

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 August 2006 (10.08.2006)

PCT

(10) International Publication Number
WO 2006/083090 A1

(51) International Patent Classification:
A61K 38/16 (2006.01)

(21) International Application Number:
PCT/KR2006/000289

(22) International Filing Date: 25 January 2006 (25.01.2006)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
10-2005-0009875 3 February 2005 (03.02.2005) KR

(71) Applicant (for all designated States except US): **EWHA UNIVERSITY-INDUSTRY COLLABORATION FOUNDATION** [KR/KR]; Ewha Womans University, 11-1,, Daehyeon-dong, Seodaemoon-ku,, Seoul 120-750 (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KANG, Sang-Won** [KR/KR]; 72-408, Hyundai Apt., 455, Apgujeong-dong,

Kangnam-ku,, Seoul (KR). **CHOI, Min-Hee** [KR/KR]; 308-102, Poshometown Apt., Shinchonmaeul, Bo-jeong-dong, Gihyeung-gu,, Yongin, Kyunggi-do 446-566 (KR).

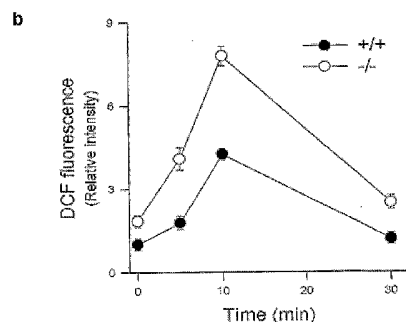
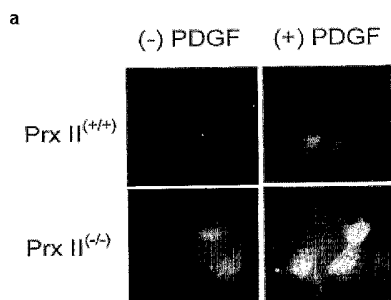
(74) Agent: **DARAE PATENT FIRM**; 10th Floor, KIPS,, 647-9, Yeoksam-dong, Kangnam-ku,, Seoul 135-980 (KR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: PHARMACEUTICAL COMPOSITION FOR PREVENTING AND TREATING RESTENOSIS COMPRISING PEROXIREDOXIN 2 PROTEIN AS AN ACTIVE INGREDIENT AND COMPOSITION FOR SCREENING THERAPEUTICS OF RESTENOSIS COMPRISING PEROXIREDOXIN 2 GENE OR ITS PROTEIN PRODUCT, AND METHOD FOR SCREENING THERAPEUTICS OF RESTENOSIS USING SAID COMPOSITION



(57) Abstract: Disclosed herein is a pharmaceutical composition for the prophylaxis and treatment of restenosis, comprising a peroxiredoxin 2(Prx II) protein as an active ingredient. Optionally, the composition may contain other active ingredients suppressive of restenosis at the carotid artery, the coronary artery, the peripheral artery, and the renal artery. Also, a composition and a method are provided for screening therapeutics for restenosis. This screening composition comprises a peroxiredoxin 2 gene or protein. Together with the method, the composition is useful for searching and developing therapeutics for restenosis.



European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

— with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Description

PHARMACEUTICAL COMPOSITION FOR PREVENTING AND TREATING RESTENOSIS COMPRISING PEROXIREDOXIN 2 PROTEIN AS AN ACTIVE INGREDIENT AND COMPOSITION FOR SCREENING THERAPEUTICS OF RESTENOSIS COMPRISING PEROXIREDOXIN 2 GENE OR ITS PROTEIN PRODUCT, AND METHOD FOR SCREENING THERAPEUTICS OF RESTENOSIS USING SAID COMPOSITION

Technical Field

- [1] The present invention relates to a pharmaceutical composition for the prophylaxis and treatment of restenosis (renarrowing), a composition for screening therapeutics for restenosis, and a method for screening therapeutics for restenosis using the same.

[2]

Background Art

- [3] Peroxiredoxin (Prx) is a scavenger of hydrogen peroxide and alkyl hydroperoxides in living organisms (Chae, H. Z. et al., Proc. Nat. Acad. Sci. 91: 7017-7021, 1994). There are six distinct mammalian Prx isozymes, types I to VI, that are detected in a wide range of tissues (Rhee, SG et al., IUBMB Life, 52:35~41, 2001). These have been found to have potent antioxidant activities in vivo. All of the known mammalian Prxs, except for type VI, utilize thioredoxin as an electron donor, and thus were formerly known as thioredoxin peroxidases.
- [4] In addition to their antioxidant activity, Prxs have been implicated in various cellular functions including cell proliferation and differentiation, enhancement of natural killer cell activity, protection of radical-sensitive proteins, heme metabolism, and intracellular signaling (Nemoto Y, et al., Gene, 91:261~265, 1990; Prosperi MT, et al., Genomics, 19:236~241, 1994; Tsuji K, et al., Biochem J. 307:377~381, 1995; Shau H, et al., Immunogenetics, 40:129~134, 1994; Watabe S, et al., Biochem Biophys Res Commun. 213:1010~1016, 1995; Iwahara S, et al., Biochemistry, 34:13398~13406, 1995; Wen ST, et al., Genes Dev. 11:2456~2467, 1997). The biochemical characteristics revealed from cultured animal cell studies show that Prx may be one of those that play important roles in maintaining cellular redox potential.
- [5] Of these antioxidant proteins, 2-cys peroxiredoxin type II (Prx II) is a cellular peroxidase that eliminates endogenous H_2O_2 produced in response to growth factors including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF).

- [6] It is known that Prx II is located in abundance in the cytoplasm of cells, binds to integral membrane proteins or cell membranes via its C-terminal region, and has a high affinity for H_2O_2 (K_m for H_2O_2 $<10 \mu M$).
- [7] Also, Prx II is found to be highly expressed in erythrocytes and to play a protective role against reactive oxygen species (ROS)-mediated damage in these cells, and is induced at the early stages of erythroid differentiation prior to hemoglobin accumulation (Rabilloud T, et al., *Biochem J.* 312:699-705, 1995).
- [8] Platelet-derived growth factor (PDGF) is a potent mitogenic and migratory factor that regulates the tyrosine phosphorylation of a variety of signaling proteins through the generation of endogenous H_2O_2 . Particularly, PDGF stimulates the proliferation and migration of smooth muscle cells during vascular remodeling. In this regard, Prx II serves as an important regulator for the PDGF-mediated generation of H_2O_2 , but its precise function in signal transduction has yet to be proven.
- [9] Based on the observation that Prx II^{-/-} mice have hemolytic anemia, Lee TH et al., suggested that Prx II plays a major role in protecting RBCs from oxidative stress in mice (Lee TH et al., *Blood*, 101(12):pp5033-5038, 2003). U. S. Pat. Publication No. 2002/0168353 discloses a composition for the treatment of HIV infection, comprising purified type I Peroxiredoxin and type II Peroxiredoxin.
- [10] Restenosis, or renarrowing, is said to recur in as many as 50% of stenosis cases or more, as determined by coronary angiography after coronary angioplasty. Being a major obstacle to the successful treatment of cardiovascular diseases, restenosis occurs in about 30% of patients who receive coronary angioplasty (balloon dilatation and stent insertion).
- [11] Restenosis, although the precise mechanism thereof must be further revealed, is known to be attributed to the fact that growth factors and cytokines are locally secreted due to vascular endothelial cell injury during angioplasty or balloon dilatation and induce smooth muscle cell proliferation and migration through autocrine and paracrine mechanisms, leading to the narrowing of artery lumens. Hence, smooth muscle cell proliferation has recently been recognized as a clinical problem that is important in the effectiveness of coronary angioplasty (Bauters C, Isner JM, *Prog Cardiovasc Dis.* 40(2):107-116, 1997, U.S. Pat. Nos. 6,780,406 and 6,740,678).
- [12] Extensive studies have been conducted to reduce or prevent restenosis. Particularly, many attempts have been made to regulate smooth muscle cell proliferation, using angiotensin converting enzyme (ACE) inhibitors, antisense RNAs for cell cycle regulatory proteins, and thymidine kinase genes (Rakugi et al, *J. Clin. Invest.*, 93:339-346, 1994; Simons et al., *Nature*, 359:67-70, 1992; U.S. Pat. No. 6,780,406).
- [13] Leading to the present invention, the intensive and thorough study on intracellular functions of Prx II, conducted by the present inventors, resulted in the finding that Prx

II is involved in vascular muscle cell migration, thereby being used in preventing or reducing vascular restenosis.

[14]

Disclosure of Invention

Technical Solution

[15] Accordingly, the present invention has been made keeping in mind the above problems occurring in the prior art, and an object of the present invention is to provide a pharmaceutical composition for the prophylaxis and treatment of vascular restenosis, comprising Prx II as an active ingredient.

[16] Another object of the present invention is to provide a composition for screening therapeutics for restenosis.

[17] A further object of the present invention is to provide a method for screening therapeutics for restenosis using the composition.

[18] In accordance with an embodiment, the present invention pertains to a composition for the prophylaxis and treatment of vascular restenosis (renarrowing), comprising Prx II as an active ingredient.

[19] Particularly, the composition comprising Prx II as an active ingredient in accordance with the present invention may be used for the prophylaxis and treatment of restenosis in the carotid artery, the coronary artery, the peripheral artery, and the renal artery.

[20] In addition, the composition comprising Prx II as an active ingredient in accordance with the present invention is useful for preventing and treating restenosis attributable to hardening chronically occurring in grafted vessels or angioplasted segments or to short-term proliferation after angioplasty.

[21] In addition to Prx II, the composition of the present invention may further contain other ingredients having inhibitory activity against restenosis.

[22] Prx II has inhibitory activity against various cellular events including receptor tyrosine phosphorylation through growth factor signaling, vascular smooth muscle cell proliferation and migration, and neointimal thickening. This protein can be expressed by the base sequence of a prx II gene, listed in SEQ. ID. NO. 1, that encodes the amino acid sequence listed in SEQ. ID. NO. 2.

[23] Prx II (gene loci Prdx2) is a cellular peroxidase that eliminates endogenous H_2O_2 produced in response to growth factors such as PDGF and EGF. The enzyme suppresses the amplification of intracellular signal transduction by inhibiting site-specific phosphorylation at PDGFR β -PLC γ 1. Through this mechanism, Prx II leads to the suppression of the proliferation, migration and neointimal thickening of vascular smooth muscle cells.

- [24] Upon stimulation with PDGF, the intracellular levels of H_2O_2 and phosphorylated proteins in Prx II-deficient embryonic fibrocytes increase. In Prx II-deficient cells stimulated with PDGF, PLC γ 1 (phospholipase gamma 1) is phosphorylated particularly on tyrosine-783 and tyrosine-1253. Also, a phosphorylation increase is found only on tyrosine 579/581 and 857 out of the phosphorylation residues of PDGFR β . However, the re-expression of wild-type Prx II within the cells returns these cellular events back to the suppression of tyrosine inhibition.
- [25] Prx II was also found to suppress the tyrosine phosphorylation of PDGFR β in a site-specific manner, as well as cell migration in both human primary smooth muscle cells and mouse embryonic fibroblasts.
- [26] After being injured, the carotid artery of Prx II-deficient mice (Prx II^{-/-} mice) undergoes more severe neointimal thickening during restenosis than does that of wild-type mice.
- [27] Taking advantage of the fact that during vascular remodeling, the loss of Prx II functions results in increasing the migration of smooth muscle cells, a pharmaceutical composition comprising Prx II, which suppresses the migration and neointimal thickening of smooth muscle cells, can be used as a medication for the diagnosis, prevention and treatment of cardiovascular diseases caused by smooth muscle cell migration, such as restenosis (Ferns GA, et al., Science. 1991 Sep 6;253(5024):1129-32; Heldin CH, Westermark B. Physiol Rev. 1999 Oct;79(4):1283-316).
- [28] In the present invention, it is suggested that Prx II is an H_2O_2 -based signal regulator that plays a special role in PDGFR signal transduction and can be a new target protein in the treatment of cardiovascular diseases.
- [29] In addition to the active ingredient Prx II, the composition of the present invention may contain pharmaceutically acceptable and physiologically suitable additives. Vehicles, disintegrants, sweeteners, binders, coatings, dilatants, lubricants, talc, and/or flavors may be used in the composition.
- [30] The composition of the present invention comprising Prx II as an active ingredient may be formulated in combination with at least one pharmaceutically acceptable carrier.
- [31] For liquid formulations, physiological saline, sterile saline, Ringer's solutions, buffered saline, albumin injections, dextrose solutions, maltodextrose solutions, glycerol, ethanol and combinations thereof may be used as vehicles. If necessary, other conventional additives such as antioxidants, buffers, bacteriostatics, and the like, may be added. Also, together with diluents, dispersants, surfactants, binders, lubricants and the like, the composition of the present invention may be formulated into pills, capsules, granules, and tablets as well as injection forms, such as solutions,

suspensions, emulsions, etc. Further, the composition of the present invention may be formulated according to the methods described in Remington's Pharmaceutical Science, Mack Publishing Company, Easton PA.

[32] In accordance with the present invention, the composition comprising Prx II as an active ingredient may be administered in the form of granules, powders, coated tablets, capsules, suppositories, syrups, juices, suspensions, emulsions, injectable solutions, or sustained release agents.

[33] As for the administration of the composition of the present invention comprising Prx II as an active ingredient, it may be achieved in a conventional manner through one of intravenous, intraarterial, intraabdominal, intramuscular, intrathoracic, transdermal, intranasal, inhalation, local, rectal, oral, intraocular, or subcutaneous routes, or the like.

[34] Factors determining the dose of the composition comprising Prx II as an active ingredient include the type and severity of disease, the content and kind of the active and other ingredients, the dosage form of the composition, the age, body weight, physical condition, sex and diet of patients, the administration time interval, administration route, release rate of the composition, treatment time periods, co-administered drugs, etc. For adults, the composition may be administered once or many times a day in a total daily dose from 0.1 to 10 mg of Prx II per kg of body weight.

[35] In accordance with another embodiment, the present invention pertains to a method for preventing and treating cardiovascular diseases caused by smooth muscle cell migration, such as vascular restenosis, by administering Prx II.

[36] The present invention is intended to suppress the migration or neointimal thickening of smooth muscle cells after vessel injury in patients with cardiovascular diseases by administering Prx II thereto, thereby preventing vascular restenosis.

[37] In the method for the prophylaxis and treatment of cardiovascular diseases caused by smooth muscle cell migration, such as restenosis, a Prx II protein may be administered via an intravenous, an intraarterial, an intraabdominal, an intramuscular, an intrathoracic, a transdermal, an intranasal, an inhalation, a local, a rectal, an oral, an intraocular, or a subcutaneous route.

[38] In the method for the prophylaxis and treatment of cardiovascular diseases caused by smooth muscle cell migration, such as restenosis, Prx II is administered at a total daily dose from 0.01 mg to 100 mg per kg of body weight for adults one or many times a day, depending on various factors, including the type and severity of disease, the content and kind of the active and other ingredients, the dosage form of the composition, the age, body weight, physical condition, sex and diet of patients, the administration time interval, administration route, release rate of the composition, treatment time periods, co-administered drugs, etc.

[39] In accordance with a further embodiment, the present invention pertains to the use

of Prx II in the preparation of medical products for the prophylaxis and treatment of cardiovascular diseases caused by smooth muscle cell migration, such as restenosis.

[40] In accordance with still a further embodiment, the present invention pertains to a composition for screening therapeutics for restenosis, comprising a mammalian Prx II gene.

[41] The Prx II gene useful in the screening composition of the present invention may be selected from among the base sequence of SEQ. ID. NO. 1, base sequences of polymorphs of SEQ. ID. NO. 1, fragments of the base sequences, and combinations thereof.

[42] In accordance with still another embodiment, the present invention pertains to a composition for screening therapeutics for restenosis, comprising a mammalian Prx II protein.

[43] The Prx II protein useful in the screening composition of the present invention may be selected from among the amino acid sequence of SEQ. ID. NO. 2, proteins expressed from the base sequence of SEQ. ID. NO. 1, proteins expressed from the base sequences of polymorphs of SEQ. ID. NO. 1, Prx II polypeptide fragments having the same physiological activity as Prx II, and combinations thereof.

[44] The screening composition of the present invention shows a relieving effect on restenosis.

[45] In accordance with yet another embodiment, the present invention pertains to a method for screening therapeutics for restenosis using as a target material the screening composition comprising a Prx II gene.

[46] In this method, the composition for screening therapeutics for restenosis comprising a Prx II gene is brought into contact with a test material to determine whether the test material enhances or suppresses the expression of the gene contained in the composition.

[47] In accordance with yet a further embodiment, the present invention pertains to a method for screening therapeutics for restenosis using as a target material the screening composition comprising a Prx II protein.

[48] In this method, the composition for screening therapeutics for restenosis comprising a Prx II protein is brought into contact with a test material to determine whether the test material enhances or suppresses the expression of the gene contained in the composition.

[49] To screen therapeutics for restenosis with the composition comprising a Prx II gene, conventional methods useful for the analysis of reaction between DNA-DNA, DNA-RNA, DNA-protein, or DNA-compound may be used.

[50] For example, available is a hybridization test for identifying the combination between the gene and a test material in vitro, a method for measuring the expression

rate of the gene through Northern analysis after reaction between mammalian cells and a test material, quantitative PCR, and quantitative in-time PCR, or a reporter gene assay in which the gene coupled with a reporter gene is introduced into cells to react with a test material, followed by measuring the expression rate of the reporter protein.

- [51] In this regard, the composition of the present invention may contain distilled water or buffer for stabilizing the structure of nucleic acids, as well as the Prx II gene.
- [52] To screen therapeutics for restenosis with the composition comprising a Prx II protein, conventional methods useful for the analysis of reaction between proteins and proteins, or between proteins and compounds, may be used.
- [53] Available are, for example, screening methods utilizing the measurement of the activity of a test compound after reaction with the Prx II gene or protein, a yeast two-hybrid assay, a search for a phage-displayed peptide clone binding to a Prx II protein, HTS (high throughput screening) using a natural or chemical library, drug hit HTS, cell-based screening, or DNA array.
- [54] In this regard, the composition of the present invention may contain a buffer or reaction solution for safely maintaining the structure or activity of the protein in addition to the protein expressed from the Prx II gene. For in vivo assays, the composition of the present invention may further comprise a cell capable of expressing the protein or a cell containing a plasmid capable of expressing the protein in the presence of a promoter regulating the description of the gene.
- [55] In the screening method of the present invention, the test material may be a material that is assumed to have the possibility of being used as a therapeutic for restenosis according to conventional assays, or randomly selected nucleic acid sequences, proteins, extracts or natural materials.
- [56] If a test material is found to enhance the expression of the gene or the activity of the protein as measured by the screening method of the present invention, it can be a candidate therapeutic for restenosis. On the other hand, if a test material is found to suppress the expression of the gene or the activity of the protein as measured by the screening method of the present invention, its inhibitors can be also candidates for therapeutics for restenosis.
- [57] As such, the candidates for therapeutics for restenosis in accordance with the present invention may be leading compounds for the development of the therapeutics, which optimally modify the structure of the Prx II gene or protein so as to enhance the expression of the Prx II gene or the activity of the Prx protein.
- [58] Partially or fully enhancing the expression of mammalian Prx II genes or the activity of mammalian Prx II proteins, the materials thus obtained can be used to treat restenosis, coronary sclerosis, or other cardiovascular diseases caused due to the decreased expression of the Prx II gene or the decreased activity of the Prx II protein.

[59]

Brief Description of the Drawings

[60] FIG. 1 shows intracellular H_2O_2 levels in normal MEF and Prx II^(-/-) MEF in response to PDGF stimulation.

[61] FIG. 2 shows levels of phosphorylation on total proteins in normal MEF and Prx II^(-/-) MEF in response to PDGF stimulation.

[62] FIG. 3 shows levels of phosphorylation on individual signaling proteins in normal MEF and Prx II^(-/-) MEF in response to PDGF stimulation.

[63] FIG. 4 shows levels of phosphorylation on various tyrosine residues of PDGFR β in normal MEF and Prx II^(-/-) MEF in response to PDGF stimulation.

[64] FIG. 5 shows levels of phosphorylation on total proteins and PLC γ 1 when a human Prx II wild-type protein is expressed in mouse Prx II^(-/-) MEF (a) and levels of phosphorylation on PDGFR β in response to PDGF when human Prx II wild-type and inactivated mutant proteins were expressed in mouse Prx II^(-/-) MEF (b).

[65] FIG. 6 shows cell migration towards PDGF (a) and the number of cells that migrated (b) when a human Prx II wild-type protein is expressed in mouse Prx II^(-/-) MEF.

[66] FIG. 7 shows levels of phosphorylation on total proteins and tyrosine residues of PDGFR β in response to PDGF upon the overexpression of a Prx II wild-type protein in human VSMCs in which Prx II wild-type protein is expressed.

[67] FIG. 8 shows cell migration towards PDGF (a) and the number of cells that migrated (b) when a Prx II protein is overexpressed in human VSMCs.

[68] FIG. 9 shows the tyrosine phosphorylation of PDGFR β when VSMCs isolated from Prx II^(-/-) mice are treated with PDGF.

[69] FIG. 10 shows neointimal thickening after vessel injury in normal and Prx II^(-/-) mice.

[70] FIG. 11 shows neointimal thickening after vessel injury in Prx II^(-/-) mice injected with control IgG and an anti-PDGF antibody.

[71]

Mode for the Invention

[72] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

[73]

[74] EXAMPLE 1: Effect of Prx II on Tyrosine Phosphorylation of Endogenous Proteins

[75]

[76] 1-1) Observation of H_2O_2 generation and tyrosine phosphorylation in response to

PDGF with respect to Prx II expression

[77] In this example, changes in endogenous H_2O_2 generation in response to PDGF were observed with respect to Prx II expression.

[78] Wild-type Prx II^(+/+) MEF (mouse embryonic fibroblasts) and Prx II^(-/-) MEF were prepared according to a typical process well known in the art after Prx II^(+/+) mice were mated with each other and embryos were excised from the mice on the 10th day of pregnancy (Kang, S. W. et al., J Biol Chem, 279, 2535-43 (2004)). PDGF-BB (25 ng/ml, Upstate) was used for PDGF stimulation.

[79] The intracellular level of H_2O_2 was measured using 2',7'-dichlorofluorescein diacetate (H2DCFDA, Molecular Probes Inc.).

[80] When applied to cells, this compound crosses the plasma membrane and is transported into cells wherein it is hydrolyzed into impenetrable 2',7'-dichlorofluorescein (H2DCF) by endogenous esterases. This hydrolysate is oxidized by endogenous H_2O_2 to form 2',7'-dichlorofluorescein (DCF) that has an excitation peak at 488 nm and an emission peak at 515 nm, enabling fluorescence detection.

[81] Prx II^(+/+) MEF and Prx II^(-/-) MEFs were cultured in DMEM supplemented with 10% FBS (fetal bovine serum) in the presence of an antibiotic. Prior to PDGF stimulation, these cells were synchronized by serum starvation for 24 hours in DMEM supplemented with 0.5% FBS. PDGF stimulation was performed by treating the cells with PDGF at a density of 25 ng/ml for 5, 10, or 30 min. After completion of PDGF stimulation, the cells were washed with HBSS (HEPES-buffered saline solution) and allowed to react for an additional 5 min in 5 mM DCFH-DA. Fluorescence was measured using a confocal laser microscope (Kang, S. W. et al. J Biol Chem 273, 6297-302 (1998)).

[82] In FIG. 1a, there are photographs of 2',7'-DCF fluorescence taken 10 min after the PDGF-BB stimulation, showing that Prx II^(-/-) MEFs are higher in endogenous H_2O_2 level than Prx II^(+/+) MEFs both before and after PDGF stimulation.

[83] In FIG. 1b, relative intensities of DCF are plotted against time, showing that the generation of H_2O_2 in the Prx II-deficient mouse cells was about twice as large as that in wild-type cells in response to PDGF and that the generation of H_2O_2 was temporally increased and returned to the base line within 30 min.

[84]

[85] 1-2) Observation of tyrosine phosphorylation of endogenous proteins

[86] Following the treatment of Prx II^(+/+) MEF and Prx II^(-/-) MEF with PDGF as in Example 1-1, the phosphorylation of intracellular proteins over time was observed.

[87] Proteins extracted from each cell were immunoblotted with an anti-phosphorylation antibody (4G10, Upstate) so as to analyze them for tyrosine phosphorylation. An anti-

Prx II antibody (Labfrontier Co., Korea), and an anti-tubulin antibody (Sigma-Aldrich Co.) was used for immunoblotting and the results are given in FIG. 2.

- [88] Coincident with Example 1-1 with respect to H_2O_2 generation over time, the PDGF-induced tyrosine phosphorylation on proteins in the Prx II^(-/-) MEFs was observed to dramatically increase compared to that in the wild-type MEFs (FIG. 2).
- [89]
- [90] 1-3) Observation of the phosphorylation of phospholipase C gamma 1
- [91] Using phospho-specific antibodies, Prx II expression was analyzed for the activation of downstream signaling molecules.
- [92] After treatment with PDGF, Prx II^(+/+) MEFs and Prx II^(-/-) MEFs were immunoblotted to determine phosphorylation on Src, PLCγ1, ERK2, and Akt proteins with time. In this regard, phospho-Src antibodies (Biosource), phospho-Akt antibodies (Cell Signaling Technology), and phospho-ERK antibodies (Cell Signaling Technology) were used and c-Src, Akt, PDGFRβ (M-20), and ERK2 were purchased from Santa Cruz Biotechnology. Anti-phospho-PLCγ1 (pY783 and pY1253) antibodies were prepared according to well-known methods (Sekiya F, et al., J Biol Chem. 2004 279(31):32181-90).
- [93] As seen in FIG. 3, the residues Tyr783 and Tyr1253, known to be responsible for the activity of PLCγ1, were phosphorylated at higher rates in Prx II^(-/-) MEFs than in the wild-type cells, but there were no changes in c-Src, ERK and Akt activation-dependent phosphorylation.
- [94]
- [95] 1-4) Regulation of Prx II in site-specific phosphorylation for PDGFR activation
- [96] 1-4-1) Antibody preparation
- [97] Phosphor-specific antibodies against peptide regions corresponding to the seven well-known tyrosine residues of PDGFR (providing binding sites for signaling molecules having SH2 domains, such as c-Src, p85 subunit of PI-3K, GAP, Grb2, SHP-2, and PLCγ, respectively) were prepared.
- [98] Phospho-peptide antigens (SynPep, USA) used for the production of phospho-specific PDGFR antibodies were as follows: pY579, DGHEpYIYVDPMQ; pY716, SAELpY-SNALPVG; pY740, SDGGpYMDMSKDE; pY751, ESVDpYVPMLDMK; pY771, ESSNpYMAPYDNY; pY857, RDSNpYISKGSTF; pY1009, SSVLpYTAVQPNE; pY1021, GDNDpYIPLPDP; (the phosphorylated tyrosine residue in each human PDGFR amino acid sequence was italicized).
- [99] The phospho-peptides were conjugated with keyhole limpet hemocyanin via glutaraldehyde and injected into rabbits. Rabbit anti-serum was purified using sequential affinity chromatography in columns filled with non-phosphorylated and phosphorylated peptide-conjugated Affigel-15 (Bio-Rad) agarose resins.

- [100] 1-4-2) Regulation of Prx II in site-specific phosphorylation for PDGFR β activation
- [101] After being treated with PDGF, Prx II^(+/+) MEF and Prx II^(-/-) MEF cells were analyzed for PDGFR β phosphorylation over time through an immunoblotting assay. Using anti-phospho -PDGFR β antibodies, site-specific regulation was observed on the tyrosine residues.
- [102] As seen in FIG. 4, a significant increase in phosphorylation was observed only on Tyr 579/581 and Tyr 857 in Prx II^(-/-) MEFs as compared with wild-type cells.
- [103] When Prx II is deficient, as described above, endogenous H₂O₂ is not eliminated so that downstream signaling molecules are activated. In this regard, site-specific phosphorylation was observed on the residues Y783 and Y1253 of PLC γ 1 and the residues Y579/581 and Y857 of PDGFR. Also, the peroxidase activity of Prx II is essential for the site-specific regulation of PDGFR phosphorylation.
- [104]
- [105] EXAMPLE 2: Observation of Cell Migration in Prx II-Reexpressed Prx II^(-/-) MEF
- [106]
- [107] An add-back rescue experiment, in which retrovirus was used to re-express human Prx II-wt in Prx II^(-/-) MEFs, was performed to examine the influence of the re-expression on protein phosphorylation and migration.
- [108]
- [109] 2-1) Re-expression of human Prx II-wt in Prx II^(-/-) MEFs and observation of protein phosphorylation
- [110] Prx II^(-/-) MEF was infected with retrovirus carrying a control gene or a human Prx II wild-type gene in order to examine the phosphorylation of cellular proteins.
- [111] Control retrovirus (C) and human Prx II-encoding retrovirus (P) were obtained from a stably transformed PT67 cell line and a transiently transformed Phoenix-ampho packing cell line (<http://www.stanford.edu/group/nolan/index.html>).
- [112] In advance of serum starvation, Prx II^(-/-) MEFs were infected with human Prx II-encoding retrovirus (P) at 10 M.O.I.(multiplicity of infection) for two days to express Prx II therein.
- [113] Proteins were extracted from the prepared cells and analyzed for total phosphorylation and tyrosine phosphorylation on PLC γ 1 and PDGFR β through immunoblotting assay.
- [114] In response to PDGF, as is apparent from the data of FIG. 5a, the Prx II-reexpressed cell was phosphorylated to a lesser extent than was the control cell, not only with respect to total proteins but also with respect to the Y783 and Y1253 of PLC γ 1.
- [115] Also, as seen in FIG. 5b, the level of the re-expressed Prx II^(-/-) MEF was similar to the wild-type MEF (Prx II^(+/+)) with respect not only to intracellular Prx II level, but also to tyrosine phosphorylation of PDGFR β in response to PDGF. Also, Prx II-

reexpressed cells were found to have the Y579/581 and Y857 of PDGFR phosphorylated to a lesser extent.

[116] Therefore, the re-expression of Prx II leads to the suppression of the tyrosine phosphorylation on PLC gamma and PDGFR β .

[117]

[118] 2-2) Test for Migration of mouse embryonic fibroblast (MEF)

[119] The Prx II^(-/-) MEF, in which Prx II was reexpressed in the same manner as above, was examined for cell migration.

[120] An assay for MEF cell migration was performed in a 24-well Transwell culture chamber (Costar; 8- μ m pore size). The membrane of the Transwell culture chamber was coated with gelatin B (1 μ g/ μ l) and placed on a 24-well plate. In the lower compartment of the culture chamber, PDGF-BB (25 ng/ml) and DMEM, containing 0.1% bovine serum albumin were filled.

[121] In the upper compartment of the chamber, MEFs (5×10^4) were incubated at 37°C/5% CO₂ for 6 hours. The cells that moved toward the lower part of the filter were stained with 0.6% hematoxylin and 0.5% eosin and counted under an optical microscope. Cell counts were performed in four fields of view selected at random through two independent experiments and were expressed as means $\pm$ S.D.

[122] As understood from data of FIG. 6, lower counts resulted for the wt Prx II-reexpressed cells than for the control, implying that Prx II suppressed PDGF-induced fibroblast proliferation and migration.

[123]

[124] **EXAMPLE 3: Effect of Prx II on Vascular Smooth Muscle Cell Migration**

[125]

[126] PDGF is an important factor for smooth muscle cell proliferation and migration during vascular remodeling. The selective regulation of Prx II in the PDGFR β -PLC γ 1 pathway of human and murine vascular smooth muscle cells (VSMCs) was observed in vitro.

[127]

[128] 3-1) Prx II expression and cellular protein phosphorylation in human VSMCs

[129] Human aortic smooth muscle cells were cultured according to the instructions of the manufacturer (Clonetics Co., USA).

[130] The cells were sub-cultured four times and immunoblotted with an antibody against α -smooth muscle actin (Sigma). Prior to PDGF-BB (25 ng/ml, Upstate) stimulation, the primary cells were subjected to serum starvation (0.5% FBS) for 24 hours.

[131] Control retrovirus (C) and human Prx II-encoding retrovirus (P) were obtained from a stably transformed PT67 cell line and a transiently transformed Phoenix-ampho packing cell line (<http://www.stanford.edu/group/nolan/index.html>).

- [132] In advance of serum starvation, VSMCs were infected with human Prx II-encoding retrovirus (P) at 10 M.O.I. (multiplicity of infection) for two days.
- [133] As understood from data of FIG. 7, neither the control (C) nor the human Prx II-encoding retrovirus-treated group (P) was phosphorylated on tyrosine residues 579/581, 857, and 716 of PDGFR β before PDGF stimulation. 10 min after PDGF stimulation, phosphorylation on the Y579/581, and Y857 residues of PDGFR β occurred to a significantly lesser extent in the human Prx II-encoding retrovirus-treated group (P) than in the control (C).
- [134] The overexpression of wild-type Prx II in human aortic VSMCs about three times as high as the endogenous expression of Prx II resulted in a significant decrease in phosphorylation on PLC γ 1 and the Y579/581 and Y857 of PDGFR.
- [135]
- [136] 3-2) Test for human VSMC migration
- [137] In the human aortic smooth muscle cells prepared in Example 3-1), Prx II was examined for cell migration.
- [138] An assay for the human aortic smooth muscle cell migration was performed in a 24-well Transwell culture chamber (Costar; 8- μ m pore size). The membrane of the Transwell culture chamber was coated with gelatin B (1 μ g/ μ l) and placed on a 24-well plate. In the lower compartment of the culture chamber, PDGF-BB (25 ng/ml) and DMEM, containing 0.1% bovine serum albumin, were charged.
- [139] In the upper compartment of the chamber, VSMCs (5×10^4) were incubated at 37°C/5% CO₂ for 6 hours. The cells that moved toward the lower part of the filter were stained with 0.6% hematoxylin and 0.5% eosin and counted under an optical microscope. Cell counts were performed in four fields of view selected at random through two independent experiments and were expressed as means $\pm$ S.D.
- [140] As can be understood from data of FIG. 8, lower counts resulted for the wt Prx II-reexpressed cells than for the control, implying that Prx II suppressed PDGF-induced smooth muscle cell migration.
- [141]
- [142] 3-3) Observation of phosphorylation in murine VSMCs
- [143] From wild-type mice and Prx II-deficient mice, both 10 weeks old, aortic smooth muscle cells were prepared according to a method described in the reference (Ohmi, K. et al., Biochem Biophys Res Commun, 238, 154-8 (1997)).
- [144] Proteins were extracted from each cell treated with PDGF and analyzed for the tyrosine phosphorylation of PDGFR β by immunoblotting.
- [145] When treated with PDGF, as seen in FIG. 9, VSMCs prepared from the artery of Prx II^(-/-) mice showed more intensive site-specific phosphorylation of PDGFR β than did those prepared from the artery of wild-type mice.

[146]

[147] 3-4) Effect of Prx II on Restenosis of mouse carotid artery after vessel injury

[148] After wild-type and Prx II^(-/-) mouse carotid arteries were injured, the effect of Prx II on the neointimal thickening of VSMCs during restenosis was observed in vivo.

[149] Transluminal wire injuries to mouse left common carotid arteries were performed as specified in the literature (Schober, A. et al., Circulation, 109, 380-5 (2004)).

[150] 10-week-old male mice were anesthetized, followed by exposing the left external carotid artery and coagulating its branches using electric currents. A 0.016-inch flexible angioplasty guide wire was advanced 1 cm through the transverse arteriotomy of the external carotid artery, and endothelial denudation was achieved by 6 passes along the common carotid artery. After one week, the common carotid artery was excised after transcardiac perfusion-fixation with 3.7% formaldehyde and heparinized saline and then paraffin-embedded.

[151] 5 consecutive tissue slices (taken at 100 µm intervals and each 3 µm thick) were obtained from the middle region of the common carotid artery. For morphological observation, each slice was stained with hematoxylin and eosin. Cross-sectional areas of the medial and neointimal layers were analyzed with a computerized analysis system and program (MetaMorpho imaging v5.0, Molecular Devices) with the aid of a digital camera system (CoolSNAP camera system with BX51 microscopy, Olympus).

[152] Neither the wild-type mice nor the Prx II^(-/-) mice underwent the neointimal thickening of the carotid artery that was not injured. When injured with the wire, the carotid artery of the Prx II^(-/-) mice had neointimal layers significantly thicker than those of the wild-type mice, as shown in FIG. 10a. VSMCs were accumulated on the neointimal layer, as indicated by the arrow in FIG. 10a.

[153] The bar graph of FIG. 10b was drawn on the basis of percentage increase of neointimal plaque area relative to medial area (mean±S.E.M., n=5, P<0.01). As seen in the graph, the % increase of neointimal area of Prx II^(-/-) mice increased to about three times that of wild-type mice.

[154] Therefore, the loss of Prx II function leads to the promotion of VSMC migration during vascular remodeling.

[155]

[156] EXAMPLE 4: Plasma PDGF Neutralization

[157]

[158] In order to prevent the neointimal thickening caused by PDGF-induced signal transduction activation, a test for plasma PDGF neutralization was performed.

[159] control IgG or an anti-PDGF antibody (Cat. No. AF-220-NA, R&D systems) was injected into Prx II^(-/-) three times a day over one week starting one day before injuring the carotid artery. At a dose of 5 µg per 20 g of body weight per injection, the antibody

was injected intravenously on -1 and 0 day and then subcutaneously until +4 day. Afterwards, the left external carotid artery was observed for change in neointimal thickness and the results were expressed as percentages of neointimal plaque thickness relative to the medial layer (mean $\pm$ S.E.M., n=4, P<0.01).

[160] As is understood from data of FIG. 11, anti-PDGF antibody administration significantly reduces the neointimal thickening.

[161] Prx II suppresses PDGF-induced smooth muscle cell migration while the loss of Prx II functions results in improving smooth muscle cell migration during vascular remodeling. Thus, the pharmaceutical composition of the present invention can be useful for the prophylaxis and treatment of restenosis.

[162] Also, the composition comprising a Prx II gene or protein in accordance with the present invention can be used for screening materials, that can react therewith, as candidates for developing therapeutics for restenosis.

[163]

Industrial Applicability

[164] As described above, the present invention provides a pharmaceutical composition, comprising Prx II as an active ingredient, for the prophylaxis and treatment of restenosis, based on the research finding that the function of Prx II is highly responsible for the migration of smooth muscle cells during vascular remodeling.

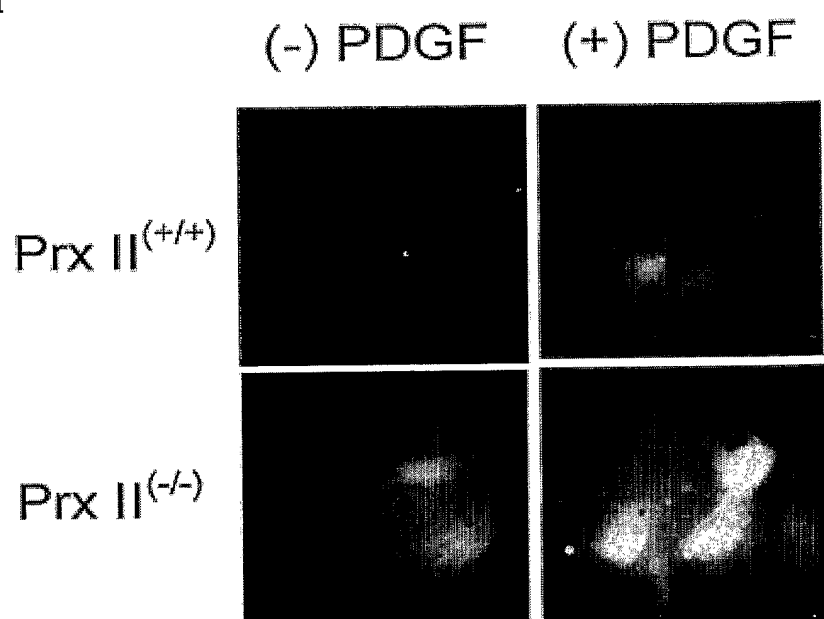
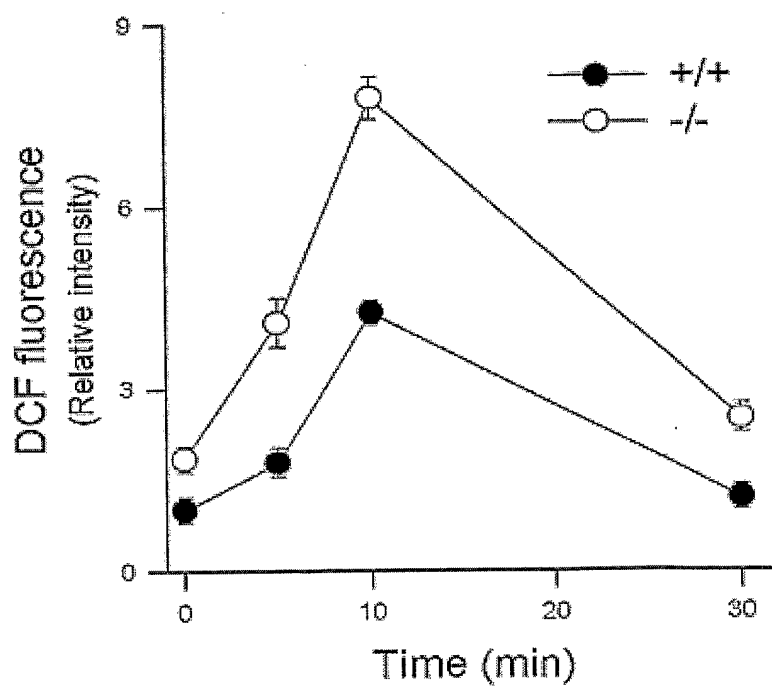
[165] Further, the screening composition and method according to the present invention is very useful in searching for and developing therapeutics for restenosis.

[166] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

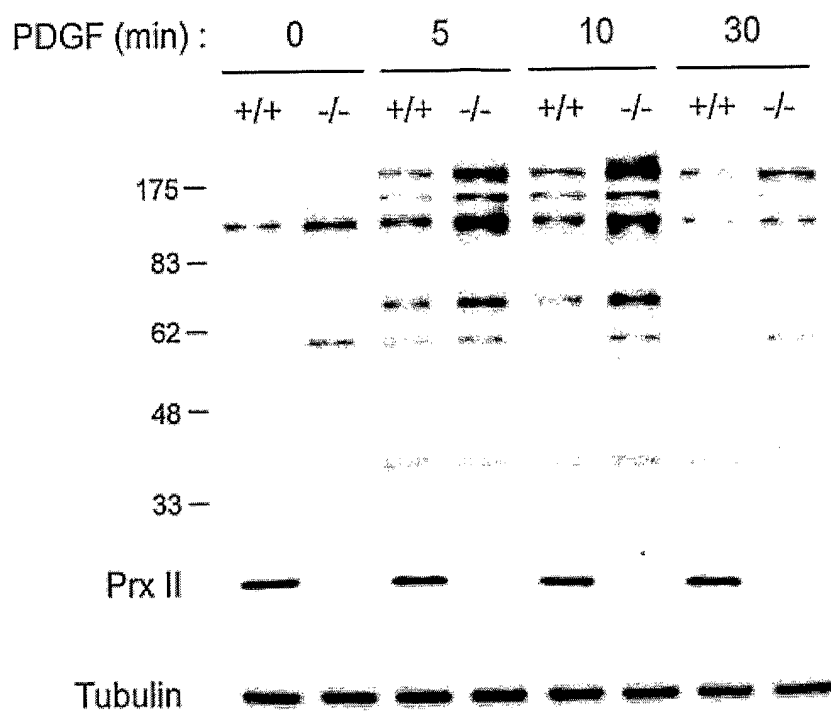
Claims

- [1] A pharmaceutical composition for the prophylaxis and treatment of restenosis, comprising peroxiredoxin 2 (Prx II) as an active ingredient.
- [2] The pharmaceutical composition as set forth in claim 1, wherein the Prx II has an amino acid sequence listed in SEQ. ID. NO. 2.
- [3] The pharmaceutical composition as set forth in claim 1, wherein the restenosis occurs in a carotid artery, a coronary artery, a peripheral artery, and/or a renal artery.
- [4] The pharmaceutical composition as set forth in claim 1, wherein the restenosis is caused due to the chronic hardening process performed in grafted vessels or angioplasted segments or to short-term cell proliferation after angioplasty.
- [5] A composition for screening therapeutics for restenosis, comprising a mammalian Prx II gene.
- [6] The composition as set forth in claim 5, wherein the Prx II gene is selected from a group consisting of the base sequence of SEQ. ID. NO. 1, base sequences of polymorphs of SEQ. ID. NO. 1, fragments of the base sequences, and combinations thereof.
- [7] A composition for screening therapeutics for restenosis, comprising a mammalian Prx II protein.
- [8] The composition as set forth in claim 7, wherein the mammalian Prx II protein is selected from a group consisting of an amino acid sequence of SEQ. ID. NO. 2, proteins expressed from the base sequence of SEQ. ID. NO. 1, proteins expressed from the base sequences of polymorphs of SEQ. ID. NO. 1, Prx II polypeptide fragments having the same physiological activity as Prx II, and combinations thereof.
- [9] A method for screening therapeutics for restenosis, comprising reacting the composition of claim 5 or 6 with a test material to examine whether the test material activates or suppresses the expression of the gene.
- [10] A method for screening therapeutics for restenosis, comprising reacting the composition of claim 7 or 8 with a test material to examine whether the test material enhances or inhibits function of the Prx II protein.

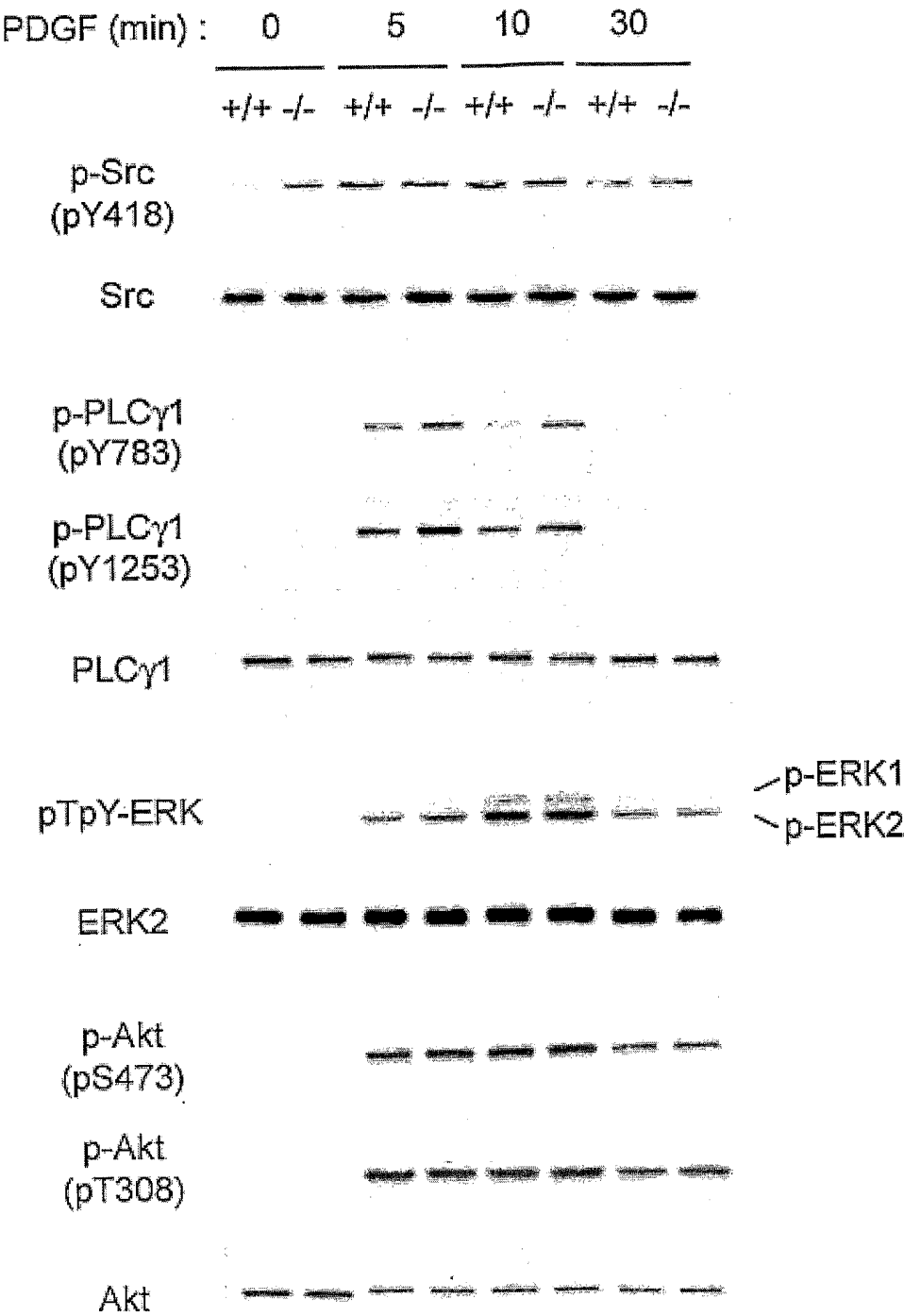
[FIG 1]

a**b**

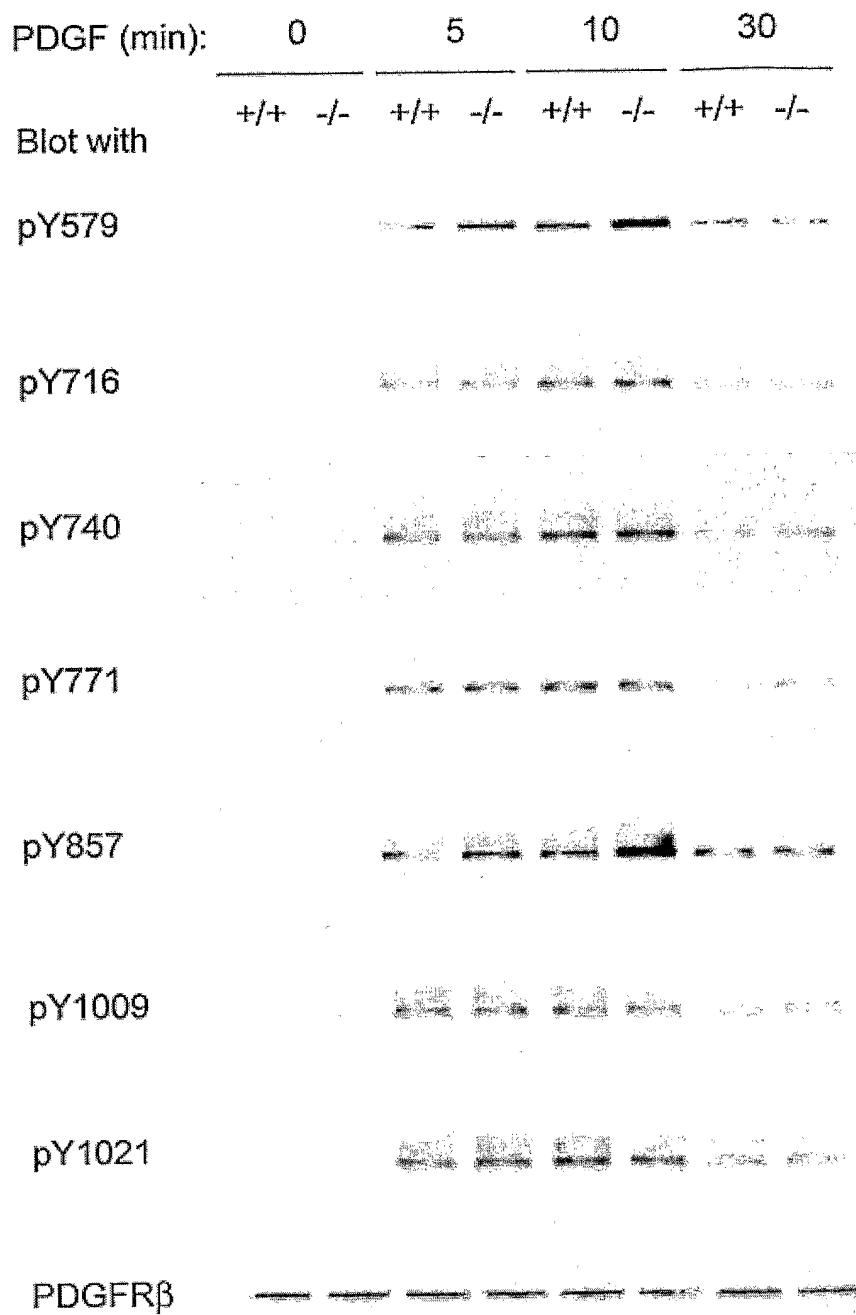
[FIG. 2]



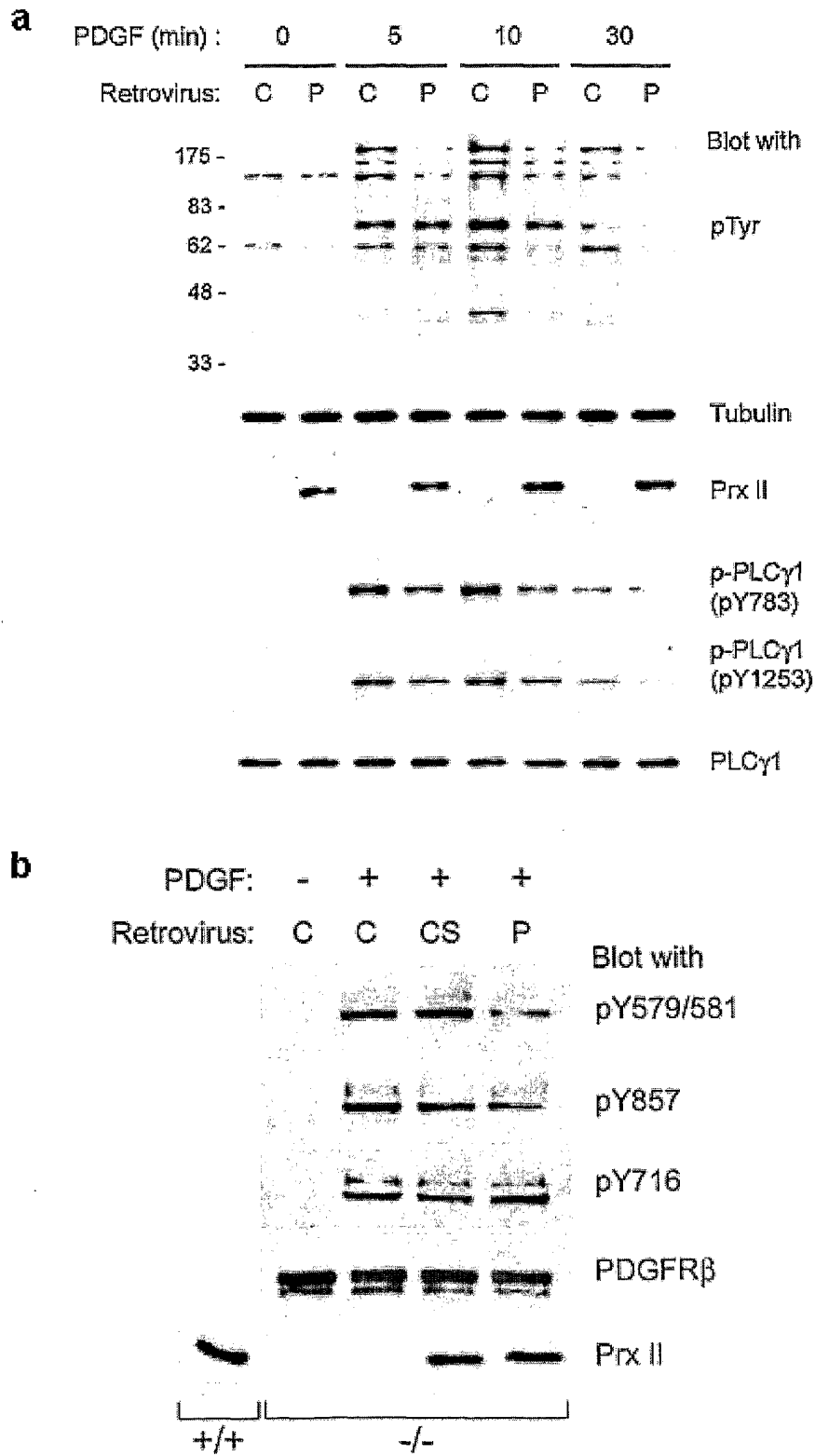
[FIG. 3]



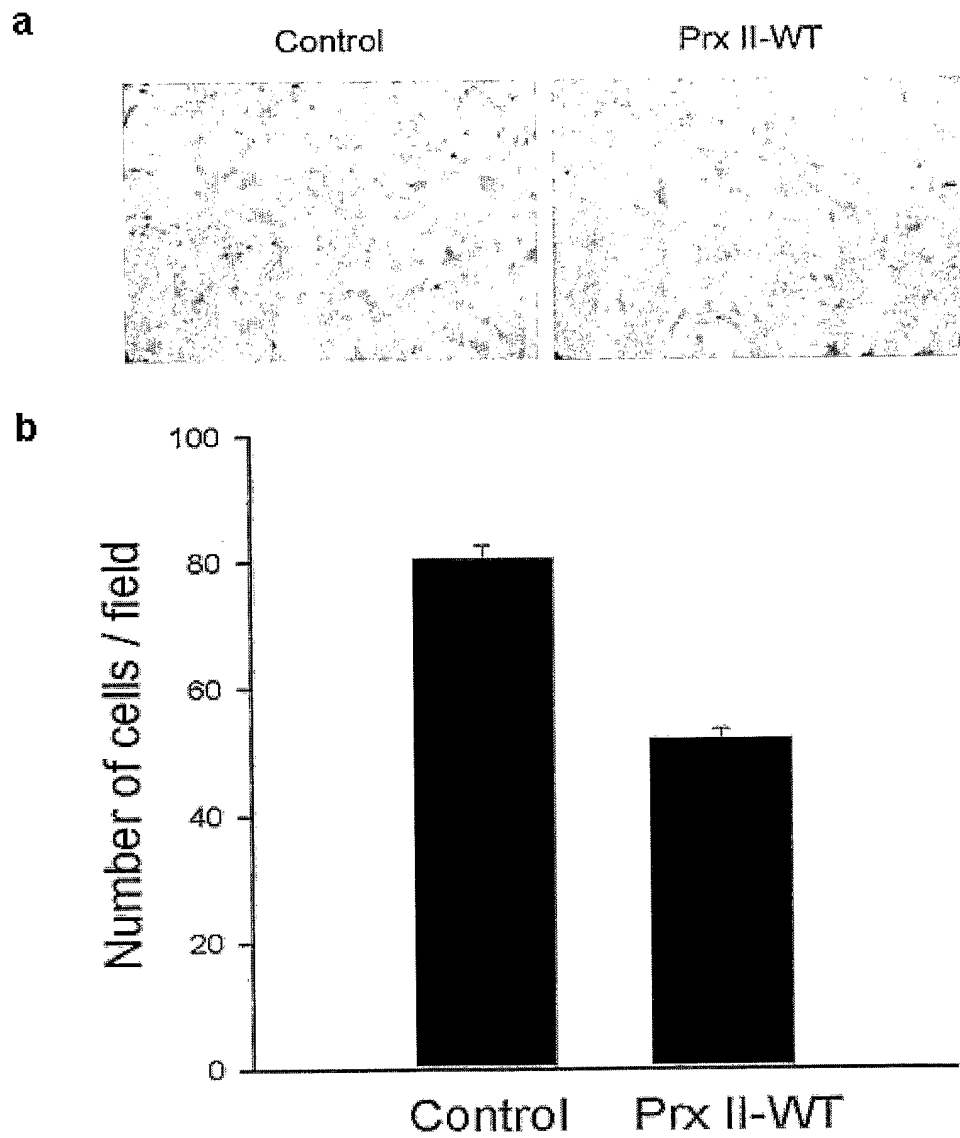
[FIG. 4]



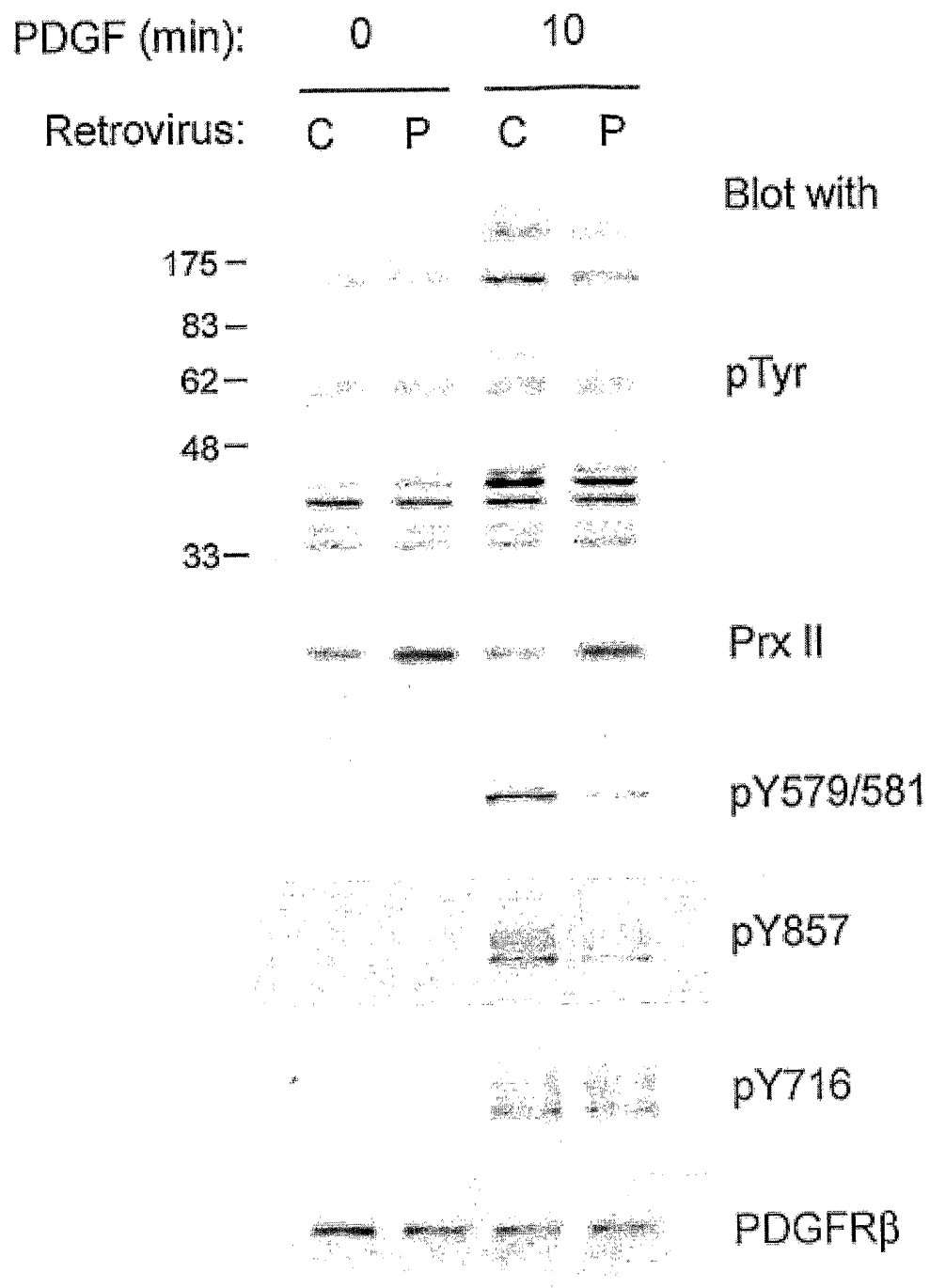
[FIG. 5]



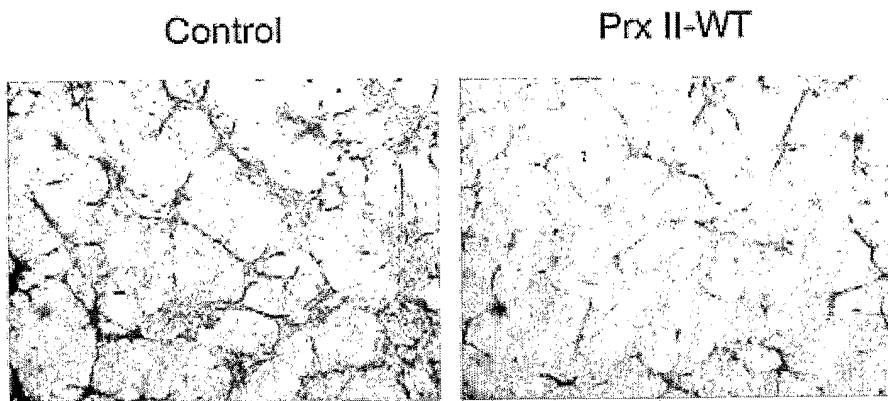
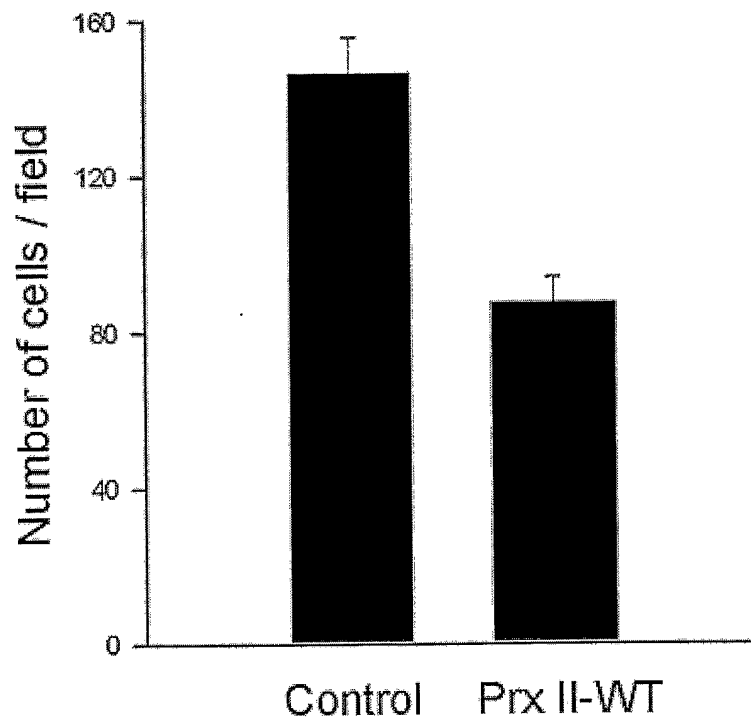
[FIG. 6]



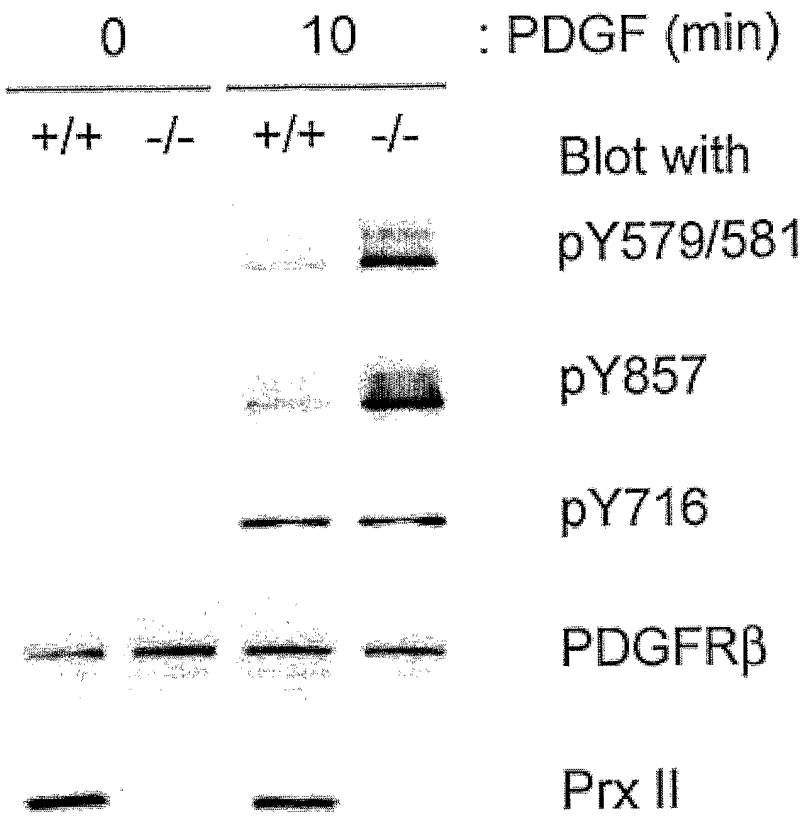
[FIG. 7]



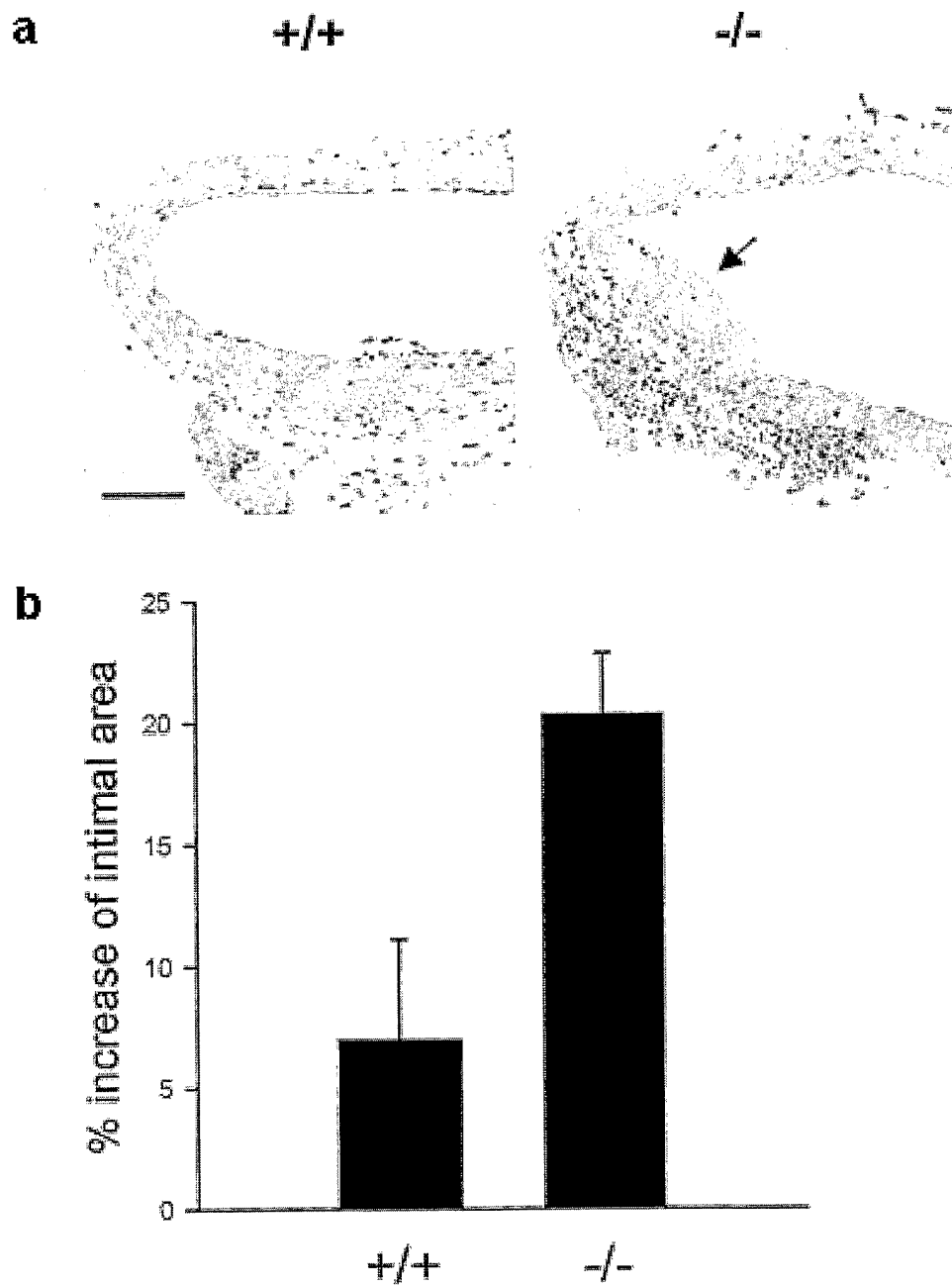
[FIG. 8]

a**b**

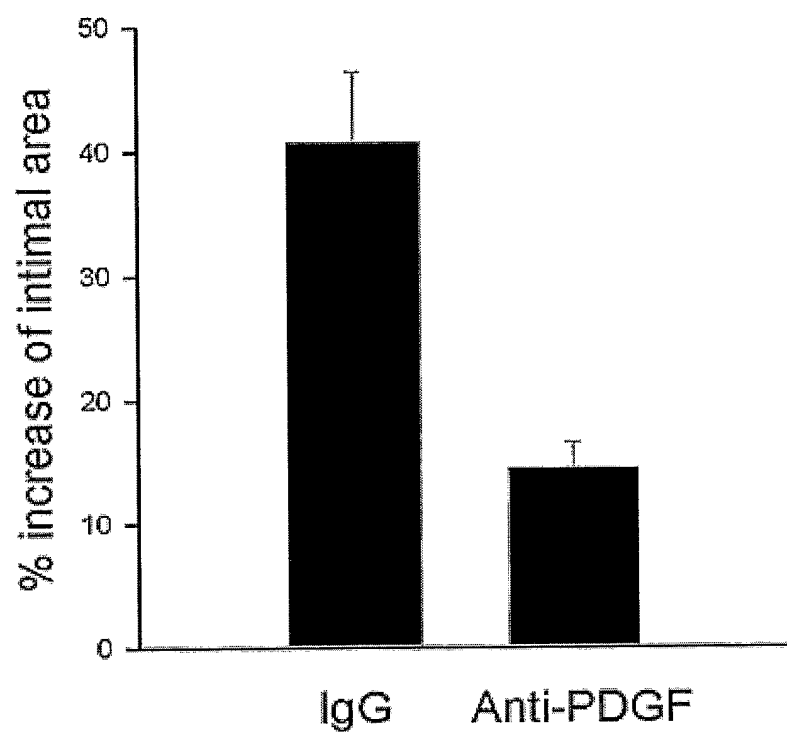
[FIG. 9]



[FIG. 10]



[FIG. 11]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000289**A. CLASSIFICATION OF SUBJECT MATTER***A61K 38/16(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI pubmed database, NCBI blastn database, Delphion Research Intellectual Property network database,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ten Berge D et al 'Prx1 and Prx2 are upstream regulators of sonic hedgehog and control cell proliferation during mandibular arch morphogenesis.'. ' In Development 2001 Vol.128, No. 15, pp2929-38 see the whole document	1-10
A	Jones FS et al. 'Prx1 controls vascular smooth muscle cell proliferation and tenascin-C expression and is upregulated with Prx2 in pulmonary vascular disease.. ' In Circ Res 2001, Vol. 89, No. 2. pp131-8. see the whole document	1-10
A	Kim H et al 'Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide-induced apoptosis in thyroid cells.. In J. Biol Chem, 2000, Vol. 275. No. 24, pp18266-70 see the whole document	1-10

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 MAY 2006 (08.05.2006)

Date of mailing of the international search report

08 MAY 2006 (08.05.2006)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5600



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/000289

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: