US 20110123538A1

(19) **United States**

(12) **Patent Application Publication**

(10) **Pub. No.: US 2011/0123538 A1**

(43) **Pub. Date: May 26, 2011**

Kim et al.

(54) **COMPOSITION COMPRISING EXPRESSION OR ACTIVITY INHIBITORS OF NINJURIN 1 FOR THE PREVENTION AND TREATMENT OF INFLAMMATORY DISEASE**

(75) Inventors: **Kyu-Won Kim**, Seoul (KR); **Hyo-Jong Lee**, Seoul (KR)

(73) Assignee: **SNU R&DB FOUNDATION**, Seoul (KR)

(21) Appl. No.: **12/999,005**

(22) PCT Filed: **Dec. 24, 2008**

(86) PCT No.: **PCT/KR2008/007629**

§ 371 (c)(1),
(2), (4) Date: **Dec. 14, 2010**

(30) **Foreign Application Priority Data**

Dec. 9, 2008 (KR) 10-2008-0124555

Publication Classification

(51) **Int. Cl.**

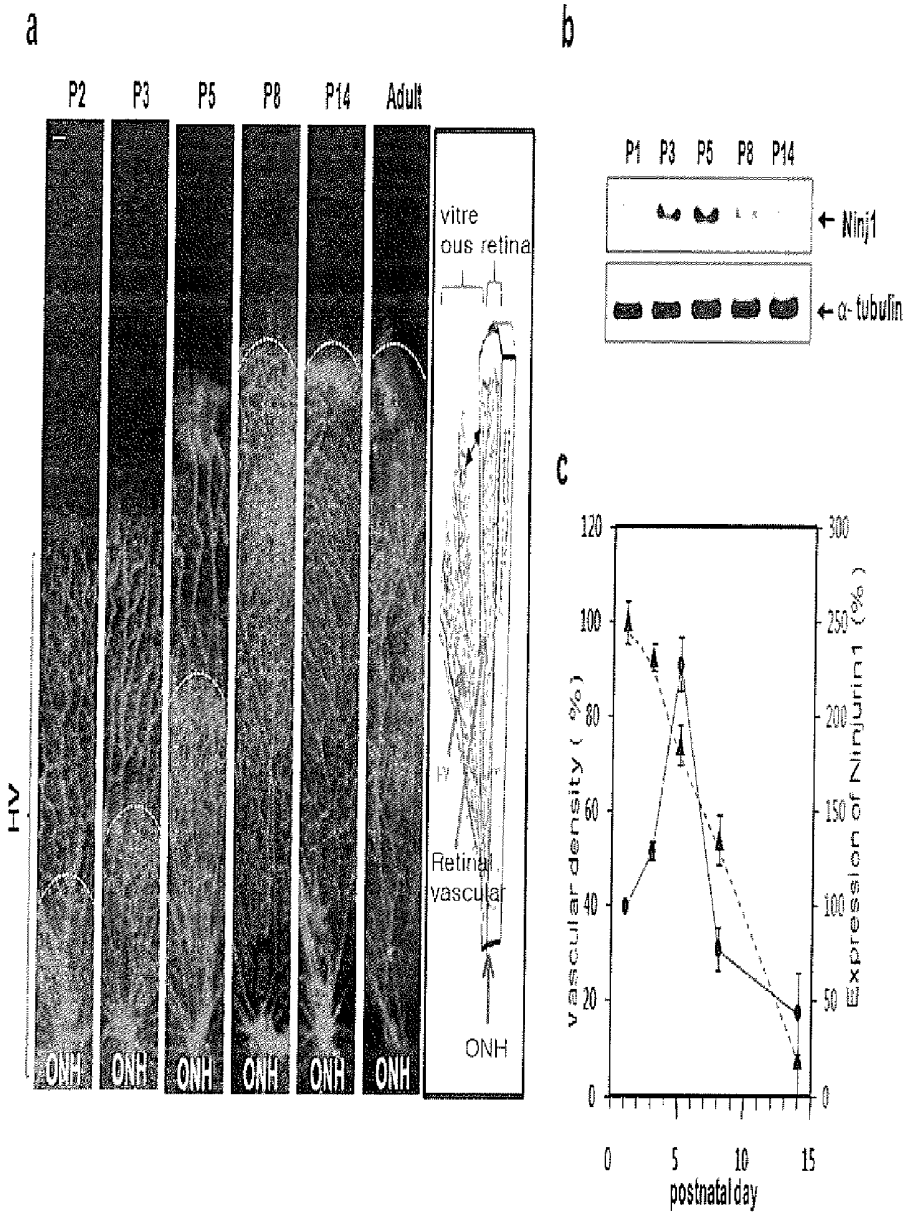
A61K 39/395	(2006.01)
C07H 21/02	(2006.01)
C07K 16/28	(2006.01)
A61K 38/02	(2006.01)
A61K 31/713	(2006.01)
A61P 25/28	(2006.01)
A61P 17/00	(2006.01)
A61P 17/06	(2006.01)
A61P 9/10	(2006.01)
A61P 29/00	(2006.01)
C12Q 1/68	(2006.01)
G01N 33/53	(2006.01)

(52) **U.S. Cl.** ..... **424/139.1**; 536/24.5; 530/389.1; 514/1.1; 514/1.9; 514/17.9; 514/18.7; 514/44 A; 424/172.1; 435/6; 435/7.1; 435/7.92

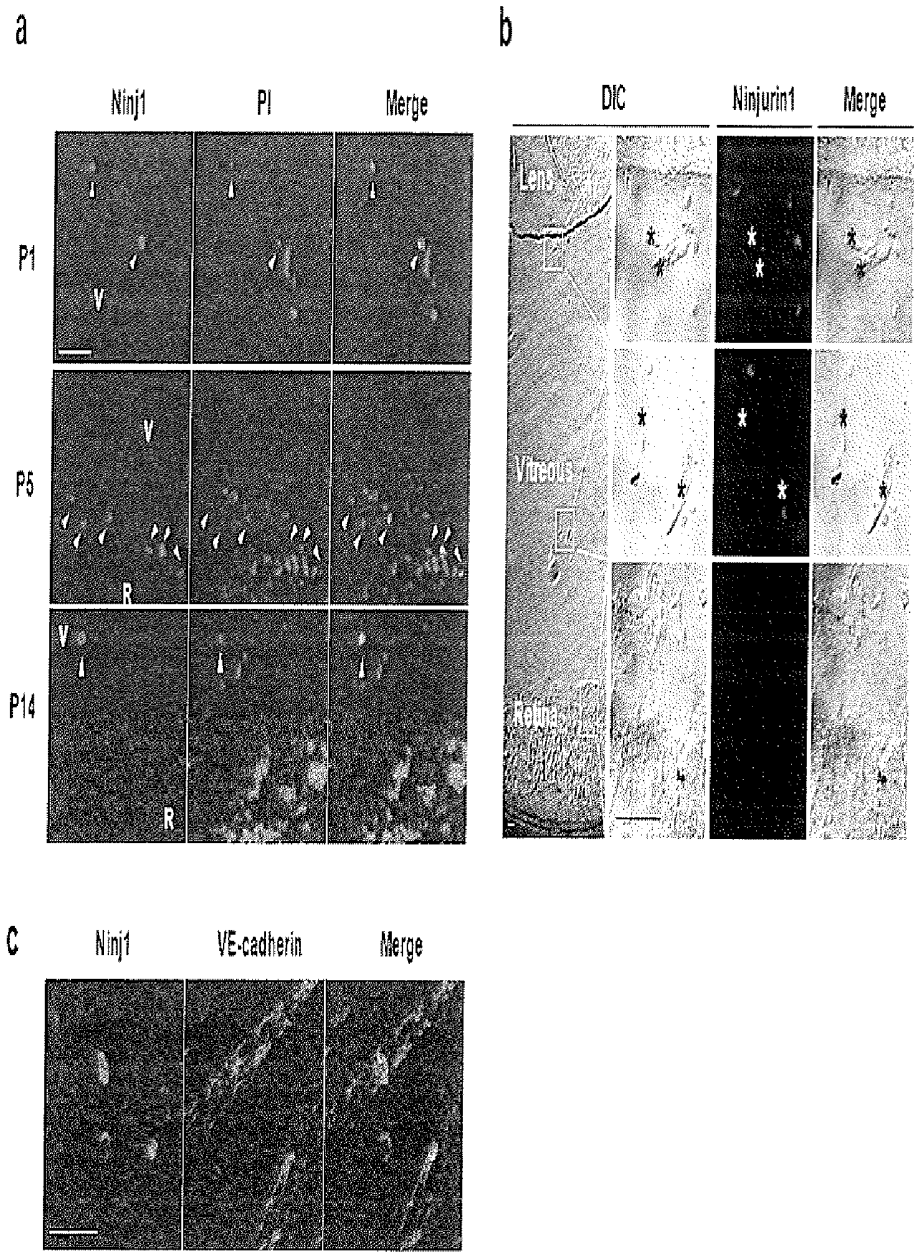
(57) **ABSTRACT**

The present invention relates to a composition comprising a Ninjurin 1 expression or activity inhibitor for the prevention and treatment of inflammatory disease. Ninjurin 1 protein is specifically expressed in macrophages around blood vessels, increases cell-cell adhesion and cell-matrix adhesion, increases expressions of Wnt7b (Wingless-type MMTV integration site family, member 7B) and Ang2 (angiopoietin-2), but reduces expression of Ang1 to induce apoptosis of vascular endothelial cells. In addition, Ninjurin 1 protein is up-regulated when inflammation is induced and induces iNOS expression as well as increased NO generation. Therefore, the Ninjurin 1 protein expression or activation inhibitor can be effectively used as an active ingredient of a composition for the prevention and treatment of inflammatory disease.

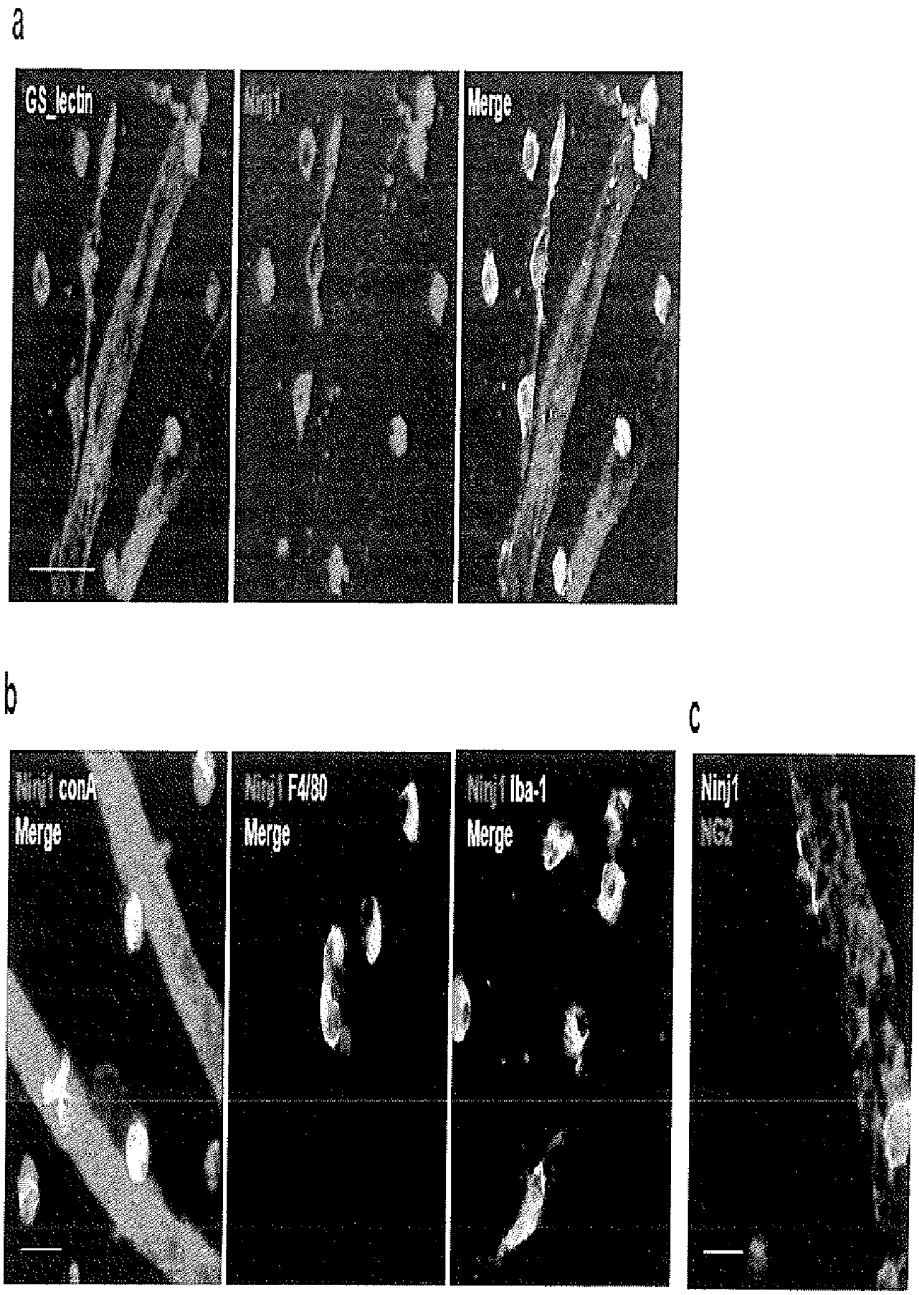
[Fig. 1]


a
P2
P3
P5
P8
P14
Adult
HV
ONH
vitre
ous retina
Retinal
vascular
ONH
b
P1 P3 P5 P8 P14
Ninj1
α-tubulin
c
vascular density (%)
Expression of Ninjurin1 (%)
postnatal day

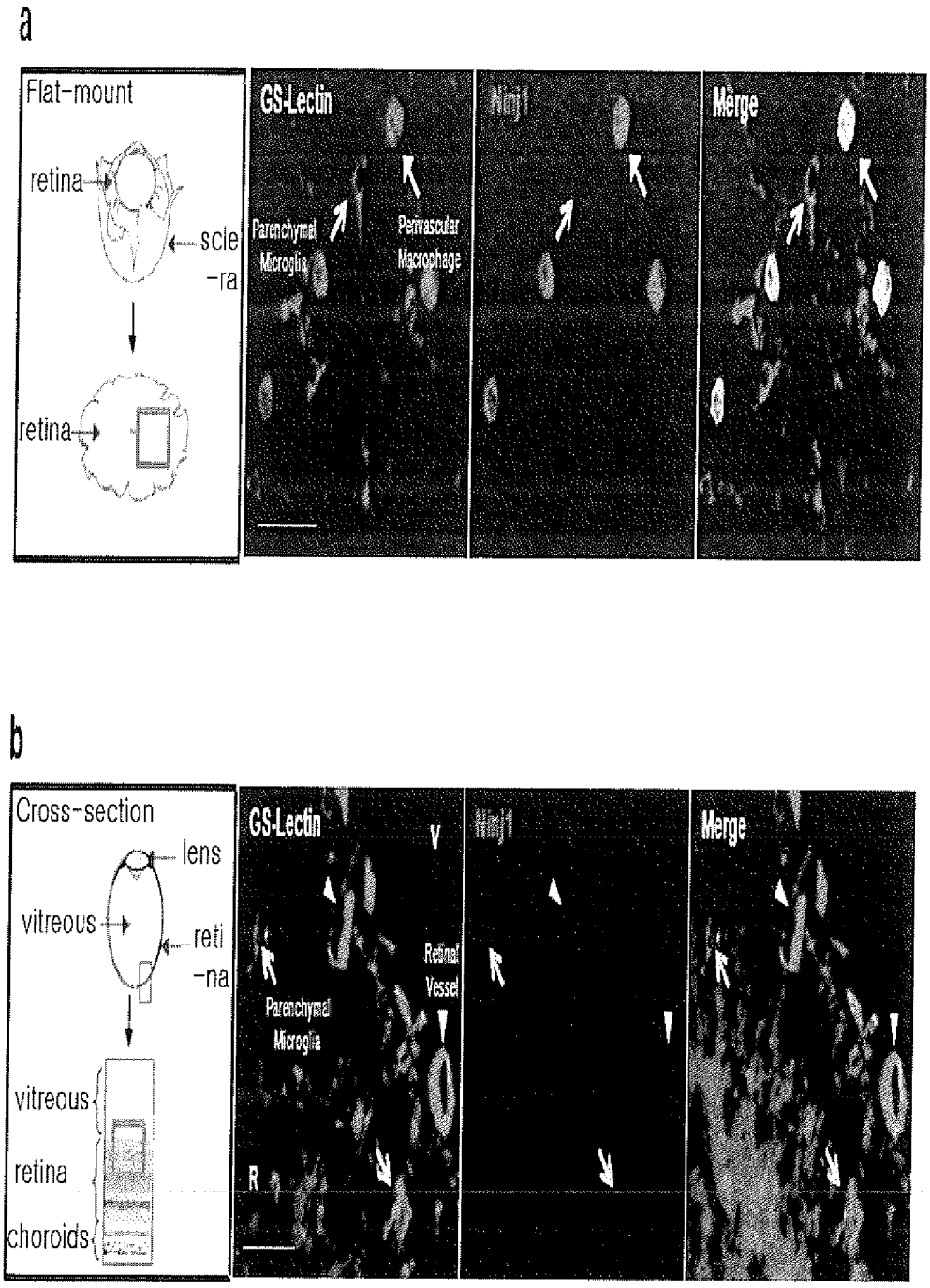
[Fig. 2]


a
Ninj1
PI
Merge
P1
P5
P14
V
R
b
DIC
Ninjurin1
Merge
Lens
Vitreous
Retina
c
Ninj1
VE-cadherin
Merge

[Fig. 3]

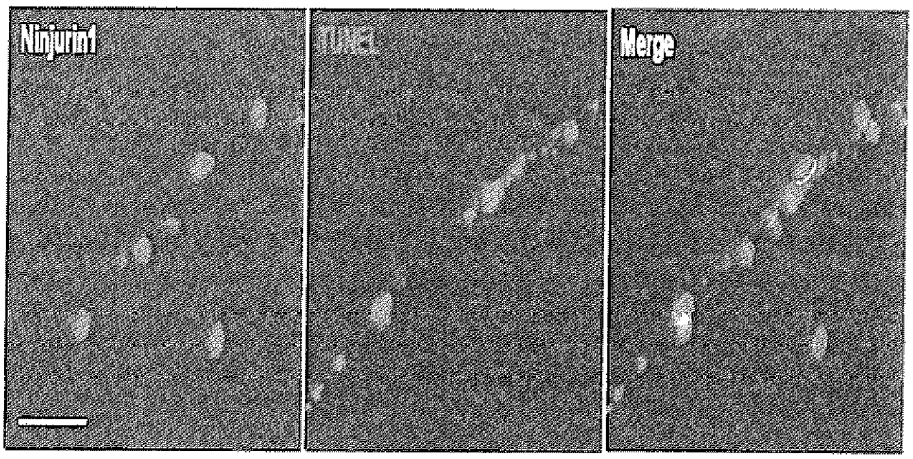

a
GS_lectin
Ninj1
Merge
b
Ninj1 conA
Merge
Ninj1 F4/80
Merge
Ninj1 Iba-1
Merge
c
Ninj1
NG2

[Fig. 4]

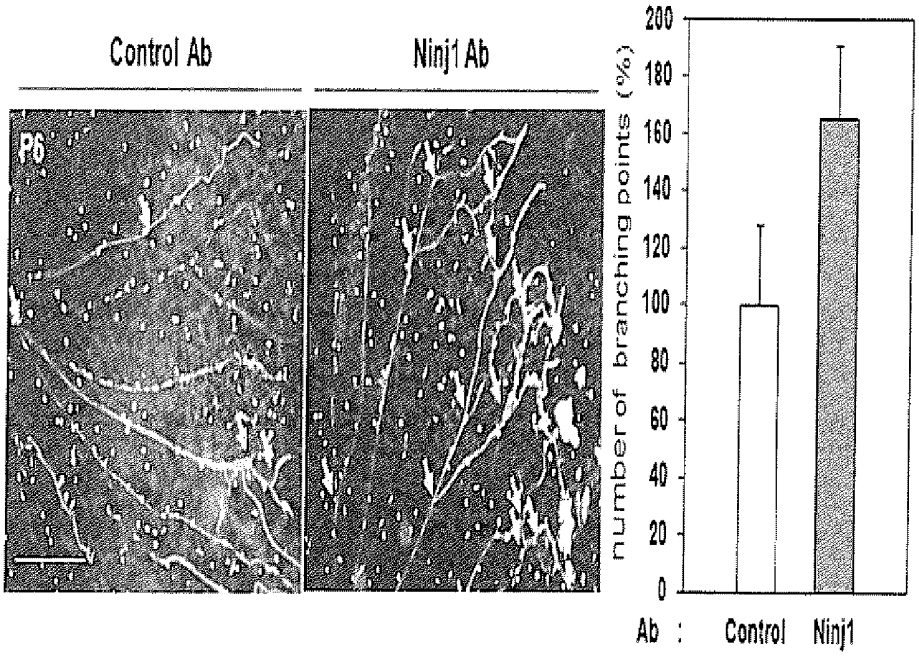

a
Flat-mount
retina
scle
-ra
retina
GS-Lectin
Ninj1
Merge
Parenchymal Microglia
Perivascular Macrophage
b
Cross-section
lens
vitreous
reti
-na
vitreous
retina
choroids
GS-Lectin
Ninj1
Merge
V
Retinal Vessel
Parenchymal Microglia
R

[Fig. 5]

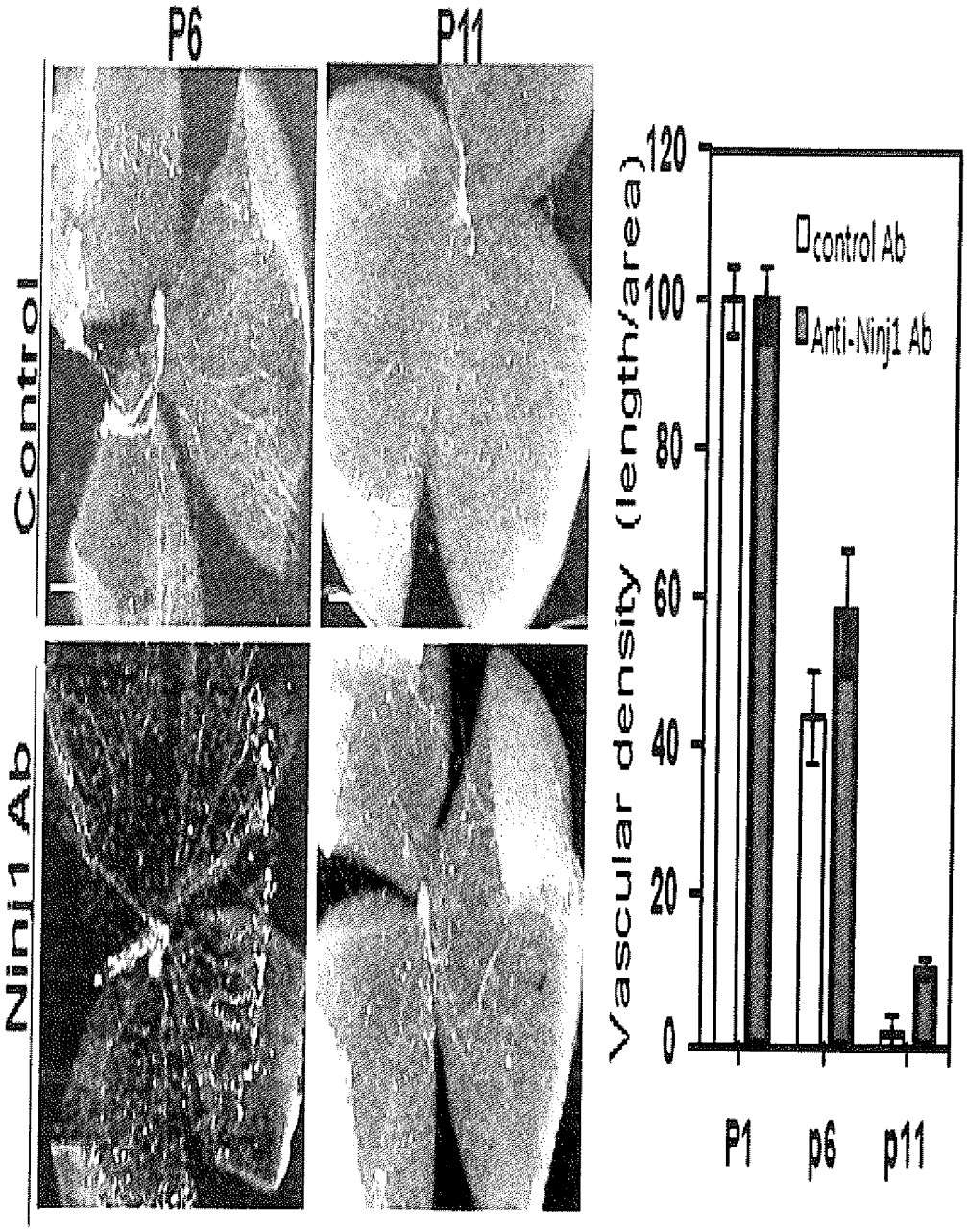
a


Ninjurin1
TUNEL
Merge

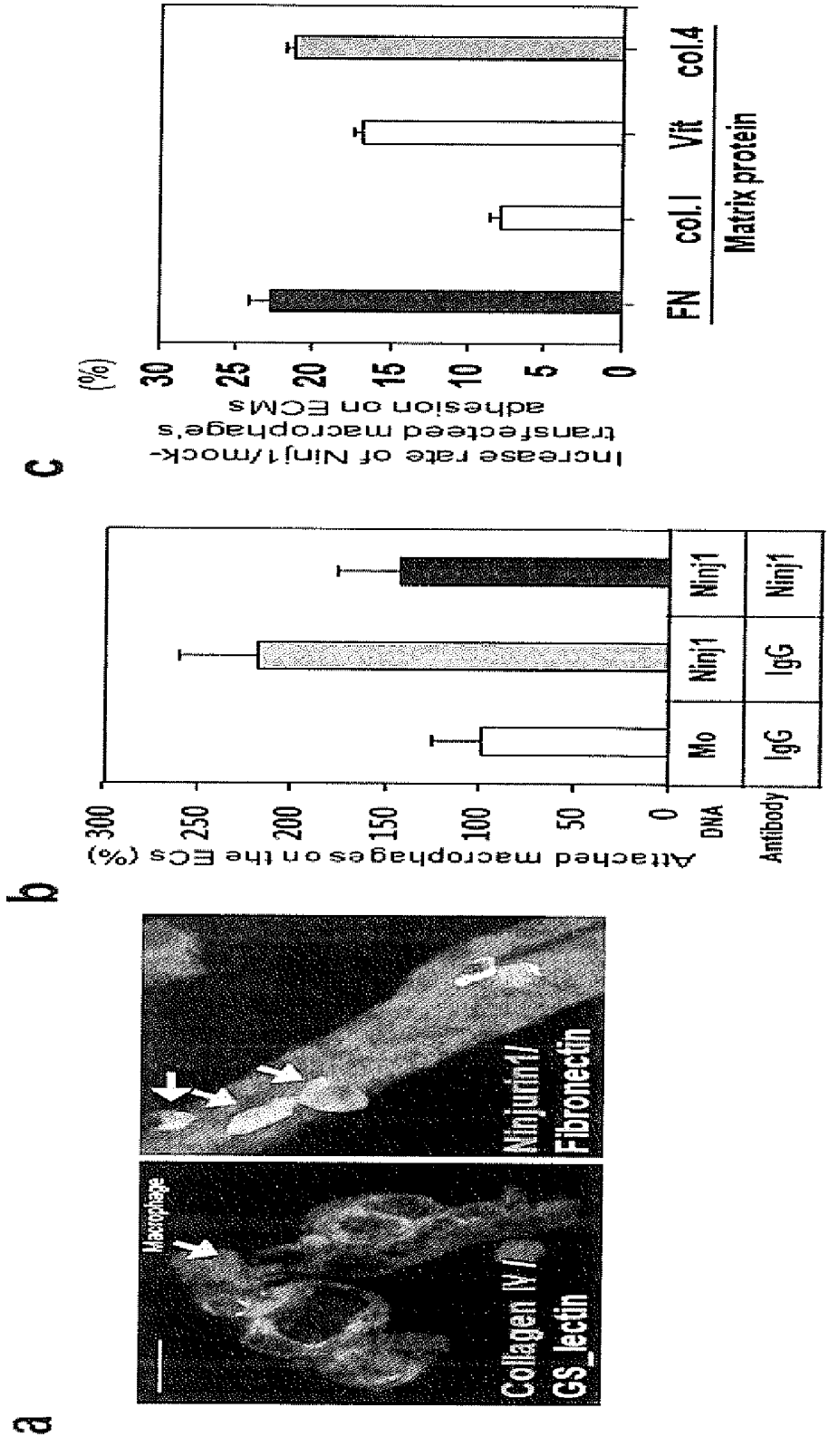
b


Control Ab
Ninj1 Ab
P6
number of branching points (%)
200
180
160
140
120
100
80
60
40
20
0
Ab :
Control
Ninj1

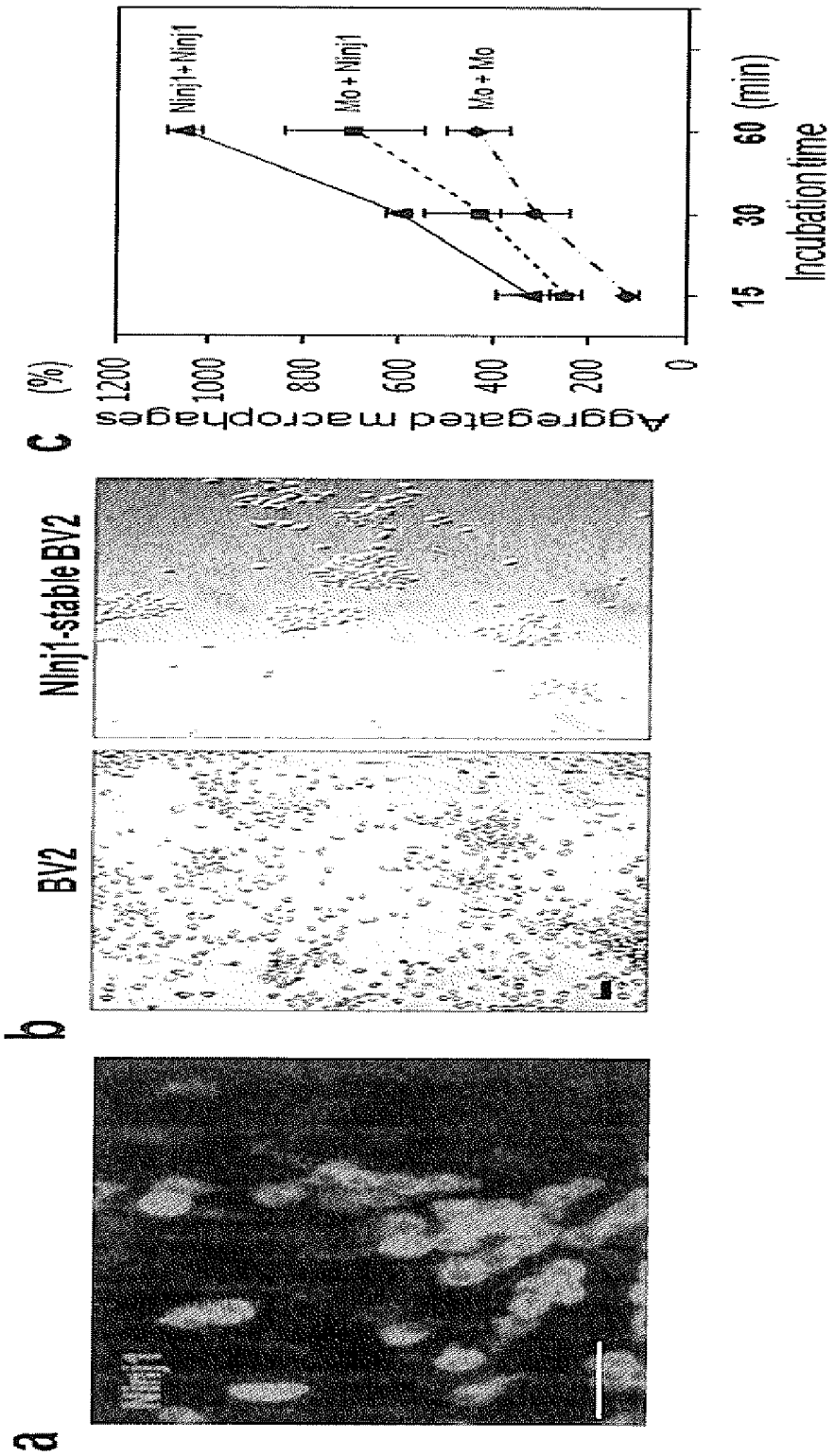
[Fig. 6]


P6
P11
Control
Ninj1 Ab
Vascular density (length/area)
120
100
80
60
40
20
0
control Ab
Anti-Ninj1 Ab
P1
p6
p11

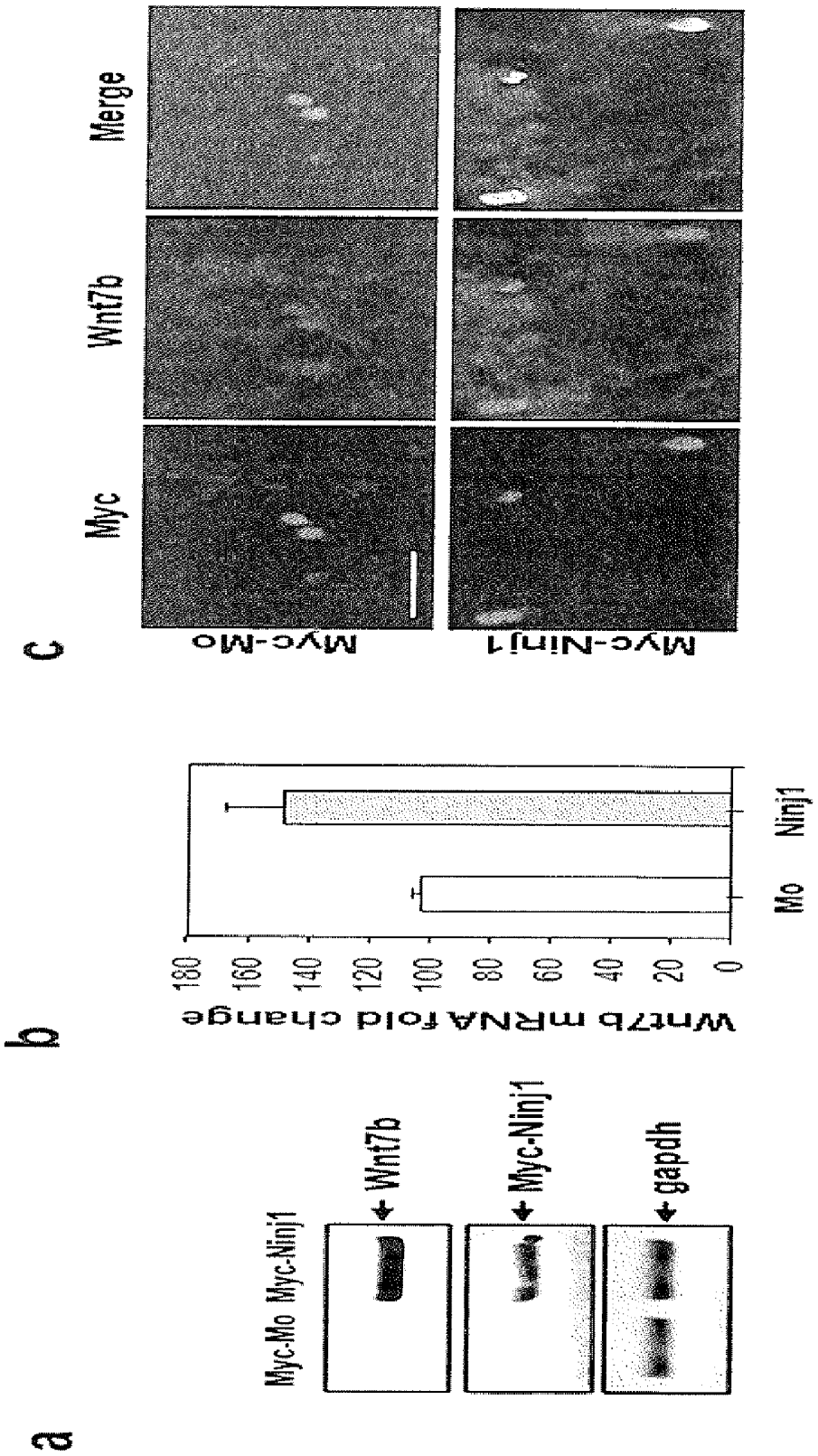
[Fig. 7]


a
Collagen IV / GS_lectin
Ninjurin1/ Fibronectin
b
Attached macrophages on the ECs (%)
300
250
200
150
100
50
0
DNA
Antibody
Mo
IgG
Ninj1
Ninj1
IgG
Ninj1
c
Increase rate of Ninj1/mock-transfectced macrophage's adhesion on ECMs
(%)
30
25
20
15
10
5
0
FN
col.I
Vit
col.4
Matrix protein

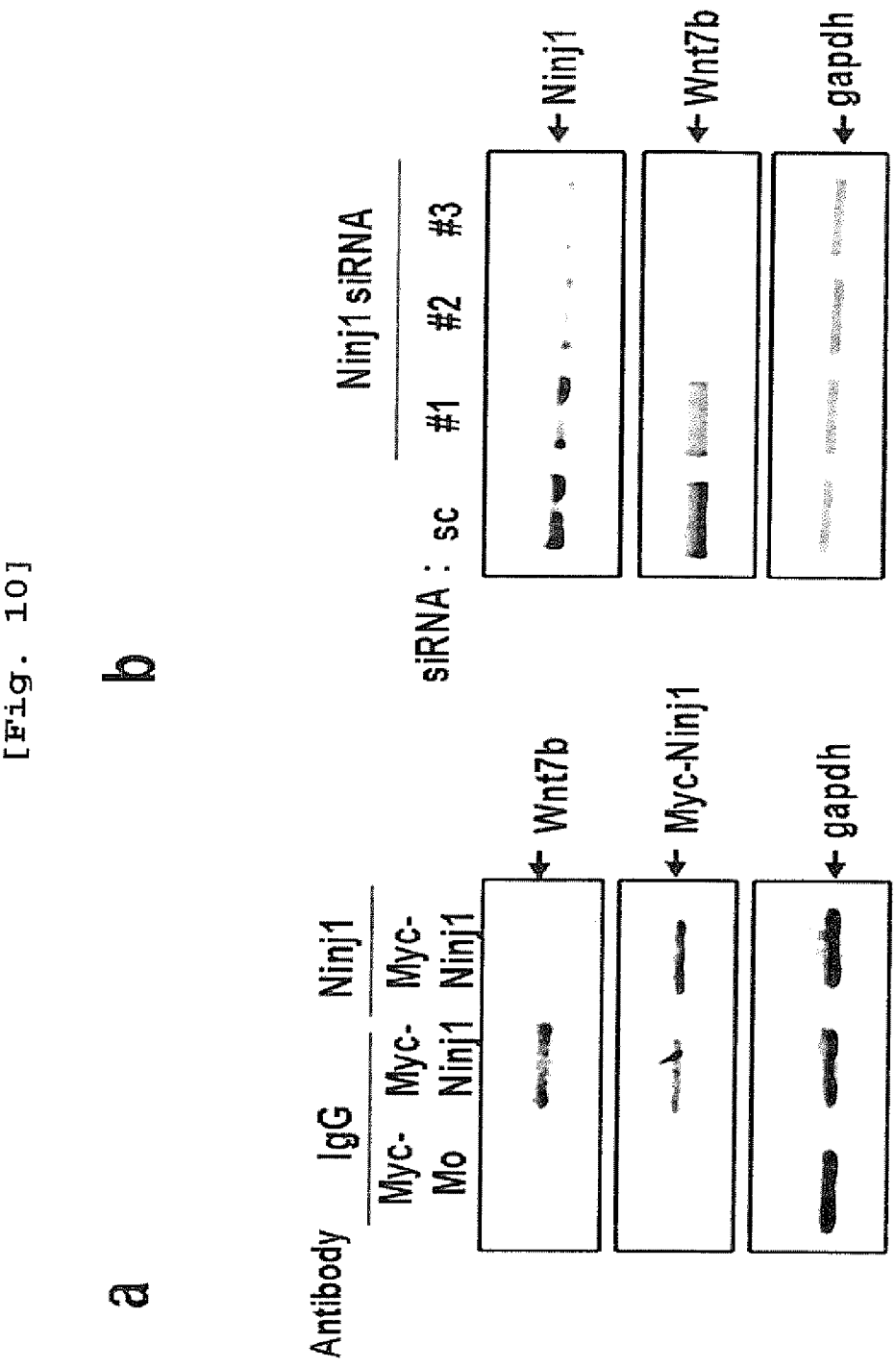
[Fig. 8]


a
Ninj1
b
BV2
Ninj1-stable BV2
c
Aggregated macrophages (%)
1200
1000
800
600
400
200
0
15
30
60 (min)
Incubation time
Ninj1+Ninj1
Mo+Ninj1
Mo+Mo

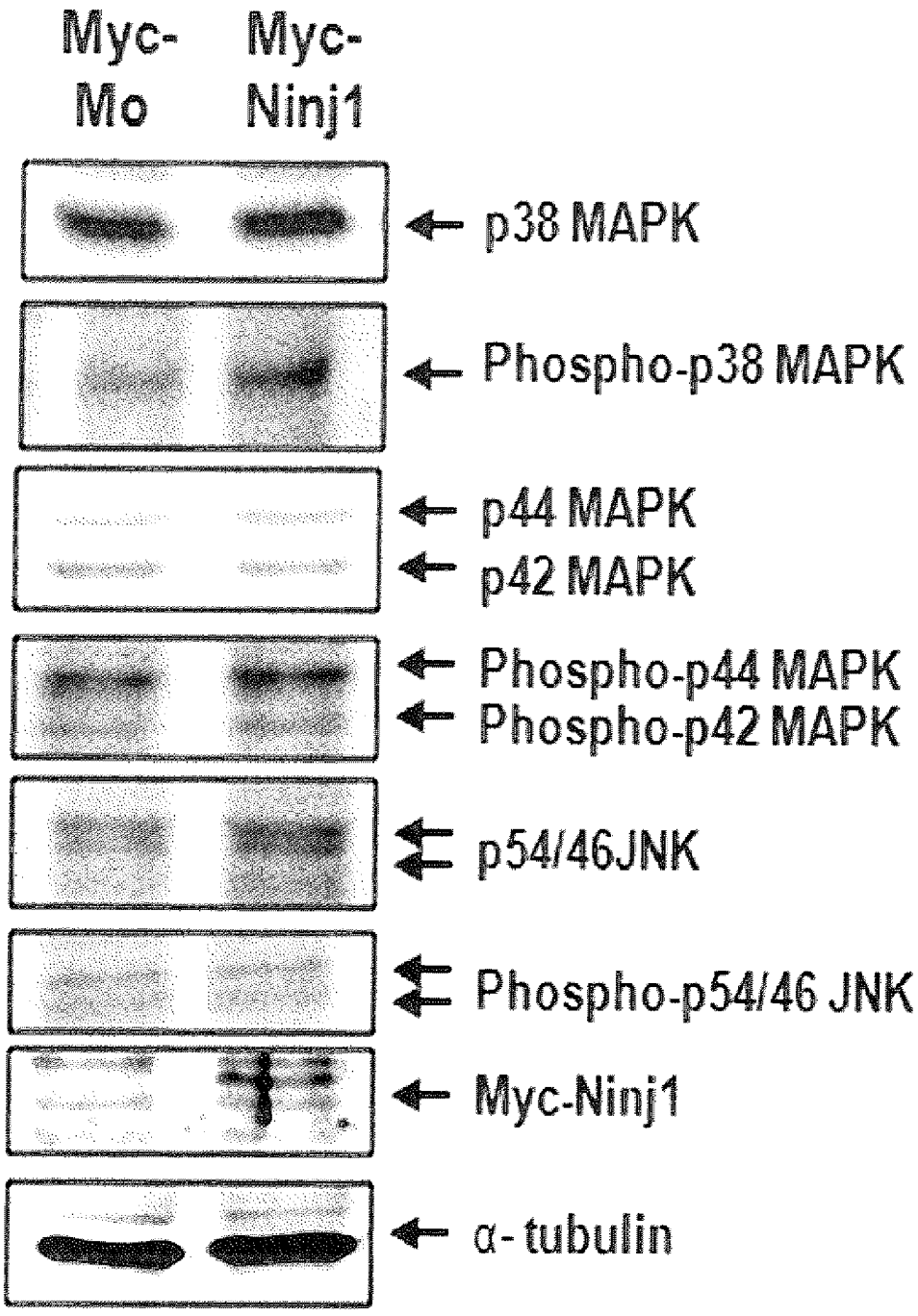
[Fig. 9]


a
Myc-Mo Myc-Ninj1
Wnt7b
Myc-Ninj1
gapdh
b
Wnt7b mRNA fold change
180
160
140
120
100
80
60
40
20
0
Mo
Ninj1
c
Myc
Wnt7b
Merge
Myc-Mo
Myc-Ninj1

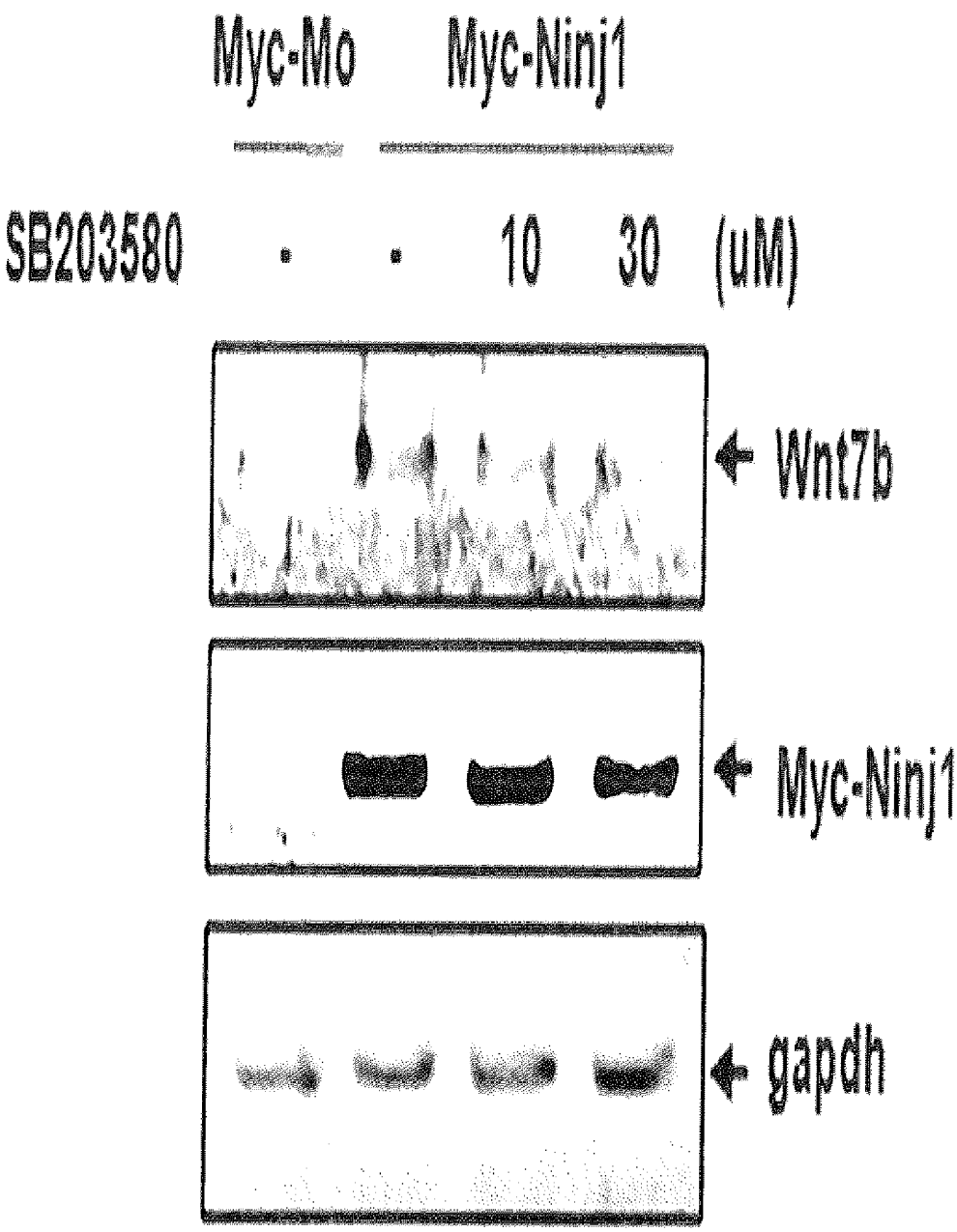
[Fig. 10]


a
Antibody
IgG
Myc-Mo
Myc-Ninj1
Ninj1
Myc-Ninj1
Wnt7b
Myc-Ninj1
gapdh
b
Ninj1 siRNA
siRNA : sc
#1
#2
#3
Ninj1
Wnt7b
gapdh

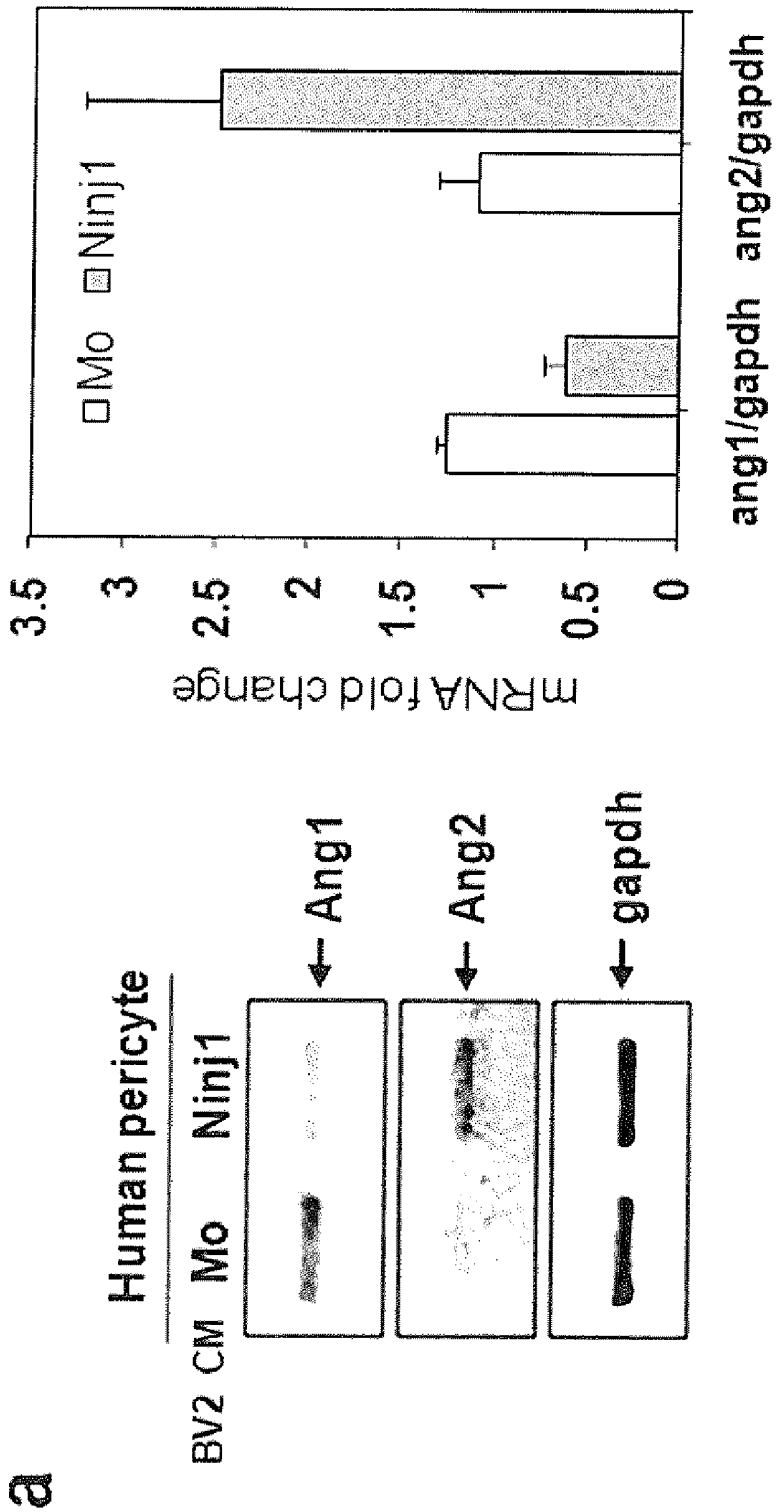
[Fig. 11]


Myc-Mo
Myc-Ninj1
p38 MAPK
Phospho-p38 MAPK
p44 MAPK
p42 MAPK
Phospho-p44 MAPK
Phospho-p42 MAPK
p54/46JNK
Phospho-p54/46 JNK
Myc-Ninj1
α-tubulin

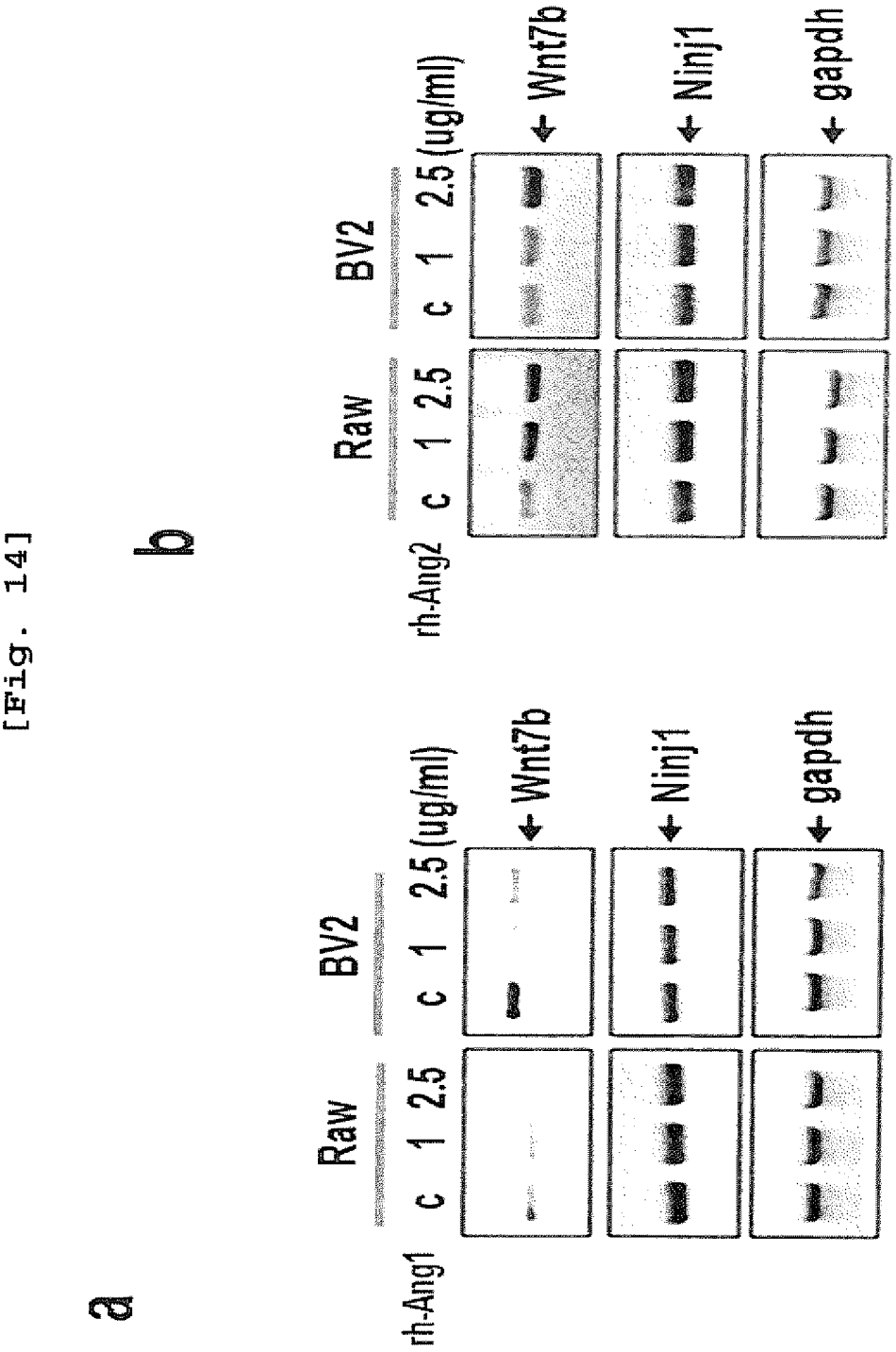
[Fig. 12]



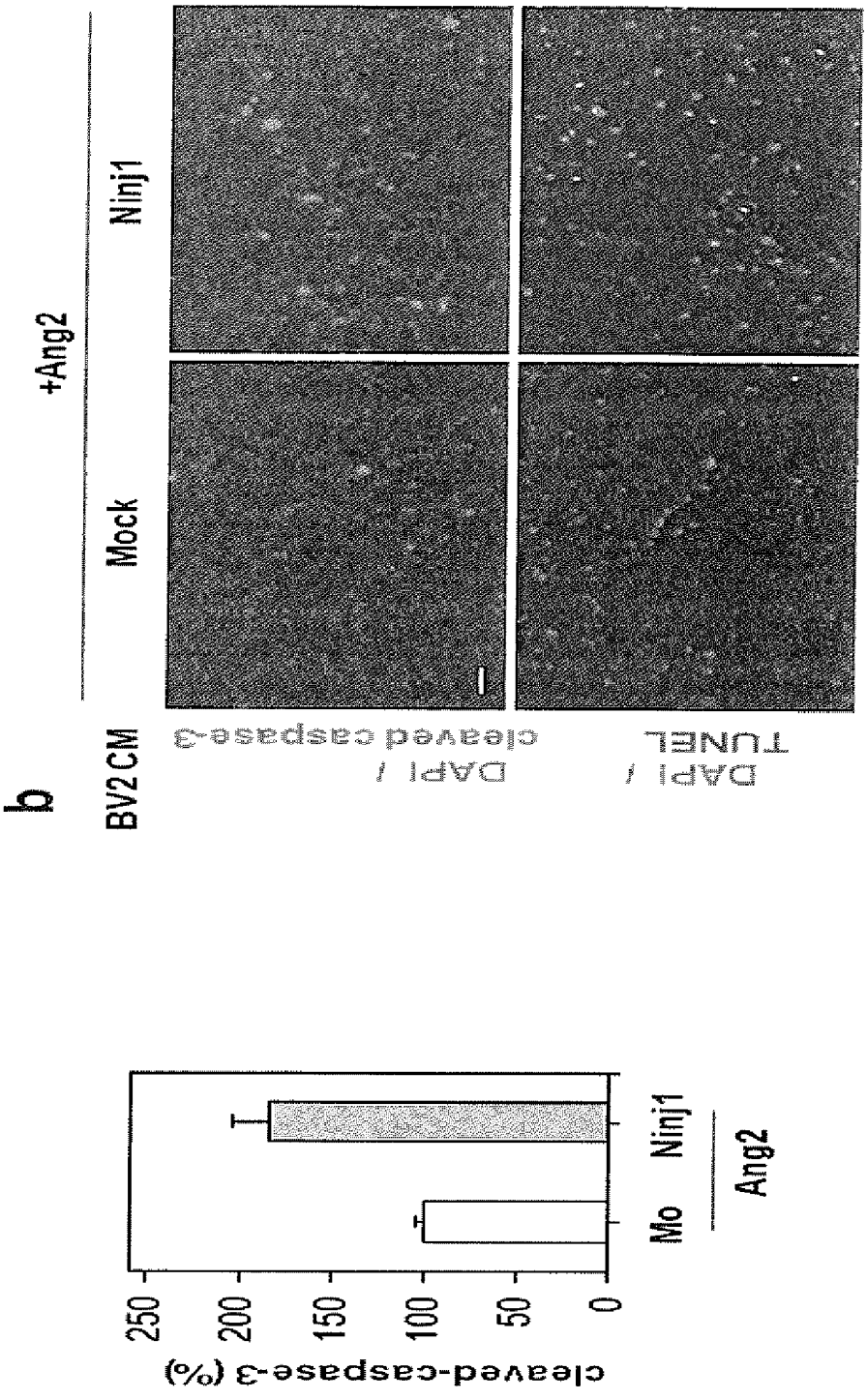
[Fig. 13]


a
Human pericyte
BV2 CM Mo Ninj1
Ang1
Ang2
gapdh
b
mRNA fold change
3.5
3
2.5
2
1.5
1
0.5
0
Mo
Ninj1
ang1/gapdh
ang2/gapdh

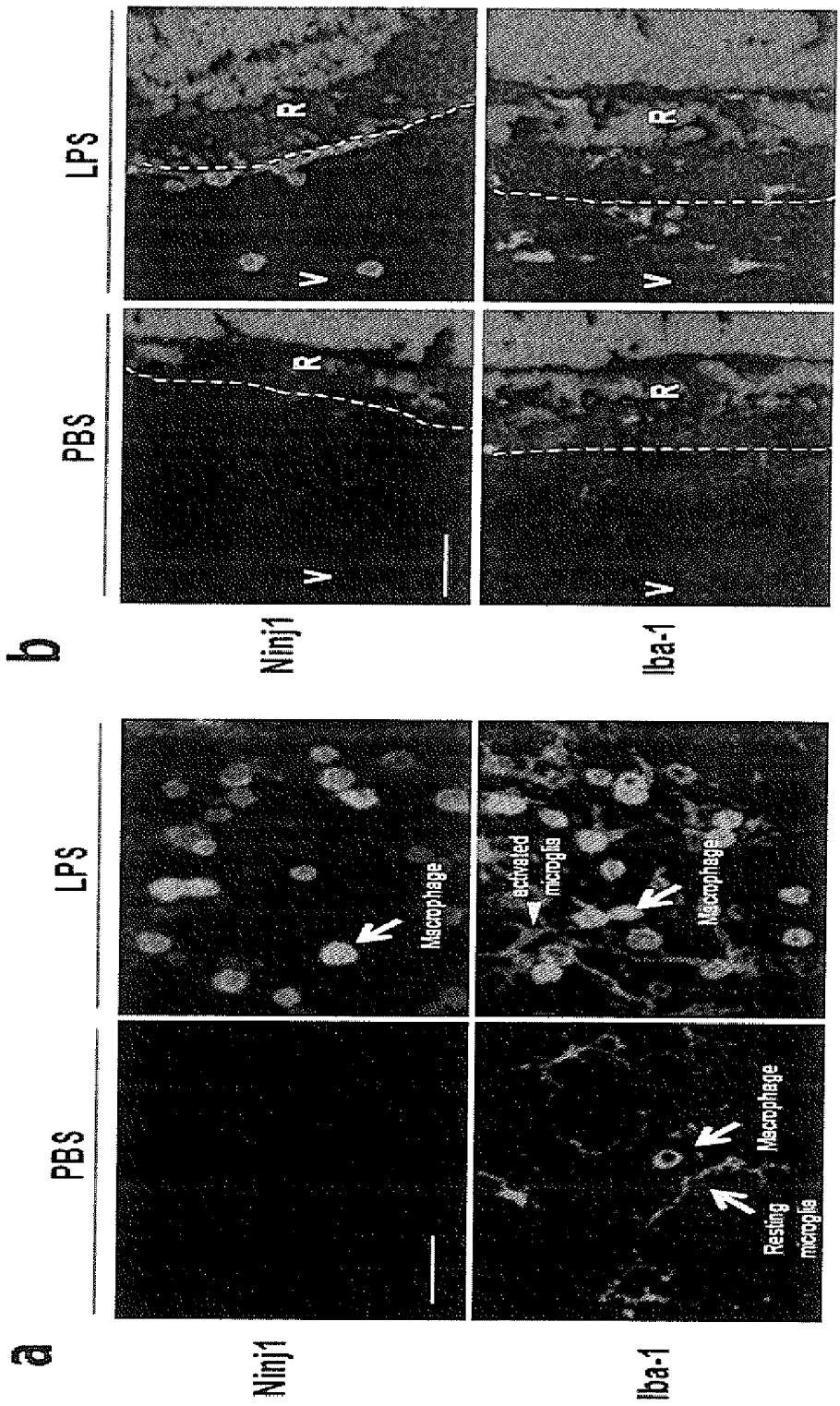
[Fig. 14]



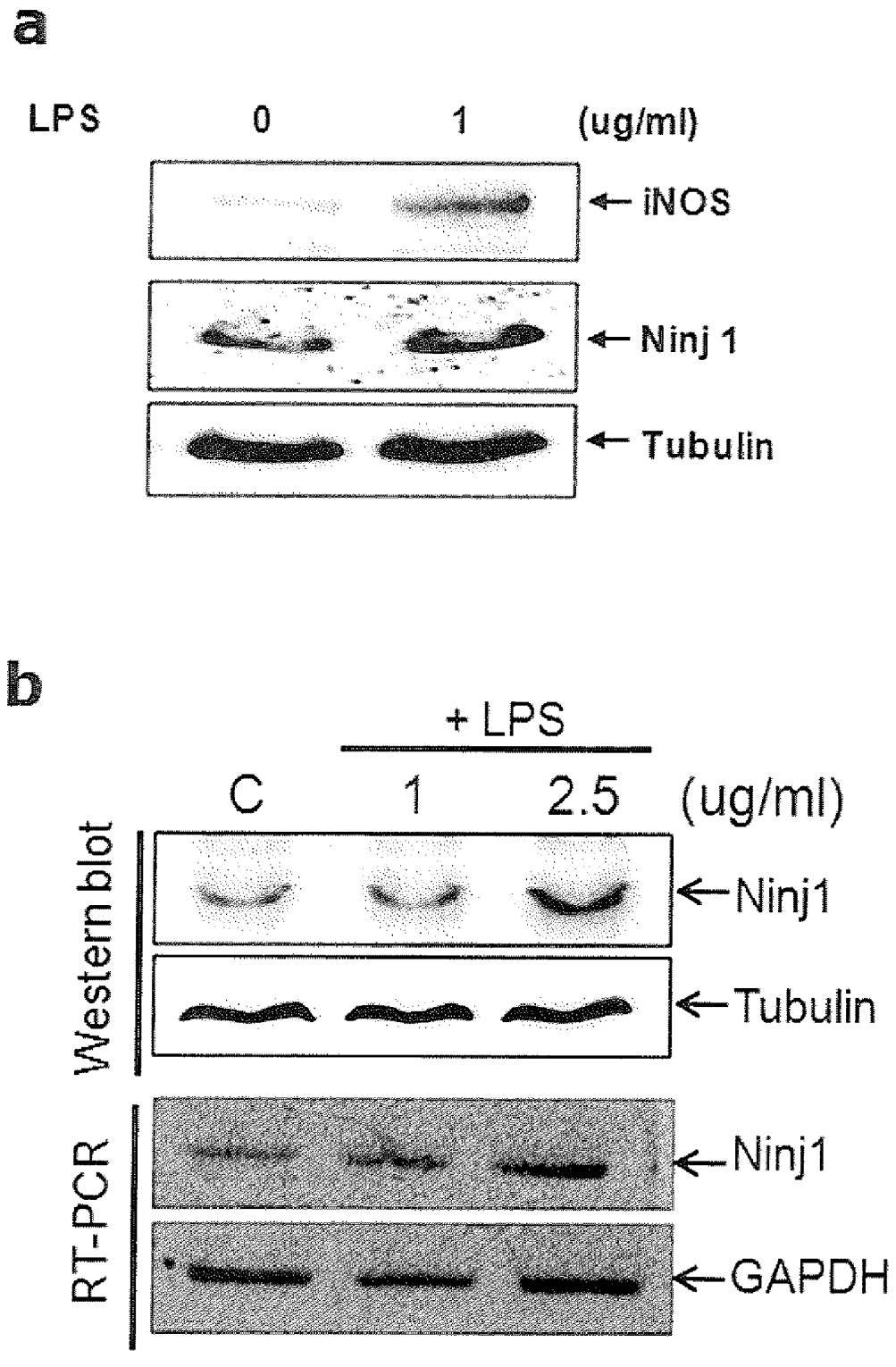
[Fig. 15]


a
cleaved-caspase-3 (%)
250
200
150
100
50
0
Mo
Ninj1
Ang2
b
BV2CM
Mock
Ninj1
+Ang2
cleaved caspase-3
DAPI /
TUNEL
DAPI /

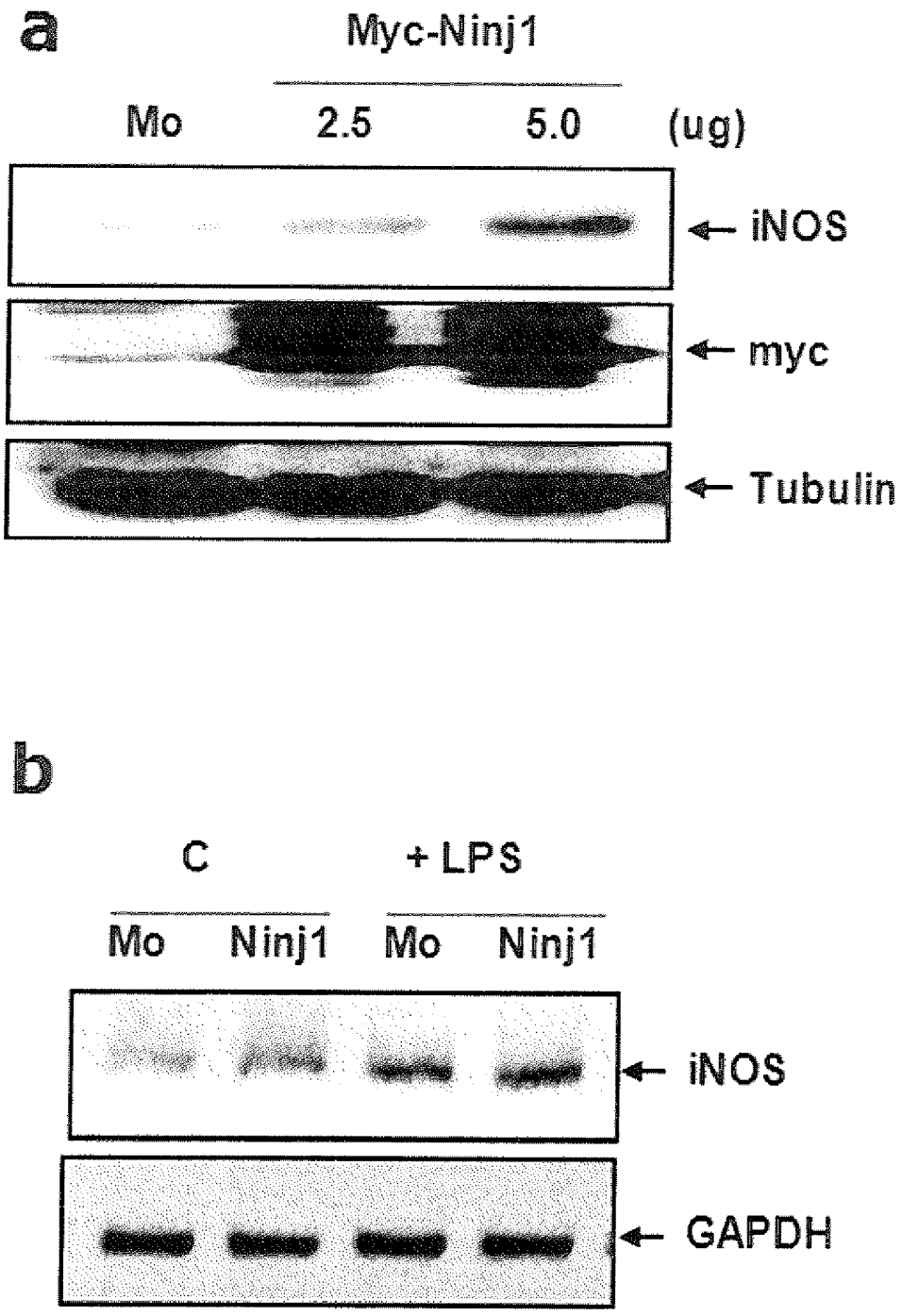
[Fig. 16]


a
PBS
LPS
Ninj1
Iba-1
Macrophage
activated microglia
Resting microglia
b
PBS
LPS
Ninj1
Iba-1
R
V

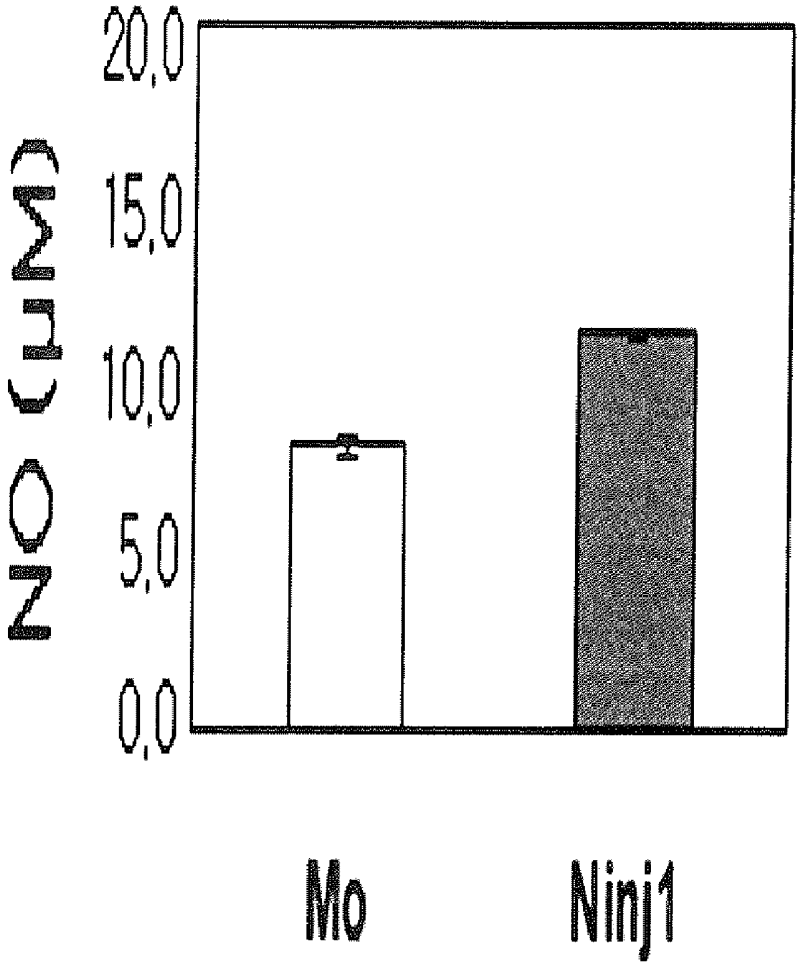
[Fig. 17]


a
LPS
0
1
(ug/ml)
iNOS
Ninj 1
Tubulin
b
+ LPS
C
1
2.5
(ug/ml)
Western blot
Ninj1
Tubulin
RT-PCR
Ninj1
GAPDH

[Fig. 18]


a
Myc-Ninj1
Mo
2.5
5.0
(ug)
iNOS
myc
Tubulin
b
C
+ LPS
Mo
Ninj1
Mo
Ninj1
iNOS
GAPDH

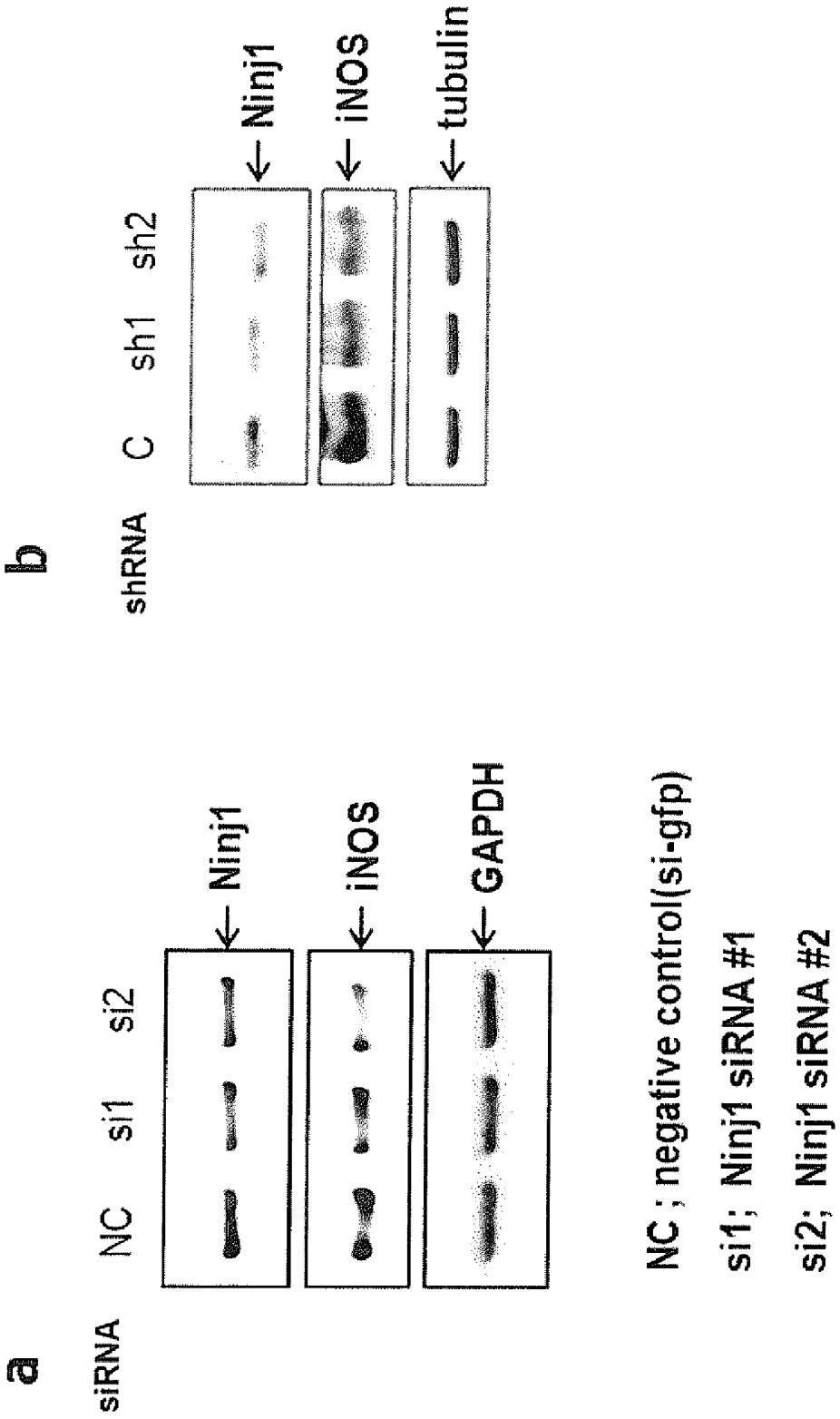
[Fig. 19]



Mo ; pCS2+myc DNA

Ninj1(Myc-Ninj1) ; pCS2+myc-Ninj1 DNA

[Fig. 20]


a
siRNA
NC
si1
si2
Ninj1
iNOS
GAPDH
NC ; negative control(si-gfp)
si1; Ninj1 siRNA #1
si2; Ninj1 siRNA #2
b
shRNA
C
sh1
sh2
Ninj1
iNOS
tubulin

COMPOSITION COMPRISING EXPRESSION OR ACTIVITY INHIBITORS OF NINJURIN 1 FOR THE PREVENTION AND TREATMENT OF INFLAMMATORY DISEASE

TECHNICAL FIELD

[0001] The present invention relates to a composition comprising a Ninjurin 1 expression or activity inhibitor for the prevention and treatment of inflammatory disease. Particularly, Ninjurin 1 protein specifically expressed in macrophages around blood vessels interacts with vascular endothelial cells to induce apoptosis of the cells via Wnt7b-Ang signal transduction pathway, increases iNOS expression and promotes NO generation to induce inflammation at last. And the present invention relates to a composition comprising such a Ninjurin 1 expression or activity inhibitor as an active ingredient for the prevention and treatment of inflammatory disease.

BACKGROUND ART

[0002] Ninjurin 1 was first reported by Toshiyuki Araki et al in 1996 (Araki, T. & Milbrandt, J., *Neuron* 17, 353-361, 1996). It was found during the screening of a gene up-regulated in Schwann cells after damage caused by transection or crush in sciatic nerve.

[0003] Phylogenetic tree was made with proteins having homology with Ninjurin based on protein information in GeneBank (Zhang, S. et al., *Genes & development* 20, 1899-1910, 2006). It was reported that vertebrate have two Ninjurin proteins, Ninjurin 1 and Ninjurin 2. In fact, Ninjurin 1 and Ninjurin 2 were found in vertebrate such as human, mouse and rat. Ninjurin identified in invertebrate is classified into three types, A, B, and C, and specifically identified in *drosophila*, mosquito, and so on. Human Ninjurin 1 shows 90% homology with mouse Ninjurin 1 (Chadwick, B. P. et al., *Genomics* 47, 58-63, 1998). In the meantime, human Ninjurin 1 shows 55% homology with human Ninjurin 2 (Araki, T. & Milbrandt, J., *J Neurosci* 20, 187-195, 2000).

[0004] Human Ninjurin 1 is located at chromosome 9q22 and is composed of 152 amino acids. Mouse Ninjurin 1, in the meantime, is located at chromosome 13 and is composed of 152 amino acids. Two transmembrane domains are predicted in the amino acid sequence of Ninjurin 1. It was also confirmed by experiments that Ninjurin 1 is the protein located in cell membrane (Araki, T. & Milbrandt, J., *Neuron* 17, 353-361, 1996). Accordingly, it can be predicted that N-terminal region of Ninjurin 1 is stretched long out of cell.

[0005] Ninjurin 1 is expressed in diverse tissues. For example, it is expressed at RNA level in the heart, brain, placenta, lung, liver, SK. Muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small int., colon, blood, adrenal gland and dorsal root ganglia (DRG). It is expressed at protein level in the liver, kidney, thymus, uterus, adrenal gland, retina and dorsal root ganglia (Araki, T. et al., *The Journal of biological chemistry* 272, 21373-21380, 1997).

[0006] The functions of Ninjurin 1 known so far are in relation to 1) cell adhesion, 2) neurite outgrowth, 3) cellular senescence, and 4) cancer.

[0007] Particularly, regarding the function of Ninjurin 1 in relation to cell adhesion 1), it was reported via cell aggregation experiment performed with Jurkat T cell leukemia that Ninjurin 1 increased aggregation among cells (Araki, T. & Milbrandt, J., *Neuron* 17, 353-361, 1996). For the cell adhesion, polymerization of actin filaments, oxidative phosphorylation, divalent cation and proper pH (pH 7-11) are required (Araki, T. et al., *The Journal of biological chemistry* 272, 21373-21380, 1997). In the study using *drosophila*, Ninjurin A protein digested with MMP1 (matrix metalloproteinase 1) acted as a signal molecule to inhibit cell adhesion (Zhang, S. et al., *Genes & development* 20, 1899-1910, 2006).

[0008] To investigate the function of Ninjurin 1 in relation to neurite outgrowth 2), CHO cells were monolayer-cultured, on which DRG neurite cells were cultured. When CHO cells over-expressing Ninjurin 1 were used, neurite cell proliferation was increased (Araki, T. & Milbrandt, J., *Neuron* 17, 353-361, 1996). When the amino acid sequence ranging from the 26^{th} to the 37^{th} residue of Ninjurin 1 protein was modified, neurite cell proliferation was inhibited (Zhang, S. et al., *Genes & development* 20, 1899-1910, 2006). When DRG neurite cells and skin-derived fibroblast-like cells (FLCs) were co-cultured, when Ninjurin 1 was expressed, neurite cells were being proliferated but when Ninjurin 1 was inhibited by an antibody not to be functioning, neurite cell proliferation was inhibited (Jerregard, H. et al., *Journal of neurocytology* 30, 327-336, 2001).

[0009] Regarding cellular senescence 3), when Ninjurin 1 was over-expressed, cell cycle was arrested in G1 stage after $p21^{WAF1/Cip1}$ transcription, resulting in a significant inhibition of cell proliferation. Besides, when Ninjurin 1 was over-expressed, senescence-associated β-galactosidase activity and autofluorescence pigment were increased. Ninjurin 1 is also up-regulated in hepatocellular carcinoma tissue, suggesting that Ninjurin 1 might be involved in cellular senescence which is the target of anti-cancer treatment (Toyama, T. et al., *Journal of hepatology* 41, 637-643, 2004).

[0010] In studies of Ninjurin 1 in relation to cancer 4), Ninjurin 1 was confirmed to be up-regulated in hepatocellular carcinoma including virus infection in the liver or cirrhosis (Kim, J. W. et al., Molecules and cells 11, 151-157, 2001). Ninjurin 1 was also increased in acute lymphocytic leukemia. Ninjurin 1 was directly increased during the screening of a gene regulated by the tumor suppressing protein p53 using microarray (Kannan, K. et al., *Oncogene* 20, 2225-2234, 2001).

[0011] However, it has not been disclosed yet whether Ninjurin 1 is involved in the functions of macrophages, vascular decrease and inflammation induction.

[0012] So, the present inventors have been studied on the involvement and mechanism of Ninjurin 1 in relation to macrophages, during which the inventors confirmed that Ninjurin 1 was expressed specifically in macrophages around blood vessels, increased cell-matrix and cell-cell adhesion, increased Wnt7b (Wingless-type MMTV integration site family, member 7B) and Ang2 (angiopoietin-2) expressions, and accelerated apoptosis of vascular endothelial cells (VECs) by reducing Ang1 (angiopoietin-1) expression. Further, the present inventors completed this invention by confirming that Ninjurin 1 was up-regulated when inflammation was induced by LPS in vivo and in vitro and increased iNOS expression and NO generation and accordingly confirming that Ninjurin 1 increased the activity of macrophages to induce inflammation.

DISCLOSURE

Technical Problem

[0013] It is an object of the present invention to provide a method for preventing and treating inflammatory disease

caused by over-activation of macrophages containing the step of inhibiting the expression or activity of Ninjurin 1 protein.

Technical Solution

[0014] To achieve the above object, the present invention provides a composition comprising a Ninjurin 1 protein expression or activity inhibitor for the prevention and treatment of inflammatory disease.

[0015] The present invention also provides a method for treating inflammatory disease containing the step of administering a pharmaceutically effective dose of the said composition to a subject with inflammatory disease.

[0016] The present invention also provides a method for preventing inflammatory disease containing the step of administering a pharmaceutically effective dose of the said composition to a subject.

[0017] The present invention also provides a use of a Ninjurin 1 expression or activity inhibitor for the preparation of a composition for the prevention and treatment of inflammatory disease.

[0018] In addition, the present invention provides a screening method of a preventive and therapeutic agent for inflammatory disease comprising the following steps:

[0019] 1) treating samples to a cell line expressing Ninjurin 1 protein;

[0020] 2) measuring the expression of Ninjurin 1 protein in the cell line; and

[0021] 3) selecting a sample that inhibited the expression of Ninjurin 1 protein, compared with the expression level in the control.

[0022] Hereinafter, the present invention is described in detail.

[0023] The present invention provides a composition comprising a Ninjurin 1 protein expression or activity inhibitor for the prevention and treatment of inflammatory disease.

[0024] The said Ninjurin 1 protein preferably has the amino acid sequence represented by SEQ. ID. NO: 1, but not always limited thereto.

[0025] The Ninjurin 1 protein expression inhibitor is preferably selected from the group consisting of an antisense nucleotide complementarily binding to Ninjurin 1 mRNA, short interfering RNA and short hairpin RNA, but not always limited thereto.

[0026] The Ninjurin 1 protein activity inhibitor is preferably selected from the group consisting of a compound complementarily binding to Ninjurin 1 protein, a peptide, a peptide mimetix and an antibody, but not always limited thereto.

[0027] The inflammatory disease herein is preferably selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis, but not always limited thereto and any inflammatory disease caused by over-activation of macrophages can be included.

[0028] In this invention, to investigate the relation of Ninjurin 1 with macrophages, mice at different days after birth were prepared and Ninjurin 1 protein expression and location where Ninjurin 1 protein was expressed particularly in oculus were investigated under immunofluorescence microscope and by Western blotting during ocular development stage. As a result, Ninjurin 1 was largely expressed in the early ocular development stage and specifically in macrophages around blood vessels (see FIGS. **1**-**4**). The above result indicates that Ninjurin 1 is expressed specifically in macrophages and is involved in the interaction of macrophages with blood vessel cells.

[0029] In this invention, to investigate the effect of Ninjurin 1 on vascular endothelial cells, oculus was extracted respectively from a mouse at some days after birth and a mouse where Ninjurin was neutralized by a Ninjurin 1 antibody. Then, the locations and amounts of macrophages and vascular endothelial cells in the oculus were observed under immunofluorescence microscope. As a result, macrophages expressing Ninjurin 1 were adhered on vascular endothelial cells which were being through apoptosis, and the level of vitreous vascular endothelial cells in the mouse neutralized by a Ninjurin 1 antibody was higher than that in the control (see FIGS. **5** and **6**). The above results indicate that macrophages expressing Ninjurin 1 are involved in apoptosis of vitreous vascular endothelial cells.

[0030] In this invention, to investigate the effect of Ninjurin 1 on cell-cell adhesion and cell-matrix adhesion, cells transformed or not transformed with Ninjurin 1 were used for the experiment examining cell adhesion to different matrixes and coagulation. As a result, over-expression of Ninjurin 1 increased macrophage-vascular endothelial cell adhesion and also increased macrophage binding to such matrixes as collagen, fibronectin and vitronectin, and at the same time increased coagulation near blood vessel cells (see FIGS. **7** and **8**). Therefore, it was confirmed that Ninjurin 1 was involved in cell-cell adhesion and cell-matrix adhesion.

[0031] In this invention, to investigate the effect of Ninjurin 1 on Wnt-Ang signal transduction system, expression patterns of Wnt7b, p38, MAPK (p44/p42), JNK (p54/46), Ang1 and Ang2 over the Ninjurin 1 expression patterns were investigated in Ninjurin 1 over-expressing cells and in Ninjurin 1 expression inhibited cells by RT-PCR and real-time PCR. As a result, when Ninjurin 1 expression was increased, expressions of Wnt7b, phosphorylated p38, MAPK (p44/p42) and JNK (p54/46) were also increased (see FIG. **9**-FIG. **13**). To investigate the relation of Ninjurin 1 and Wnt7b with Ang1 and Ang2, intracellular concentrations of Wnt7b and Ninjurin 1 were investigated in the presence of different concentrations of Ang1 or Ang2. As a result, Wnt7b was down-regulated as Ang1 concentration was increased, while Wnt7b was up-regulated as Ang2 concentration was increased (see FIG. **14**). To investigate the effect of Ninjurin 1 on apoptosis mediated by Ang2, pericytes were treated with the cell culture solution in which Ninjurin 1 was over-expressed in the presence of Ang2, followed by examining digestion by caspase 3 and apoptosis. As a result, over-expression of Ninjurin 1 increased digestion by caspase3 and apoptosis (see FIG. **15**). In conclusion, Ninjurin 1 increased the expressions of Wnt7b and Ang2 but reduced the expression of Ang1, suggesting that Ninjurin 1 induced apoptosis of vitreous vascular endothelial cells by regulating Wnt-Ang signal transduction system.

[0032] That is, Ninjurin 1 is specifically expressed in macrophages around blood vessels, so that it mediates direct interaction between macrophages and vascular endothelial cells and thereby activates Wnt-Ang pathway, resulting in the induction of apoptosis and decrease of blood vessels. Therefore, apoptosis in blood vessel cells can be inhibited by suppressing expression or activation of Ninjurin 1.

[0033] In this invention, to investigate Ninjurin 1's involvement in inflammation reaction, lipopolysaccharide (LPS) was intraperitoneally injected into a rat to induce inflammation. Then, oculus was extracted, followed by observation by

immunofluorescence staining. As a result, LPS administration increased the number of macrophages expressing Ninjurin 1 (see FIG. **16**).

[0034] In this invention, cells were treated with LPS to induce inflammation. Then, Ninjurin 1 expression and iNOS expression, the index of inflammation, were investigated and also iNOS expression patterns varying from Ninjurin 1 expression and generation of nitric oxide (NO), the inflammatory mediator, were measured. As a result, as LPS concentration was increased, Ninjurin 1 expression was also increased. And as Ninjurin 1 expression was increased, iNOS expression and NO generation were increased (see FIG. **17**-FIG. **20**). In conclusion, Ninjurin 1 was up-regulated when inflammation was induced and the increase of Ninjurin expression resulted in the increase of iNOS expression and NO generation, suggesting that Ninjurin 1 is involved in inflammation inducing mechanism.

[0035] That is, Ninjurin 1 increases iNOS and NO levels to induce inflammation. So, inflammation can be inhibited by suppressing Ninjurin 1 expression or activation.

[0036] As explained hereinbefore, Ninjurin 1 increases the activity of macrophages, and over-activation of macrophages causes diverse diseases including rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis (Morand, E. F. et al., *Intern Med J* 35, 419-426, 2005), atherosclerosis (Choudhury, R. P., et al., *Nat Clin Pract Cardiovasc Med* 2, 309-315, 2005), and multiple sclerosis (Minagar, A. et al., *J Neurol Sci* 202, 13-23, 2002), etc. Therefore, such diseases, particularly inflammatory disease caused by over-activation of macrophages can be prevented and treated by inhibiting Ninjurin 1 expression or activation.

[0037] The composition for the prevention and treatment of inflammatory disease comprising a Ninjurin 1 protein expression or activation inhibitor as an active ingredient can include the said active ingredient by 0.0001-50 weight % by the total weight of the composition.

[0038] The composition of the present invention can include one or more effective ingredients having the same or similar function to the Ninjurin 1 protein expression or activation inhibitor.

[0039] The composition of the present invention can include one or more pharmaceutically acceptable carriers such as saline, sterilized water, Ringer's solution, buffered saline, dextrose solution, maltodextrin solution, glycerol, ethanol, liposome and a mixture comprising one or more of those components in addition to the said active ingredient. If necessary, a general additive such as an antioxidant and buffer can be additionally added. The composition of the present invention can be formulated in different forms including aqueous solutions, suspensions and emulsions for injection, pills, capsules, granules or tablets by mixing with diluents, dispersing agents, surfactants, binders and lubricants. A target organ specific antibody or other ligands can be mixed with one of the said carriers to be delivered to the target organ. The composition can further be prepared in suitable forms according to ingredients by following the method represented in Remington's Pharmaceutical Science (the newest edition), Mack Publishing Company, Easton Pa.

[0040] Nucleotide or nucleic acid used in this invention can be formulated for oral, local, parenteral, intranasal, intravenous, intramuscular, hypodermic, ophthalmic or transdermal administration. It is more preferred to prepare nucleic acid or vector as an injectable formulation. For direct injection, the injectable composition can be mixed with a pharmaceutically acceptable carrier. The composition of the present invention can also include a freeze-dried composition facilitating injection using sterilized isotonic solution or distilled water or saline. Direct injection of nucleic acid into a tumor of a patient brings the effect of focusing the treatment effect on infected tissues, which favors the treatment. Dosage of the nucleic acid can be regulated according to diverse parameters, particularly a gene or a vector, administration method, target disease and required treatment period, etc. in addition, weight, age, gender, health condition, administration times, administration method, excretion and severity of a disease. The preferable dosage is 0.0001~100 mg/kg per day and more preferably 0.001~10 mg/kg per day, and administration frequency is once a day or preferably a few times a day.

[0041] The present invention also provides a method for treating inflammatory disease containing the step of administering a pharmaceutically effective dose of the said composition to a subject with inflammatory disease.

[0042] The present invention also provides a method for preventing inflammatory disease containing the step of administering a pharmaceutically effective dose of the said composition to a subject.

[0043] The inflammatory disease herein is preferably selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis, but not always limited thereto and any inflammatory disease caused by over-activation of macrophages can be included.

[0044] The composition of the present invention can include one or more effective ingredients having the same or similar function to the Ninjurin 1 protein expression or activation inhibitor.

[0045] The composition of the present invention can be administered orally or parenterally and be used in general forms of pharmaceutical formulation.

[0046] The composition of the present invention can be prepared for oral or parenteral administration by mixing with generally used diluents or excipients such as fillers, extenders, binders, wetting agents, disintegrating agents and surfactant. Formulations for parenteral administration are sterilized aqueous solutions, water-insoluble excipients, suspensions, emulsions, lyophilized preparations, suppositories and injections. Water insoluble excipients and suspensions can contain, in addition to the active compound or compounds, propylene glycol, polyethylene glycol, vegetable oil like olive oil, injectable ester like ethylolate, etc. Suppositories can contain, in addition to the active compound or compounds, witepsol, macrogol, tween 61, cacao butter, laurin butter, glycerogelatin, etc.

[0047] The effective dosage of the composition can be determined according to weight, age, gender, health condition, diet, administration frequency, administration method, excretion and severity of a disease. The preferable dosage is 0.0001~100 mg/kg per day and more preferably 0.001~10 mg/kg per day, and administration frequency is once a day or preferably a few times a day.

[0048] The composition of the present invention can be administered alone or treated together with surgical operation, hormone therapy, chemo-therapy and biological regulators.

[0049] The present invention also provides a use of a Ninjurin 1 expression or activity inhibitor for the preparation of a composition for the prevention and treatment of inflammatory disease.

[0050] The said Ninjurin 1 protein preferably has the amino acid sequence represented by SEQ. ID. NO: 1, but not always limited thereto.

[0051] The Ninjurin 1 protein expression inhibitor is preferably selected from the group consisting of an antisense nucleotide complementarily binding to Ninjurin 1 mRNA, short interfering RNA and short hairpin RNA, but not always limited thereto.

[0052] The Ninjurin 1 protein activity inhibitor is preferably selected from the group consisting of a compound complementarily binding to Ninjurin 1 protein, a peptide, a peptide mimetix and an antibody, but not always limited thereto.

[0053] The inflammatory disease herein is preferably selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis, but not always limited thereto and any inflammatory disease caused by over-activation of macrophages can be included.

[0054] Ninjurin 1 protein of the present invention is expressed specifically in macrophages around blood vessels, increases cell-cell adhesion and cell-matrix adhesion, increases the expressions of Wnt7b and Ang2, reduces Ang1 expression and accelerates apoptosis of vascular endothelial cells. Ninjurin 1 protein is up-regulated when inflammation is induced, and then it increases iNOS expression and NO generation. Therefore, a Ninjurin 1 protein expression or activation inhibitor can be effectively used as an active ingredient of a composition for the prevention and treatment of inflammatory disease.

[0055] In addition, the present invention provides a screening method of a preventive and therapeutic agent for inflammatory disease comprising the following steps:

[0056] 1) treating samples to a cell line expressing Ninjurin 1 protein;

[0057] 2) measuring the expression of Ninjurin 1 protein in the cell line; and

[0058] 3) selecting a sample that inhibited the expression of Ninjurin 1 protein, compared with the expression level in the control.

[0059] In this method, the Ninjurin 1 protein of step 1) preferably has the amino acid sequence represented by SEQ. ID. NO: 1, but not always limited thereto.

[0060] In this method, the protein expression of step 2) is measured by one of the methods selected from the group consisting of immunofluorescence method, ELISA, Western blotting, and RT-PCR, but not always limited thereto.

[0061] In this method, the inflammatory disease of step 2) is preferably selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis, but not always limited thereto.

Advantageous Effect

[0062] Ninjurin 1 protein of the present invention is expressed specifically in macrophages around blood vessels and then mediated direct interaction between macrophages and vascular endothelial cells. By which, it activates Wnt-Ang pathway to induce apoptosis. Ninjurin 1 also increases iNOS expression and NO generation, suggesting that it is directly involved in inflammation reaction. Therefore, the said Ninjurin 1 expression or activation inhibitor can be effectively used as an active ingredient of a composition for the prevention and treatment of inflammatory disease.

DESCRIPTION OF DRAWINGS

[0063] The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

[0064] FIG. **1** is a set of diagrams illustrating the ocular development stage of a mouse and Ninjurin 1 expression over the time: (a) vitreous and incipient retinal vessel stained with GS-lectin (green) of mice at 2 days (P2), at 3 days (P3), at 5 days (P5), at 8 days (P8), and at 14 days (P14) from birth and an adult mouse; (b) graph illustrating Ninjurin 1 protein expression in oculus of those mice examined by Western blotting; (c) graph illustrating blood vessel density (▲) and Ninjurin 1 expression (●) over the ocular development in those mice (considering the value at P1 as 100%).

[0065] FIG. **2** is a set of diagrams illustrating the Ninjurin 1 expression and location in the mouse oculus: (a) diagram showing cellular location where Ninjurin 1 expression is observed in cross-sections of oculus of mice at 1 day (P1), at 5 days (P5) and at 14 days (P14) from birth. At this time, Ninjurin 1 was stained green and nucleus was stained with propidium iodide (PI) (red): V: vitreous, R: retina, white arrow: cells expressing Ninjurin 1. (b) diagram illustrating the DIC microscope imaging of the oculus, in which Ninjurin 1 was stained red; (c) diagram illustrating Ninjurin 1 (red) and VE-cadherin (green) in the oculus extracted from the mouse at 5 days (P5), observed by double immunostaining.

[0066] FIG. **3** is a set of diagrams illustrating the location of Ninjurin 1 expression around blood vessels in the mouse oculus: (a) diagram illustrating GS-lectin (green) and Ninjurin 1 (red) (if overlapped: yellow) in the oculus of the mouse at 5 days (P5), observed under fluorescent microscope after double immunostaining; (b) diagram illustrating Ninjurin 1 (red) and ConA (green) (if overlapped: yellow), Ninjurin 1 (red) and F4/80 (green) (if overlapped: yellow), and Ninjurin 1 (red) and Iba-1 (green) (if overlapped: yellow) in the oculus, observed under fluorescent microscope after double immunostaining; and (c) diagram illustrating Ninjurin 1 (green) and NG2 (red) (if overlapped: yellow) in the oculus, observed under fluorescent microscope after double immunostaining.

[0067] FIG. **4** is a set of diagrams illustrating the location of Ninjurin 1 expression and macrophages around blood vessels in the mouse oculus: (a) diagram illustrating Ninjurin 1 (red) and GS-lectin (green) in the oculus of the mouse at 5 days (P5), observed under fluorescent microscope after double immunostaining. At this time, hyaloid vessels were eliminated from the oculus using 5% gelatin. (b) diagram illustrating Ninjurin 1 (red) and parenchymal microglia (green) in the retina, observed under fluorescent microscope after double immunostaining (white arrow: retinal vessel; yellow arrow: parenchymal microglia stretching its arm).

[0068] FIG. **5** is a set of diagrams illustrating the changes of vascular endothelial cells over Ninjurin 1 expression in the mouse vitreous; (a) diagram illustrating that macrophages expressing Ninjurin 1 in vitreous of the mouse at 8 days (P8) were adhered to vascular endothelial cells being through apoptosis, which was observed under immunofluorescent microscope; and (b) diagram illustrating that vitreous of the mouse at 6 days (P6) neutralized with Ninjurin 1 antibody was stained with GS-lectin (green), followed by observation under fluorescent microscope (yellow arrow: branching point of blood vessel).

[0069] FIG. **6** is a diagram illustrating the changes of blood vessel density over Ninjurin 1 expression, in which oculus of a mouse at 6 days (P6) belonging to the control and oculus of a mouse at 6 days (P6) neutralized with a Ninjurin 1 antibody were stained with GS-lectin (green), followed by observation under fluorescent microscope.

[0070] FIG. **7** is a set of diagrams illustrating the cell-matrix adhesion over Ninjurin 1 expression: (a) diagram illustrating Type IV collage (red) and GS-lectin (green), and Ninjurin 1 (red) and fibronectin (green) in the vitreous, observed under fluorescent microscope after double immunostaining; (b) graph illustrating the cell-matrix adhesion in BV2 cells transformed with pCS2+-Ninjurin 1 and in BV2 cells transformed with pCS2+-Mock which were both neutralized by a Ninjurin 1 antibody and control IgG respectively; and (c) graph illustrating the result of investigation of adhesion of BV2 cells transformed with pCS2+-Ninjurin 1 and BV2 cells transformed with pCS2+-Mock to different matrixes (FN: fibronectin; Col. I: type I collagen; Vit: vitronectin; Col. IV: type IV collagen).

[0071] FIG. **8** is a set of diagrams illustrating the cell-cell adhesion over Ninjurin 1 expression; (a) diagram illustrating that Ninjurin 1 (red) in the vitreous of the mouse at 3 days (P3) was stained, followed by observation under fluorescent microscope; (b) diagram illustrating the coagulation of wild type BV2 cells and BV2 cells (Ninj1-stable BV2) expressing Ninjurin 1 constantly, observed after 2 day culture; and (c) graph illustrating the coagulation of cultured Mock-Ninjurin 1 expressing cell mixture.

[0072] FIG. **9** is a set of diagrams illustrating the Wnt7b expression over Ninjurin 1 expression: (a) diagram illustrating the result of RT-PCR, in which Wnt7b expressions in BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1 were shown; (b) diagram illustrating the result of real-time PCR; and (c) diagram illustrating the result of immunostaining examining the expressions of Wnt7b (green) and c-Myc (red).

[0073] FIG. **10** is a set of diagrams illustrating the Wnt7b expression over Ninjurin 1 expression: (a) diagram illustrating the result of RT-PCR investigating Wnt7b expression in BV2 cells expressing Myc-Ninjurin 1 neutralized with Ninjurin 1 antibody; and (b) diagram illustrating the result of RT-PCR examining Wnt7b expression in BV2 cells in which Ninjurin 1 was suppressed by siRNA.

[0074] FIG. **11** is a diagram illustrating the expressions of p38, MAPK (p44/p42) and JNK (p54/46) over Ninjurin 1 expression. This picture shows the result of Western blotting with cell lysate of BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1.

[0075] FIG. **12** is a diagram illustrating the Wnt7b expression according to the inhibition of p38 expression. BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1 were treated with SB203580, the p38 inhibitor, at different concentrations, followed by RT-PCR examining the expression of Wnt7b.

[0076] FIG. **13** is a diagram illustrating the expressions of Ang1 and Ang2 over Ninjurin 1 expression. Pericytes were treated with culture solutions of BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1, followed by RT-PCR (left) and real-time PCR (right) to investigate Ang1 and Ang2 expressions.

[0077] FIG. **14** is a diagram illustrating the levels of Wnt7b and Ninjurin 1 according to the concentrations of Ang1 and Ang2 in rodent originated macrophages (Raw264.7, BV2) and BV2 cells. RT-PCR was performed to quantify Wnt7b and Ninjurin 1 affected by different concentrations of recombinant human Ang1 (rh-Ang1) (left) and by different concentrations of recombinant human Ang2 (rh-Ang2) (right).

[0078] FIG. **15** is a set of diagrams illustrating the caspase 3 digestion and apoptosis by Ninjurin 1 in HUVEC cells in the presence of rh-Ang2: (a) diagram illustrating the result of Western blotting examining caspase 3 digestion in HUVEC cells treated with culture solutions of BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1; and (b) diagram illustrating the result of immunostaining showing caspase 3 (red), TUNEL (green) and nucleus (blue) in HUVEC cells treated with culture solutions of BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1.

[0079] FIG. **16** is a set of diagrams illustrating the Ninjurin 1 expression under the inflammation condition induced by intraperitoneal injection of LPS into Sprague-Dawley (SD) line SPF rat: (a) diagram illustrating the result of immunostaining of the rat oculus with Iba-1 (green). The rat was administered with LPS to induce systemic inflammation; and (b) diagram illustrating the result of immunostaining of vitreous of the oculus with Iba-1, in which Ninjurin 1 was stained red.

[0080] FIG. **17** is a set of diagrams illustrating the Ninjurin 1 expression and iNOS expression, the index of inflammation, in BV2 cells treated with LPS: (a) diagram illustrating the iNOS expression examined by Western blotting with BV2 cells treated with different concentrations of LPS; and (b) diagram illustrating the Ninjurin 1 expression examined by RT-PCR and Western blotting with BV2 cells treated with different concentrations of LPS.

[0081] FIG. **18** is a set of diagrams illustrating the iNOS expression over Ninjurin 1 expression: (a) diagram illustrating the result of Western blotting examining iNOS expressions in BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1; and (b) diagram illustrating the result of RT-PCR examining iNOS expressions in BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1.

[0082] FIG. **19** is a diagram illustrating the generation of NO, the inflammatory mediator, over Ninjurin 1 expression. NO levels in BV2 cells expressing Myc-mock and BV2 cells expressing Myc-Ninjurin 1 were quantified by using Griess reagent.

[0083] FIG. **20** is a set of diagrams illustrating the iNOS expression according to the decrease of Ninjurin 1 expression: (a) diagram illustrating the result of RT-PCR examining iNOS expression when Ninjurin 1 expression was inhibited by siRNA in BV2 cells expressing Ninjurin 1; and (b) diagram illustrating the result of RT-PCR examining iNOS expression when Ninjurin 1 expression was inhibited by shRNA in BV2 cells expressing Ninjurin 1.

MODE FOR INVENTION

[0084] Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

[0085] However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1

Preparation of Test Animals and Conditions Thereof

[0086] Specific pathogen-free (SPF) pregnant Sprague-Dawley (SD) line rats and male ICR mice/SD rats were pur-

chased from Samtaco BioKorea Co., and then maintained in an animal facility at College of Pharmacy Seoul National University under germ-free conditions during the whole experimental period. All the animal tests were approved by Institute of Laboratory Animal Resources Seoul National University (http://ilar.snu.ac.kr).

Example 2

Cell Culture and Preparation of Cell Lysate and Cell Culture Solution and Conditions Thereof

[0087] Primary cultured human microvascular pericytes (Applied Cell Biology Research Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM); Raw 264.7 cells (TIB-71™) and BV2 cells were cultured in DMEM; and HUVEC cells were cultured in M199 supplemented with 20% FBS (Invitrogen, Grand Island, N.Y.). Those cells were cultured at 37° C. in humid air composed of 95% O_2 and 5% CO_2. Raw cells and BV2 cells were transformed by using Lipofectamine Plus Reagent (Invitrogen). Cell lysate was prepared by using lysis buffer (40 mM Tris-Cl pH 8.4, 10 mM EDTA, 120 mM NaCl, 0.1% NP-40). To prepare culture solution to treat human microvascular pericytes, transformed macrophages were cultured in serum(FBS)-free DMEM for 24 hours, which were then filtered with 0.22 μm filter paper (Millipore), followed by 10 fold concentration using centrifugation filtering tube (Millipore). For the chemical treatment, cells were cultured at the density of 60-70% and then treated with Ang1 and Ang2 (500-1000 ng/ml) independently or together in serum free condition for 14-16 hours. To understand signal transduction pathway, cells were transformed with Mock or Ninjurin 1 cDNA. Serum was depleted for 14-16 hours and then the cells were treated with SB203580 (Sigma), the p38 inhibitor, for 24 hours.

Example 3

Construction of Ninjurin 1 Protein Expression Vector

[0088] Wild-type mouse Ninjurin 1 cDNA was synthesized from NIH-3T3 fibroblasts by RT-PCR. At this time, the primer represented by SEQ. ID. NO: 2 (5'-GG GAATTCCATGGAGTCGGGCACTGAGGA-3', upstream containing EcoRI restriction enzyme site) and the primer represented by SEQ. ID. NO: 3 (5'-CTC CTCGAGTTCTACTGCCGGGGCGCCACGT-3', downstream containing Xho I restriction enzyme site) were used. The synthesized cDNA was inserted in pCS2+ vector (labeled with Myc) to express the cDNA in mammalian cells.

Experimental Example 1

Ninjurin 1 Expression Over the Time During Ocular Development

[0089] The present inventors sacrificed those mice at 2 days (P2), at 3 days (P3), at 5 days (P5), at 8 days (P8), and at 14 days (P14) and adult mice prepared in Example 1 and extracted their oculus, followed by GS-lectin immunostaining to observe vitreous and incipient retinal vessel development.

[0090] Particularly, immunostaining was performed as follows. The antibodies used herein were Ninjurin 1 (1:500, provided from Dr. J. Milbrandt), VE-cadherin, GS-lectin (1:500, Santa Cruz), Iba-1 (1:500, Wako), NG2 (1:300, Chemicon) and cleaved-caspase3 (1:500, Cell Signaling). Nuclei were stained with DAPI and propidium iodide (Molecular Probes). Tissues and cells were reacted with the said primary antibody, followed by reaction with secondary antibody exemplified by Alexa488 conjugated IgG or Alexa546 conjugated IgG (Molecular Probes). Images were obtained by using Axiovert M200 microscope (Zeiss, Oberkochen, Germany), followed by analysis with NIH-image J program. Immunostaining was further performed under the same conditions.

[0091] Ninjurin 1 protein expression in each oculus of mice having different days was investigated by Western blotting.

[0092] Particularly, Western blotting was performed as follows. At this time, Ninjurin 1 (BD Phaminogen), c-Myc (Santa Cruz), α-tubulin (BioGenex), caspase-3 (Cell signaling), iNOS (Santa Cruz) specific primary antibodies were used. Anti-mouse/rabbit horseradish peroxidase conjugated secondary antibody was purchased from Pierce Chemical Co. Color development was performed by using ECL Plus reagent (Amersham Biosciences), followed by detection with LAS-3000 (Fujifilm). Recombinant Ang1 and Ang2 were purchased from R&D Systems INC. Ponceau S solution was purchased from Sigma. Western blotting was performed under the same conditions.

[0093] Analysis of results and statistics were performed as follows. Band strength was quantified by using ImageJ (http://rsb.info.nih.gov/ij/), for which stained gapdh, alpha-tubulin or ponceau S band strength was used as standard. The result was presented as mean value±standard deviation after being converted as relative percentage. The value of the protein showing the highest strength was considered as 100%. Comparison of statistics between two groups was performed by using Student's t-test. When $P<0.05$, it was judged as statistically significant. Analysis of results and statistics were performed under the same conditions.

[0094] Ninjurin 1 and propidium iodide were double stained in the cross-section oculus extracted from mice at 1 day (P1), at 5 days (P5) and at 14 days (P14). Ninjurin 1 and VE-cadherin in the oculus of the mouse at 5 days (P5) were double immuno-stained, followed by observation under fluorescent microscope.

[0095] As a result, as shown in FIG. **1** and FIG. **2**, as ocular development progressed, density of blood vessel was reduced. Ninjurin 1 protein expression was gradually increased up to 5 days from birth and the highest Ninjurin 1 expression was observed in mice at 3 days and at 5 days, but thereafter the expression was gradually decreased (FIG. **1** and FIG. **2**). The above results indicate that Ninjurin 1 protein is most expressed in the early stage of ocular development.

Experimental Example 2

Ninjurin 1 Expression Specific in Macrophages Around Blood Vessels

[0096] The present inventors performed double immunostaining of GS-lectin and Ninjurin 1, Ninjurin 1 and ConA, Ninjurin 1 and F4/80, Ninjurin 1 and Iba-I, and Ninjurin 1 and NG2 in the cross-section oculus of the mouse at 5 days (P5). Then, expressions of those proteins were observed under fluorescent microscope.

[0097] Hyaloid vessels and structures were eliminated from the oculus using 5% gelatin and then GS-lectin and Ninjurin 1 in the whole mount oculus were immuno-stained, followed by observation under fluorescent microscope.

[0098] As a result, as shown in FIG. **3** and FIG. **4**, Ninjurin 1 was expressed in macrophages around blood vessels but not in parenchymal microglia (FIG. **3** and FIG. **4**). Therefore, Ninjurin 1 protein was confirmed to be expressed specifically in macrophages around blood vessels.

Experimental Example 3

Effect of Ninjurin 1 on Apoptosis of Vascular Endothelial Cells

[0099] GS-lectin and Ninjurin 1 in vitreous of the mouse at 8 days (P8) were stained, followed by observation under fluorescent microscope.

[0100] Vitreous bodies of mice at 6 days (P6) and at 11 days (p11) were observed as the controls and oculus of mice at 6 days (p6) and at 11 days (p11) neutralized with a Ninjurin 1 antibody were observed as the experimental group under immunofluorescent microscope.

[0101] Particularly, to block Ninjurin 1 by using an antibody, 1 mg/kg of Ninjurin 1 (BD) mouse neutralizing antibody or 1 mg/kg of mouse isotype control antibody (Santa Cruz) were intraperitoneally injected into mice at day 1 (P1). The mice were sacrificed at day 6 (P6) and at day 11 (P11). Blood vessels and macrophages in oculus were stained by using GS-lectin.

[0102] As a result, as shown in FIG. **5** and FIG. **6**, macrophages expressing Ninjurin 1 were adhered on TUNEL positive vascular endothelial cells (proceeded to apoptosis) and the number of vascular endothelial cells was higher in the mouse group neutralized with a Ninjurin 1 antibody than in the control group (FIG. **5** and FIG. **6**). From the above results, it was confirmed that Ninjurin 1 affected apoptosis of vitreous vascular endothelial cells and in the meantime Ninjurin 1 neutralization by a Ninjurin 1 antibody resulted in the decrease of vitreous vascular endothelial cells.

Experimental Example 4

Effect of Ninjurin 1 on Cell-Cell Adhesion and Cell-Matrix Adhesion

[0103] The present inventors investigated cell adhesion by using BV2 cells transformed with pCS2+-Ninjurin 1 or pCS2+-Mock.

[0104] Particularly, to investigate cell-cell adhesion, BV2 cells were stained with Hoechst (H33342) for 10 minutes, followed by washing with DMEM. The cells were detached by using trypsin/EDTA and those cells were distributed together with a neutralizing antibody in a 96-well plate (black bottom) on which a single layer of mouse brain microvascular endothelial cells was coated. The cells were lysed with 0.2% NP-40 and fluorescence of the lysate was measured at 340 nm by ELISA. To investigate cell-matrix adhesion, BV2 cells were loaded in a well plate coated with matrixes such as fibronectin (FN, Invitrogen), type I/IV collagen (col. I/col. IV, BD), gelatin (Sigma) and vitronectin (Vit, Invitrogen). After reaction for 15 minutes, the BV2 cells were washed with PBS twice. The adhered cells were stained with crystal violet, followed by washing twice again. The cells were lysed with 0.2% NP-40 and fluorescence of the lysate was measured at 590 nm by ELISA. At last, the cells transformed with Ninjurin 1 and the cells not-transformed were mixed, followed by investigation of aggregate formation therein according to the conventional method (Araki T, et al., Neuron. 1996 August; 17 (2): 353-61).

[0105] As a result, as shown in FIG. **7**, Ninjurin 1 expression increased macrophage-vascular endothelial cell adhesion. And, cell-matrix adhesion in BV2 cells expressing Ninjurin 1 was greater than in BV2 cells expressing mock. When BV2 cells were neutralized with a Ninjurin 1 antibody, cell-matrix adhesion capacity of the cells was reduced (FIG. **7**). As shown in FIG. **8**, BV2 cells expressing Ninjurin 1 formed an aggregate around vascular cells, that is the cells were growing with forming an aggregate (FIG. **8**).

[0106] Therefore, it was confirmed that Ninjurin 1 induced cell-cell aggregation and accelerated cell-matrix adhesion.

Experimental Example 5

Effect of Ninjurin 1 on Wnt-Ang Signal Transduction System

[0107] The present inventors performed RT-PCR and real-time PCR to investigate Wnt7b expression in BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA and Wnt7b expression in cells in which Ninjurin 1 expression was inhibited by using siRNA.

[0108] Particularly, RT-PCR and real time PCR were performed as follows: Transformed BV2 cells were cultured in DMEM supplemented with 1% serum for 2 days, and then RNA was extracted by using Trizol reagent (Invitrogen). Following primers were used for RT-PCR: Gapdh: forward primer 5'-ACCACAGTCCATGCCATCAC-3' (SEQ. ID. NO: 4), reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' (SEQ. ID. NO: 5); Ninjurin 1: forward primer 5'-GAGTCGGGCACTGAGGA-3' (SEQ. ID. NO: 6), reverse primer: 5'-GTTGCAGGGGTCTGGTCA-3' (SEQ. ID. NO: 7); Ang1: forward primer 5'-AGGCTTGGTTTCTCGT-CAGA-3' (SEQ. ID. NO: 8), reverse primer: 5'-TCTGCA-CAGTCTCGAAATGG-3' (SEQ. ID. NO: 9); Ang2: forward primer 5'-GCTGCTGGTTTATTACTGAAGAA-3' (SEQ. ID. NO: 10), reverse primer: 5'-TCAGGTGGACTGGGAT-GTTTAG-3' (SEQ. ID. NO: 11); Wnt7b: forward primer 5'-AAGAACTCCGAGTAGGGAGTCG-3' (SEQ. ID. NO: 12), reverse primer: 5'-TGCGTTGTACTTCTCCTTGAGC-3' (SEQ. ID. NO: 13); Wnt7b: 2^{nd} round forward primer 5'-CCGAGTAGGGAGTCGAGAGG-3' (SEQ. ID. NO: 14), reverse primer: 5'-CACACCGTGACACTTACATTCC-3' (SEQ. ID. NO: 15). The PCR products were separated on 1.2% agarose gel containing EtBr (ethidium bromide), followed by analysis by digital imaging. Primers used for real-time PCR were as follows: Ang2: forward primer 5'-TGT-GATCTTGTCTTGGCCGC-3'(SEQ. ID. NO: 16), reverse primer: 5'-AGAGGGAGTGTTCCAAGAAGC-3' (SEQ. ID. NO: 17); Gapdh, Ang1 and Wnt7: same primers as used for RT-PCR were used. Conditions for RT-PCR and real-time PCR were the same.

[0109] Wnt7b expression in BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA was observed under immunofluorescent microscope.

[0110] As a result, as shown in FIG. **9** and FIG. **10**, Wnt7b expression was increased in BV2 cells expressing Ninjurin 1, compared with in BV2 cells expressing mock. In the meantime, when Ninjurin 1 expression was inhibited, Wnt7b expression was reduced (FIG. **9** and FIG. **10**).

[0111] To investigate the effect of Ninjurin 1 on the expressions of p38, MAPK (p44/p42) and JNK (p54/46), Western blotting was performed with the lysate of BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA. The BV2 cells transformed with Myc-mock or Myc-Ninjurin 1

labeled DNA were treated with SB203580, the p38 inhibitor, at different concentrations (0, 10, and 30 uM), followed by investigation of Wnt7b expression.

[0112] As a result, as shown in FIG. **11**, expressions of phosphorylated p38, MAPK (p44/p42) and JNK (p54/46) were increased in BV2 cells expressing Ninjurin 1, compared with in BV2 cells expressing mock (FIG. **11**). As shown in FIG. **12**, Wnt7b expression stimulated by Ninjurin 1 was reduced by the p38 inhibitor dose-dependently (FIG. **12**).

[0113] To investigate the effect of Ninjurin 1 on the expressions of Ang1 and Ang2, the culture solution of BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA were treated to human pericytes, followed by RT-PCR and real-time PCR to examine the expressions of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2).

[0114] As shown in FIG. **13**, Ang1 expression was reduced in pericytes treated with culture solution of BV2 cells expressing Ninjurin 1, compared with in pericytes treated with culture solution of BV2 cells expressing mock, but Ang2 expression was increased in pericytes treated with culture solution of BV2 cells expressing Ninjurin 1, compared with in pericytes treated with culture solution of BV2 cells expressing mock (FIG. **13**).

[0115] To investigate interaction among Wnt7b, Ninjurin 1, Ang1 and Ang2, Wnt7b and Ninjurin 1 were quantified by RT-PCR in the presence of recombinant human Ang1 (rh-Ang1) and rh-Ang2 at different concentrations (0, 1 and 2.5 ug/ml) in rodent originated macrophages (Raw264.7, BV2).

[0116] As shown in FIG. **14**, Wnt7b and Ninjurin 1 expressions were reduced by rh-Ang1 dose-dependently, but increased by rh-Ang2 dose-dependently (FIG. **14**).

[0117] HUVEC cells were treated with culture solution of BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA in the presence of rh-Ang2 for 24 hours. Then, caspase 3 digestion in HUVEC cells was confirmed by Western blotting. And the caspase 3 digestion and TUNEL were confirmed by immunofluorescence staining.

[0118] As shown in FIG. **15**, caspase 3 digestion and apoptosis were increased in HUVEC cells treated with culture solution of BV2 cells expressing Ninjurin 1, compared with in HUVEC cells treated with culture solution of BV2 cells expressing mock in the presence of rh-Ang2 (FIG. **15**).

[0119] Therefore, it was confirmed that Ninjurin 1 expression increases Wnt7b expression, reduces Ang1 expression but increases Ang2 expression, and activates p38, MAPK (p44/42) and JNK (p54/46). That is, Ninjurin 1 regulates Wnt-Ang signal transduction system and causes apoptosis of vascular endothelial cells by Ang2.

Experimental Example 6

Ninjurin 1 Expression Pattern Under Inflammatory Condition Induced by LPS

[0120] To induce inflammation in vivo, lipopolysaccharide (LPS) (lipopolysaccharides from *Escherichia coli* 0111:B4, Sigma) was intraperitoneally injected into Sprague-Dawley (SD) line SPF rat. Oculus was extracted from the rat, followed by immunofluorescence staining (Ninjurin 1 and Iba-1 were stained) to investigate Ninjurin 1 expression (Ninjurin 1 stained), and inflow and activation of macrophages and microglias (Iba-I stained).

[0121] As a result, as shown in FIG. **16**, the cells expressing Ninjurin 1 were observed in vitreous after LPS injection and inflow of ovoid macrophages was accelerated and at the same time microglias stretching their arms were changed into activated microglias. That is, LPS injection resulted in the increase of inflow and activation of macrophages and microglias and round shaped macrophages expressing Ninjurin 1 were observed in retina (FIG. **16**).

[0122] Therefore, it was confirmed that when inflammation was induced by LPS, the number of macrophages expressing Ninjurin 1 were increased and Ninjurin 1 expression was also increased.

Experimental Example 7

Inflammation Related Protein Expression Over Ninjurin 1 Expression

[0123] To induce inflammation in vitro, BV2 cells were treated with LPS at different concentrations (0, 1, and 2.5 ug/ml), followed by Western blotting and RT-PCR to investigate iNOS expression, the index for inflammation, and Ninjurin 1 expression.

[0124] As shown in FIG. **17**, iNOS was up-regulated by LPS injection and Ninjurin 1 expression was also increased by LPS dose-dependently (FIG. **17**).

[0125] To investigate the effect of Ninjurin 1 expression on iNOS expression, Western blotting and RT-PCR were performed to measure iNOS expression in BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA.

[0126] As shown in FIG. **18**, iNOS expression was increased in BV2 cells over-expressing Ninjurin 1, compared in BV2 cells expressing mock. iNOS expression was increased as Ninjurin 1 expression was increased (FIG. **18**).

[0127] To investigate the effect of Ninjurin 1 expression on the generation of NO, the inflammatory mediator, NO generation was measured in BV2 cells transformed with Myc-mock or Myc-Ninjurin 1 labeled DNA.

[0128] Particularly, NO generation was calculated by measuring nitrite, the stable reaction product of NO in the cell culture solution, by using Griess reagent. 100 μl of cell culture solution was mixed with 100 μl of Griess reagent [1% sulfanilamide dissolved in 30% acetate and 0.1% N-(1-naphthyl) ethylenediamine dissolved in 60% acetate, 1:1]. 10 minutes later, OD_{570} was measured by ELISA (Ebert S, et al., *J. Neuroimmunol.* 2005 February; 159(1-2): 87-96). The acetate, sulfanilamide and N-(1-naphthyl)ethylenediamine were purchased from Sigma.

[0129] As shown in FIG. **19**, NO generation was increased in BV2 cells over-expressing Ninjurin 1, compared in BV2 cells expressing mock (FIG. **19**).

[0130] To investigate the effect of Ninjurin 1 expression inhibition on iNOS expression, siRNA and shRNA were respectively inserted into a vector, which were inserted into BV2 cells. Then, Ninjurin 1 and iNOS expressions in the BV2 cells where Ninjurin 1 expression was inhibited were quantified by Western blotting and RT-PCR.

[0131] As a result, as shown in FIG. **20**, Ninjurin 1 expression was significantly reduced by siRNA and shRNA and accordingly iNOS expression was reduced (FIG. **20**).

[0132] Therefore, it was confirmed that Ninjurin 1 overexpression increased expression of iNOS, the inflammation index and increased generation of NO, the inflammatory mediator. On the other hand, inhibition of Ninjurin 1 expression resulted in the decrease of iNOS expression. The above results suggest that Ninjurin 1 is deeply involved in inflammation reaction.

[0133] The Manufacturing Examples of the composition for the present invention are described hereinafter.

Manufacturing Example 1

Preparation of Pharmaceutical Formulations

<1-1> Preparation of Powders

[0134]

Kluyveromyces	2 g
Lactose	1 g

[0135] Powders were prepared by mixing all the above components, which were filled in airtight packs according to the conventional method for preparing powders.

<1-2> Preparation of Tablets

[0136]

Kluyveromyces	100 mg
Corn starch	100 mg
Lactose	100 mg
Magnesium stearate	2 mg

[0137] Tablets were prepared by mixing all the above components by the conventional method for preparing tablets.

<1-3> Preparation of Capsules

[0138]

Kluyveromyces	100 mg
Corn starch	100 mg
Lactose	100 mg
Magnesium stearate	2 mg

[0139] Capsules were prepared by mixing all the above components, which were filled in gelatin capsules according to the conventional method for preparing capsules.

<1-4> Preparation of Pills

[0140]

Kluyveromyces	1 g
Lactose	1.5 g
Glycerin	1 g
Xylitol	0.5 g

[0141] Pills were prepared by mixing all the above components according to the conventional method for preparing pills. Each pill contained 4 g of the mixture.

<1-5> Preparation of Granules

[0142]

Kluyveromyces	150 mg
Soybean extract	50 mg
Glucose	200 mg
Starch	600 mg

[0143] All the above components were mixed, to which 100 mg of 30% ethanol was added. The mixture was dried at 60° C. and the prepared granules were filled in packs.

INDUSTRIAL APPLICABILITY

[0144] The present invention can be effectively applied in the development of drugs for diverse diseases caused by over-activation of macrophages induced by over-expression of Ninjurin 1 such as rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis.

[0145] Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

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His Gly Pro Ile Asn Val Asn His Tyr Ala Ser Lys Lys Ser Ala Ala
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65                  70                  75                  80

Val Val Leu Ile Ser Ile Ser Leu Val Leu Gln Ile Gly Val Gly Val
                85                  90                  95

Leu Leu Ile Phe Leu Val Lys Tyr Asp Leu Asn Asn Pro Asp Lys His
            100                 105                 110

Ala Lys Leu Asp Phe Leu Asn Asn Leu Ala Thr Gly Leu Val Phe Ile
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<220> FEATURE:
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<400> SEQUENCE: 16

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<223> OTHER INFORMATION: reverse primer of Ang2

<400> SEQUENCE: 17

agagggagtg ttccaagaag c 21

1. A composition for the prevention and treatment of inflammatory disease comprising a Ninjurin 1 protein expression or activation inhibitor.

2. The composition according to claim **1**, wherein the Ninjurin 1 protein has the amino acid sequence represented by SEQ. ID. NO: 1.

3. The composition according to claim **1**, wherein the Ninjurin 1 protein expression inhibitor is selected from the group consisting of an antisense nucleotide complementarily binding to Ninjurin 1 mRNA, short interfering RNA and short hairpin RNA.

4. The composition according to claim **1**, wherein the Ninjurin 1 protein activation inhibitor is selected from the group consisting of a compound complementarily binding to Ninjurin 1 protein, a peptide, a peptide mimetix and an antibody.

5. The composition according to claim **1**, wherein the inflammatory disease is selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis.

6. A method for treating or preventing inflammatory disease comprising administering a pharmaceutically effective dose of the composition of claim **1** to a subject with inflammatory disease.

7. (canceled)

8. The method according to claim **6**, wherein the inflammatory disease is selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis.

9. (canceled)

10. The method according to claim **6**, wherein the Ninjurin 1 protein has the amino acid sequence represented by SEQ. ID. NO: 1.

11. The method according to claim **6**, wherein the Ninjurin 1 protein expression inhibitor is selected from the group consisting of an antisense nucleotide complementarily binding to Ninjurin 1 mRNA, short interfering RNA and short hairpin RNA.

12. The method according to claim **6**, wherein the Ninjurin 1 protein activation inhibitor is selected from the group consisting of a compound complementarily binding to Ninjurin 1 protein, a peptide, a peptide mimetix and an antibody.

13. The method according to claim **6**, wherein the inflammatory disease is selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis.

14. A method for screening a preventive and therapeutic agent for inflammatory disease comprising:

1) treating samples to a cell line expressing Ninjurin 1 protein;
2) measuring the expression of Ninjurin 1 protein in the cell line; and
3) selecting a sample that inhibited the expression of Ninjurin 1 protein, compared with the expression level in the control.

15. The method according to claim **14**, wherein the Ninjurin 1 protein of step 1) has the amino acid sequence represented by SEQ. ID. NO: 1.

16. The method according to claim **14**, wherein the protein expression of step 2) is measured by one of the methods selected from the group consisting of immunofluorescence method, ELISA, Western blotting, and RT-PCR.

17. The method according to claim **14**, wherein the inflammatory disease of step 2 is selected from the group consisting of rheumatic arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis, atherosclerosis and multiple sclerosis.

* * * * *

专利名称(译)	包含水蛭素1的表达或活性抑制剂的组合物，用于预防和治疗炎性疾病		
公开(公告)号	US20110123538A1	公开(公告)日	2011-05-26
申请号	US12/999005	申请日	2008-12-24
[标]申请(专利权)人(译)	首尔大学校产学协力团		
申请(专利权)人(译)	首尔大学R＆DB基金会		
当前申请(专利权)人(译)	首尔大学R＆DB基金会		
[标]发明人	KIM KYU WON LEE HYO JONG		
发明人	KIM, KYU-WON LEE, HYO-JONG		
IPC分类号	A61K39/395 C07H21/02 C07K16/28 A61K38/02 A61K31/713 A61P25/28 A61P17/00 A61P17/06 A61P9/10 A61P29/00 C12Q1/68 G01N33/53		
CPC分类号	C12N15/113 C12N2310/14 C12N2310/11 A61P9/10 A61P17/00 A61P17/06 A61P25/28 A61P29/00		
优先权	1020080124555 2008-12-09 KR		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及包含Ninjurin 1表达或活性抑制剂的组合物，用于预防和治疗炎性疾病。 Ninjurin 1蛋白在血管周围的巨噬细胞中特异性表达，增加细胞 - 细胞粘附和细胞 - 基质粘附，增加Wnt7b（Wingless型MMTV整合位点家族，成员7B）和Ang2（血管生成素-2）的表达，但减少表达Ang1诱导血管内皮细胞凋亡此外，当诱导炎症时，Ninjurin 1蛋白被上调并诱导iNOS表达以及增加的NO产生。因此，Ninjurin 1蛋白质表达或活化抑制剂可以有效地用作用于预防和治疗炎性疾病的组合物的活性成分。