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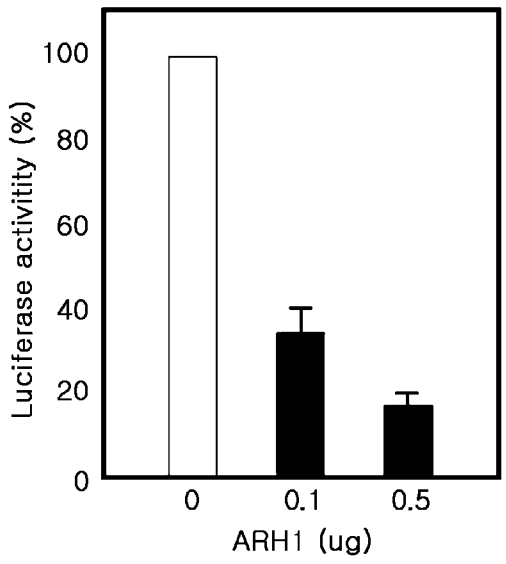
*[Continued on next page]*

(54) Title: NF-κB INHIBITOR CONTAINING ARH1 PROTEIN OR GENE ENCODING THE SAME

[Fig. 1]




(57) Abstract: The present invention relates to novel uses of ARH1 protein or a gene encoding the same, more particularly, to an NF-κB inhibitor comprising the ARH1 protein or the gene encoding the same as an active ingredient and a composition for the treatment of angiogenesis-related diseases, inflammatory diseases, autoimmune diseases, or viral diseases, comprising the inhibitor.

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# Description

# Title of Invention: NF-κB INHIBITOR CONTAINING ARH1 PROTEIN OR GENE ENCODING THE SAME

## Technical Field

[1] The present invention relates to novel uses of ARH1 protein or a gene encoding the same, and more particularly, to an NF-κB inhibitor comprising the ARH1 protein or the gene encoding the same as an active ingredient, and a composition for the treatment of angiogenesis-related diseases, inflammatory diseases, autoimmune diseases, or viral diseases, comprising the inhibitor.

[2]

## Background Art

[3] NF-κB (nuclear factor kappa B) has been one of the most highly studied transcription factors since it was first discovered in 1986 as a transcription factor in mature B cells which constitutively binds to the immunoglobulin κ light chain (Baeuerle and Baltimore, *Cell*, 87: 13-20, 1996).

[4] NF-κB is an important transcription factor that regulates genes involved in the inhibition of apoptosis and in the activation of immune and inflammatory responses (Beg and Baltimore, *Science*, 274: 782-784, 1996), and consists of five subunits; p52/p100(NF-κB2), c-Rel, RelB, and p65(RelA). NF-κB forms either homodimers or heterodimers of p50 subunit (p50, p52) and p65 subunit (p65, c-Rel, RelB) in cells, and the p50/p65 (p50/relA) heterodimer of NF-κB is the most abundant, active form. NF-κB is maintained in an inactive state in the cytoplasm bound to its inhibitors, IκB proteins (IκBα, IκBβ, IκBγ, IκBε, and Bcl3). However, the IκB proteins are phosphorylated and subsequently degraded in response to various external signals such as cytokines (TNF-α, IL-1, etc.), bacterial/viral infection (LPS, dsRNA, etc.), and stress (ROI, UV, adriamycin, radiation, etc.), thereby releasing NF-κB. The free NF-κB translocates to the nucleus, where it binds to the specific NF-κB binding sites of target genes and activates their expression (Rothwarf et al., *Nature*, 395:297-300, 1998; Yamaoka et al., *Cell*, 93:1231-1240, 1998).

[5]

[6] NF-κB is a crucial transcription factor involved in several physiological processes including embryogenesis and immune responses, but aberrant NF-κB activation has been implicated in the development of many diseases. Since regulation of aberrant NF-κB activation is able to inhibit development or progression of degenerative/intractable diseases, it has attracted much attention as a target for drug development.

[7]

[8] By engaging TNFR1, Tumor Necrosis Factor (TNF) activates the transcription factors NF-κB and AP-1, leading to induction of proinflammatory and immunomodulatory genes. TNF binds to TNFR1 in a trimeric state inducing receptor oligomerization. TNFR1 recruits the cytosolic protein TRADD (TNFR-associated death domain) through its death domain in the cytoplasmic side. TRADD functions as a mediator that recruits various signal transducers into the activated TNFR1. That is, TRADD recruits TRAF2, RIP, or FADD into the TNFR1. TRAF2 (TNF-associated factor-2) and RIP (receptor-interacting protein) stimulate pathways leading to activation of NF-κB and JNK/AP-1, whereas FADD mediates activation of apoptosis. TRAF2 activates NIK (NF-κB-inducing kinase), in turn, NIK activates IKK (I-κB kinase complex), leading to phosphorylation and degradation of I-κB (inhibitor of κB). Subsequently, the liberated NF-κB translocates to the nucleus, where it activates the transcription of target genes (Hsu, H. et al., *Cell*, 81, 495-504, 1995; Van Antwerp, DJ. et al., *Science*, 274, 787-789, 1996; Hsu, H. et al., *Cell*, 84, 299-308, 1996b; Locksley, RM. et al., *Cell*, 104, 487-501, 2001). This suggests that intracellular protein interactions are actively engaged in cell apoptosis, inhibition of protein degradation, and nuclear translocation of proteins. Accordingly, it is expected that the NF-κB activation can be regulated by affecting interactions between specific proteins in the TNF signaling pathway.

[9]

[10] On the other hand, ARH1, a gene containing a GTP-binding domain of about 26 kDa, is known to be present in eukaryotic cells derived from diverse species. It is also reported that its expression is low in breast and ovarian cancer, and it is able to induce the inhibition of cell growth (Yu, Y. et al., *Proc. Natl. Acad. Sci. USA*, 96, 214-219, 1999; Luo, RZ. et al., *Oncogene*, 22, 2897-2909, 2003).

[11]

## Disclosure of Invention

## Technical Problem

[12] The present inventors have explored genes capable of inhibiting NF-κB activity by regulating interactions between specific proteins in the TNF signaling pathway. They found that ARH1 protein interacts with an essential component of TNF signaling, TRADD, and thus competitively blocks the TRADD-TRAF2 interaction, so as to inhibit nuclear translocation and activation of NF-κB, thereby completing the present invention.

[13]

## Solution to Problem

[14] It is an object of the present invention to provide an NF-κB inhibitor, comprising an

ARH1 protein or gene encoding the same as an active ingredient.

[15] It is another object of the present invention to provide a composition for the treatment of diseases associated with aberrant NF-κB activation, comprising the NF-κB inhibitor.

[16] It is still another object of the present invention to provide a method for inhibiting NF-κB activity in cells using the ARH1 protein or gene encoding the same.

[17] It is still another object of the present invention to provide a method for treating diseases associated with aberrant NF-κB activation using the NF-κB inhibitor.

## Advantageous Effects of Invention

[18] The ARH1 protein according to the present invention interacts with the TRADD protein to inhibit the nuclear translocation of NF-κB, thereby inhibiting the NF-κB activation in cells. Thus, the ARH1 protein acts as an NF-κB inhibitor. Substantially, the NF-κB inhibitor of the present invention showed inhibitory effects on angiogenesis, which is a disease associated with aberrant NF-κB activation, at the cell and tissue level.

[19]

## Brief Description of Drawings

[20] FIG. 1 is a graph showing that ARH1 expression in cells decreases the activity of NF-κB-reporter gene in a concentration-dependent manner;

[21] FIG. 2 is a graph showing that ARH1 expression in cells inhibits the NF-κB-mediated transcriptional activity in a concentration-dependent manner;

[22] FIG. 3 shows the accumulation of p65 subunit of NF-κB in the cytoplasm due to the inhibitory effect of ARH1 protein on the nuclear translocation of NF-κB;

[23] FIG. 4 shows a photograph (left) and a graph (right) showing the results of yeast two-hybrid analysis, which was performed to examine whether the TRADD obtained by screening an ovarian cDNA library directly interacts with the ARH1 protein in cell;

[24] FIG. 5 is a photograph showing the results of Western blot analysis, in which genes were constructed using different expression vector systems, and their interaction in vitro was examined using ARH1 and TRADD antibodies;

[25] FIG. 6 is a photograph showing the comparative in vitro competitive binding abilities of proteins obtained using different expression vector systems, and purified;

[26] FIG. 7 is a photograph showing the localization of nucleus (DAPI), ARH1 and TRADD in human cell line, which was visualized by fluorescence microscopy;

[27] FIG. 8 is a photograph showing the ARH1 mRNA expression level and the morphology of ARH1-expressing cell under a fluorescence microscope (ARH1 expression interfered by siARH1), in which RT-PCR was performed to examine the mRNA level according to RNA interference, and the ARH1-expressing cell was vi-

sualized using a GFP-expressing vector;

[28] FIG. 9 is a graph showing the inhibitory effects of ARH1 on HUVEC cell proliferation (recovered upon siARH1 treatment);

[29] FIG. 10 is a photograph (left) and a graph (right) showing the inhibitory effects of ARH1 on HUVEC cell migration (recovered upon siARH1 treatment);

[30] FIG. 11 is a photograph (left) and a graph (right) showing the inhibitory effects of ARH1 on HUVEC cell invasion (recovered upon siARH1 treatment);

[31] FIG. 12 is a photograph showing the inhibitory effects of ARH1 protein on the vascular endothelial growth factor;

[32] FIG. 13 is a photograph (left) and a graph (right) showing the inhibitory effects of ARH1 protein on tube formation of HUVEC cell (recovered upon siARH1 treatment);

[33] FIG. 14 is a photograph (upper) and a graph (lower) showing the ex vivo inhibitory effects of ARH1 protein on blood vessel formation in mouse muscle (recovered upon siARH1 treatment); and

[34] FIG. 15 is a photograph and a graph showing the in vivo inhibitory effects of ARH1 protein on blood vessel formation in human ovarian cancer cell.

[35]

## Best Mode for Carrying out the Invention

[36] In accordance with one aspect, the present invention relates to an NF-κB inhibitor, comprising an ARH1 protein or gene encoding the same as an active ingredient.

[37] As used herein, the term "NF-κB inhibitor" refers to agents capable of reducing the expression or activity of NF-κB in cells, in particular, to agents that directly act on NF-κB or indirectly act on upstream modulators of the NF-κB pathway to reduce NF-κB expression at the transcription level, to increase degradation of expressed NF-κB, or to impair its activity, thereby reducing the expression level or activity of NF-κB. When NF-κB is activated in cells, it translocates to the nucleus, where it binds to target sites, leading to the enhanced expression of other genes. In the present invention, the "NF-κB activation" means substantial expression of the other genes, and the "inhibition of NF-κB activation" means that the translocation of NF-κB into the nucleus is inhibited to suppress the expression of the other genes.

[38]

[39] ARH1, a gene containing a GTP-binding domain of about 26 kDa, is known to be present in eukaryotic cells derived from diverse species. In the present invention, the ARH1 protein or gene encoding the same encompasses ARH1 proteins derived from diverse mammalian species including human, genes encoding the same, or functionally equivalent variants thereof, as long as they inhibit NF-κB activity. As used herein, the term "functionally equivalent variants" means that they function to inhibit NF-κB

activity, including even variants having mutations as compared to the wild-type protein derived from specific species or base sequence thereof.

[40] The ARH1 protein or gene encoding the same that can be used in the present invention may be derived from animals including human, for example, monkeys, pigs, horses, cows, sheep, dogs, cats, mice, rabbits, etc., preferably ARH1 derived from human, and more preferably the ARH1 protein represented by SEQ ID NO: 1 (NIH GenBank accession number AAG35625) or a gene encoding the ARH1 protein represented by SEQ ID NO: 2 (NIH GenBank accession number AF202543), but is not limited thereto.

[41]

[42] The ARH1 protein of the present invention includes a protein having the natural amino acid sequence as well as amino acid sequence variants thereof. The term "ARH1 protein variant" is intended to refer to ARH1 proteins, which are different in amino acid sequence from the wild- type due to the deletion, insertion, non-conservative or conservative substitution of one or more amino acid residues, or combinations thereof. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the art (H.Neurath, R.L.Hill, The Proteins, Academic Press, New York, 1979). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly, in both directions. The variant, if desired, may be modified by phosphorylation, sulfation, acetylation, glycosylation, methylation, farnesylation or the like.

[43] The ARH1 protein or variants thereof may be obtained by isolation from natural sources, artificial synthesis (Merrifleld, *J. Amer. chem. Soc.* 85:2149-2156, 1963) or by recombinant means on the basis of the DNA sequences (Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory Press, New York, USA, second edition, 1989).

[44]

[45] Further, in the present invention, the ARH1 protein-encoding gene means a nucleic acid molecule encoding ARH1 protein, which may be isolated from nature or artificially synthesized.

[46] The nucleic acid molecule that can be used as a gene encoding the ARH1 protein of the present invention includes functionally equivalent nucleic acids, which are modified by deletion, substitution or insertion of bases in the ARH1 nucleic acid molecule, but function in substantially the same manner as the ARH1 nucleic acid.

[47] Further, the nucleic acid molecule encoding the ARH1 protein of the present invention may include genomic DNAs, cDNAs, and chemically synthesized DNAs. The preparation of the genomic DNAs and cDNAs can be conducted by using methods

commonly known to one skilled in the art.

[48] A genomic DNA can be prepared, for example, by extracting genomic DNAs from a cell comprising the ARH1 gene, constructing and developing a genomic library (plasmids, phages, cosmids, BAC, PAC or the like can be used as vectors), and then obtaining the genomic DNA by colony or plaque hybridization using a probe prepared based on a DNA (for example, SEQ ID NO: 2) that encodes the protein of the present invention. Alternatively, the genomic DNA can also be prepared by constructing a primer specific to a DNA (for example, SEQ ID NO: 2) that encodes the protein of the present invention, and carrying out PCR (Polymerase Chain Reaction) using this primer.

[49] A cDNA can be prepared, for example, by synthesizing cDNAs from mRNA extracts of a cell that comprises the ARH1 gene, constructing and expanding a cDNA library by inserting the cDNAs into vectors such as λZAP, and then using colony or plaque hybridization as above, or PCR procedures, to obtain the cDNA.

[50] It is preferable that DNAs thus isolated have a high homology at the amino acid level to the amino acid sequence of the ARH1 protein (SEQ ID NO: 1). Here, high homology means that at least 50% of the entire amino acid sequence is identical, preferably 70% or more, and more preferably 90% or more. The sequence identity of amino acids or nucleotides can be analyzed by programs including BLASTN or BLASTX (Altschul, SF. et al., *J. Mol. Biol.*, 215, 403-410, 1990) that have been developed based on the algorithm of BLAST (Karlin, S and Altschul, SF. *Proc. Natl. Acad. Sci. USA*, 90, 5873-5877, 1993). The specific techniques of these analytical methods are widely known (http://www.ncbi.nlm.nih.gov.).

[51]

[52] Meanwhile, the gene encoding the ARH1 protein of the present invention may be included in an expression vector for transfer into target cells.

[53] The gene encoding the ARH1 protein of the present invention can be introduced into cells by a variety of means, including DNA/DEAE-dextran complex, DNA/nuclear protein complex, and DNA/lipid complex, for which the ARH1 gene may be included in a carrier for efficient transfer into cells. The carrier is preferably a vector, including viral vector and non-viral vector, for example, plasmid, phage, cosmid, and viral vector, and these vectors may be self-replicating, or may be integrated into the DNA of a host cell.

[54]

[55] The term "introduction" as used herein refers to the introduction of foreign DNA into cells by transfection or transduction. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, mi-

croinjection, liposome fusion, and lipofectamine and protoplast fusion. The term "transduction" refers to the process whereby foreign DNA is introduced into cells using a virus or viral vector as a means for infection.

[56] In the expression vector, the ARH1 gene may be linked to an expression regulatory sequence, such as promoter/enhancer sequences, and other sequences required for transcription, translation or processing. The regulatory sequences indicate constitutive expression of nucleic acids, as well as containing tissue-specific regulatory and/or inducible sequences. The design of the expression vector may be determined depending on host cells to be transfected and other factors such as desired expression levels.

[57] Further, the vector that comprises the ARH1 gene of the present invention may be introduced into suitable eukaryotic cells in cell culture system to express the ARH1 protein directly or transformed into prokaryotic cells to express the ARH1 protein, followed by purification for use. The ARH1 protein of the present invention may include, for example, purified protein, water-soluble protein, or protein linked to a carrier for transfer or administration into target cells, or protein fused with amino acid residues.

[58]

[59] Meanwhile, the ARH1 protein of the present invention exhibits an NF-κB inhibitory activity, which is preferably achieved by inhibiting nuclear translocation of p50 or p65 subunit of NF-κB.

[60] Further, the inhibition of NF-κB activation is preferably achieved by interactions between ARH1 protein and TRADD (TNF receptor-associated death domain) protein. Such interactions are able to inhibit activation or nuclear translocation of NF-κB.

[61] Specifically, the ARH1 protein of the present invention is able to bind with the TRADD protein, preferably TRADD N-domain. Subsequently, the binding competitively blocks the interaction between TRADD and TRAF2 (TNF receptor-associated factor 2), so that the NF-κB activation via TRAF2 can be inhibited.

[62] The TRADD protein is a protein that functions as a mediator to recruit various signal transducers into the activated TNFR1. The TRADD protein functions to recruit TRAF2, RIP, or FADD into the TNFR1. In particular, TRAF2 (TNF-associated factor-2) and RIP (receptor-interacting protein) stimulate pathways leading to activation of NF-κB and JNK/AP-1. TRAF2 and RIP activate NIK (NF-κB-inducing kinase), in turn, NIK activates IKK (I-κB kinase complex), leading to phosphorylation and degradation of I-κB (inhibitor of κB). Subsequently, the liberated NF-κB translocates to the nucleus, where it activates the transcription of target genes.

[63] The ARH1 protein of the present invention binds to the TRADD protein to competitively block the TRADD-TRAF2 interaction, so that the NF-κB activation via

TRAF2 can be inhibited. Consequently, the nuclear translocation of p50 or p65 subunit of NF-κB is inhibited to suppress the transcription of target genes.

[64]

[65] In the specific Example of the present invention, the present inventors confirmed the inhibitory activity of ARH1 protein on NF-κB activation. They also confirmed that the binding of ARH1 protein to TRADD protein blocks TRADD-TRAF2 interaction, and thus the NF-κB activation via TRAF2 is inhibited to impair the nuclear translocation of p50 or p65 subunit of NF-κB, thereby achieving the inhibition of NF-κB activation.

[66] Further, the present inventors examined the effect of ARH1 protein on NF-κB-induced angiogenesis. They found that the ARH1 protein is able to inhibit angiogenesis, and also inhibit expression of the known angiogenesis-stimulating factor, VEGF.

[67]

[68] In accordance with another aspect, the present invention relates to a composition for the treatment of diseases associated with aberrant NF-κB activation, comprising the NF-κB inhibitor that comprises the ARH1 protein or gene encoding the same as an active ingredient.

[69] The ARH1 protein or gene encoding the same of the present invention interacts with the TRADD protein in cells, and thus inhibits nuclear translocation of p50 or p65 subunit of NF-κB, thereby exhibiting the NF-κB inhibitory activity. Accordingly, it can be provided as a composition for the treatment and prevention of diseases associated with aberrant NF-κB activation.

[70] The NF-κB inhibitor of the present invention may be applied to any disease caused by aberrant NF-κB activation without limitation, exemplified by angiogenesis-related diseases, inflammatory diseases, autoimmune diseases or viral diseases, more specifically, asthma, allergic rhinitis, atopic dermatitis, urticaria, conjunctivitis, psoriasis, ulcerative colitis, systemic inflammatory response syndrome, sepsis, polymyositis, dermatomyositis, polyarteritis nodosa, mixed connective tissue disease, Sjogren's syndrome, gout, dementia of the Alzheimer's type, Parkinson's disease, amyotrophic lateral sclerosis, chronic rheumatism, type I diabetes mellitus, type II diabetes mellitus, diabetic retinopathy, multiple sclerosis, Crohn's disease, chronic thyroiditis (Hashimoto's thyroiditis), celiac disease, myasthenia gravis, pernphigus vulgaris, systemic erythematodes, viral diseases (HIV, Herpes, Sendai rotavirus, Influenza virus, Rabies virus, Vesicular Stomatitis Virus, Rhino virus, Hepatitis A Virus, Hepatitis B Virus, Rubella virus, etc.), bacterial diseases, radiation injury, arteriosclerosis, hemangiomas, angiofibroma, reperfusion injury, and cardiac hypertrophy.

[71] As used herein, the term "prevention" means all of the actions in which the disease is restrained or retarded by the administration of the composition. As used herein, the

term "treatment" means all of the actions in which the disease condition has been improved or modified favorably by the administration of the composition.

[72]

[73] The composition for the treatment of diseases associated with aberrant NF-κB activation, which comprises the NF-κB inhibitor comprising the ARH1 protein or gene encoding the same as an active ingredient, may additionally include a pharmaceutically acceptable carrier, and be formulated together with the carrier. As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. For formulation of the composition into a liquid preparation, a pharmaceutically acceptable carrier which is sterile and biocompatible may be used such as saline, sterile water, Ringer's solution, buffered physiological saline, albumin infusion solution, dextrose solution, maltodextrin solution, glycerol, and ethanol. These materials may be used alone or in any combination thereof. If necessary, other conventional additives may be added such as antioxidants, buffers, bacteriostatic agents, and the like. Further, diluents, dispersants, surfactants, binders and lubricants may be additionally added to the composition to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions, or pills, capsules, granules, or tablets.

[74]

[75] The composition for the treatment of diseases associated with aberrant NF-κB activation may be prepared into any formulation, oral or parenteral formulation. The pharmaceutical formulations of the present invention include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), subcutaneous, vaginal or parenteral (including intramuscular, subcutaneous, and intravenous) administration, or for administration by inhalation or insufflation.

[76] The formulations for oral administration comprising the composition of the present invention as an active ingredient include tablets, troches, lozenges, water-soluble or oil suspensions, powders or granulates, emulsions, hard or soft capsules, syrups or elixirs, etc. In order to prepare the formulation in the form of tablet and capsule, the composition further includes: binders such as lactose, saccharose, sorbitol, mannitol, starch, amylopectin, cellulose or gelatin; excipients such as dicalcium phosphate; disintegrants such as corn starch or potato starch; and lubricants such as magnesium stearate, calcium stearate, sodium stearyl fumarate or polyethylene glycol wax. For capsules, a liquid carrier, such as a lipid, may be further used in addition to the above-mentioned compounds.

[77] The formulations for non-oral administration comprising the composition of the present invention as an active ingredient are formulated into injections for sub-

cutaneous, intravenous or intramuscular routes, suppositories, or sprays inhalable via the respiratory tract, such as aerosols. Injection preparations may be obtained by dissolving or suspending the composition of the present invention, together with a stabilizer or a buffer, in water and packaging the solution or suspension in ampules or vial units. Suppositories are typically made of a suppository base, such as cocoa butter or another glyceride, or a therapeutic laxative. For sprays, such as aerosol, a propellant for spraying a water-dispersed concentrate or wetting powder may be used in combination with an additive.

[78]

[79] In accordance with still another aspect, the present invention relates to a method for treating diseases associated with aberrant NF-κB activation using the NF-κB inhibitor.

[80] In the present invention, the treatment of the diseases may include a step of administering the pharmaceutical composition for the treatment of diseases associated with aberrant NF-κB activation, comprising the NF-κB inhibitor. As used herein, the term "administration" is intended to refer to the introduction of the pharmaceutical composition of the present invention into a patient in a suitable manner, and includes delivery of the ARH1 gene by viral or non-viral techniques or transplantation of ARH1-expressing cell.

[81] As long as it introduces the composition of the present invention to a desired tissue, any administration route, either oral or parenteral, may be adopted. Specifically, the composition may be administered via oral, rectal, topical, intravenous, intraperitoneal, intramuscular, intraarterial, transdermal, intranasal, inhalation, intraocular, or intracutaneous route in a typical manner, preferably, topical administration into target tissues.

[82] The therapeutic method includes administering the composition of the present invention in a pharmaceutically effective amount. It will be apparent to those skilled in the art that the suitable total daily dose may be determined by an attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient may vary depending on a variety of factors, including the kind and degree of a desired reaction, the specific composition, including the use of any other agents according to the intended use, the patient's age, weight, general health, gender, and diet, the time of administration, route of administration, and rate of the excretion of the composition; the duration of the treatment; other drugs used in combination or coincidentally with the specific composition; and like factors well known in the medical arts. Accordingly, it is preferable that the effective amount of the pharmaceutical composition, suitable for the purposes of the present invention, is properly determined by taking into consideration of the above factors.

[83] Further, the therapeutic method of the present invention may be applied to any

animal being susceptible to diseases associated with aberrant NF-κB activation, and the term "animal" includes human and primate, as well as livestock and pets including cattle, pigs, sheep, horses, dogs and cats, etc.

[84]

[85] In accordance with still another aspect, the present invention relates to a method for inhibiting NF-κB activity in cells using the ARH1 protein or gene encoding the same.

[86] If cells are treated with the ARH1 protein of the present invention directly or treated with the gene encoding the ARH1 protein to express the protein, nuclear translocation of NF-κB is inhibited, thereby impairing the NF-κB activity. Specifically, the ARH1 protein interacts with the TRADD (TNF receptor-associated death domain) protein to competitively block interaction between TRADD and TRAF2 (TNF receptor-associated factor 2). Consequently, the nuclear translocation of p50 or p65 subunit of NF-κB is inhibited to effectively inhibit the aberrant NF-κB activation in cells.

[87] In this connection, the method for treating cells with the ARH1 gene of the present invention may be performed using viral or non-viral gene delivery technique to introduce nucleic acid molecules into cells. Viral delivery mechanism may include lentivirus, retrovirus, adenovirus, herpes virus, avipoxvirus or the like, but is not limited thereto. Non-viral delivery mechanism may include lipid-mediated transfection, liposome, immunoliposome, lipofectin, cationic amphiphiles, and combinations thereof. In conclusion, ARH1-expressing eukaryotic cells, resulting from introduction of the ARH1 gene, can be used as a cell therapeutic agent against diseases associated with aberrant NF-κB activation.

[88]

[89] Hereinafter, the present invention will be described in detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[90]

## Mode for the Invention

[91] **Example 1. Cell culture**

[92] Human cervical carcinoma-derived HeLa cell, ovarian carcinoma cell lines 2774, SKOV-3, and OVCA-3, and breast carcinoma cell line MCF-7 (American Type Culture Collection, USA) were cultured in DMEM and RPMI media (Life Technologies, USA) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (100 unit/ml) at 5% $CO_2$ and 37℃. HUVEC cell line (human umbilical vein endothelial cells, Clonetics, San Diego, USA) was cultured in EGM media (Clonetics, San Diego, USA) supplemented with 2% FBS (Fetal Bovine Serum) at 5% $CO_2$ and 37℃.

[93]

[94] **Example 2: Construction of ARH1, TRADD, TRAF2-expressing vectors**

[95] Expression vectors containing human ARH1 gene and other genes were constructed. Prokaryotic expression vectors, pGEX4T-1-ARH1 (FIG. 6), pGEX4T-1-TRAF2 (FIG. 6), and pET28-TRADD(N) (FIG. 6), and eukaryotic expression vectors, pcDNA3.1/ARH1 (FIGs. 2, 10, 11 and 13), pcDNA4/HisMax-ARH1 (FIG. 5), pEGFPN3-ARH1 (FIGs. 7 and 10), pEGFPC1-TRADD(N) (FIGs. 5 and 7) and pcDNA4/HisMax-TRADD (FIG. 5) were each cloned by the following method.

[96] Briefly, the ARH1 gene fragment was obtained by PCR, and then inserted into the *EcoR*I and *Xho*I sites of pcDNA3.1 (Invitrogen, USA), pcDNA4/HisMax-ARH1 (Invtrogen), pGEX4T-1 (Amersham Biosciences, Sweden) and pEGFPN3 (Clonetech, USA) expression vectors, and into the *Bgl*II and *EcoR*I sites of pEGFPC1 and pEGFPN3 expression vectors, respectively. The TRAF2-encoding gene was inserted into the *BamH*I and *Sal*I sites of pGEX4T-1 expression vector, and the recombinant protein was separated and purified. The fragment of TRADD-encoding gene was inserted into the *EcoR*I and *Xho*I sites of pET28 (Novagen, USA) and pcDNA4/HisMax (Invitrogen) expression vectors, respectively. Each of the plasmids containing the gene fragments was subjected to DNA sequencing analysis (Applied Biosystems, USA) according to the manufacturer's recommendations.

[97]

[98] *E.coli* (*E.coli* BL21DE3) transformed with the prokaryotic expression vectors were cultured in LB culture media containing 100 ㎍/ml ampicillin and 75 ㎍/ml kanamycin at 37℃ until the optical density at 595 nm reached 0.5~0.6. The cells were induced to express each recombinant protein using 0.5 mM IPTG (isopropyl-b-D-thiogalactopyranoside) at 37℃ for 3~4 hr, depending on each gene, and then resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.5 mM DTT), followed by sonication. Each of the denatured proteins was separated and purified using a GST or $Ni^+$ column.

[99]

[100] **Example 3: Examination of inhibitory activity of ARH1 protein on NF-κB transcriptional activity**

[101] To examine inhibitory activity on NF-κB mediated transcriptional activity, the normal cell line, HEK293 was cultured in culture plates (24 well, Costar Co.), and transformed by electroporation with 250 ng of the NF-κB luciferase reporter construct, pNFB-Luc (purchased from Stratagen) and 25 ng of Renilla luciferase vector, pRL-TK (Promega) together with 0.1 and 0.5 ㎍ of ARH1, or without ARH1 as a control. After 24 hrs, the cells were cultured in antibiotic-free media for at least 2 hrs, and then stimulated by the addition of 10 ng/ml human TNF-α. The cells were harvested and

treated with lysis buffer according to dual-luciferase assay manual (Promega) to assess luciferase activity using a luminometer. As a result, it was found that NF-κB mediated transcriptional activity was inhibited in the cells transformed with the ARH1 gene (FIGs. 1 and 2).

[102]

[103] Meanwhile, to examine the effect of ARH1 on the nuclear translocation of NF-κB, the ovarian carcinoma cell line SKOV-3 was transfected with the expression vector harboring the ARH1 gene. Then the present inventors examined the p65 protein expression level in the cells. Briefly, SKOV-3 cells transfected with the ARH1-expressing vector or empty vector (containing no ARH1 gene) were lysed with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, 10 mmol/L sodium deoxylate]. 20 ㎍ of total cell lysate were separated on SDS-10% polyacrylamide gel, and transferred onto a nylon membrane. The membrane was incubated with anti-p65 antibody (Santa Cruz, USA), and visualized using an ECL Chemiluminescent Kit (Amersham, UK) according to the manufacturer's instructions. To quantify the total amount of protein, HSP 90 (Santa Cruz, USA) protein was used as a cytosolic marker, and Histone H1 (Santa Cruz, USA) antibody was used as a nuclear marker to perform Western blot analysis.

[104] As a result, higher expression of cytosolic p65 protein and lower expression of nuclear p65 protein were observed in the ARH1-expressing cells, compared to the control group (FIG. 3), suggesting that overexpression of ARH1 blocks the nuclear translocation of NF-κB, and ARH1 promotes protein degradation of NF-κB (p65) in the cytoplasm, and thus it functions to promote instability and inactivation of NF-κB (p65) protein, and to inhibit the nuclear translocation of NF-κB (p65) protein.

[105]

[106] **Example 4: Interaction between ARH1 protein and TRADD protein**

[107] To understand the inhibition mechanism of ARH1 protein on NF-κB activation at the molecular level, yeast two-hybrid analysis was performed according to Gyuris et al. (Gyuris et al., *Cell*, 75: 791-803, 1993; Park et al., *Cancer Res.*, 65: 749-757, 2005), so as to screen the proteins that interact with the ARH1 protein. Briefly, for protein expression, the ARH1 gene fragment was inserted into the *EcoR*I and *Xho*I sites of pGilda vector (Clontech), which can be used in both yeast and *E.coli* and contains the LexA regulatory gene. Next, the construct was introduced into the yeast strain EGY48 (Clontech), and cultured in histidine-free media for primary selection to obtain colonies. Subsequently, secondary screening was performed using HeLa cell cDNA library (Clontech) to select colonies. To examine the binding proteins, a variety of methods (growth on medium containing 2% galactose but lacking leucine, blue colony formation on synthetic media containing 2% galactose and 2% raffinose) were used to

select the binding genes, and their base sequences were analyzed using the BLAST program provided by NCBI. As a result, it was found that ARH1 interacts with TRADD (FIGs. 4 and 5). For further confirmation, the TRADD-encoding DNA fragment was obtained by PCR, and cloned into the EcoRI and XhoI sites of pJG4-5 (Clontech). The yeast strain EGY48 was transformed with the construct to conduct various assays. The direct interaction between TRADD and ARH1 proteins was examined by co-immunoprecipitation, whereby endogenous TRADD binds with exogenous GFP-tagged ARH1 (GFP-ARH1) protein. These results confirmed the direct binding of ARH1 to TRADD (FIG. 6). In addition, the nuclear localization of ARH1 and TRADD in the cell were confirmed by fluorescence microscopy (FIG. 7).

[108] These results indicate that the TRADD N-domain plays an important role in the interaction between ARH1 and TRADD, and thus the N-domain is necessary for the interaction with ARH1. Furthermore, the results suggest a new function of N-domain, which is engaged in transcriptional regulation via protein-protein interaction, in addition to the previously reported function of serving as a motif for TRAF2 binding.

[109] Taken together with the results of Examples 3 and 4, it was found that the ARH1 overexpression blocks the interaction of TRADD with TRAF2 (FIGs. 4 and 5), and inhibits the nuclear translocation of p50 or p65 subunit of NF-κB, and thus p65/p50 protein is accumulated in the cytoplasm, thereby inhibiting NF-κB activity (FIG. 3). These results suggest a new function of ARH1, namely, that ARH1 competitively blocks TRADD-TRAF2 interaction, so as to inhibit the transcriptional regulation mechanism and the nuclear translocation of p50/p65 subunit of NF-κB, thereby inhibiting the NF-κB activity.

[110]

[111] **Example 5: Inhibitory effects of ARH1 on angiogenesis and metastasis in human ovarian cancer cell**

[112] There have been reports that inhibition of NF-κB activation suppresses angiogenesis. There is a report that angiogenesis in epithelial cells is associated with NF-κB activation, which is regulated by integrin-linked kinase (Lee, SP. et al., *Circulation*, 114, 150-159, 2006). ILK is an intracellular protein, which interacts with the cytoplasmic domain of integrin, and is an important regulator of cell structure, survival and proliferation. This article demonstrated that in response to tissue ischemia, both the endogenous amount and kinase activity of ILK are upregulated in epithelial cells under hypoxia. The upregulated ILK regulates nuclear translocation of the transcription factors, HIF-1 and NF-κB via Akt, Erk, and IB under hypoxia. Subsequently, HIF-1 and NF-κB control the expression of ICAM-1 (intercellular adhesion molecule-1) and SDF-1 (stromal cell-derived factor-1), key molecules shown to be involved in selective recruitment of EPCs to ischemic tissue. These facts suggest that modulation of ILK

activity would be a good target to control the process of vasculogenesis. In addition, it was recently reported that up-regulation of Angiopoietin-1 (Ang-1), which is a critical angiogenic factor, is mediated by the activation of the transcription factor NF-κB (Mitola, S. et al., *Blood*, 112, 1154-1157, 2008), and a tumor and vascular associated protein, angiocidin induces phosphorylation of IB, p50, and p65 for the nuclear translocation of NF-κB (Gaurnier-Hausser, A. et al., *Cancer Res.*, 68, 5905-5914, 2008).

[113]

[114] Accordingly, the present inventors examined whether the ARH1 protein inhibits angiogenesis. Angiogenesis requires the process of tube formation, including growth, migration, and proliferation of epithelial cells. That is, the potent angiogenic capacity is attributed to proliferation, migration and invasion, and tube formation of epithelial cells. First, to examine the inhibitory activity of ARH1 protein on angiogenesis in vitro, the ARH1 protein was expressed in epithelial cells. The specific procedures are as follows.

[115] First, the ARH1 gene was overexpressed in epithelial cells, and, for accuracy, its mRNA level was determined by RT-PCR, and its protein level was also determined by Western blot analysis. In addition, as a control group, siARH1 (siRNA inhibiting ARH1 gene expression) was constructed to examine its inhibitory effects on the expression of ARH1 mRNA and protein in the same manner (FIG. 8). Furthermore, to examine the effects of ARH1 protein on the proliferation of epithelial cells, [$^3$H] thymidine incorporation analysis was performed to measure DNA synthesis as follows. The epithelial cells (4.5 x $10^3$ cells/well) were transferred to a gelatin-coated 96-well plate (Nunc), and cultured at 37°C for one day. In cell growth media [(Medium 199/10% FBS/10mM HEPES (pH 7.4)], the epithelial cells were pretreated with ARH1 protein, mock (not including ARH1 protein), or siRNA peptide inhibiting ARH1 expression, and VEGF (10 ng/ml) was added thereto. After 48 hrs, the epithelial cells were treated with [$^3$H] thymidine (0.5 μCi/well, 76 Ci/mmol, AP Biotech) for 24 hrs, and washed with phosphate buffer supplemented with 0.1% albumin. The cells were lysed using 0.4 N NaOH at room temperature for 20 min, and neutralized with 2N HCl. Then, DNA synthesis was examined by measuring the amount of radioactivity using a liquid scintillation counter. As a result, it was confirmed that the ARH1 protein inhibits the proliferation of epithelial cells (FIG. 9).

[116] Further, to examine its effects on the migration and invasion of epithelial cells, a cell migration test was performed. Specifically, the cell migration test was performed using a transwell plate (pore size: 8 ㎛, Costar, USA). The bottom surface of transwell plate was precoated with each ARH1 solution (10 ㎍/ml) at 4°C overnight, and blocked with VEGF (25 ng/ml) in M199 supplemented with 1% FBS for 1 hr and 30 min. HUVEC

cells (3 x $10^5$ cells/ml) were suspended in culture media, and 0.1 ml of suspending cells were precultured with each of the ARH1 protein and siARH1 peptide at 37℃ for 30 min. Next, the cells were allowed to migrate at 37℃ for 6~8 hrs. The cells remaining on the upper surface of the filter were removed with a cotton swab to terminate the migration. The filter was fixed with 8% glutaraldehyde and stained with crystal violet for visualization under a light microscope. The number of cells was counted in 8 randomly selected HPF (microscopic high power fields, x 200).

[117] As a result, the migration and invasion of epithelial cells were promoted by VEGF in the transwell system, which was inhibited by the ARH1 protein (FIGs. 10 and 11).

[118]

[119] Furthermore, the inhibitory effects of ARH1 protein on the expression of the angiogenesis-stimulating factor VEGF were examined, whereby it was seen that treatment of ARH1 protein inhibited the VEGF expression in both HUVEC and ovarian cancer cells (FIG. 12). These results suggest that the ARH1 protein reduces VEGF expression, thereby preventing angiogenesis.

[120]

[121] **Example 6: Examination of inhibitory activity of ARH1 protein on epithelial tube formation in human ovarian cancer cell**

[122] To examine the inhibitory activity of ARH1 protein on angiogenesis, the present inventors analyzed its effects on epithelial tube formation. Specifically, the wells of 96-well plates were coated with 100 ㎕ of matrigel (Chemicon, USA), and the gel was allowed to polymerize. HUVEC cells (3 x $10^5$ cells/ml) were suspended in culture media, and then 0.1 ml of the suspending cells was added to each well coated with matrigel. The protein and anti-ARH1 were added thereto, and the cells were cultured at 37℃ for 16~18 hrs. The cells were photographed, and the lengths of the tube structures were measured and the mean value was calculated. As a result, the epithelial tube formation was selectively inhibited by ARH1 and anti-ARH1 in matrigel. However, the tube formation inhibited by ARH1 was recovered in the control group, siRNA-treated cells (FIG. 13). Taken together, these results suggest the potential of ARH1 protein as a powerful angiogenesis inhibitor, which was also examined using an ex vivo system prior to in vivo mouse model. In this regard, a vessel sprouting assay was performed, in which significant inhibition of angiogenesis was seen(FIG. 14).

[123]

[124] **Example 7: Examination of inhibitory activity of ARH1 protein on angiogenesis in vivo**

[125] To examine the inhibitory activity of ARH1 protein in vivo, the ovarian cancer cell line 2774 was injected subcutaneously into mice to prepare tumor mouse models. When tumor size reached 70-100 $mm^3$, the ARH1 protein was directly injected to the

tumor three times at 3-day intervals. As a result, the average tumor size increased rapidly in the control group (PBS-treated mouse) and reached above 1500 $mm^3$, whereas the tumor size did not increase in the ARH1-treated group (FIG. 15). In addition, the blood vessel formation in tumor section was examined by immunostaining for CD31, whereby weak blood vessel formation was observed in the ARH1-treated group, whereas many blood vessels were observed in the control group (FIG. 15).

[126] These results indicate that ARH1 expression can inhibit angiogenesis to cause tumor cell apoptosis, and also show that ARH1 expression inhibits the NF-κB activation, and thus suppresses angiogenesis caused by aberrant NF-κB activation at the tissue level.

[127]

## Industrial Applicability

[128] The ARH1 protein according to the present invention is able to inhibit the activation of NF-κB in cells. Thus, an NF-κB inhibitor comprising the ARH1 protein or gene encoding the same can be effectively used for the development of therapeutic agents for various diseases caused by aberrant NF-κB activation, for example, angiogenesis-related diseases, inflammatory diseases, autoimmune diseases, and viral diseases.

[129]

# Claims

[Claim 1] An NF-κB inhibitor, comprising an ARH1 protein or gene encoding the same as an active ingredient.

[Claim 2] The NF-κB inhibitor according to claim 1, wherein the ARH1 protein inhibits nuclear translocation of p50 or p65 subunit of NF-κB.

[Claim 3] The NF-κB inhibitor according to claim 1, wherein the NF-κB inhibition is achieved by binding of ARH1 protein to TRADD (TNF receptor-associated death domain) protein.

[Claim 4] The NF-κB inhibitor according to claim 3, wherein the ARH1 protein binds with TRADD to competitively block interaction between TRADD and TRAF2 (TNF receptor-associated factor 2).

[Claim 5] The NF-κB inhibitor according to claim 1, wherein the ARH1 protein or gene encoding the same is derived from human.

[Claim 6] The NF-κB inhibitor according to claim 1, wherein the ARH1 gene is contained in an expression vector for intracellular delivery.

[Claim 7] A composition for the treatment of diseases associated with aberrant NF-κB activation, comprising the NF-κB inhibitor of claim 1.

[Claim 8] The composition according to claim 7, wherein the disease is angiogenesis-related diseases, inflammatory diseases, autoimmune diseases, or viral diseases.

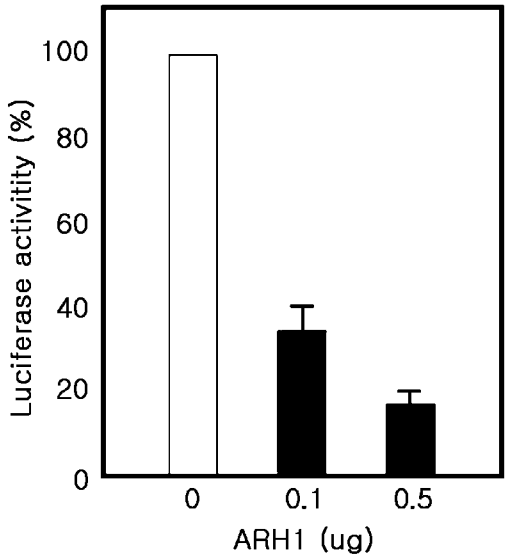
[Claim 9] The composition according to claim 7 or 8, wherein the disease is selected from the group consisting of asthma, allergic rhinitis, atopic dermatitis, urticaria, conjunctivitis, psoriasis, ulcerative colitis, systemic inflammatory response syndrome, sepsis, polymyositis, dermatomyositis, polyarteritis nodosa, mixed connective tissue disease, Sjogren's syndrome, gout, dementia of the Alzheimer's type, Parkinson's disease, amyotrophic lateral sclerosis, chronic rheumatism, type I diabetes mellitus, type II diabetes mellitus, diabetic retinopathy, multiple sclerosis, Crohn's disease, chronic thyroiditis (Hashimoto's thyroiditis), celiac disease, myasthenia gravis, pernphigus vulgaris, systemic erythematodes, viral diseases (HIV, Herpes, Sendai rotavirus, Influenza virus, Rabies virus, Vesicular Stomatitis Virus, Rhino virus, Hepatitis A Virus, Hepatitis B Virus, Rubella virus, etc.), bacterial diseases, radiation injury, arteriosclerosis, hemangiomas, angiofibroma, reperfusion injury, and cardiac hypertrophy.

[Claim 10] The composition according to claim 7, further comprising a pharmaceutically acceptable salt.

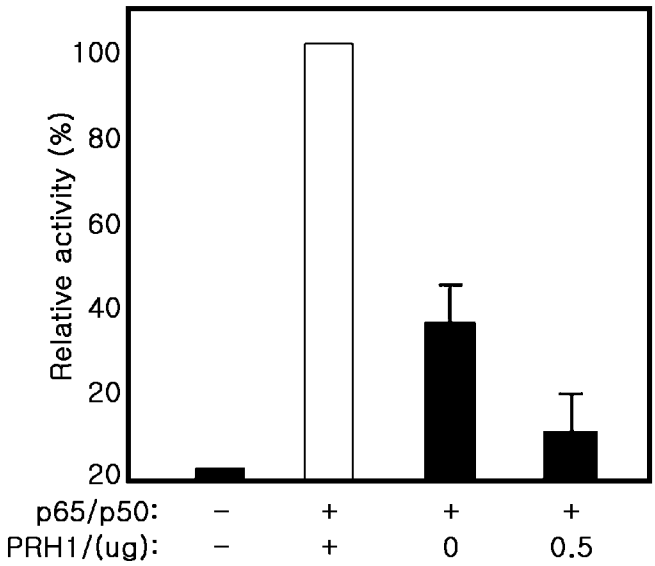
[Claim 11] A method for inhibiting cellular NF-κB activation using an ARH1 protein or gene encoding the same.

[Claim 12] The method according to claim 11, wherein the ARH1 protein interacts with TRADD (TNF receptor-associated death domain) to competitively block interaction between TRADD and TRAF2, thereby inhibiting nuclear translocation of p50 or p65 subunit of NF-κB.

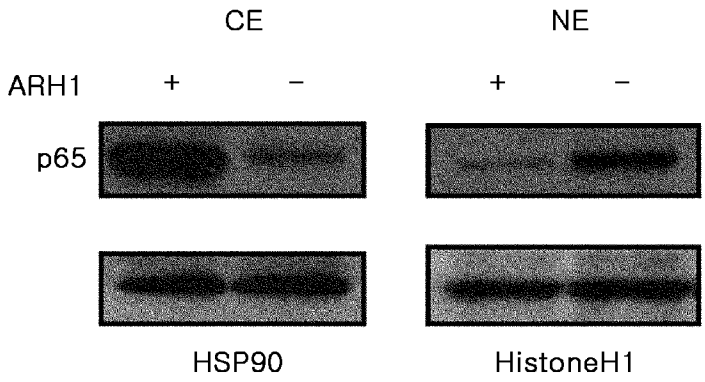
[Fig. 1]



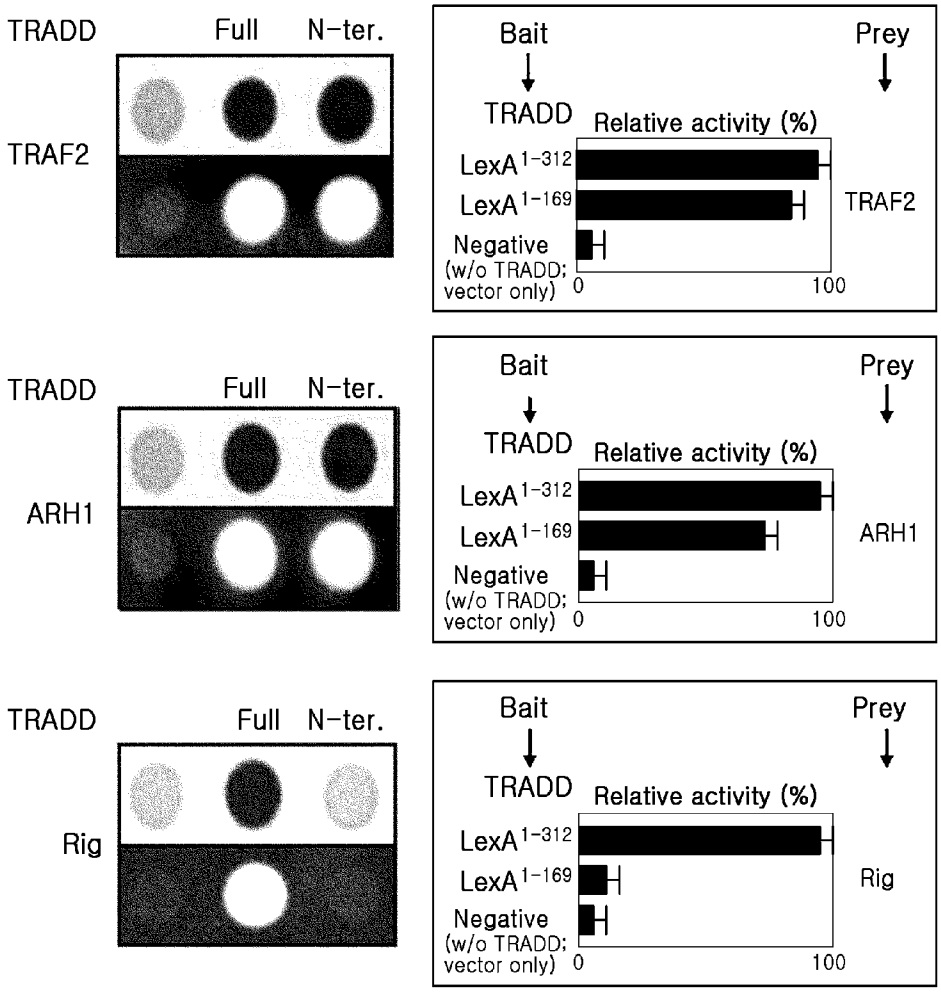

[Fig. 2]



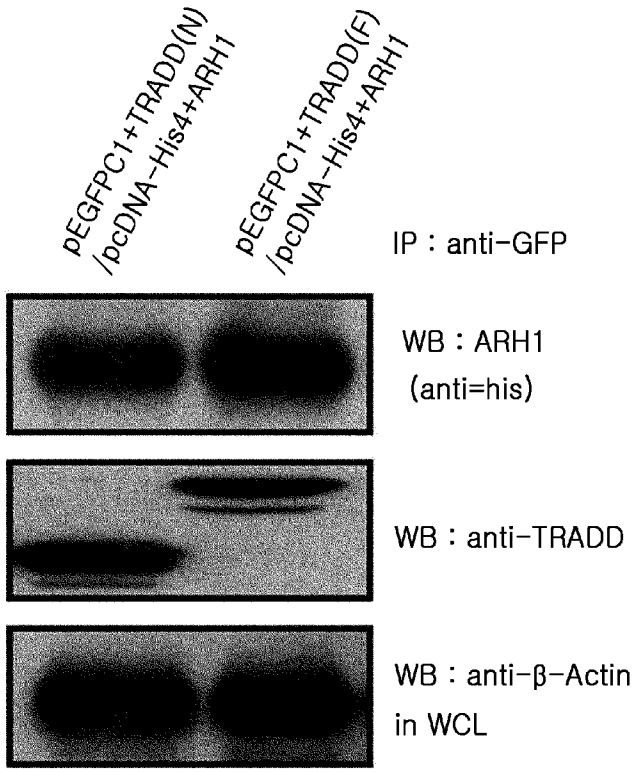

[Fig. 3]



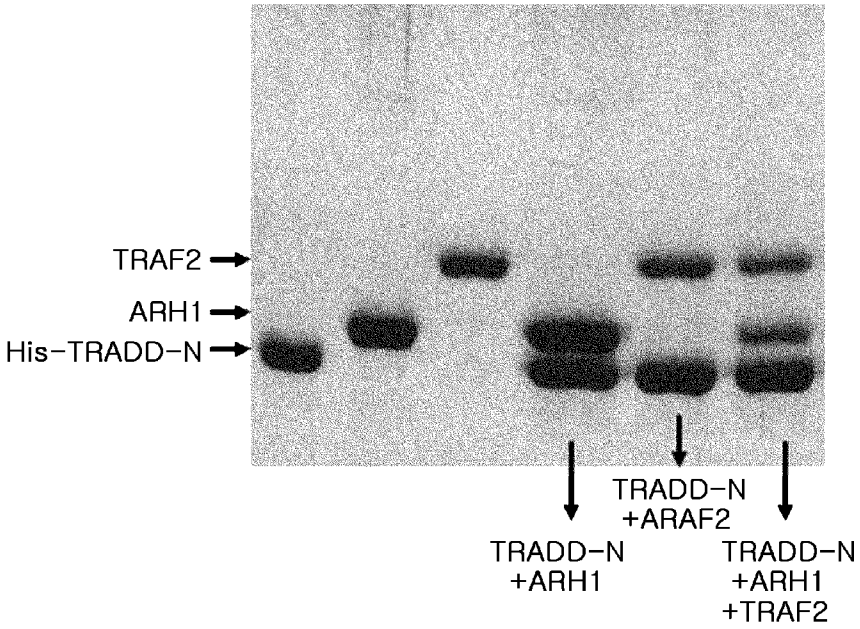
[Fig. 4]


TRADD
Full
N-ter.
TRAF2
Bait
Prey
TRADD
Relative activity (%)
LexA 1-312
LexA 1-169
Negative
(w/o TRADD; vector only)
TRAF2
0
100
TRADD
Full
N-ter.
ARH1
Bait
Prey
TRADD
Relative activity (%)
LexA 1-312
LexA 1-169
Negative
(w/o TRADD; vector only)
ARH1
0
100
TRADD
Full
N-ter.
Rig
Bait
Prey
TRADD
Relative activity (%)
LexA 1-312
LexA 1-169
Negative
(w/o TRADD; vector only)
Rig
0
100

[Fig. 5]

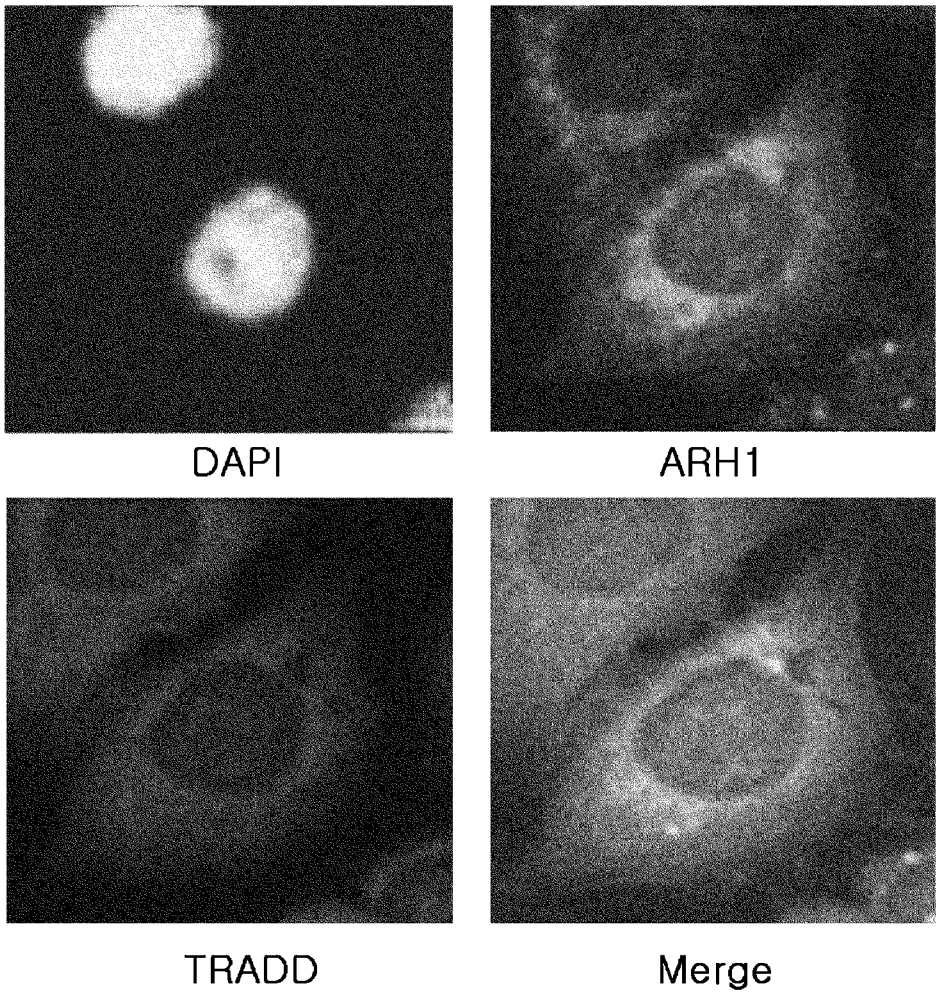

pEGFPC1+TRADD(N) /pcDNA-His4+ARH1
pEGFPC1+TRADD(F) /pcDNA-His4+ARH1
IP : anti-GFP
WB : ARH1 (anti=his)
WB : anti-TRADD
WB : anti-β-Actin in WCL

[Fig. 6]

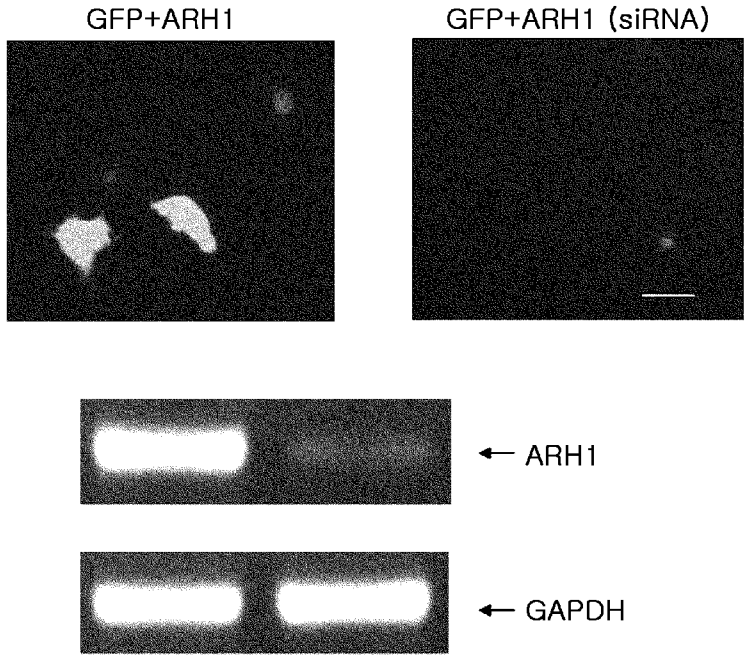



-His-TRADD(N) (5+18=23kDa),ARH1 (26kDA), TRAF2 (53kDa)

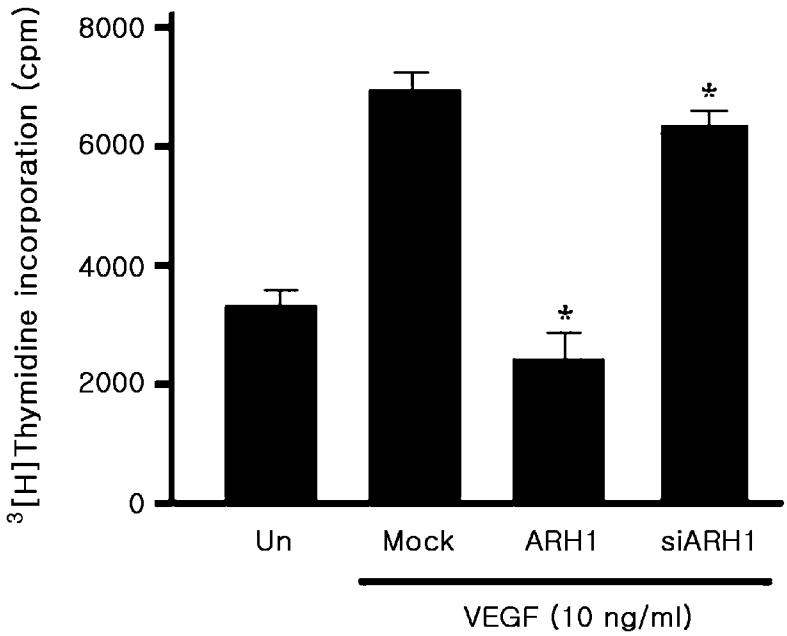
[Fig. 7]



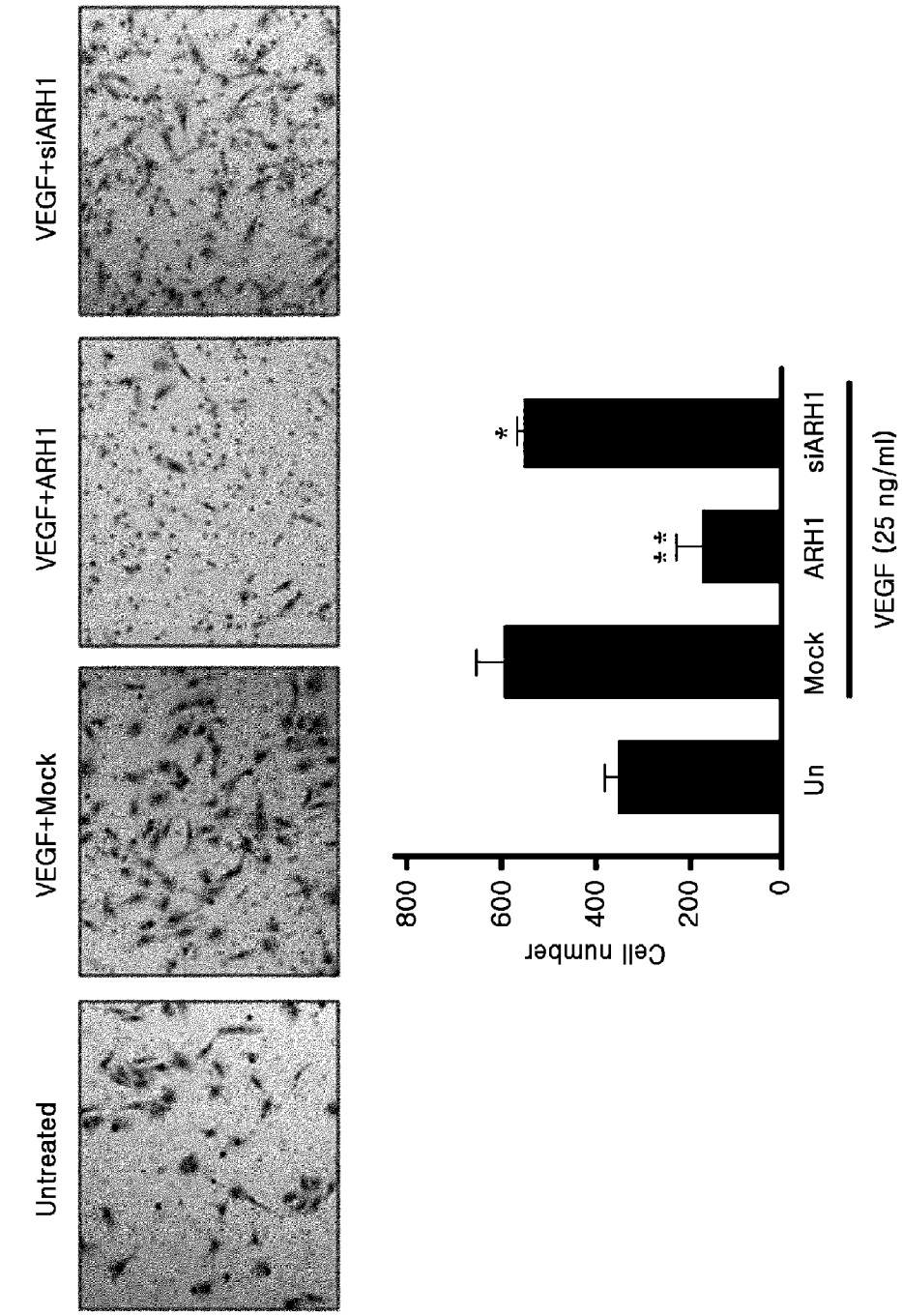
[Fig. 8]


GFP+ARH1
GFP+ARH1 (siRNA)
ARH1
GAPDH

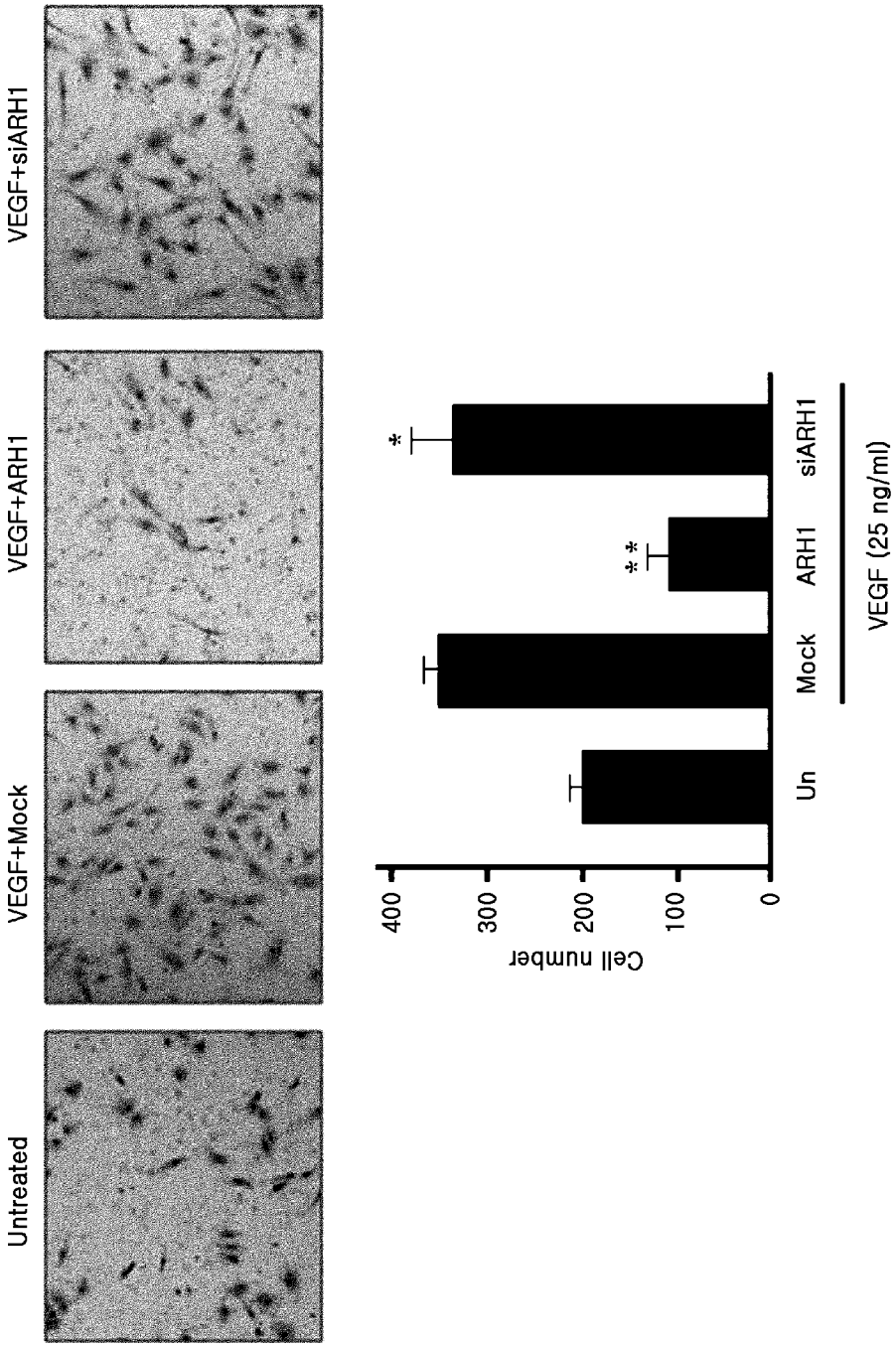
[Fig. 9]


$^{3}$[H]Thymidine incorporation (cpm)
8000
6000
4000
2000
0
Un
Mock
ARH1
siARH1
VEGF (10 ng/ml)

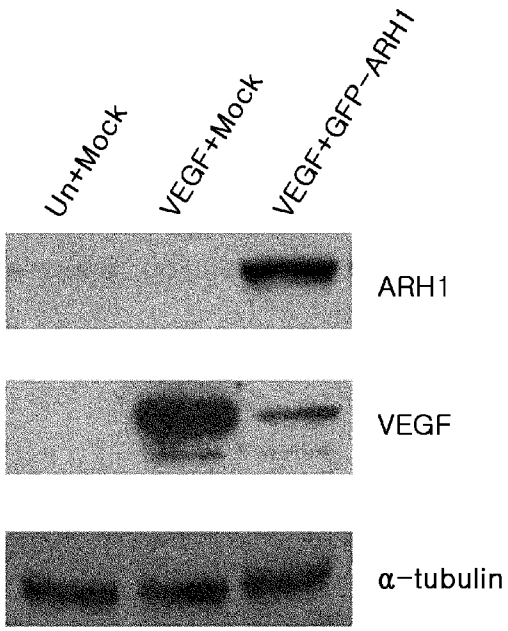
[Fig. 10]


Untreated
VEGF+Mock
VEGF+ARH1
VEGF+siARH1
Cell number
0
200
400
600
800
Un
Mock
ARH1
siARH1
VEGF (25 ng/ml)
*
**

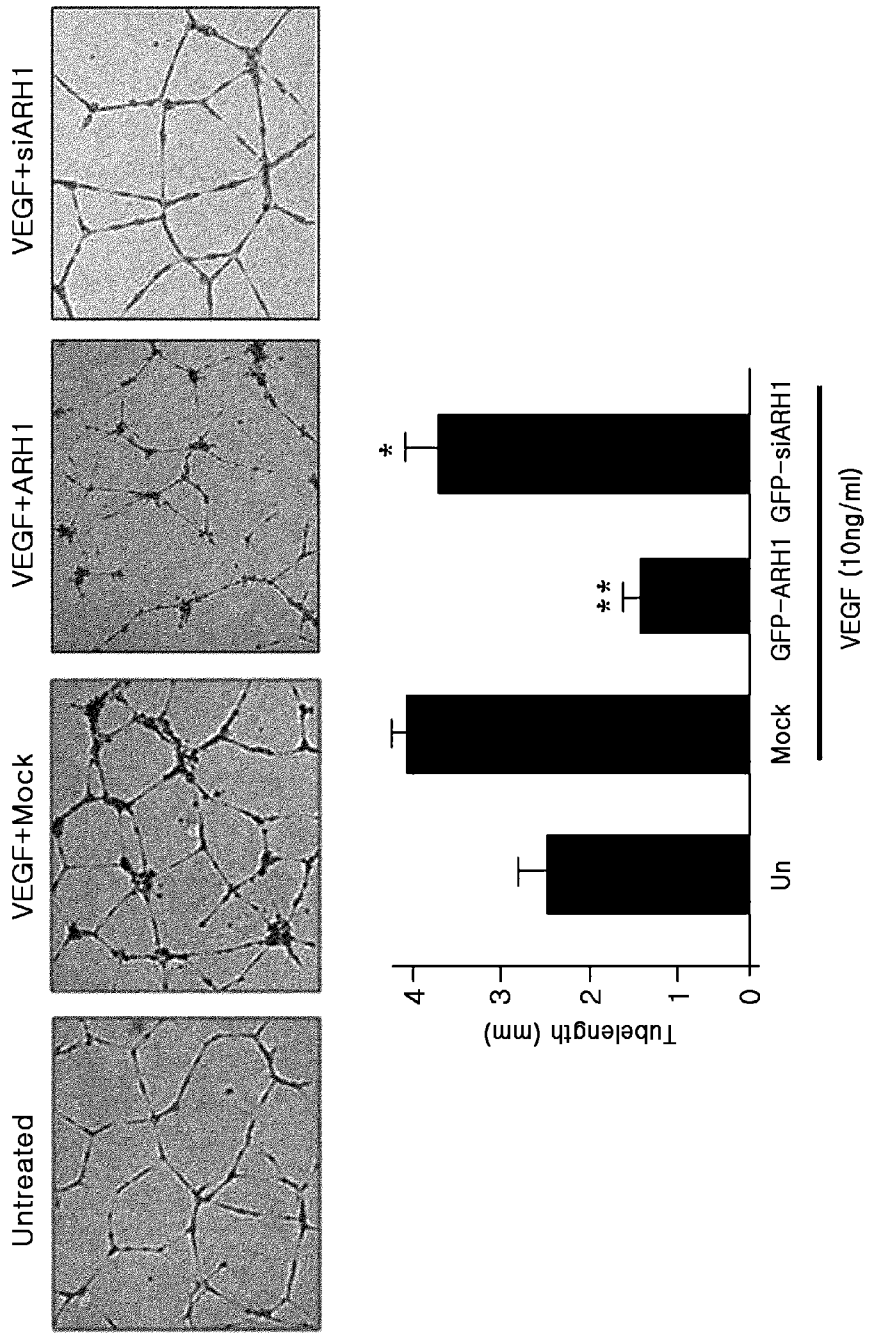
[Fig. 11]


Untreated
VEGF+Mock
VEGF+ARH1
VEGF+siARH1
Cell number
0
100
200
300
400
*
**
Un
Mock
ARH1
siARH1
VEGF (25 ng/ml)

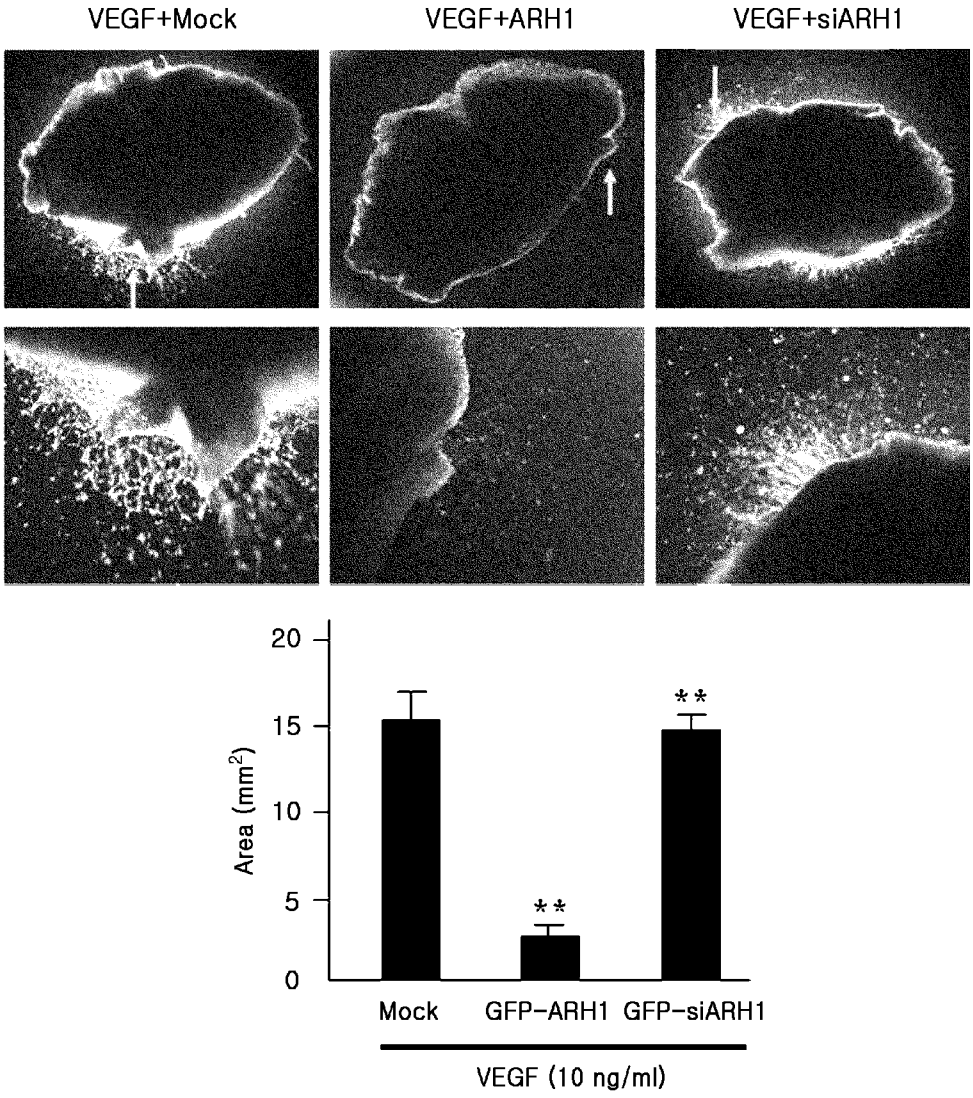
[Fig. 12]



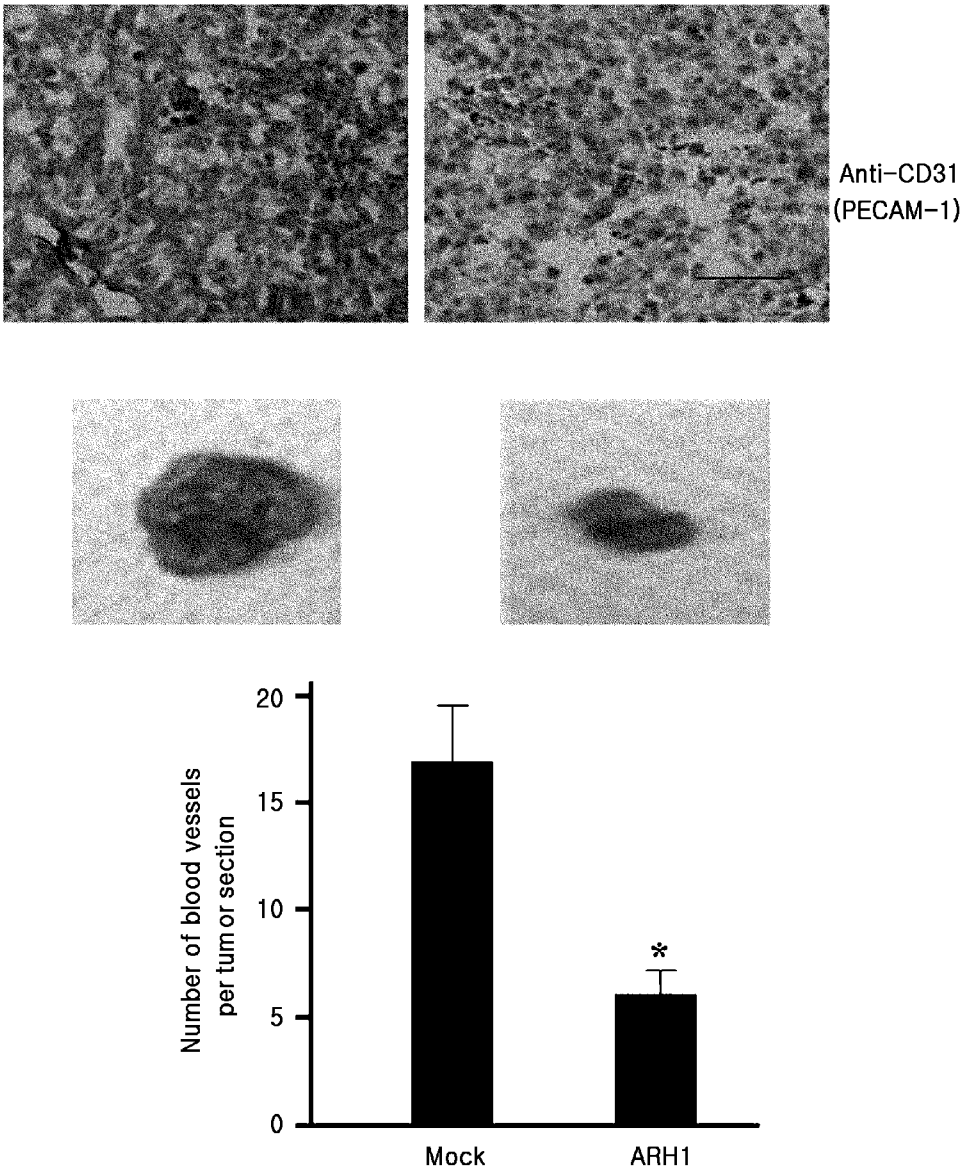
[Fig. 13]


Untreated
VEGF+Mock
VEGF+ARH1
VEGF+siARH1
Tubelength (mm)
0
1
2
3
4
*
**
Un
Mock
GFP-ARH1
GFP-siARH1
VEGF (10ng/ml)

[Fig. 14]


VEGF+Mock
VEGF+ARH1
VEGF+siARH1
Area (mm2)
20
15
10
5
0
**
**
Mock
GFP-ARH1
GFP-siARH1
VEGF (10 ng/ml)

[Fig. 15]


Anti-CD31
(PECAM-1)
Number of blood vessels per tumor section
20
15
10
5
0
*
Mock
ARH1