAC ACC A 3′; β-actin primers, (sense) 5′ ACA TCT GCT GGA AGG TGG AC 3′ and (antisense) 5′ GGT ACC ACC ATG TAC CCA GG 3′. The thermocycling programs were as follows: HEY1 and β-actin (32 cycles at 94°C for 1 min, at 55°C for 45 s and at 72°C for 1 min), HEY2, Nrp-2 and Nr-cam (40 cycles at 94°C for 1 min, at 55°C for 1 min and at 72°C for 1 min), MyoX and Cdc42 (25 cycles at 96°C for 15 s, at 60°C for 15 s and at 72°C for 30 s). The amplified products were electrophoresed with 1% agarose gel and visualized by GoldView™ (SBS Genetech, Beijing, China) and ultraviolet irradiation.[21]

Western blot analysis

Protein Notch 1, Cleaved Notch1 and GAPDH were detected by Western blot analysis as described previously.[22] GAPDH was used as an internal standard. HUVECs were treated with different concentrations of NOR (1, 3, 10 and 30 μmol/L) or curcumin (10 μmol/L) in the presence or absence of VEGF (10 ng/mL) for three hours. The cells were then washed twice with ice-cold PBS buffer (pH 7.4), and the proteins were extracted with lysis buffer (50 mmol/L Tris-HCL, 150 mmol/L NaCl, 0.02% NaN_3 and 1% NP_{40}) for 30 min on ice. The lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4°C. The concentration of protein in the supernatants was determined using the bicinchoninic acid protein assay. Equal amounts of lysate protein were separated by 8% SDS-polyacrylamide gel electrophoresis and then transferred onto poly(vinylidene difluoride) membranes. After being blocked with 10% non-fat dry milk for one hour at room temperature, the membranes were washed three times with PBS Tween-20 (PBST) buffer and incubated with monoclonal antibodies or phosphor-specific antibodies overnight at 4°C. Subsequently, they were washed three times with PBST buffer and incubated with peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies for one hour at room temperature. The bands were visualized using film exposure with enhanced chemiluminescence detection reagents.

GLIDE docking

The three-dimensional structure of the proteins used in the docking study was downloaded from the Protein Data Bank (PDB). The potential binding pocket was predicted using Discovery Studio 2.5 (Accelrys Inc., San Diego, CA, USA) and the Site Map module in Maestro 8.0 (Schrödinger LLC, New York, NY, USA). The PDB structure was prepared using the 'Protein Prepare Wizard' automatically, and subsequently its grid file was generated in Maestro 8.0.[23] Only the top-ranked pocket was used in the subsequent docking study.

For GLIDE docking, the PDB structure was prepared the same as mentioned above. The initial conformation of the compound used was obtained by a conformational search in water with a force field of OPLS_2005 based on the mixed torsional/low-mode sampling method in Maestro 8.0. The binding site was defined as a cubic (20 $Å^3$) with the center at points (−106.98, −48.436, −42.231), (19.747, 16.264, 15.771), (72.965, 11.9, 8.394) and (−1.107, 5.739, −1.31) for 3NBN, 3IAG, 1TTU and 1TK7, respectively. The XP mode (extra precision) was selected, and a post-docking minimization was conducted to penalize highly strained ligand geometries and eliminate poses with eclipsing interactions.

Immunohistochemistry staining

The tissues of knee joints from AA rats were sectioned, and endogenous peroxidase was blocked with 0.3% H_2O_2 for 10 min. The section was then treated with 10 mol/L urea and compositus protease for antigen repair and blocked with 1% BSA, followed by incubation with antibodies (1:100; Santa Cruz Biotechnology) or control (rabbit IgG) overnight at 4°C. The SABC Kit (Boster, Wuhan, China) was used according to the manufacturer's instructions.[24]

Statistical analysis

The data are expressed as mean ± SD of three independent experiments. Comparisons between multiple groups were performed using one-way analysis of variance and Dunnett's test. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of NOR on synovial angiogenesis in AA rats

Arthritis-related angiogenesis in synovium was evaluated by immunohistochemistry assay. Factor VIII, a marker for endothelial cells and blood vessels, was quantified using a specific antibody. As shown in Figures 2a and b, immune-reactive blood vessels increased in the hyperplasia knee synovium of AA rats. NOR (7.5, 15 and 30 mg/kg), orally administered for 10 days, dose-dependently reduced blood vessel formation in the synovium consistent with the results that NOR could diminish secondary footpad swelling and prevent joint destruction (data not shown). Notably, NOR (30 mg/kg) and dexamethasone (0.5 mg/kg) reduced blood vessel numbers to basal levels.

To confirm the above observations, the synovial expressions of VEGF and basic fibroblast growth factor (bFGF), two well-identified proangiogenic growth factors, were examined by immunohistochemical staining. Figures 2c–f showed a significant increase in the expressions of both VEGF and bFGF in synovial tissues in AA rats. Surprisingly, these expressions were nearly completely inhibited by administration of NOR (30 mg/kg) and dexamethasone (0.5 mg/kg) for 10 days. These findings demonstrated that NOR was able to prevent synovial angiogenesis in AA rats, which might serve as a key mechanism responsible for its antirheumatoid action.

Effect of NOR on VEGF-induced angiogenesis *in vitro*

Angiogenesis is a multistep process, including the migration, proliferation and tube formation of endothelial cells. The effect of NOR on angiogenesis was studied in HUVECs, and VEGF was used as an inducer. Curcumin, a natural product with good antiangiogenesis activity, was used as a positive control.[25,26]

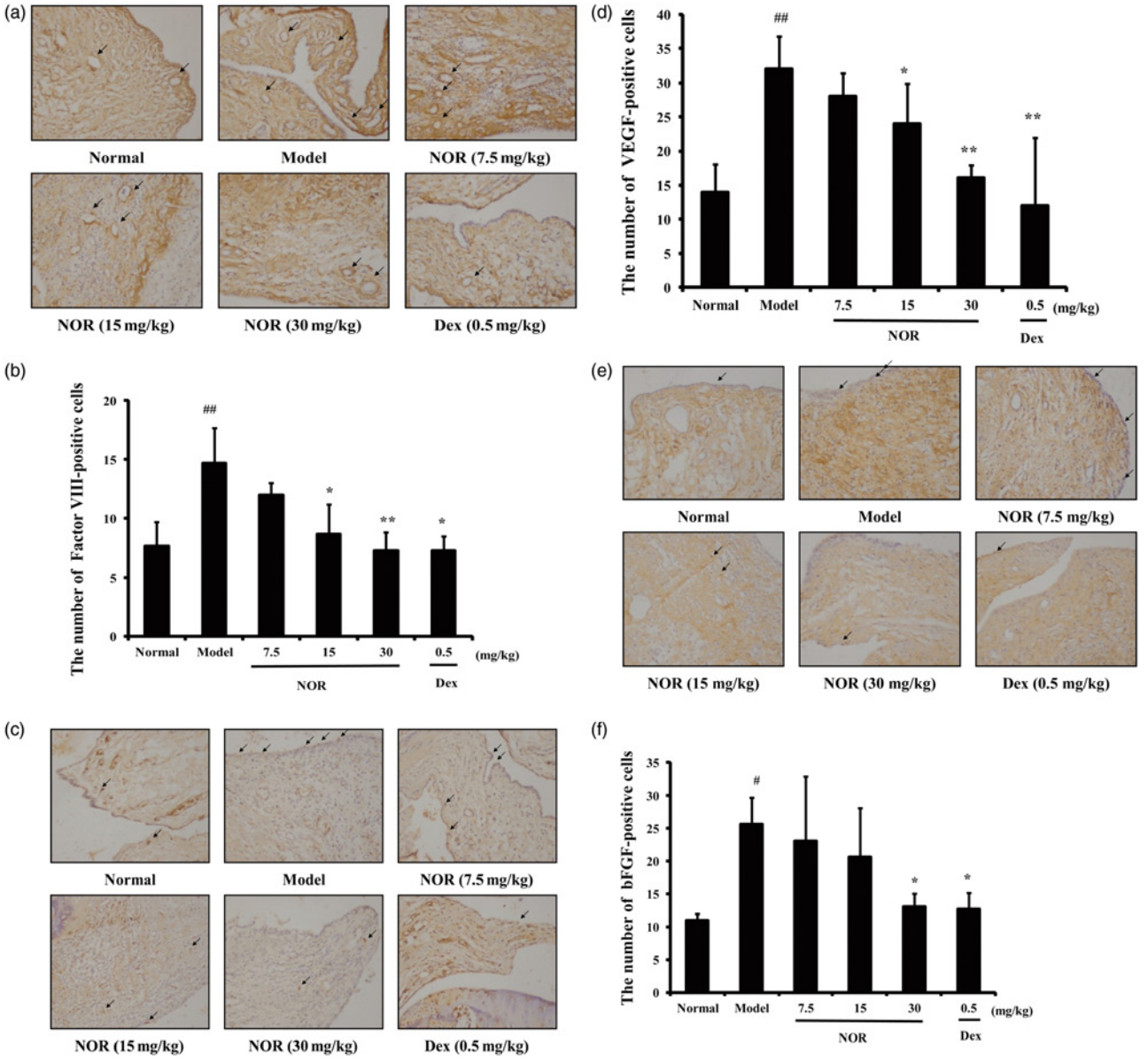

Figure 2 Effect of NOR on synovial angiogenesis in AA rats. (a) Effect of NOR on blood vessels in synovial tissues. Representative sections are synovial tissues stained with Factor VIII antibody. (b) Quantification of the number of new blood vessels that express Factor VIII in synovial tissues. (c) Effect of NOR on VEGF expression in synovial tissues. (d) Quantification of the positive cells that express VEGF in synovial tissues. (e) Effect of NOR on bFGF expression in synovial tissues. (f) Quantification of the positive cells that express bFGF in synovial tissues. The data are expressed as mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus model group (original magnification: ×200 in a, c, e). The arrows indicated blood vessels (a), VEGF-positive cells (c) and bFGF-positive cells (e), respectively. NOR, norisoboldine; Dex, dexamethasone; AA, adjuvant-induced arthritis; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor. (A color version of this figure is available in the online journal)

The proliferation of HUVECs was evaluated by immunofluorescence for EdU. As shown in Figures 3a and b, treatment with VEGF (10 ng/mL) for 24 h significantly increased the number of proliferating HUVECs. However, NOR (1, 3, 10 and 30 μmol/L) did not affect the proliferation of HUVECs. In contrast, curcumin (10 μmol/L) nearly abrogated the proliferation, with an inhibitory percentage of 73.9%.

The migration of HUVECs was assessed using a transwell chamber. Figures 3c and d showed that numerous HUVECs migrated to the underside of the filters after stimulation with VEGF for six hours. NOR (1, 3, 10 and 30 μmol/L) exhibited concentration-dependent inhibition of migration, with inhibitory percentages of 14.1%, 36.5%, 49.3% and 69.9%, respectively. Interestingly, NOR (10 and 30 μmol/L) and curcumin (10 μmol/L) completely inhibited the migration of HUVECs caused by VEGF. The tube formation of HUVECs and microvessel sprouting of rat aortic rings, two migration-related processes, were tested on the surface of matrigel. VEGF (10 ng/mL) treatment not only led to the formation of an extensive and enclosed network of tubes in HUVECs (Figures 3e and f), but also significantly enhanced endothelial sprouting from rat aortic ring segments (Figures 3g and h). NOR (1, 3, 10 and 30 μmol/L) concentration-dependently disrupted the tube formation and sprouting induced by VEGF. Notably, NOR (10 and

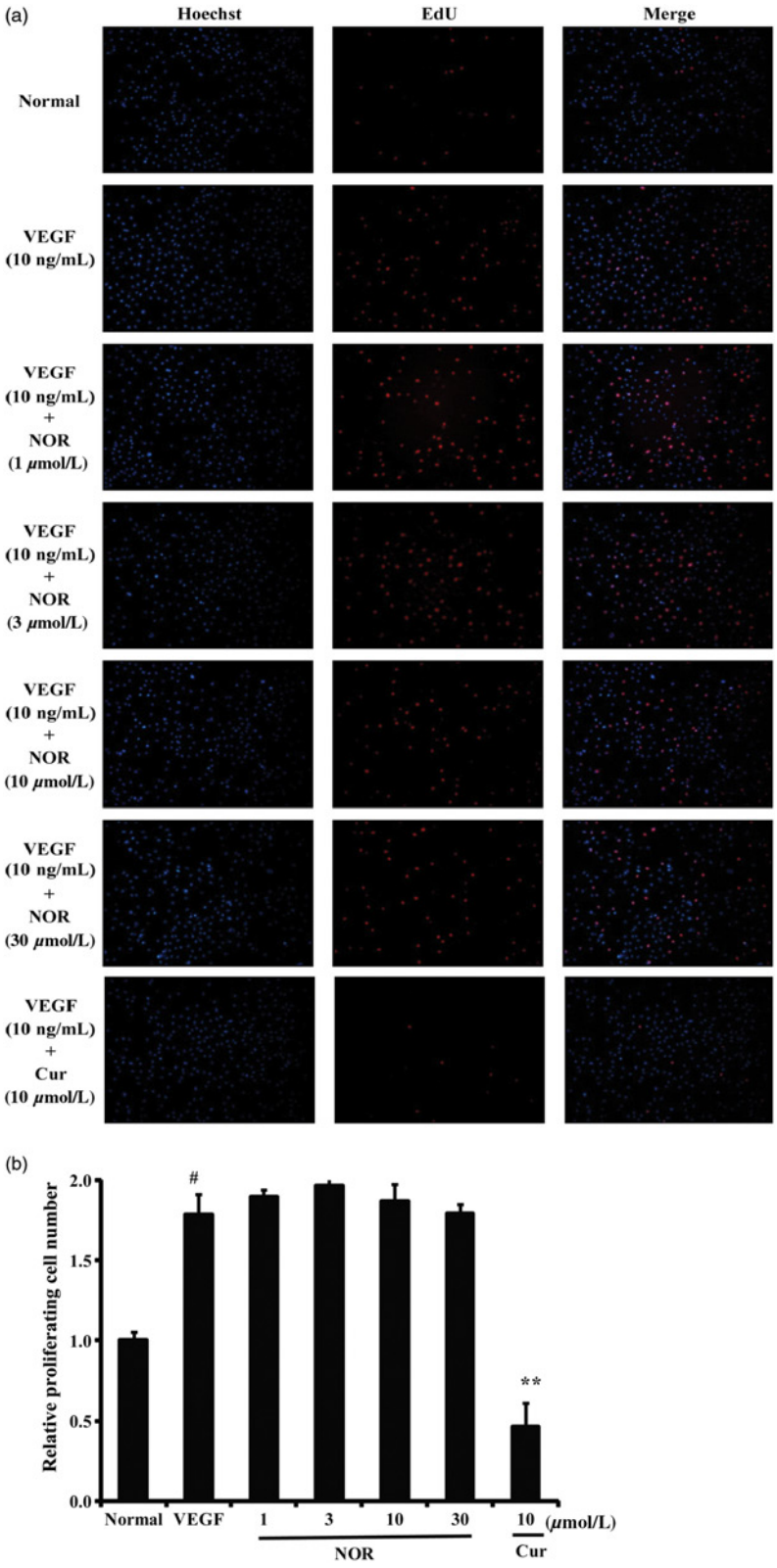

Figure 3 Effect of NOR on VEGF-induced *in vitro* angiogenesis. (a) Effect of NOR on VEGF-induced proliferation of HUVECs. Cells were cultured with NOR or curcumin in the presence or absence of VEGF for 24 h. (b) Quantification of HUVEC proliferation. (c) Effect of NOR on VEGF-induced migration of HUVECs. Cells were cultured with NOR or curcumin in the presence or absence of VEGF for six hours. (d) Quantification of HUVEC migration. (e) Effect of NOR on VEGF-induced tube formation of HUVECs. Cells were cultured with NOR or curcumin in the presence or absence of VEGF for 24 h. (f) Quantification of HUVEC tube formation. (g) Effect of NOR on VEGF-induced aortic ring sprouts. Rat aorta rings were exposed to NOR or curcumin in the presence or absence of VEGF for six days, and then the microvascular outgrowths were photographed. (h) Quantification of the vascular outgrowths. The data are expressed as mean $\pm$ SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus VEGF group (original magnification: $\times$100 in a, c, e, g). NOR, norisoboldine; Cur, curcumin; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell. (A color version of this figure is available in the online journal)

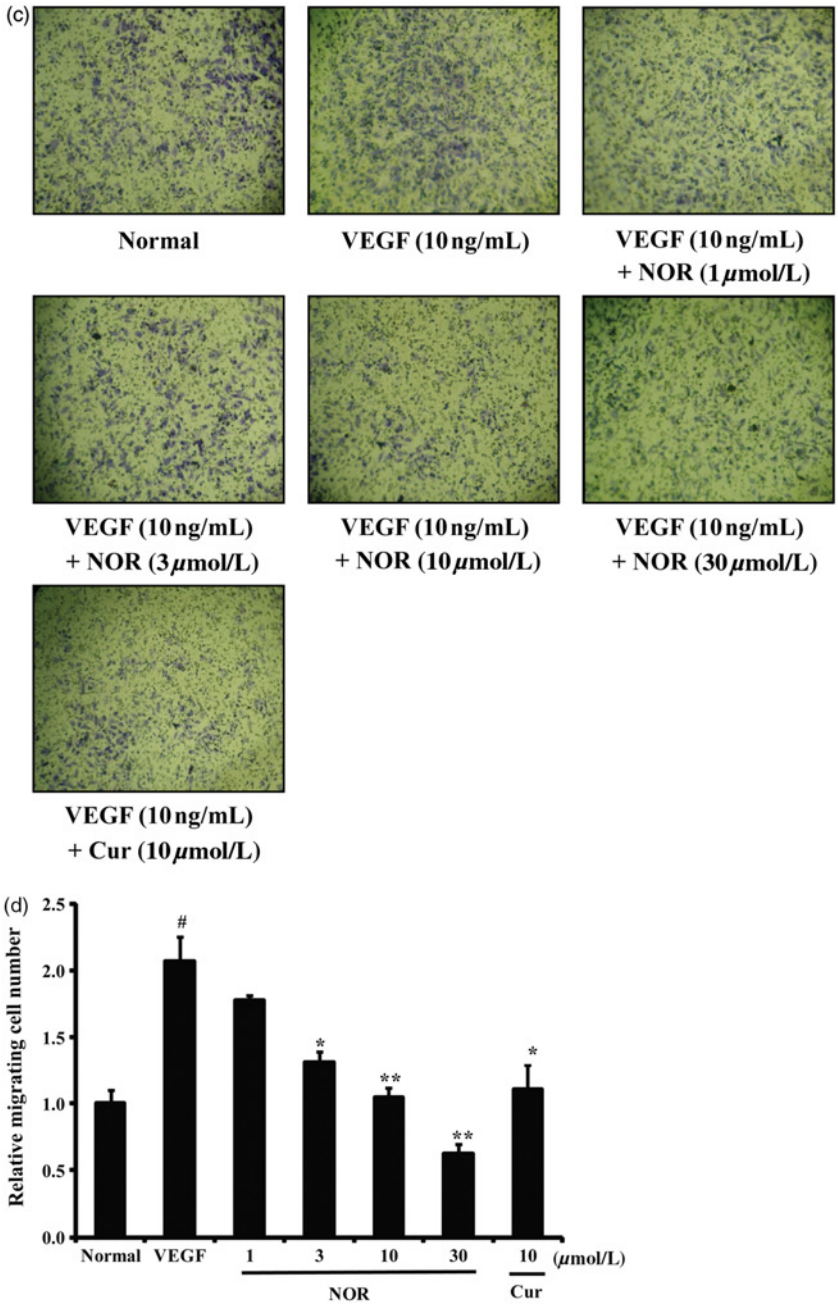

Figure 3 *(Continued)*

30 μmol/L) and curcumin (10 μmol/L) completely reversed the effect of VEGF on the tube formation and microvessel sprouting of endothelial cells.

The aforementioned results indicated that NOR possessed antiangiogenesis activity, which could be attributable to the prevention of endothelial migration or migration-related processes, such as tube formation and microvessel sprouting, but not endothelial cell proliferation.

Effect of NOR on VEGF-induced endothelial tip cell phenotype

The formation of new blood vessels depends on two endothelial cell populations with distinct functions, namely 'tip cells' and 'stalk cells.' The former, tip cells, develops a phenotype with migration and leads to sprout extension. In contrast, stalk cells favor proliferation and account for sprout elongation.[27] To determine if NOR can reduce endothelial cell migration by preventing the tip cell phenotype, we employed the fibrin bead assay and measured filopodia length and the gene expression of the tip cell markers Nrp-2, Nr-cam, MyoX and Cdc42.

The endothelial cells began to migrate away from the bead on day 3. These endothelial cells tended to the so-called 'tip cells' and began to form sprouts on day 6 (Figures 4a and b). In contrast to just a few tip cells in the normal group, the number of tip cells and consequent sprouting dramatically increased in the 10 ng/mL

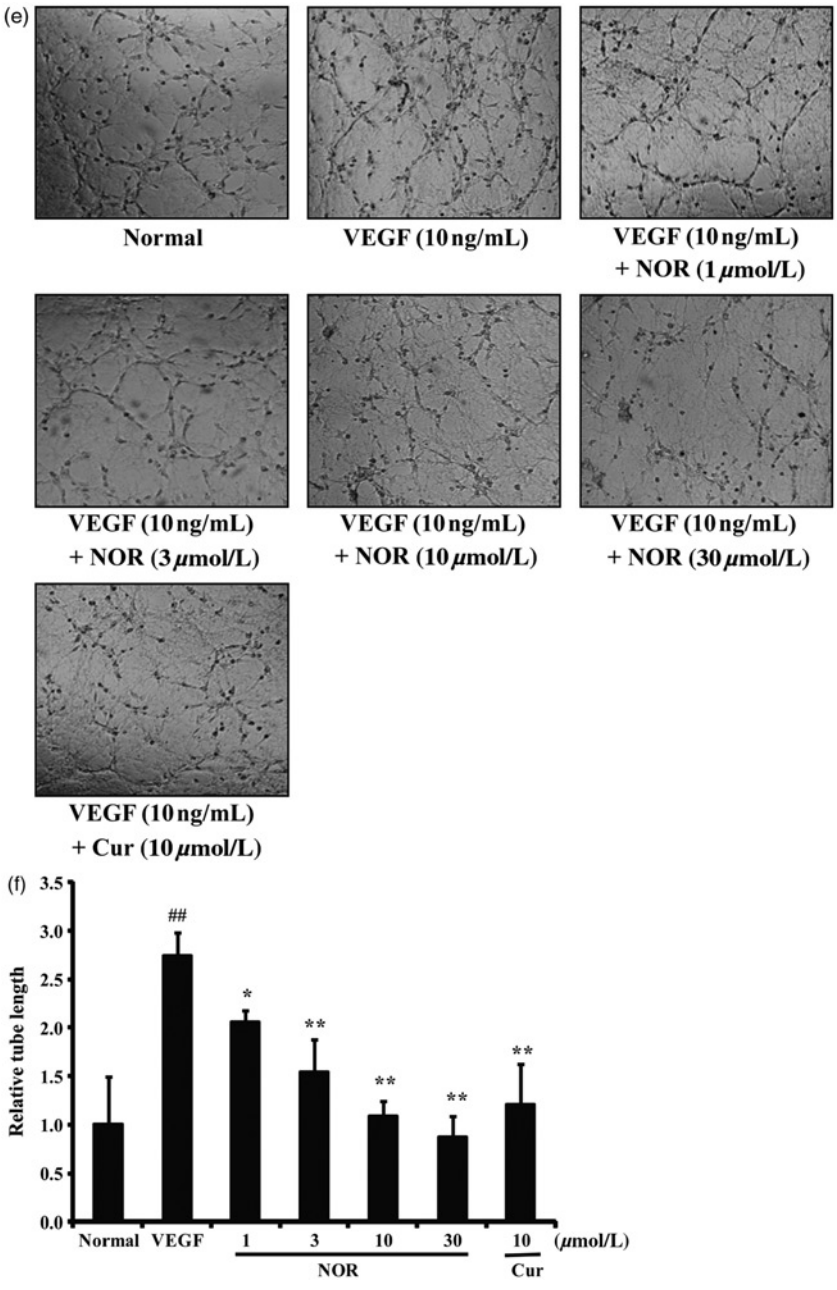

Figure 3 *(Continued)*

VEGF-treated group, which was consistent with the report from Nakatsu *et al.*[15] NOR (3, 10 and 30 μmol/L) concentration-dependently inhibited tip cells and sprouts, with inhibitory percentages of 17.7%, 42.2% and 59.5%, respectively. Compared with curcumin, NOR (10 μmol/L) induced a similar effect on the tip cell phenotype and sprouts. In addition, treatment with NOR (30 μmol/L) completely reversed the increase in tip cells and reduced the number of sprouts significantly. In a follow-up study on elongated filopodia that facilitated tip cells to migrate (Figures 4c and d), VEGF significantly elongated filopodia. However, the length of filopodia induced by VEGF was moderately shortened by NOR (10 μmol/L) and curcumin (10 μmol/L).

Expression of numerous genes, including Nrp-2, Nr-cam, MyoX and Cdc42, are enriched in tip cells, which are closely associated with cell migration and thus considered as typical markers for tip cell. To gain insights into the effect of NOR on tip cells, the mRNA expressions of these markers were assayed by reverse transcription polymerase chain reaction. Figures 4e and f showed that Nrp-2, Nr-cam, MyoX and Cdc42 gene expressions were moderately increased after VEGF stimulation. Treatment with various concentrations of NOR (1, 3, 10 and 30 μmol/L) significantly decreased the expressions of the four genes. Curcumin (10 μmol/L) also markedly inhibited VEGF-induced expressions of Nrp-2, Nr-cam, MyoX and Cdc42.

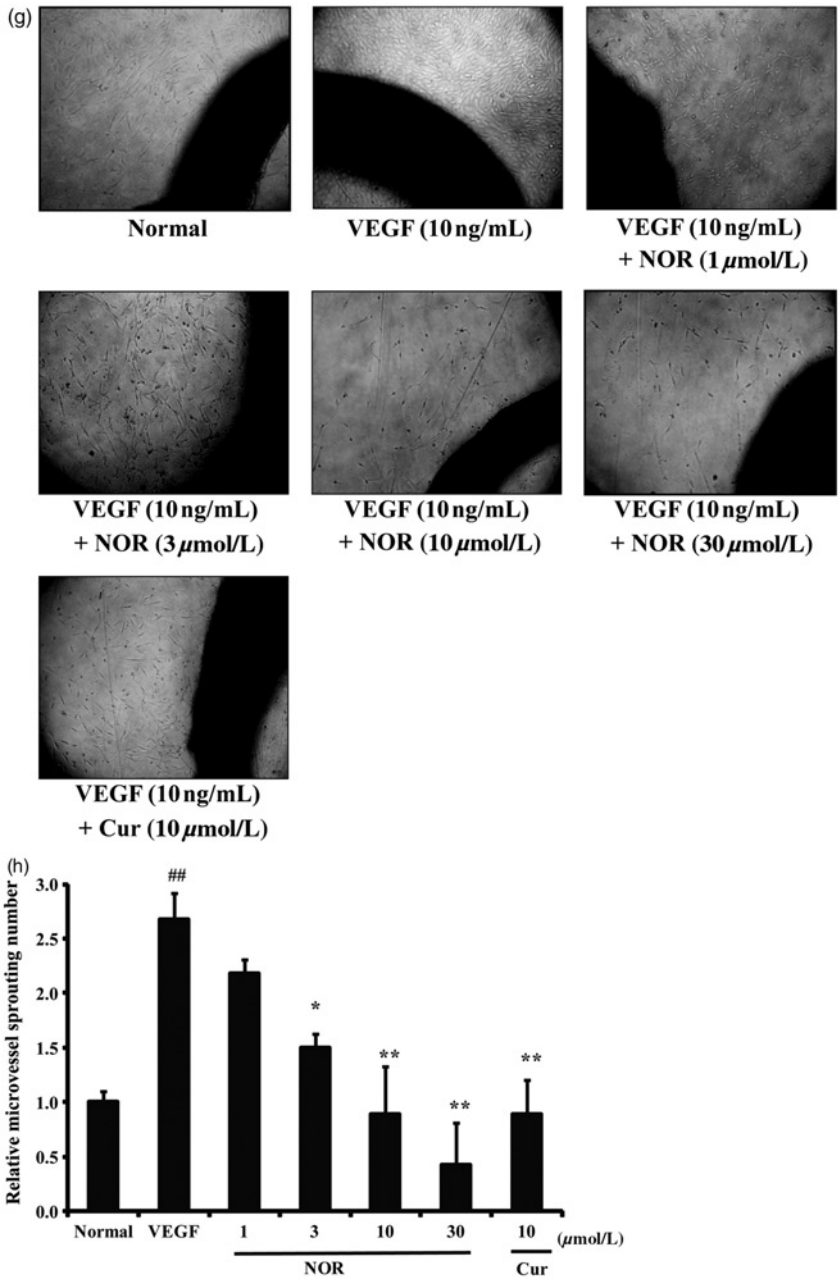

Figure 3 *(Continued)*

Altogether, the above data demonstrated that NOR prevented endothelial cell differentiation into the tip cell phenotype and reduced the length of filopodia in their morphology.

Effect of NOR on VEGF-induced activation of Notch1 pathway in HUVECs

The Notch1 pathway plays a critical role in maintaining the tip cell phenotype and moderating branching morphogenesis and angiogenesis.[27,28] Therefore, in the present study, we used DAPT, a γ-secretase inhibitor that blocks Notch1 function, to determine whether NOR regulates the tip cell phenotype via the Notch1 pathway. Although DAPT itself (25 μmol/L) induced an increase in the tip cell phenotype, pretreatment with DAPT partially reversed the inhibitory effects of NOR on tip cell number, filopodia elongation and the expression of related genes induced by VEGF in HUVECs (Figures 4a–f).

To further elucidate the effect of NOR on Notch1 activation, Notch1 and Cleaved Notch1 (Notch intracellular domain [NICD]) proteins were examined by Western blot analysis. As shown in Figures 5a and b, a stable level of Notch1 protein was observed in HUVECs after exposure to different treatments, but NOR barely affected it. Cleaved Notch1 protein levels were moderately increased by VEGF stimulation and significantly promoted by NOR but not curcumin. NOR at concentrations of 10 and 30 μmol/L increased Cleaved Notch1 expression 2.1-fold and 2.4-fold, respectively (Figures 5a and b). HEY1 and HEY2 are downstream target genes that represent activation

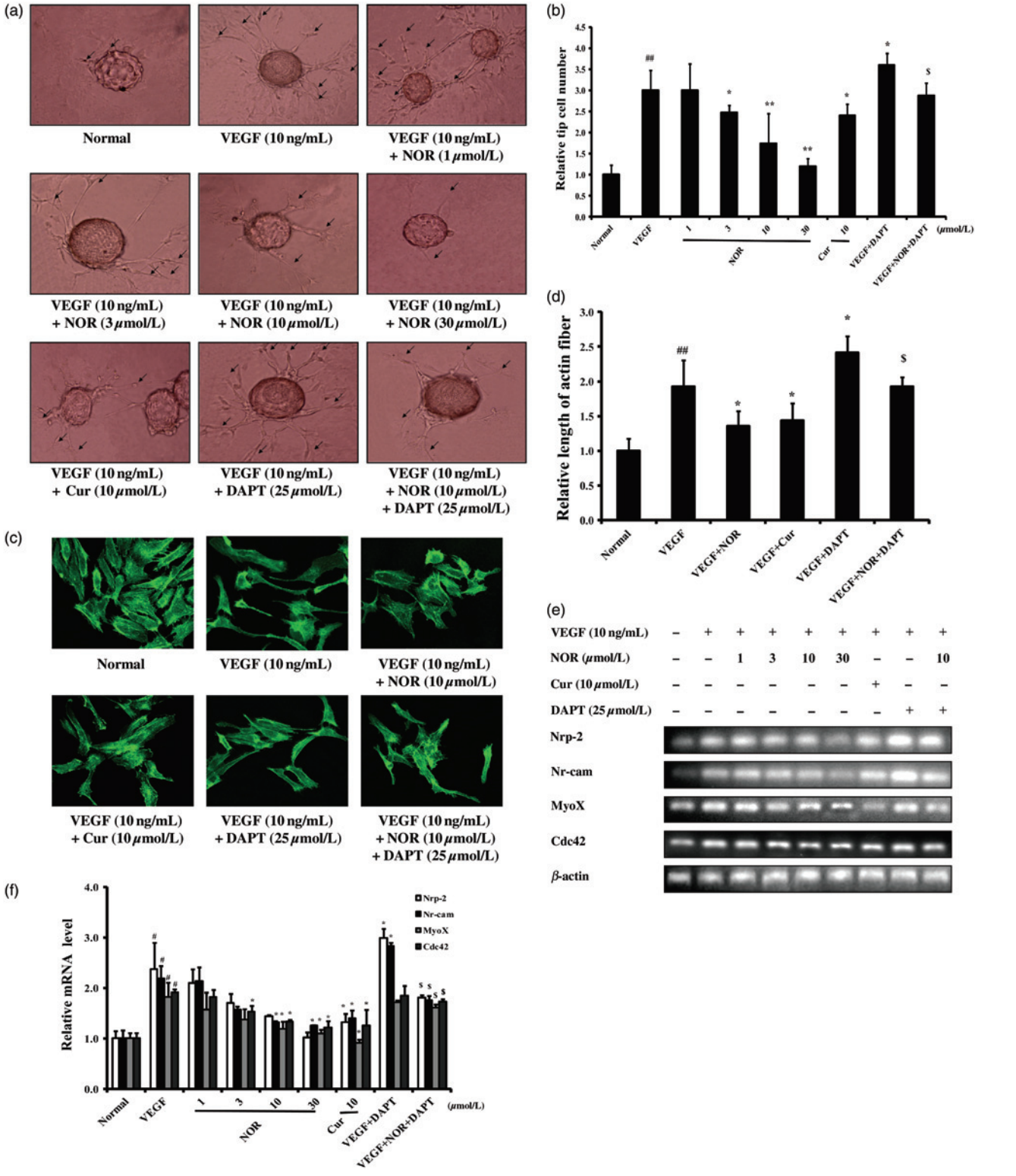

Figure 4 Effect of NOR on VEGF-induced endothelial tip cell phenotype. (a) Effect of NOR on VEGF-induced sprouting of endothelial-cell-coated beads in fibrin gel. Cells on beads were exposed to different treatments, and photographs were taken on day 14. (b) Quantification of migrating endothelial tip cells. (c) Effect of NOR on VEGF-induced filopodia elongation. (d) Quantification of filopodia elongation. (e) Effect of NOR on VEGF-induced Nrp-2, Nr-cam, MyoX and Cdc42 gene expressions. The cells were cultured with NOR or curcumin in the presence or absence of VEGF for three hours and then harvested for PCR analysis. (f) Quantification of Nrp-2, Nr-cam, MyoX and Cdc42 gene expressions. The data are expressed as mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus VEGF group; $^{\$}P < 0.05$, versus VEGF and NOR (10 μmol/L) group (original magnification: ×200 in a, ×400 in c). The arrows indicated tip cells in A. NOR, norisoboldine; Cur, curcumin; VEGF, vascular endothelial growth factor; PCR, polymerase chain reaction; DAPT, *N*-{*N*-[2-(3,5-difluorophenyl) acetyl]-(*S*)-alanyl}-(*S*)-phenylglycine tert-butyl ester. (A color version of this figure is available in the online journal)

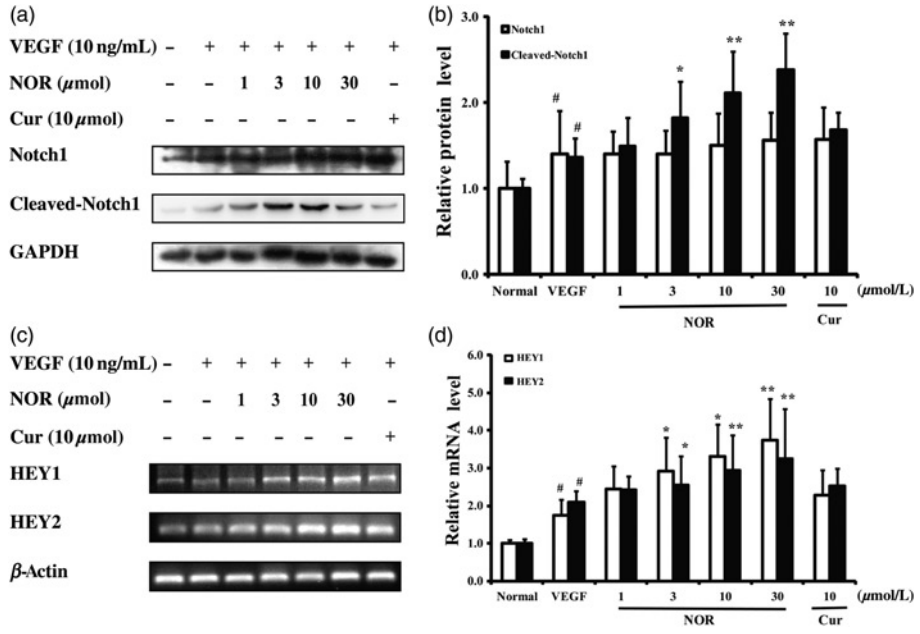

Figure 5 Effect of NOR on VEGF-induced activation of Notch1 pathway in HUVECs. Cells were cultured with NOR or curcumin in the presence or absence of VEGF for three hours and then harvested for Western blot or PCR analysis. (a) Effect of NOR on VEGF-induced Notch1 and Cleaved Notch1 protein expression. (b) Quantification of Notch1 and Cleaved Notch1 protein expression. (c) Effect of NOR on VEGF-induced HEY1 and HEY2 gene expression. (d) Quantification of HEY1 and HEY2 gene expression.The data are expressed as mean ± SD of three independent experiments. $^{\#}P < 0.05$, versus normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus VEGF group. NOR, norisoboldine; Cur, curcumin; VEGF, vascular endothelial growth factor; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

of the Notch signaling pathway.[14] Figures 5c and d show that NOR (1, 3, 10 and 30 μmol/L) promoted the expression of the Notch1 target genes HEY1 and HEY2 in a concentration-dependent manner. The fact that curcumin (10 μmol/L) treatment had little effect on both the Cleaved Notch1 protein and Notch1 target genes suggested that the Notch1 pathway might not be implicated in the mechanisms responsible for the effect of curcumin on the tip cell phenotype. These findings indicated that Notch1 played a very important role in NOR-mediated inhibition of the tip cell phenotype induced by VEGF in HUVECs.

Potential target of NOR on Notch1 pathway

To further understand how NOR affects the Notch1 pathway, a preliminary *in silico* study was performed to clarify the potential targets. The three-dimensional structures of Notch1 and its homologs are not available at present. It is difficult to study the interaction between Notch1 and NOR either by crystal structure-based or homology modeling-based simulation. In this situation, downstream proteins of the Notch1 receptor are thus of interest. A systematic search in the PDB for proteins related to Notch1 and subsequent potential binding pocket prediction on them was conducted. Four proteins were selected for further docking studies because of the existence of potential binding pockets on them. These proteins included suppressor of deltex (PDB ID: 1TK7), Notch1 transcription complex trimers on HES1 DNA (PDB ID: 3NBN) and two nuclear effectors of Notch signaling (CSL, CBF-1/RBP Jκ/Suppressor of Hairless/LAG-1) bound to different target DNA (PDB ID: 1TTU and 3IAG). The extra precision docking study of NOR was made with the four proteins in GLIDE 4.5, and its resultant GLIDE score is shown in Table 1. The GLIDE score for 3NBN was −6.139 (the highest value) and the score for 3IAG was somewhat lower (−5.580). The scores for 1TTU and 1TK7 were obviously low. This fact suggested that 3NBN should deserve more discussion. Figures 6a and b show that NOR oriented to a shallow pocket. The oxygen atoms of 9-hydroxyl and 10-methoxyl were involved in hydrogen bonding with the NH of Lys145, and 1-hydroxyl formed another hydrogen bond with the carbonyl of Glu323. In addition, the hydrophobic and van der Waals interactions between NOR and the surrounding residues strengthened the binding (Figure 6c).

This finding suggested that NOR might exert effects on the Notch1 transcription complex and thus affect the function of the Notch1 pathway.

Discussion

Angiogenesis is the development of new vessels from pre-existing vessels. In the synovium of RA, there are various

Table 1 GLIDE score for norisoboldine and four proteins

	Score	evdw	ecoul	Hbond
3NBN	−6.139	−29.164	−9.776	−2.438
3IAG	−5.580	−28.549	−5.542	−1.302
1TTU	−3.509	−22.896	−4.384	−1.334
1TK7	−3.382	−23.127	−0.152	0

evdw, van der Waals energy; ecoul, coulomb energy; Hbond, hydrogen-bonding term

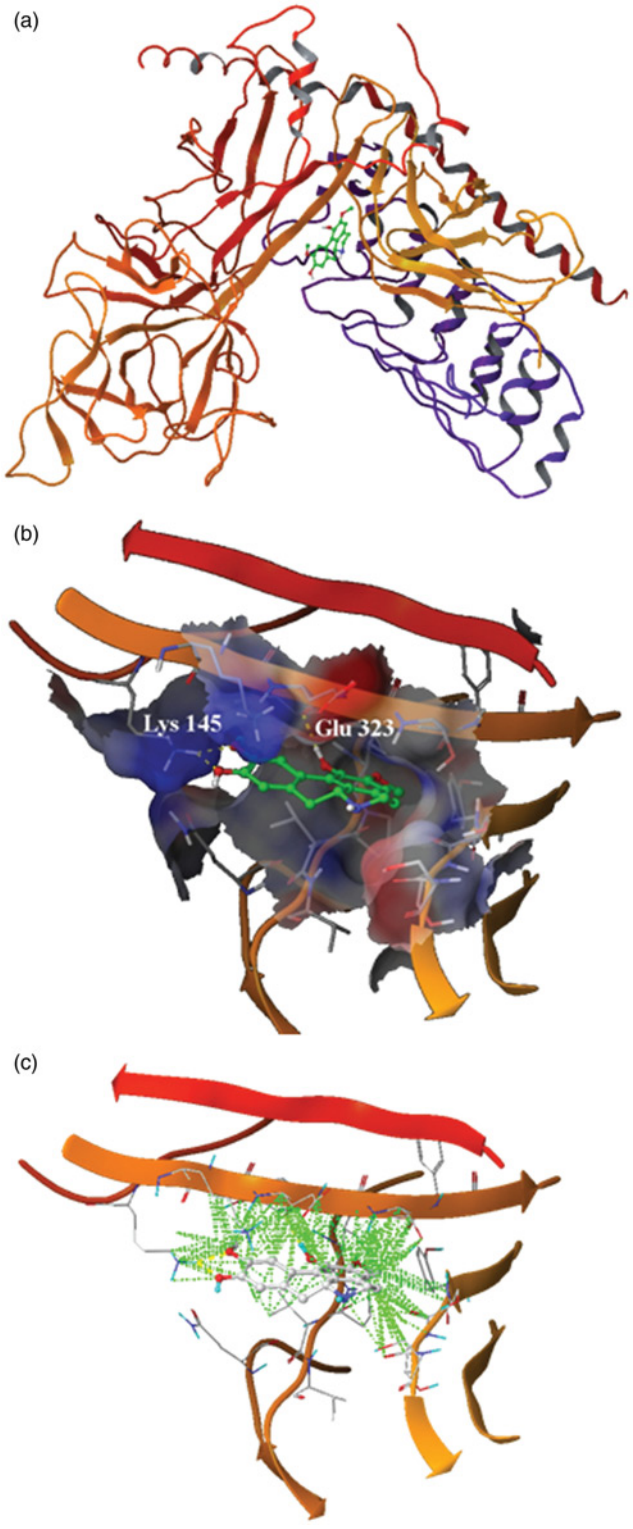

Figure 6 Potential target of NOR on Notch1 pathway. (a) Overview of the docking mode for NOR to 3NBN. (b) Interaction mode for NOR in 3NBN. (c) Hydrophobic and van der Waals interactions between NOR and surrounding residues. The hydrogen bond and contact between ligand and residues are depicted as dotted lines. NOR, norisoboldine. (A color version of this figure is available in the online journal)

types of cells, such as lymphocytes, fibroblast-like synoviocytes and macrophages, which can release angiogenic factors to activate synovial endothelial cells. The activated endothelial cells should produce proteolytic enzymes to degrade the underlying endothelial basement membrane and synovial extracellular matrix, which allows endothelial cells emigrating into interstitial tissue and forming the primary capillary sprouts. Subsequently, the new basement membrane is formed with these new residential endothelial cells, and new capillaries are generated.[2,29] Thus, the migration and proliferation of endothelial cells is the most fundamental and important process in angiogenesis.

NOR was previously shown to be able to ease arthritis and reduce joint injury in CIA mice. In this study, NOR was found not only to reduce the number of blood vessels and the expression of growth factors in the synovium of AA rats, but also to inhibit VEGF-induced *in vitro* angiogenesis in HUVECs. Notably, NOR (10 and 30 μmol/L) almost completely reversed VEGF-induced migration, tube formation and sprouting of endothelial cells; however there is little effect on the proliferation of endothelial cells. These findings indicated that the action of NOR was distinct from that of curcumin (a positive control) and it might function through selectively preventing the migration or migration-related processes of endothelial cells.

Prior to migration, partial endothelial cells at the forefront of vessel branches can be driven to the tip cell phenotype after exposure to angiogenic stimuli, such as hypoxia-induced growth factors. 'Tip cell' comprise a highly polarized cell population with the ability of migration and guiding a sprouting vessel by filopodia. The subsequent elongation of the new branch relies on the proliferation of endothelial 'stalk cells,' which trail behind the pioneering tip cells. Once these new branches connect with other branches via tip-cell fusion to form a lumen, the nascent plexus becomes a mature functional network.[27,30] Therefore, it is a crucial step for an endothelial cell to develop into the tip cell phenotype that initiates migration. With the transformation into tip cells, some genes (e.g. Nrp-2, Nr-cam, MyoX and Cdc42) in endothelial cells are highly expressed, and are generally considered as tip cell markers.[14,31–34]

In this study, we explored the possibility that the inhibitory effect of NOR on the migration of endothelial cells was mediated by preventing the endothelial tip cell phenotype. A series of experiments, including the fibrin bead assay, filopodia length assay and tip cell marker assay, were conducted to evaluate the tip cell phenotype. It was shown that NOR was indeed able to reduce the number of tip cells, length of filopodia and expressions of Nrp-2, Nr-cam, MyoX and Cdc42 genes, confirming that prevention of endothelial tip cell phenotype substantially contributed to the selective inhibition of NOR on endothelial cell migration induced by VEGF.

Multiple signal pathways participate in the process of VEGF-induced angiogenesis. Among them, the Notch pathway, a conserved ligand–receptor signaling pathway that modulates cell fate and differentiation, plays a critical role in determining whether endothelial cells transfer into a tip cell phenotype.[14,28,35] Notch1–4 proteins are single-pass transmembrane proteins that consist of a signal peptide, an extracellular domain, a transmembrane domain and an intracellular signaling domain. The activation of the Notch pathway initiates when the ligand binds to the Notch

receptor for site-specific cleavage, and then its intracellular domain can be released. This cleaved Notch protein or NICD translocates into the nucleus and interacts with the members of the DNA-binding transcription factor CSL (CBF-1/RBP Jκ/Suppressor of Hairless/LAG-1) to drive transcription.[27,35] The Notch1 pathway has received increasing attention by researchers in recent years. However, reports concerning the impact of Notch1 pathway activation on the tip cell phenotype and subsequent angiogenesis are still controversial.[27,36–39] For example, Patel *et al.*[36] found that downregulation of the Notch1 pathway could lead to the inhibition of proliferation, migration and network formation of HUVECs. Williams *et al.*[37] found that upregulation of the Notch1 ligand Delta-like 4 could inhibit endothelial cell function induced by VEGF. Chen *et al.*[38] found that inhibition of the Notch1 signal transduction pathway could stimulate the proliferation of HUVECs and facilitate angiogenesis. Dong *et al.*[39] reported that upregulation of the Notch1 pathway in RF/6A cells significantly increased proliferation and tube formation but decreased cell migration. Therefore, the influences of the Notch1 pathway on the angiogenesis process are closely related to the species, microenvironment and specific cell type. In addition, Cleaved Notch1 and Notch1 target genes, such as HEY1 and HEY2, locate at the downstream of Notch1. Their expressions strongly depend on the activation or inactivation of Notch1, and can be used to indicate the degree of Notch1 activation.[14,40,41]

To determine if NOR-mediated inhibition of the tip cell phenotype of endothelial cells depends on the Notch1 pathway, the γ-secretase inhibitor DAPT was used to selectively block Notch1 function. It was found that DAPT itself could significantly increase the number of tip cells, the filopodia length and the gene expressions of some tip cell markers. Pretreatment of DAPT largely attenuated the inhibitory effect of NOR on HUVECs. These results indicated that the inhibition of NOR on the tip cell phenotype was deeply associated with the Notch1 pathway. To further clarify the impact of NOR on the Notch1 pathway, we examined the expressions of Notch1, Cleaved Notch1 and the Notch1 target genes HEY1 and HEY2. NOR was found to increase the expressions of Cleaved Notch1, HEY1 and HEY2 in a concentration-dependent manner, suggesting the activation of the Notch1 pathway by NOR contributing to the prevention of angiogenesis. Furthermore, given that multiple factors are probably involved in the Notch1 pathway, an insight into the potential target sites of NOR should be valuable. Due to the lack of information about the three-dimensional structure of Notch1 and its homologs, we were not able to design the experiment based on the crystal structure or homology modeling. We selected four proteins that exist downstream of the Notch1 receptor from the PDB for the *in silico* docking study, and found that the potential target of NOR was the Notch1 transcription complex.[42–46]

In summary, the present study demonstrated that NOR could significantly reduce synovial angiogenesis in AA rats, which might be achieved by selectively inhibiting endothelial cell migration. The anti-migration action of NOR could be attributed, at least partially, to the prevention of the Notch1 pathway-related tip cell phenotype of endothelial cells with a potential action target of Notch1 transcription complex. The findings reinforce our understandings about the antirheumatoid mechanisms of NOR.

Author contributions: All the authors participated in the design and interpretation of the experiments. QL and YD conceived and designed the experiments. QL performed the experiments, analyzed the data and wrote the paper. YX, GC and ZW provided the compound. BT and ZW collected the animal samples. SL and TL performed molecular docking studies. YL and XG had a scientific discussion for this study. All the authors read and approved the final manuscript.

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