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(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL** [US/US]; 308 Bynum Hall, Campus Box 4105, Chapel Hill, North Carolina 27599 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CLEMMONS, David R.** [US/US]; 349 Tenney Circle, Chapel Hill, North Carolina 27514 (US). **MAILE, Laura A.** [GB/US]; 9113 Coachway, Chapel Hill, North Carolina 27516 (US).

(74) Agent: **MYERS BIGEL SIBLEY & SAJOVEC, P.A.**; P.O. Box 37428, Raleigh, North Carolina 27627 (US).

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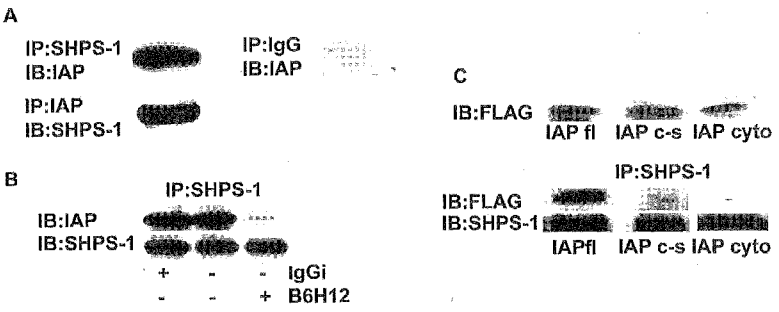
(54) **Title**: METHOD FOR INHIBITING CELLULAR ACTIVATION BY INSULIN-LIKE GROWTH FACTOR-1

Fig. 1



(57) **Abstract**: A method of inhibiting cellular activation by Insulin-like Growth Factor-1 (IGF-1) in a subject in need thereof (e.g., a subject afflicted with cancer, atherosclerosis, diabetic retinopathy or other disease) comprises administering an antagonist that inhibits the binding of IAP to SHPS-1 to the subject in an amount effective to inhibit cellular activation by IGF-1. Compounds and compositions for carrying out such methods are also described.

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METHOD FOR INHIBITING CELLULAR ACTIVATION BY INSULIN-LIKE GROWTH FACTOR-1

Priority Statement

This application claims priority to U.S. Application Serial No. 13/219,276, filed August 26, 2011, the entire contents of which are incorporated herein by reference.

Statement of Government Support

This invention was made with government support under grant number AG02331 from the National Institutes of Health. The U.S. Government has certain rights to this invention.

Field of the Invention

The present invention concerns methods for inhibiting IGF-1 activity in subjects in need thereof, such as subjects afflicted with cancer, atherosclerosis, nephropathy and/or retinopathy.

Background of the Invention

Insulin-like growth factor-I is required for generalized somatic growth, that is the normal growth and development that occurs throughout childhood requires IGF-1. If the IGF-1 gene is deleted from mice, the mice are born at half of a normal size and grow poorly after birth reaching approximately 30% of normal adult size. Therefore this growth factor is an important mitogen for all known cell types.

Interest has emerged in inhibiting IGF-1 activation of mitogenesis in cells because it has been shown that high concentrations of IGF-1 are linked to the development of cancer whereas low concentrations of IGF-1 appear to be cancer protective. For example, U.S. Patent No. 6,340,674 to Baserga et al. describes an antisense method of inhibiting proliferation of cancer cells by contacting the cancer cells with an oligonucleotide substantially complementary to a region of IGF-1 receptor RNA and which specifically hybridizes to IGF-1 receptor RNA.

In addition, IGF-1 is synthesized in the local microenvironment in several diseases that involve abnormal cellular repair. An important disease of this type is atherosclerosis, which is the leading cause of death in the United States. Cells in the

atherosclerotic lesion synthesize excess IGF-1 and therefore excess IGF-1 signaling leads to enlargement of lesions. Several studies have shown that if the effect of this IGF-1 is inhibited, lesion progression is retarded. Therefore there is significant interest in inhibiting IGF-1 action in vessel wall cell types such as smooth muscle cells.

Traditional approaches to inhibiting IGF-1 such as blocking ligand binding to the IGF-1 receptor have failed for two reasons: first, the binding site is quite large and therefore it is difficult to design compounds that will effectively inhibit binding; second, there is a significant structural overlap between the IGF-1 receptor and the insulin receptor, and approaches that have attempted to alter IGF-1 receptor activity by blocking the activity of the receptor have invariably led to toxicity due to coinhibition of the insulin receptor. Antisense techniques present the problem of delivering the active agent to the interior of target cells. Thus there is a need for new ways to inhibit IGF-1 activity or production in cells of subjects in need of such treatment.

Summary of the Invention

The present invention provides a method of inhibiting cellular activation by Insulin-like Growth Factor-1 (IGF-1) in a subject in need thereof [for example, a subject that has or is at increased risk of having a cancer, a tumor, atherosclerosis, nephropathy (e.g., diabetic nephropathy) and/or retinopathy (e.g., diabetic retinopathy)]. The method comprises administering an antagonist that inhibits the binding of IAP to SHPS-1 to the subject in an amount effective to inhibit cellular activation by IGF-1 (for example, an amount effective to treat the condition or disorder or a treatment effective amount).

One aspect of the present invention is a method of treating a tumor in a subject (e.g., a subject in need thereof), comprising administering to the subject an IAP to SHPS-1 binding antagonist (e.g., an antibody of this invention) in an amount effective to treat the tumor (e.g., an amount effective to inhibit the effect of IGF-1 on the tumor). Also included herein is a method of treating cancer in a subject (e.g., a subject in need thereof), comprising administering to the subject an effective amount of an IAP to SHPS-1 binding antagonist (e.g., an antibody of this invention). Nonlimiting examples of cancers that may be treated according to the methods of this invention include breast cancer, colon cancer, lung cancer, prostate cancer, acute

myelogenous leukemia, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, bladder cancer, leiomyosarcoma, ovarian cancer, glioblastoma and hepatocellular carcinoma. Tumors to be treated include those that are associated with any of the cancers listed above as well as any tumors that express IGF-1 receptors.

Another aspect of the present invention is an improvement in a method of treating a tumor in a subject in need thereof by administering a treatment effective amount of an antineoplastic compound (i.e., a chemotherapeutic agent) and/or radiation therapy to the subject, the improvement comprising administering to the subject an to IAP to SHPS-1 binding antagonist in an amount effective to inhibit IGF-1 mediated rescue of tumor cells (i.e., inhibit the anti-apoptotic effect of IGF-1 on tumor cells).

A further aspect of the present invention is a method of treating atherosclerosis in a subject in need thereof, comprising administering to the subject an IAP to SHPS-1 binding antagonist in an amount effective to treat the atherosclerosis. Any type of atherosclerotic lesion may be treated, such as coronary, carotid and/or femoral atherosclerosis. In general, atherosclerotic lesions to be treated are those in which the lesion cells express IGF-1 receptors.

A further aspect of the present invention is a method of treating diabetic nephropathy (e.g., diabetic nephropathy) in a subject in need thereof, comprising administering to the subject an IAP to SHPS-1 binding antagonist in an amount effective to treat the nephropathy.

A further aspect of the present invention is a method of treating retinopathy (e.g., diabetic retinopathy) in a subject in need thereof, comprising administering to the subject an IAP to SHPS-1 binding antagonist in an amount effective to treat the retinopathy.

A further aspect of the present invention is a method of treating coronary artery disease in a subject in need thereof, comprising administering to the subject an IAP to SHPS-1 binding antagonist in an amount effective to treat the coronary artery disease.

Antagonists that may be used in carrying out the methods described herein, sometimes referred to herein as active agents, may be of any suitable type, including proteins or peptides, such as antibodies or antigen binding fragments thereof. Particular examples of antagonists that can be used to carry out the present invention include but are not limited to antibodies that antagonize IAP to SHPS-1 binding,

SHPS-1 fragments comprising, consisting of or consisting essentially of the IAP binding domain, IAP fragments comprising, consisting of or consisting essentially of the SHPS-1 binding domain, analogs thereof, and/or non-peptide mimetics or analogs thereof. In one embodiment of this invention, the antibody can be the monoclonal antibody B6H12.

A further aspect of the present invention is a pharmaceutical formulation comprising an active agent (e.g., an antibody or antigen binding fragment thereof as described herein in a pharmaceutically acceptable carrier.

A further aspect of the present invention is the use of an active agent as described herein for the manufacture of a medicament for carrying out a method of treatment as described herein.

A further aspect of the present invention is an *in vitro* method of screening compounds for activity in *(i)* inhibiting cellular activation by Insulin-like Growth Factor-I (for example, inhibiting cell growth by IGF-1, *(ii)* treating cancers or tumors (as described above), and/or *(iii)* treating atherosclerosis (as described above), the method comprising the steps of: (a) adding or contacting a test compound to an *in vitro* system comprising the SHPS-1 protein and the IAP protein; then (b) determining whether the test compound is an antagonist of IAP to SHPS-1 binding; and then (c) identifying the test compound as active or potentially active in *(i)* inhibiting cellular activation by Insulin-like Growth Factor -1, *(ii)* treating cancers or tumors, and/or *(iii)* treating atherosclerosis when the test compound is an antagonist of IAP to SHPS-1 binding.

The present invention also provides a monoclonal antibody that specifically binds an epitope within amino acids 71-80 (i.e., the peptide ALNKSTVPTD, SEQ ID NO:6) of the human IAP protein (amino acid numbering is based on the amino acid sequence of SEQ ID NO:7) and is an antagonist of IAP to SHPS-1 binding. A further characteristic of the antibody is that it does not disrupt IAP binding to a β_3 protein. The numbering of the amino acids for human IAP is based on the reference amino acid sequence of GenBank® Database Accession No. NP_942088 (incorporated by reference herein) and is as follows, with the first amino acid numbered 1 and the last amino acid numbered 305. Amino acid residues 71-80 are bolded in the sequence below.

MWPLVAALLL GSACCGSAQL LFNKTKSVEF TFCNDTVVIP CFVTNMEAQN TTEVYVKWKF KGRDIYTFDG **ALNKSTVPTD** FSSAKIEVSQ LLKGDASLKM

DKSDAVSHTG NYTCEVTELT REGETIIELK YRVVSWFSPN ENILIVIFPI FAILLFWGQF GIKTLKYRSG GMDEKTIALL VAGLVITVIVIV GAILFVPG EYSLKNATGL GLIVTSTGIL ILLHYYVFST AIGLTSFVIA ILVIQVIAYI LAVVGLSLCI AACIPMHGPL LISGLSILAL AQLLGLVYMK FVASNQKTIQ PPRNN (SEQ IN NO:7).

The monoclonal antibody described above can be the monoclonal antibody produced by hybridoma NPG-1, or a monoclonal antibody that competes for binding to the same epitope as the epitope bound by a monoclonal antibody produced by the hybridoma NPG-1. Hybridoma NPG-1 was deposited with the American Type Culture Collection (ATCC; Manassas, VA) on August 17, 2012 and assigned Accession No. _____.

Additionally provided herein is a method of inhibiting IGF-1 actions in a subject in need thereof, comprising administering to the subject an antibody or antigen binding fragment thereof of this invention.

Further provided is a method of treating diabetic retinopathy, diabetic atherosclerosis, diabetic nephropathy and/or coronary artery disease in a subject (e.g., a subject in need thereof), comprising administering to the subject an effective amount of the antibody or antigen binding fragment thereof of this invention. In such methods the antibody can, in some embodiments be administered by subcutaneous injection and/or intravenous infusion.

Brief Description of the Drawings

Figures 1A-C. Co-precipitation of IAP with SHPS-1 and disruption with anti-IAP antibody, B6H12. **A.** Cell lysates were immunoprecipitated with an anti IAP antibody and co-precipitation of SHPS-1 determined by immunoblotting with anti SHPS-1 antiserum or immunoprecipitated with SHPS-1 and co-precipitation of IAP determined by immunblotting with an anti IAP antibody. As a control, cell lysates were also immunoprecipitated with an irrelevant polyclonal antibody (IgG) and immunoblotted with an anti IAP antibody. **B.** Quiescent pSMCs were incubated for two hours ± the addition of the anti IAP monoclonal antibody, B6H12, or an irrelevant control monoclonal antibody (both at 4 μg/ml). Co-precipitation of IAP with SHPS-1 was then determined by immunoprecipitating with an SHPS-1 antibody and immunoblotting with an anti IAP antibody. The amount of SHPS-1 protein in

each lane is shown in the lower panel. **C.** Expression of FLAG labeled IAP and association with SHPS-1. Top panel: Expression of FLAG labeled IAP was determined by immunblotting whole cell lysates from cells transfected with each of the IAP cDNA constructs using an anti FLAG antibody. The results as scanning units are: Lane 1:38018, Lane 2:39274, Lane 3:46779. Lower panels: Cell lysates were immunoprecipitated with an anti-SHPS-1 antibody then co-precipitation of FLAG labeled IAP was determined by immunoblotting with an anti FLAG antibody. The amount of SHPS-1 that was immunoprecipitated in each lane is shown in the lower panel.

Figures 2A-C. A. SHPS-1 phosphorylation and SHP-2 recruitment to SHPS-1 in response to IGF-1 following disruption of the association between IAP and SHPS-1 by the anti IAP antibody, B6H12. Quiescent cells were incubated for two hours ± B6H12 antibody or irrelevant control monoclonal antibody (both at 4 μg/ml) then exposed to IGF-1 (100 ng/ml) as indicated. Cell lysates were immunoprecipitated with an anti-SHPS-1 antibody then SHPS-1 phosphorylation was determined by immunoblotting with an antiphosphotyrosine antibody (p-Tyr). The association of SHP-2 with SHPS-1 was visualized by immunoblotting using an anti SHP-2 antibody. The amount of SHPS-1 protein in each lane is shown in the lower panel. The increase in SHPS-1 phosphorylation and SHP-2 recruitment following IGF-1 stimulation as determined by scanning densitometry analysis of western immunoblots from three separate experiments is shown. ** $p < 0.05$ when cells preincubated with B6H12 are compared with cells preincubated in SFM alone. **B** SHPS-1 phosphorylation and SHP-2 recruitment in response to IGF-1 following disruption of the association between IAP and SHPS-1 in cells expressing mutated forms of IAP. Cells were exposed to IGF-1 (100 ng/ml) for various periods. Cell lysates were immunoprecipitated with an anti-SHPS-1 antibody and SHPS-1 phosphorylation was determined by immunoblotting with an antiphosphotyrosine antibody (pTyr). The association of SHP-2 was visualized by immunoblotting using an anti SHP-2 antibody. The amount of SHPS-1 protein in each lane is shown in the lower panel. The increase in SHPS-1 phosphorylation and SHP-2 recruitment following IGF-1 stimulation as determined by scanning densitometry analysis of western immunoblots from three separate experiments is shown. ** $p < 0.05$ when cells expressing mutant forms of IAP are compared with cells expressing IAP fl. **C.** SHPS-1 phosphorylation

in response to PDGF. Cells were exposed to PDGF (10 ng/ml) for 5 minutes. Following cell lysis and immunoprecipitation with an anti SHPS-1 antibody SHPS-1 phosphorylation was determined by immunoblotting with an anti phosphotyrosine antibody (pTyr).

Figures 3A-B. IGF-1R phosphorylation time course and SHP-2 recruitment following disruption of the interaction between IAP and SHPS-1. **A.** Quiescent cells were incubated ± anti-IAP antibody, B6H12 (4 μg/ml) then exposed to IGF-1 (100 ng/ml) for various lengths of time. Following lysis and immunoprecipitation with an anti IGF-1R antibody phosphorylation of the receptor was determined by immunoblotting with an anti phosphotyrosine antibody (pTyr). The association of SHP-2 was determined by immunoblotting with an anti SHP-2 antibody. The amount of IGF-1R protein in each lane is shown in the lower panel. The level of tyrosine phosphorylation of IGF-1R as a percentage of maximum phosphorylation detected as determined by scanning densitometry analysis of western immunoblots from three separate experiments is shown. The increase in SHP-2 recruitment following IGF-1 stimulation as determined by scanning densitometry analysis of western immunoblots from three separate experiments is also shown. ** $p < 0.05$ when cells preincubated with B6H12 are compared with cells preincubated in SFM alone. **B.** Cells were incubated with IGF-1 (100 ng/ml) for various times. Following lysis and immunoprecipitation with an anti IGF-1R antibody phosphorylation of the receptor was determined by immunoblotting with an anti phosphotyrosine antibody (pTyr). The association of SHP-2 was determined by immunoblotting with an anti SHP-2 antibody. The amount of IGF-1R protein in each lane is shown in the lower panel. The changes in IGF-1R phosphorylation and SHP-2 recruitment following IGF-1 stimulation as determined by scanning densitometry analysis of western immunoblots from three separate experiments are shown. **$p < 0.05$ when cells expressing IAPc-s are compared with cells expressing IAP fl.

Figures 4A-B. **A.** Phosphorylation of MAPK in response to IGF-1. Cells were plated and grown prior to a 2-hour incubation ± the anti-IAP antibody, B6H12 or irrelevant control monoclonal antibody (both at 4 μg/ml) and then treated with IGF-1 (100 ng/ml) for 10 minutes. The level of p42/44 MAPK phosphorylation was determined by immunoblotting with a phosphospecific MAPK antibody. The total amount of MAPK in each sample was determined by immunoblotting with a MAPK

antibody. **B.** Cells were plated and grown prior to a 2 hour incubation ± B6H12 or an irrelevant control monoclonal antibody (both at a concentration of 4 μg/ml) and then treated with IGF -I (100 ng/ml) for 48 hours. Cell number in each well was then determined. Each data point represents the mean of three independent experiments. **p = < 0.05 when cell number in the cultures incubated in the presence of B6H12 are compared with cell number in the cultures incubated in the absence of antibody.

Figure 5. IGF-1 stimulated cell migration in cells expressing full-length IAP and IAP C-S. Confluent cells were wounded and then incubated ± IGF-1 (100 ng/ml) for 48 hours. The number of cells migrating across the wound edge in at least 5 pre-selected regions was counted. Each data point represents the mean ± S.E.M. of three independent experiments. ** p <0.05 when migration in the presence of IGF-1 is compared with incubation in SFM alone.

Figures 6A-D. Human endothelial cells were exposed to the anti-IAP antibody and lysates were immunoprecipitated with anti-SHPS-1 and then immunoblotted for IAP or Shc. **A**. The monoclonal antibody NPG-1 can disrupt IAP binding to SHPS-1. Cells were preincubated with NPG-1 and then SHPS-1 was immunoprecipitated and the immunoprecipitate was immunoblotted for IAP. **B**. Shc, which has to bind to SHPS-1 to be activated in endothelial cells does not bind normally if the cells are pre-incubated with NPG-1 antibody. **C**. The NPG-1 antibody specifically disrupts IAP binding to SHPS-1 without disrupting IAP binding to β_3. **D.** SHPS-1 association with IAP was determined in the aorta homogenates from control (Con), diabetic (D) and diabetic rats treated with the anti-IAP antibody (R569) (D + AB).

Figures 7A-C. *In vitro* assay of capillary formation. **A**. Cell permeability measured *in vitro* based on dextran blue permeation. IGF-1 stimulates permeation and this is inhibited in the presence of the NPG-1 antibody (IAPab). **B**. The tight junction protein occludin, which allows endothelial cells to form a permeability barrier is disrupted in the presence of IGF-1, resulting in occludin leaving the junctional complex that is normally formed and diffusing out into the cell. In the presence of NPG-1 antibody, this effect of IGF-1 is completely inhibited. **C**. Photomicrographs of endothelial cell tube formation. In IGF-1 treated cells, tube formation can be seen (upper right panel), where the capillary cells are joining each other with capillary tubes. In the presence of NPG-1 antibody, this is completely

disrupted, as shown in the lower two panels and on the bar graph, where the number of tubes per cm^2 is shown.

Figures 8A-B .Rat endothelial cells were cultured in 25mM glucose. Following overnight in SFM cells were incubated with (AB) or without (Con) the anti-IAP antibody (R569), prepared using the rat IAP sequence NKNSTTREQN (SEQ ID NO:8), which are amino acids 71-80 of the rat IAP sequence; numbering is based on the amino acid sequence of NCBI Reference Sequence Accession No. NP_062068 [MWPLAAALLL GSCCCGSAQL LLSKVKSVEF TSCNDTVVIP CKVLNVEAQS TDEMFVKWKL NKSYIFIYDG **NKNSTTREQN** FTSAKISVSD LLKGIASLTM DTHEAVVGNY TCEVTELSRE GKTVIELKNR PVSWFSTNEK ILIVIFPILA ILLFWGKFGI LTLKYKSSHT NKRIILLLVA GLALTLIVVV GAILFIPGEK PVKNASGLGL IVISTGILIL LQYNVFMTAF GMTSFTIAIL ITQVLGYVLA VVGMCLCIMA CEPVHGPLLI SGLGIIALAE LLGLVYMKFV ASNQRTIQPP RNN (SEQ ID NO:9)]. For antibody production, this sequence was linked to KLH as an immunogen according to known methods. **A**. Control antibody had no effect on IAP/SHPS-1 association, whereas the anti-rat IAP antibody completely disrupted this association. B. Following IGF-1 stimulation, there is tyrosine phosphorylation of SHPS-1, stimulation of AKT and MAPK activation. These are inhibited in the presence of the anti-rat IAP antibody.

Figure 9. Vascular permeability. Non diabetic rats are shown as Control (N=5). The diabetic rats were treated with control IgG (N=12) or IgG purified from antiserum that contained the anti-rat IAP antibody described herein (N=14) by protein A sepharose chromatography. After three weeks the animals were anesthetized and then vascular permeability was measured. The anti-rat IAP IgG significantly inhibited retinal vein vascular permeability, which is one of the first changes that occurs in diabetic retinopathy.

Detailed Description of the Invention

The present invention is explained in greater detail below. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations

and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure which do not depart from the instant invention. Hence, the following specification is intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Subjects that may be treated by the present invention include both human subjects for medical purposes and animal subjects for veterinary and drug screening and development purposes. Other suitable animal subjects are, in general, mammalian subjects such as humans, primates, bovines, ovines, caprines, porcines, equines, felines, canines, lagomorphs, rodents (*e.g.*, rats and mice), *etc.* Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile and adult subjects.

"IGF-1" as used herein means insulin-like growth factor-1.

"IGF-1R" as used herein means an IGF-1 receptor.

"IAP" as used herein means integrin associated protein. IAP may be of any type but is preferably mammalian IAP (*e.g.,* human, mouse, rat, rabbit, monkey, pig, etc.), and is most preferably human IAP. IAP (sometimes also called CD47) is known and described in, for example, E. Brown et al. *J Cell Biol* **111:**2785-94 (1990); C. Rosales et al. *J Immunol* **149:**2759-64 (1992); D. Cooper et al. *Proc Natl Acad Sci USA* **92:**3978-82 (1995); P. Jiang et al. *J Biol Chem* **274:**559-62 (1999); P. Oldenborg et al. *Science* **288:**2051-4 (2000); M. Seiffert et al. *Blood* **94:**3633-43 (1999); E. Vernon-Wilson et al. *Eur J Immunol* **30:**2130-2137 (2000); H. Yoshida et al. *J Immunol* **168:**3213-20 (2002); and I. Babic et al. *J Immunol* **164:**3652-8 (2000).

"SHPS-1" as used herein means src homology 2 domain containing protein tyrosine phosphatase substrate 1. SHPS-1 may be of any type but is preferably mammalian SHPS-1 (*e.g.,* human, mouse, rat, rabbit, monkey, pig, etc.), and is most preferably human SHPS-1. SHPS-1 (sometimes also called P84) is known and described in, for example, T. Noguchi et al., *J Biol Chem* **271,** 27652-8 (1996); Y. Fujioka et al., *Mol Cell Biol* **16,** 6887-99 (1996); A. Kharitonenkov et al., *Nature* **386,** 181-6 (1997); M. Stofega et al., *J Biol Chem* **273,** 7112-7 (1998); and T. Takada et al., *J Biol Chem* **273,** 9234-42 (1998).

"SHP-2" as used herein means src homology 2 containing protein tyrosine phosphatase–2.

"Treat," "treating" or "treatment" as used herein refers to any type of action that imparts a benefit to a subject that has a disease or disorder or is at risk of developing the disease or disorder, including improvement in the condition of the subject (e.g., in one or more symptoms), delay in the progression of the disease, delay in the onset of symptoms and/or slowing of the progression of symptoms, etc. As such, in some embodiments, the term "treatment" can include prophylactic treatment of the subject to prevent the onset of symptoms. As used herein, the terms "treat" or "treatment" are not necessarily meant to imply cure or complete abolition of symptoms.

"Treatment effective amount," "amount effective to treat," "effective amount" or the like as used herein means an amount of an antagonist (e.g., an antibody or antigen binding fragment thereof) sufficient to produce a desirable and/or beneficial effect upon a subject that has a cancer, a tumor, atherosclerosis, retinopathy, nephropathy, or other undesirable medical condition in which IGF-1 is inducing abnormal cellular growth. This includes improvement in the condition of the patient (*e.g.,* in one or more symptoms), delay in the progression of the disease, etc.

"Pharmaceutically acceptable" as used herein means that the compound or composition is suitable for administration to a subject to achieve a beneficial and/or treatment effective outcome as described herein, without unduly deleterious side effects in light of the severity of the disease or disorder and necessity of the treatment.

Applicants specifically intend that all patents, patent publications, international patent publications and non-patent references cited herein be incorporated by reference herein in their entirety.

A. Antibodies.

The term "antibody" as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. The term "immunoglobulin" includes the subtypes of these immunoglobulins, such as IgG_1, IgG_2, IgG_3, IgG_4, etc. Of these immunoglobulins, IgM and IgG are preferred, and IgG is particularly preferred. The antibodies may be of any species of origin, including (for example) mouse, rat, rabbit, horse, or human, or may be chimeric antibodies. See, *e.g.*, M. Walker et al. *Molec. Immunol.* 26, 403-11 (1989). An antibody of this invention can be a polyclonal antibody or a monoclonal antibody. Such antibodies are produced in accordance with

known techniques. The term "antibody" as used herein also includes antibody fragments that retain the capability of binding to a target antigen (e.g., an antigen binding fragment thereof, for example, Fab, $F(ab')_2$, and Fv fragments, and the corresponding fragments obtained from antibodies other than IgG. Such fragments are also produced by known techniques.

Antibody fragments included within the scope of the present invention include, for example, Fab, Fab', $F(ab')_2$, and Fv fragments; domain antibodies, diabodies; vaccibodies, linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Such fragments can be produced by known techniques. For example, $F(ab')_2$ fragments can be produced by pepsin digestion of the antibody molecule, and Fab fragments can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse *et al. Science 254*:1275 (1989)).

Monoclonal antibodies may be recombinant monoclonal antibodies produced according to the methods disclosed, for example, in Reading U.S. Pat. No. 4,474,893, or Cabilly et al., U.S. Pat. No. 4,816,567. The antibodies may also be chemically constructed by specific antibodies made according to the method disclosed in Segel et al., U.S. Pat. No. 4,676,980.

Monoclonal Fab fragments may be produced in *Escherichia coli* by recombinant techniques known to those skilled in the art. See, e.g., W. Huse, *Science* 246:1275-81 (1989).

Antibodies for use in the present invention specifically bind to their target with a relatively high binding affinity, for example, with a dissociation constant of about 10^{-6} or 10^{-8}, up to 10^{-12} or 10^{-13}.

Humanized monoclonal antibodies that are antagonists of IAP to SHPS-1 binding are a further aspect of the present invention. A humanized antibody of the present invention may be produced from antibodies as described herein by any suitable technique, using a conventional complementarity determining region (CDR)-grafting method as disclosed, for example, in EPO Publication No. 0239400 and in U.S. Patent Nos. 6,407,213; 6,180,370; and 5,693,762, all of which are incorporated herein by reference in their entirety. Alternatively, a humanized antibody may be produced by directly modifying antibody variable regions without diminishing the

native affinity of the domain for antigen while reducing its immunogenicity with respect to a heterologous species (see, *e.g.*, U.S. Patent No. 5,766,886, which is incorporated herein by reference in its entirety).

Using a CDR-grafting method, the humanized antibody is generally produced by combining a human framework region (FR) with one or more CDRs from a non-human (usually a mouse or rat) immunoglobulin that are capable of binding to a predetermined antigen.

Typically, the humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody may be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG_1, IgG_2, IgG_3 and IgG_4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR of the humanized antibody need not correspond precisely to the parental sequences. At least about 75% of the humanized antibody residues can correspond to those of the parental FR and CDR sequences, in some embodiments, about 90%, and in some embodiments, greater than about 95%.

Antibodies of the invention may be altered or mutated for compatibility with species other than the species in which the antibody was produced. For example, antibodies may be humanized or camelized. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-

human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework (FR) regions (*i.e.*, the sequences between the CDR regions) are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones *et al.*, *Nature 321*:522 (1986); Riechmann *et al.*, *Nature, 332*:323 (1988); and Presta, *Curr. Op. Struct. Biol. 2*:593 (1992)).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can essentially be performed following the method of Winter and co-workers (Jones *et al., Nature 321*:522 (1986); Riechmann *et al., Nature 332*:323 (1988); Verhoeyen *et al., Science 239*:1534 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues (*e.g.*, all of the CDRs or a portion thereof) and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, *J. Mol. Biol. 227*:381 (1991); Marks *et al., J. Mol. Biol. 222*:581 (1991)). The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies

(Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al., J. Immunol. 147*:86 (1991)). Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, *e.g.*, mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al., Bio/Technology 10*:779 (1992); Lonberg *et al., Nature 368*:856 (1994); Morrison, *Nature 368*:812 (1994); Fishwild *et al., Nature Biotechnol. 14*:845 (1996); Neuberger, *Nature Biotechnol. 14*:826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol. 13*:65 (1995).

Polyclonal antibodies used to carry out the present invention can be produced by immunizing a suitable animal (*e.g.*, rabbit, goat, *etc.*) with an antigen to which a monoclonal antibody to the target binds, collecting immune serum from the animal, and separating the polyclonal antibodies from the immune serum, in accordance with known procedures.

Monoclonal antibodies used to carry out the present invention can be produced in a hybridoma cell line according to the technique of Kohler and Milstein, *Nature 265*:495 (1975). For example, a solution containing the appropriate antigen can be injected into a mouse and, after a sufficient time, the mouse sacrificed and spleen cells obtained. The spleen cells are then immortalized by fusing them with myeloma cells or with lymphoma cells, typically in the presence of polyethylene glycol, to produce hybridoma cells. The hybridoma cells are then grown in a suitable medium and the supernatant screened for monoclonal antibodies having the desired specificity. Monoclonal Fab fragments can be produced in *E. coli* by recombinant techniques known to those skilled in the art. *See, e.g.*, Huse, *Science 246*:1275 (1989).

Antibodies specific to the target polypeptide can also be obtained by phage display techniques known in the art.

Monoclonal antibodies can be chimeric or "humanized" antibodies produced in accordance with known techniques. For example, chimeric monoclonal antibodies may be complementarily determining region-grafted antibodies (or “CDR-grafted antibodies”) produced in accordance with known techniques.

An example of an antibody of this invention is monoclonal antibody B6H12 (*e.g.*, B6H12.2 assigned ATCC Accession No. HB-9771).

The present invention also provides a monoclonal antibody that specifically binds an epitope within amino acids 71-80 of the human IAP protein (numbering based on the amino acid sequence of SEQ ID NO:7) and is an antagonist of IAP to SHPS-1 binding. For example the antibody can bind an epitope comprising amino acids 71-73, 71-74, 71-75, 71-76, 71-77, 71-78, 71-79, 71-80, 72-74, 72-75, 72-76, 72-77, 72-78, 72-79, 72-80, 73-75, 73-76, 73-77, 73-78, 73-79, 73-80, 74-76, 74-77, 74-78, 74-79, 74-80, 75-77, 75-78, 75-79, 75-80, 76-78, 76-79, 76-80, 77-79, 77- 80 or 78-80 of the amino acid sequence of SEQ ID NO:7.

The present invention additionally provides a monoclonal antibody produced by the hybridoma NPG-1, and called monoclonal antibody NPG-1 herein. This hybridoma was produced according to standard protocols for monoclonal antibody production as are well known in the art. The immunogen administered to the mice was the peptide ALNKSTVPTDC (SEQ ID NO:10), which represents amino acid residues 71-80 (numbering based on the amino acid sequence of SEQ ID NO:7) of the human IAP amino acid sequence as provided herein (i.e., ALNKSTVPTD, SEQ ID NO:6), with a cysteine residue added at the carboxyl terminus that was used to link the peptide to keyhole limpet hemocyanin (KLH). Therefore, the actual immunogen was a conjugate of the active peptide and KLH, linked by a cysteine. Mice were immunized and then the spleens were harvested for fusion with myeloma cells. The myeloma cell supernatants were screened by ELISA using the immunogen linked to BSA to coat the plates. The positive supernatants were then re-cloned four separate times before the final clone producing the high affinity antibody was selected. The numbering of the amino acids for human IAP is based on the reference amino acid sequence of GenBank® Database Accession No. NP_942088 (incorporated by reference herein) and is as follows, with the first amino acid numbered 1 and the last amino acid numbered 305. Amino acid residues 71-80 are bolded in the sequence below.

MWPLVAALLL GSACCGSAQL LFNKTKSVEF TFCNDTVVIP CFVTNMEAQN TTEVYVKWKF KGRDIYTFDG **ALNKSTVPTD** FSSAKIEVSQ LLKGDASLKM DKSDAVSHTG NYTCEVTELT REGETIIELK YRVVSWFSPN ENILIVIFPI FAILLFWGQF GIKTLKYRSG GMDEKTIALL VAGLVITVIVIV GAILFVPG EYSLKNATGL GLIVTSTGIL ILLHYYVFST AIGLTSFVIA ILVIQVIAYI

LAVVGLSLCI AACIPMHGPL LISGLSILAL AQLLGLVYMK FVASNQKTIQ PPRNN (SEQ IN NO:7).

In some embodiments, the antibody is (a) the monoclonal antibody produced by hybridoma NPG-1, or (b) a monoclonal antibody that competes for binding to the same epitope as the epitope bound by a monoclonal antibody produced by the hybridoma NPG-1 (i.e., a monoclonal antibody that specifically binds to the epitope bound by a monoclonal antibody produced by the hybridoma NPG-1).

Hybridoma NPG-1 was deposited with the American Type Culture Collection (ATCC) in Manassas, Virginia on August 17, 2012 and assigned Accession Number _____. This hybridoma produces an antibody of the present invention, as described herein.

The monoclonal antibody NPG-1 binds to the human IAP protein and disrupts IAP binding to SHPS-1 without disrupting IAP binding to β_3 protein. The β_3 integrin subunit is a component of the $\alpha_v\beta_3$ integrin. It is expressed abundantly on the surface of vascular endothelial cells. Agents which disrupt the function of $\alpha_v\beta_3$ integrin have been shown to lead to changes in endothelial cell function as well as inhibition of endothelial cell growth. Furthermore since this integrin subunit is expressed on platelets, agents that have been shown to inhibit its function in platelets have been shown to stimulate platelet aggregation which can lead to thrombosis. This is an important distinguishing feature of this monoclonal antibody. Many antibodies that react with human IAP also bind to $\alpha_v\beta_3$ integrin. Disrupting IAP binding to β_3 could lead to side effects, making the therapeutic use of such antibodies undesirable. The NPG-1 antibody has the unexpected benefit of binding to human IAP and disrupting its association with SHPS-1 without disrupting IAP binding to β_3.

Further provided herein is a humanized monoclonal antibody NPG-1. The humanized form of the antibody can be prepared using *in vitro* mutagenesis. The complementarity determining regions (CDRs) are left intact unless it is necessary to alter single amino acids with these regions to avoid or minimize immunogenicity. Similarly, the framework regions is scanned for regions that might confer immunogenicity and the appropriate mouse amino acid residues are changed to human amino acid residues. The immunogenicity of the entire antibody is then determined using lymphocytes prepared from human HLA donors from each of the 20 HLA haplotypes.

Various immunoassays can be used for screening to identify antibodies having the desired specificity for the polypeptides of this invention. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificity are well known in the art. Such immunoassays typically involve the measurement of complex formation between an antigen and its specific antibody (*e.g.*, antigen/antibody complex formation). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the polypeptides or peptides of this invention can be used as well as a competitive binding assay.

Antibodies can be conjugated to a solid support (*e.g.*, beads, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques. Antibodies can likewise be conjugated to detectable groups such as radiolabels (*e.g.*, ^{35}S, ^{125}I, ^{131}I), enzyme labels (*e.g.*, horseradish peroxidase, alkaline phosphatase), and fluorescence labels (*e.g.*, fluorescein) in accordance with known techniques. Determination of the formation of an antibody/antigen complex in the methods of this invention can be by detection of, for example, precipitation, agglutination, flocculation, radioactivity, color development or change, fluorescence, luminescence, *etc.*, as is well known in the art.

In various embodiments, the antibody of this invention is an antibody or a fragment thereof (*e.g.*, a monoclonal antibody) that specifically binds to IAP. In some embodiments, the antibody of this invention is an antibody or a fragment thereof (*e.g.*, a monoclonal antibody) that specifically binds to SHPS-1.

In one embodiment, the antibody is a monoclonal antibody produced by hybridoma cell line NPG-1 (ATCC Deposit No. _____; deposited August 17, 2012). In a further embodiment, the antibody is a monoclonal antibody or a fragment thereof that competes for binding to the same epitope specifically bound by the monoclonal antibody produced by hybridoma cell line NPG-1 (ATCC Deposit No. _____; deposited August 17, 2012). In another embodiment, the antibody is a monoclonal antibody or a fragment thereof that specifically binds to the same epitope specifically bound by the monoclonal antibody produced by hybridoma cell line NPG-1 (ATCC Deposit No. _____; deposited August 17, 2012). In certain embodiments, the monoclonal antibody or a fragment thereof is a chimeric antibody or a humanized antibody. In additional embodiments, the chimeric or humanized antibody comprises at least a portion of NPG-1 (ATCC Deposit No. _____;

deposited August 17, 2012). As used herein, a "portion" of a CDR is defined as one or more of the three loops from each of the light and heavy chain that make up the CDRs (*e.g.*, from 1-6 of the CDRs) or one or more portions of a loop comprising, consisting essentially of, or consisting of at least three contiguous amino acids. For example, the chimeric or humanized antibody may comprise 1, 2, 3, 4, 5, or 6 CDR loops, portions of 1, 2, 3, 4, 5, or 6 CDR loops, or a mixture thereof.

B. Protein/peptide antagonists and other antagonists.

The amino terminal Ig domain of IAP and the extracellular Ig variable domain of SHPS-1 are sufficient for their physical interaction, and these regions may serve as protein or peptide antagonists of IAP to SHPS-1 binding. Thus, a further aspect of the present invention is an active agent that is a protein or peptide comprising, consisting of, or consisting essentially of the SHPS-1 binding domain of IAP (*e.g.,* an IAP fragment; the amino terminal Ig domain of IAP). Specific examples include, but are not limited to, a polypeptide consisting of amino acids 1 to 140 of mouse IAP; a polypeptide consisting of amino acids 1 to 135 of mouse IAP; a polypeptide consisting of amino acids 5 to 135 of mouse IAP; a polypeptide consisting of amino acids 5 to 95 of mouse IAP; a polypeptide consisting of amino acids 19 to 95 of mouse IAP; a polypeptide consisting of amino acids 1 to 140 of mouse IAP; a polypeptide consisting of amino acids 1 to 135 of rat IAP; a polypeptide consisting of amino acids 5 to 135 of rat IAP; a peptide consisting of amino acids 5 to 95 of rat IAP; a polypeptide consisting of amino acids 19 to 95 of rat IAP; a peptide consisting of amino acids 1 to 10, 1 to 15, 1 to 20, 1 to 25, 1 to 30, 1 to 35, 1 to 40, 1 to 50, 1 to 60, 1 to 70, 1 to 80, 1 to 90, 1 to 100, 1 to 110, 1 to 120, 1 to 130, 1 to 135 and/or 1 to 140 of human IAP; a peptide consisting of amino acids 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 5 to 40, 5 to 45, 5 to 50, 5 to 60, 5 to 70, 5 to 80, 5 to 95, 5 to 100, 5 to 110, 5 to 120, and/or 5 to 135 of human IAP; a peptide consisting of 10 to 20, 10 to 30, 10 to 35, 10 to 40, 10 to 45, 10 to 50, 10 to 60, 10 to 70, 10 to 80, 10 to 95, 10 to 100, 10 to 110, 10 to 120, and/or 10 to 135 of human IAP; a peptide consisting of amino acids 19 to 30, 19 to 35, 19 to 40, 19 to 45, 19 to 50, 19 to 60, 19 to 70, 19 to 80, 19 to 95, 19 to 100, 19 to 110, 19 to 120, and/or 19 to 135 of human IAP, and a peptide consisting of amino acids 30 to 50, 30 to 60, 30 to 70, 30 to 80, 30 to 90, 40 to 50, 40 to 60, 40 to 70, 40 to 80, 40 to 90, 40 to 100, 50 to 60, 50 to 70, 50 to 60, 60 to 70, 60 to 80, 70 to 80, 80 to 90, 70 to 90, 50 to 80, 50 to 90 and/or 50 to 100 of human

IAP. Also provided herein are antibodies of this invention, which specifically bind any of the IAP peptides and/or epitopes within any of the IAP peptides described herein.

Mouse; human and rat IAP are all known as described above and numbering herein refers to standard numbering assigned to amino acid residues in the full length proteins. The numbering of the amino acids for human IAP is based on the reference amino acid sequence of GenBank® database Accession No. NP_942088 (incorporated by reference herein) and is as follows, with the first amino acid numbered 1 and the last amino acid numbered 305:

MWPLVAALLL GSACCGSAQL LFNKTKSVEF TFCNDTVVIP CFVTNMEAQN TTEVYVKWKF KGRDIYTFDG ALNKSTVPTD FSSAKIEVSQ LLKGDASLKM DKSDAVSHTG NYTCEVTELT REGETIIELK YRVVSWFSPN ENILIVIFPI FAILLFWGQF GIKTLKYRSG GMDEKTIALL VAGLVITVIVIV GAILFVPG EYSLKNATGL GLIVTSTGIL ILLHYYVFST AIGLTSFVIA ILVIQVIAYI LAVVGLSLCI AACIPMHGPL LISGLSILAL AQLLGLVYMK FVASNQKTIQ PPRNN (SEQ ID NO:7).

In some embodiments, the IAP peptide can comprise, consist essentially of or consist of a peptide having the amino acid sequence FVTNMEAQNTTEVYKWK (aa 42-59, SEQ ID NO:11), a peptide having the amino acid sequence KWKFKGRDIYTFDGALNK (aa 57-74, SEQ ID NO:12), a peptide having the amino acid sequence STVPTDFSSAKIEVSQLLKGD (aa 75-95, SEQ ID NO:13), a peptide having the amino acid sequence YTFDGALNKSTVPTDFS (aa 66-92, SEQ ID NO:14) and any combination thereof.

A still further aspect of the present invention is an active agent that is a protein or peptide comprising, consisting of, or consisting essentially of the IAP binding domain of SHPS-1 (*e.g.,* an SHPS-1 fragment; the extracellular Ig variable domain of SHPS-1).

Specific examples include, but are not limited to, a polypeptide consisting of amino acids 1 to 160 of mouse SHPS-1; a polypeptide consisting of amino acids 5 to 150 of mouse SHPS-1; a polypeptide consisting of amino acids 29 to 150 of mouse SHPS-1; a polypeptide consisting of amino acids 1 to 160 of rat SHPS-1; a polypeptide consisting of amino acids 5 to 150 of rat SHPS-1; a polypeptide consisting of amino acids 29 to 150 of rat SHPS-1; a peptide consisting of amino acids 1 to 10, 1 to 15, 1 to 20, 1 to 25, 1 to 30, 1 to 35, 1 to 40, 1 to 50, 1 to 60, 1 to

70, 1 to 80, 1 to 90, 1 to 100, 1 to 110, 1 to 120, 1 to 130, 1 to 135 and/or 1 to 140 of human SHPS-1; a peptide consisting of amino acids 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 5 to 40, 5 to 45, 5 to 50, 5 to 60, 5 to 70, 5 to 80, 5 to 95, 5 to 100, 5 to 110, 5 to 120, and/or 5 to 135 of human SHPS-1; a peptide consisting of 10 to 20, 10 to 30, 10 to 35, 10 to 40, 10 to 45, 10 to 50, 10 to 60, 10 to 70, 10 to 80, 10 to 95, 10 to 100, 10 to 110, 10 to 120, and/or 10 to 135 of human SHPS-1; a peptide consisting of amino acids 19 to 30, 19 to 35, 19 to 40, 19 to 45, 19 to 50, 19 to 60, 19 to 70, 19 to 80, 19 to 95, 19 to 100, 19 to 110, 19 to 120, and/or 19 to 135 of human SHPS-1, a peptide consisting of amino acids 30 to 50, 30 to 60, 30 to 70, 30 to 80, 30 to 90, 40 to 50, 40 to 60, 40 to 70, 40 to 80, 40 to 90, 40 to 100, 50 to 60, 50 to 70, 50 to 80, 50 to 90 and/or 50 to 100 of human SHPS-1, and a peptide consisting of amino acids 100 to 120, 100 to 130, 100 to 140, 100 to 150, 120 to 140, 120 to 130, 120 to 150, 130 to 140 and/or 130 to 150 of human SHPS-1. Also provided herein are antibodies of this invention, which specifically bind any of the SHPS-1 peptides and/or epitopes within any of the SHPS-1 peptides described herein.

Mouse, human and rat SHPS-1 are all known as described above and numbering herein refers to standard numbering assigned to amino acid residues in the full length proteins. The numbering of the amino acids for human SHPS-1 is based on the reference amino acid sequence of GenBank® database Accession No. BAA12974 (incorporated by reference herein) and is as follows, with the first amino acid numbered 1 and the last amino acid numbered 503:

MEPAGPAPGR LGPLLCLLLA ASCAWSGVAG EEELQVIQPD KSVSVAAGES AILHCTVTSL IPVGPIQWFR GAGPARELIY NQKEGHFPRV TTVSESTKRE NMDFSISISN ITPADAGTYY CVKFRKGSPD TEFKSGAGTE LSVRAKPSAP VVSGPAARAT PQHTVSFTCE SHGFSPRDIT LKWFKNGNEL SDFQTNVDPV GESVSYSIHS TAKVVLTRED VHSQVICEVA HVTLQGDPLR GTANLSETIR VPPTLEVTQQ PVRAENQVNV TCQVRKFYPQ RLQLTWLENG NVSRTETAST VTENKDGTYN WMSWLLVNVS AHRDDVKLTC QVEHDGQPAV SKSHDLKVSA HPKEQGSNTA AENTGSNERN IYIVVGVVCT LLVALLMAAL YLVRIRQKKA QGSTSSTRLH EPEKNAREIT QDTNDITYAD LNLPKGKKPA PQAAEPNNHT EYASIQTSPQ PASEDTLTYA DLDMVHLNRT PKQPAPKPEP SFSEYASVQV PRK (SEQ ID NO:15).

In some embodiments, the SHPS-1 peptide can comprise, consist essentially of or consist of a peptide having the amino acid sequence RELIYNQKEGHFPRVTTVS

(aa76-93, SEQ ID NO:16), a peptide having the amino acid sequence VTSLIPVGPIQWFRG (aa57-71, SEQ ID NO:17), a peptide having the amino acid sequence VKFRKGSP (aa 122-129, SEQ ID NO:18) and any combination thereof.

IAP and SHPS-1 fragments that may serve as active agents include analogs thereof. An "analog" is a chemical compound similar in structure to a first compound, and having either a similar or opposite physiologic action as the first compound. With particular reference to the present invention, peptide analogs are those compounds which, while not having the amino acid sequences of the corresponding protein or peptide, are capable of antagonizing IAP to SHPS-1 binding. Such analogs may be peptide or non-peptide analogs, including but not limited to nucleic acid analogs, as described in further detail below.

In protein or peptide molecules which interact with a receptor (*e.g.,* on IAP or SHPS-1), the interaction between the protein or peptide and the receptor generally takes place at surface-accessible sites in a stable three-dimensional molecule. By arranging the critical binding site residues in an appropriate conformation, peptides analogs which mimic the essential surface features of the peptides described herein may be generated and synthesized in accordance with known techniques. Methods for determining peptide three-dimensional structure and analogs thereto are known, and are sometimes referred to as "rational drug design techniques". *See, e.g.*, U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; U.S. Patent No. 4,863,857 to Blalock; (applicants specifically intend that the disclosures of all U.S. Patent references cited herein be incorporated by reference herein in their entirety). *See also* Waldrop, *Science* **247**, 28029 (1990); Rossmann, *Nature* **333**, 392 (1988); Weis et al., *Nature* **333**, 426 (1988); James et al., *Science* **260**, 1937 (1993) (development of benzodiazepine peptidomimetic compounds based on the structure and function of tetrapeptide ligands).

In general, those skilled in the art will appreciate that minor deletions or substitutions may be made to the amino acid sequences of proteins or peptides of the present invention without unduly adversely affecting the activity thereof. Thus, peptides containing such deletions or substitutions are a further aspect of the present invention. In peptides containing substitutions or replacements of amino acids, one or more amino acids of a peptide sequence may be replaced by one or more other amino acids wherein such replacement does not affect the function of that sequence. Such changes can be guided by known similarities between amino acids in physical features such as charge

density, hydrophobicity/hydrophilicity, size and configuration, so that amino acids are substituted with other amino acids having essentially the same functional properties. For example: Ala may be replaced with Val or Ser; Val may be replaced with Ala, Leu, Met, or Ile, preferably Ala or Leu; Leu may be replaced with Ala, Val or Ile, preferably Val or Ile; Gly may be replaced with Pro or Cys, preferably Pro; Pro may be replaced with Gly, Cys, Ser, or Met, preferably Gly, Cys, or Ser; Cys may be replaced with Gly, Pro, Ser, or Met, preferably Pro or Met; Met may be replaced with Pro or Cys, preferably Cys; His may be replaced with Phe or Gln, preferably Phe; Phe may be replaced with His, Tyr, or Trp, preferably His or Tyr; Tyr may be replaced with His, Phe or Trp, preferably Phe or Trp; Trp may be replaced with Phe or Tyr, preferably Tyr; Asn may be replaced with Gln or Ser, preferably Gln; Gln may be replaced with His, Lys, Glu, Asn, or Ser, preferably Asn or Ser; Ser may be replaced with Gln, Thr, Pro, Cys or Ala; Thr may be replaced with Gln or Ser, preferably Ser; Lys may be replaced with Gln or Arg; Arg may be replaced with Lys, Asp or Glu, preferably Lys or Asp; Asp may be replaced with Lys, Arg, or Glu, preferably Arg or Glu; and Glu may be replaced with Arg or Asp, preferably Asp. Once made, changes can be routinely screened to determine their effects on function with enzymes.

Non-peptide mimetics of the proteins or peptides of the present invention (*i.e.,* non-peptide IAP to SHPS-1 binding antagonists) are also an aspect of this invention. Non-protein mimetics may be generated in accordance with known techniques such as using computer graphic modeling to design non-peptide, organic molecules able to antagonize IAP to SHPS-1 binding. *See, e.g.,* Knight, *BIO/Technology* **8**:105 (1990); Itzstein et al *Nature* **363**:418 (1993) (peptidomimetic inhibitors of influenza virus enzyme, sialidase). Itzstein et al. *Nature* **363**:418 (1993), modeled the crystal structure of the sialidase receptor protein using data from x-ray crystallography studies and developed an inhibitor that would attach to active sites of the model; the use of nuclear magnetic resonance (NMR) data for modeling is also known in the art and such techniques may be utilized in carrying out the instant invention. *See also* Lam et al. *Science* **263**:380 (1994) regarding the rational design of bioavailable nonpeptide cyclic ureas that function as HIV protease inhibitors. Lam et al. used information from x-ray crystal structure studies of HIV protease inhibitor complexes to design nonpeptide inhibitors.

Analogs or antagonists may also be developed by utilizing high-throughput screening of compound libraries, as discussed in further detail below. Note that such

compound libraries may be fully random libraries, or libraries generated and/or selected based upon the information based upon the antibody active agents, IAP fragment active agents, or SHPS-1 fragment active agents as described above.

Antagonists or analogs of the foregoing that may be used to carry out the invention may also be developed by generating a library of molecules, selecting for those molecules which act as antagonists, and identifying and amplifying the selected antagonists. See, e.g., Kohl et al. *Science* 260:1934 (1993) (synthesis and screening of tetrapeptides for inhibitors of farnesyl protein transferase, to inhibit ras oncoprotein dependent cell transformation). Eldred et al. (*J. Med Chem.* 37:3882 (1994)) describe nonpeptide antagonists that mimic the Arg-Gly-Asp sequence. Likewise, Ku et al. (*J. Med Chem.* 38:9 (1995)) further illustrate the synthesis of a series of such compounds. Techniques for constructing and screening combinatorial libraries of oligomeric biomolecules to identify those that specifically bind to a given receptor protein are known. Suitable oligomers include peptides, oligonucleotides, carbohydrates, nonoligonucleotides (e.g., phosphorothioate oligonucleotides; see *Chem. and Engineering News*, page 20, Feb. 7, 1994) and nonpeptide polymers (see, e.g., "peptoids" of Simon et al. *Proc. Natl. Acad. Sci. USA* 89:9367 (1992)). See also U.S. Pat. No. 5,270,170 to Schatz; Scott and Smith, *Science* 249:386-390 (1990); Devlin et al. *Science* 249:404406 (1990); Edgington, *BIO/Technology* 11:285 (1993). Peptide libraries may be synthesized on solid supports, or expressed on the surface of bacteriophage viruses (phage display libraries). Known screening methods may be used by those skilled in the art to screen combinatorial libraries to identify antagonists. Techniques are known in the art for screening synthesized molecules to select those with the desired activity, and for labeling the members of the library so that selected active molecules may be identified. See, e.g., Brenner and Lerner, *Proc. Natl. Acad. Sci. USA* 89:5381 (1992) (use of genetic tag to label molecules in a combinatorial library); PCT US93/06948 to Berger et al., (use of recombinant cell transformed with viral transactivating element to screen for potential antiviral molecules able to inhibit initiation of viral transcription); Simon et al. *Proc. Natl. Acad. Sci. USA* 89:9367 (1992) (generation and screening of "peptoids," oligomeric N-substituted glycines, to identify ligands for biological receptors); U.S. Pat. No. 5,283,173 to Fields et al. (use of genetically altered *Saccharomyces cerevisiae* to screen peptides for interactions).

As used herein, "combinatorial library" refers to a collection of diverse oligomeric biomolecules of differing sequence, which can be screened simultaneously for activity as a ligand for a particular target. Combinatorial libraries may also be referred to as "shape libraries," i.e., a population of randomized polymers which are potential ligands. The shape of a molecule refers to those features of a molecule that govern its interactions with other molecules, including Van der Waals, hydrophobic, electrostatic and dynamic. Screening procedures that may be used in conjunction with such libraries are discussed in greater detail below.

C. Formulations and administration.

For administration, the active agent of this invention (e.g., an antibody or antigen binding fragment thereof) will generally be mixed, prior to administration, with a non-toxic, pharmaceutically acceptable carrier substance (e.g. normal saline or phosphate-buffered saline), and will be administered using any medically appropriate procedure, *e.g.*, parenteral administration (*e.g.*, injection) such as by intravenous or intra-arterial injection. In some embodiments, administration can be by injection into the eye (e.g., intraocular, intraretinal and/or intravisceral injection). In some embodiments, administration can be by injection directly into the site of treatment, e.g., directly into a tumor. In some embodiments the active agent of this invention can be linked or conjugated to a carrier (e.g., polyethylene glycol) to alter the half-life or other properties of the active agent.

The active agents described above may be formulated for administration in a pharmaceutical carrier in accordance with known techniques. *See, e.g.,* Remington, *The Science And Practice of Pharmacy* (9th Ed. 1995). In the manufacture of a pharmaceutical formulation according to the invention, the active compound (including the physiologically acceptable salts thereof) is typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a liquid and is preferably formulated with the compound as a unit-dose formulation which may contain from 0.01 or 0.5% to 95% or 99% by weight of the active compound.

Formulations of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of the intended recipient.

These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient.

The active agents may be administered by any medically appropriate procedure, e.g., normal intravenous or intra-arterial administration. In certain cases, direct administration to an atherosclerotic vessel may be desired.

Active agents may be provided in lyophylized form in a sterile aseptic container or may be provided in a pharmaceutical formulation in combination with a pharmaceutically acceptable carrier, such as sterile pyrogen-free water or sterile pyrogen-free physiological saline solution.

Dosage of the active agent will depend, among other things, on the condition of the subject, the particular category or type of disorder or cancer being treated, the route of administration, the nature of the therapeutic agent employed, and the sensitivity of the tumor to the particular therapeutic agent. For example, the dosage range can be from about 0.02 to about 5000 micrograms per kilogram subject body weight. The specific dosage of the antibody or antigenic fragment thereof of this invention is not critical, as long as it is effective to result in some beneficial effect in some individuals within an affected population. In some embodiments, the dosage may be as low as about 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20 or 50 micrograms per kilogram subject body weight, or lower, and as high as about 60, 75, 90, 100, 250, 500, 1000, 2000, 3000, 4000 or 5000 micrograms per kilogram subject body weight, or even higher.

The active agents of the present invention may optionally be administered in conjunction with other, different, cytotoxic agents such as chemotherapeutic or antineoplastic compounds or radiation therapy useful in the treatment of the disorders or conditions described herein (*e.g.,* chemotherapeutics or antineoplastic compounds). The other compounds may be administered concurrently. As used herein, the word "concurrently" means sufficiently close in time to produce a combined effect (that is, concurrently may be simultaneously, or it may be two or more administrations occurring before or after each other) As used herein, the phrase "radiation therapy" includes, but is not limited to, x-rays or gamma rays which are delivered from either an externally applied source such as a beam or by implantation of small radioactive sources. Examples of other suitable chemotherapeutic agents which may be concurrently administered with active agents as described herein include, but are not limited to, Alkylating agents (including, without limitation, nitrogen mustards,

ethylenimine derivatives, alkyl sulfonates, nitrosoureas and triazenes): Uracil mustard, Chlormethine, Cyclophosphamide (Cytoxan™), Ifosfamide, Melphalan, Chlorambucil, Pipobroman, Triethylene-melamine, Triethylenethiophosphoramine, Busulfan, Carmustine, Lomustine, Streptozocin, Dacarbazine, and Temozolomide; Antimetabolites (including, without limitation, folic acid antagonists, pyrimidine analogs, purine analogs and adenosine deaminase inhibitors): Methotrexate, 5-Fluorouracil, Floxuridine, Cytarabine, 6-Mercaptopurine, 6-Thioguanine, Fludarabine phosphate, Pentostatine, and Gemcitabine; Natural products and their derivatives (for example, vinca alkaloids, antitumor antibiotics, enzymes, lymphokines and epipodophyllotoxins): Vinblastine, Vincristine, Vindesine, Bleomycin, Dactinomycin, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Ara-C, paclitaxel (paclitaxel is commercially available as Taxol®), Mithramycin, Deoxyco-formycin, Mitomycin-C, L-Asparaginase, Interferons (especially IFN-a), Etoposide, and Teniposide; Other anti-proliferative cytotoxic agents are navelbene, CPT-11, anastrazole, letrazole, capecitabine, reloxafine, cyclophosphamide, ifosamide, and droloxafine. Additional anti-proliferative cytotoxic agents include, but are not limited to, melphalan, hexamethyl melamine, thiotepa, cytarabin, idatrexate, trimetrexate, dacarbazine, L-asparaginase, camptothecin, topotecan, bicalutamide, flutamide, leuprolide, pyridobenzoindole derivatives, interferons, and interleukins. Preferred classes of antiproliferative cytotoxic agents are the EGFR inhibitors, Her-2 inhibitors, CDK inhibitors, and Herceptin® (trastuzumab). (see, e.g., US Patent No. 6,537,988; US Patent No. 6,420,377). Such compounds may be given in accordance with techniques currently known for the administration thereof.

D. Screening procedures.

As noted above, the present invention provides screening procedures which may be utilized alone or in combination with information on the various active agents described above to generate still additional active agents.

For example, active agents may also be developed by generating a library of molecules, selecting for those molecules which act as ligands for a specified target, and identifying and amplifying the selected ligands, as described herein.

Nucleic acid molecules may also act as ligands for receptor proteins. See, e.g., Edgington, *BIO/Technology* **11**:285 (1993). U.S. Patent No. 5,270,163 to Gold and Tuerk describes a method for identifying nucleic acid ligands for a given target molecule

by selecting from a library of RNA molecules with randomized sequences those molecules that bind specifically to the target molecule. A method for the *in vitro* selection of RNA molecules immunologically cross-reactive with a specific peptide is disclosed in Tsai, Kenan and Keene, *Proc. Natl. Acad. Sci. USA* **89**:8864 (1992) and Tsai and Keene, *J. Immunology* **150**:1137 (1993). In the method, an antiserum raised against a peptide is used to select RNA molecules from a library of RNA molecules; selected RNA molecules and the peptide compete for antibody binding, indicating that the RNA epitope functions as a specific inhibitor of the antibody-antigen interaction.

As noted above, potential active agents or candidate compounds as described can be readily screened for activity in *(i)* inhibiting cellular activation by Insulin-like Growth Factor-I (for example, inhibiting cell growth by IGF-1), *(ii)* treating cancers or tumors (as described above), and/or *(iii)* treating atherosclerosis (as described above) and/or diabetic neuropathy and/or retinopathy and/or any other undesirable disorder characterized by IGF-1 induced cell proliferation. The method comprises the steps of: (a) adding or contacting a test compound to an *in vitro* system comprising the SHPS-1 protein and the IAP protein (this term including binding fragments thereof sufficient to bind to the other); then (b) determining whether the test compound is an antagonist of IAP to SHPS-1 binding; and then (c) identifying the test compound as active or potentially active in *(i)* inhibiting cellular activation by Insulin-like Growth Factor -1, *(ii)* treating cancers or tumors, and/or *(iii)* treating atherosclerosis (or other disorder characterized by IGF-1 induced cell proliferation) when the test compound is an antagonist of IAP to SHPS-1 binding. The *in vitro* system may be in any suitable format, such as cells that express both the SHPS-1 protein and the IAP protein. In the alternative, the *in vitro* system may be a cell-free systems, such as an aqueous preparation of SHPS-1 and IAP, or the binding fragments thereof. The contacting, determining and identifying steps may be are carried out in any suitable manner, such as manually, semi-automated, or by a high throughput screening apparatus. The determining step may be carried out by any suitable technique, such as by precipitation, by labeling one of the fragments with a detectable group such as a radioactive group, etc., all of which may be carried out in accordance with procedures well known to those skilled in the art.

The present invention is explained in greater detail in the following non-limiting Examples, in which the following abbreviations are used: Dulbecco's modified medium (DMEM-H), Fetal bovine serum (FBS), insulin-like growth factor-I

(IGF-1), IGF-1 receptor (IGF-1R), immunoglobulin (Ig), integrin associated protein (IAP), serum free medium (SFM), smooth muscle cells (SMCs), Src homology 2 domain containing protein tyrosine phosphatase substrate 1 (SHPS-1), src homology 2 containing protein tyrosine phosphatase –2 (SHP-2).

EXAMPLE 1

The association between integrin associated protein and SHPS-1 regulates IGF-1 receptor signaling in vascular smooth muscle cells

Insulin-like growth factor-I (IGF-1) is a potent stimulator of smooth muscle cell (SMC) migration and proliferation (Jones et al. *Proc Natl Acad Sci USA* **93:**2482-7 (1996)). There is increasing evidence to show that the ability of IGF-1 to initiate intracellular signaling is regulated not only by its association with its own transmembrane receptor but also by other transmembrane proteins such as the αVß3 integrin (B. Zheng and D. Clemmons *Proc Natl Acad Sci USA* **95:**11217-22 (1998); L. Maile and D. Clemmons *J Biol Chem* **277:**8955-60 (2002)), integrin associated protein (IAP (L. Maile et al. *J Biol Chem* **277:**1800-5 (2002))) and Src homology 2 domain containing protein tyrosine phosphatase substrate-1 (SHPS-1) (Maile and Clemmons, *supra*).

SHPS-1 was identified as a tyrosine phosphorylated protein that binds to SHP-2 in v-SRC transformed fibroblasts (T. Noguchi et al. *J Biol Chem* **271:**27652-8 (1996)) and in insulin stimulated chinese hamster ovary cells (Y. Fujioka et al. *Mol Cell Biol* **16:**6887-99 (1996)). The cytoplasmic region of SHPS-1 contains 2 immunoreceptor tyrosine based inhibitory motifs (A. Kharitonenkov et al. *Nature* **386:**181-6 (1997)) that are phosphorylated in response to various mitogenic stimuli (*see, e.g.,* M. Stofega et al. *J Biol Chem* **273:**7112-7 (1998)) and integrin mediated cell attachment (*see, e.g.,* T. Takada et al. *J Biol Chem* **273:**9234-42 (1998)). This phosphorylation generates binding sites for the recruitment and activation of Src homology 2 domain tyrosine phosphatase (SHP-2) that in turn dephosphorylates SHPS-1.

In stably attached smooth muscle cells (SMCs) SHP-2 is localized to a site close to the cell membrane from where it is transferred to the SHPS-1 following IGF-1 stimulated SHPS-1 phosphorylation (L. Maile and D. Clemmons *J Biol Chem* **277:**8955-60 (2002)). This recruitment of SHP-2 is followed by the

dephosphorylation of SHPS-1 and the transfer of SHP-2 to the IGF-1R where it subsequently dephosphorylates this substrate. The importance of SHPS-1 phosphorylation in regulating IGF-1R dephosphorylation is demonstrated in cells expressing a truncated form of SHPS-1 in which the SHP-2 binding sites have been deleted. In these cells transfer of SHP-2 to both SHPS-1 and the IGF-1R is blocked and sustained phosphorylation of both molecules is evident.

IAP was first identified by its ability to associate with αVß3 (E. Brown et al. *J Cell Biol* **111:**2785-94 (1990)) and to increase the affinity of the integrin for its ligands (E. Brown et al., *J Cell Biol* **111:**2785-94 (1990)). IAP consists of a N-terminal (extracellular) Ig variable type domain followed by five membrane spanning hydrophobic helices and a cytoplasmic tail (C. Rosales et al. *J Immunol* **149:**2759-64 (1992); D. Cooper et al. *Proc Natl Acad Sci USA* **92:**3978-82 (1995)).

IAP has been shown to bind to SHPS-1 (P. Jiang et al. *J Biol Chem* **274:**559-62 (1999); P. Oldenborg et al. *Science* **288:**2051-4 (2000); M. Seiffert et al. *Blood* **94:**3633-43 (1999); E. Vernon-Wilson et al., *Eur J Immunol* **30:**2130-2137 (2000); H. Yoshida et al. *J Immunol* **168:**3213-20 (2002); I. Babic et al., *J Immunol* **164:**3652-8 (2000)). The amino terminal Ig domain of IAP and the extracellular Ig variable domain of SHPS-1 are sufficient for their physical interaction. The effect of IAP binding to SHPS-1 on growth factor stimulated SHPS-1 phosphorylation and SHP-2 recruitment has not been reported. The aim of these studies was to determine the effect of IAP association with SHPS-1 on IGF-1 stimulated SHPS-1 phosphorylation and subsequent SHP-2 recruitment and to study how this alters IGF-1R dependent SMC actions.

Experimental procedures.

Human IGF-1 was obtained from Genentech (South San Francisco, CA, USA); Polyvinyl difluoride membrane (IMMOBILON P™) was purchased from Millipore Corporation (Bedford, MA, USA). Autoradiographic film was obtained from Eastman Kodak (Rochester, NY, USA). Fetal Bovine Serum, Dulbecco's modified medium, penicillin and streptomycin were purchased from Life Technologies, (Grand Island, NY, USA). The IGF-1R ß chain antibody and the monoclonal phosphotyrosine antibody (PY99) were purchased from Santa Cruz (Santa Cruz, CA, USA). The polyclonal SHP-2 and SHPS-1 antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). The monoclonal

antibody against IAP, B6H12, was purified from a B cell hybrid purchased from the American Type Culture Collection (ATCC) and the anti FLAG monoclonal antibody was purchased from Sigma Chemical Company (St Louis, MO, USA). The antibody against the dual phosphorylated (active) form of p42/p44 MAP kinase (MAPK) and the antibody against total p42/p44 MAPK protein were purchased from Cell Signaling Technology (Beverley, MA, USA). All other reagents were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise stated.

Porcine aortic SMCs (pSMCs) were isolated as previously described (A. Gockerman et al. *Endocrinology* **136:**4168-73 (1995)) and maintained in Dulbecco's modified medium supplemented with glucose (4.5 gm/liter), penicillin (100 units/ml), streptomycin (100 μg/ml) (DMEM-H) and 10 % Fetal Bovine serum (FBS) in 10cm tissue culture plates (Falcon Laboratory, Franklin Lakes, NJ). The cells were used between passage 5 and 16.

Generation of Expression Vectors

Full-length porcine IAP with a C-terminal FLAG epitope (IAPfl). Full-length porcine IAP was cloned by RT-PCR from a cDNA library that had been derived from pSMCs that had been isolated as previously described (A. Gockerman et al. *Endocrinology* **136:**4168-73 (1995)). The 5' primer sequence 5' ATGTGGCCCTGGTGGTC (SEQ ID NO:1) corresponded to nucleotides 121-139 of the porcine sequence. The 3' primer sequence was complementary to nucleotides 1005-1030 with the addition of bases encoding the FLAG sequence (underlined) and a stop codon. The sequence was:

5' TCA<u>TTTGTCGTCGTCGTCTTTGTAGTC</u>GGTTGTATAGTCT 3' (SEQ ID NO:2).

Following sequencing, the cDNA was cloned into the pcDNA V5 his 3.1 vector (Invitrogen, Carlsbad, CA, USA).

IAP with truncation of extracellular domain at residue 135 and containing a C-terminal FLAG epitope (IAPcyto). The pcDNA V5 his 3.1 vector containing the IAPfl cDNA sequence was linearized and the mutant form of IAP was generated using PCR with a 5' oligonucleotide encoding bases 527-556 (5' TCTCCAAATGAAAAATCCTCATTGTTATT 3') (SEQ ID NO:3) and the same 3' oligonucleotide that was used to generate the IAPfl. The PCR product was cloned into pcDNA V5 his 3.1.

IAP in which cysteine 33 and 261 are substituted with serine residues containing a C-terminal FLAG epitope (IAPc-s). The IAPfl cDNA was subcloned in a pRcRSV expression vector and it was used as a template to perform single stranded mutagenesis to incorporate the two substitutions. The pRcRSV vector contains a neomycin derivative (G418) resistance gene and a bacteriophage origin of replication (F1) gene that permits direct single stranded mutagenesis of the cDNA. Two oligonucleotides encoding the base substitutions were used. They were: C33S: complementary to nucleotides 204-225 except for a base substitution to encode a serine (underlined) 5' GTAACAGTTGTATTGGAAACGGTGAATTCTA 3' (SEQ ID NO:4) and C261S: complementary to nucleotides 888-918 except for the base substitution to encode the serine residue (underlined):
5' CCATGCACTGGGGTAGACTCTGAGACGCAG (SEQ ID NO:5).

Following sequencing the DNA constructs were subcloned into pMEP4 expression vector (Invitrogen, Carlsbad, CA, USA).

Transfection of pSMCs. Cells that had been grown to 70 % confluency were transfected with one of three IAP cDNA constructs as previously described (24). Hygromycin resistant pSMCs were selected and maintained in DMEM-H containing 15 % FBS and 100 μg/ml hygromycin as described previously (Y. Imai et al., *J Clin Invest* **100,** 2596-605 (1997)). Expression of protein levels was assessed by preparing whole cell lysates and visualizing FLAG protein expression by immunoblotting as described herein. Transfected pSMCs that were obtained from two transfections performed independently were used in subsequent experiments and results obtained were consistent between the two groups of cells.

Cell lysis. Cells were plated at a density of 5 x 10^5 in a 10 cm dishes (Falcon # 3003) and then grown to 90 % confluency (approximately 5 x 10^6 cells). Cells were incubated overnight in serum free medium with 0.5 % bovine serum albumin (SFM) and then pretreated with either the monoclonal anti IAP antibody (B6H12) or an irrelevant control monoclonal antibody for 2 hours (4 μg/ml) when required and then treated with either 100 ng/ml IGF-1 or 10 ng/ml PDGF for the appropriate length of time prior to lysis in ice-cold lysis buffer: 50mM Tris HCl (pH 7.5), 150mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1mM EGTA plus 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The lysates were clarified by centrifugation at 14,000 x g for 10 minutes.

Immunoprecipitation. Cell lysates were incubated overnight at 4°C with the appropriate antibody (IGF-1R, SHPS-1 or B6H12 using a 1:500 dilution). Immune complexes were then precipitated by adding protein A sepharose and incubating for a further 2 hours at 4°C. The samples were then centrifuged at 14,000 x g for 10 minutes and the pellets washed 4 times with lysis buffer. The pellets were resuspended in 45 µl of reducing or non-reducing Laemmeli buffer, boiled for 5 minutes and the proteins were separated by SDS-PAGE, 8% gel.

Assessment of p42/p44 MAP kinase activation. pSMCS were plated at 1 x 10^6 cells/well in six well plates DMEM-H with 0.5 % FBS and incubated at 37°C for 48 hours. Plates were then rinsed and incubated for a further 2 hours in fresh DMEM-H with 0.5% FBS. Cells were then incubated in SFM with or without 4 µg/ml of B6H12 or irrelevant control monoclonal antibody for 2 hours prior to exposure to IGF-1 (100 ng/ml) for 20 minutes. Cells were then lysed with 200 µl of Laemelli buffer and the proteins in 40 µl of cell lysate were then separated by SDS-PAGE (8% gel). The activation of p42/44 MAPK was determined by immunoblotting with an antibody specific for the dual phosphorylated (threonine^{202} and tyrosine^{204}) protein (at a dilution of 1:1000) as described herein. To control for differences in protein levels, an equal volume of cell lysate from each sample was loaded on an additional 8% gel. Following separation and transfer, total p42/p44 protein levels were determined using a polyclonal p42/p44 MAPK antibody (at a dilution of 1:1000).

Western immunoblotting. Following SDS-PAGE the proteins were transferred to Immobilon P membranes. The membranes were blocked in 1% BSA in Tris-buffered saline with 0.1% Tween (TBST) for 2 hours at room temperature and then incubated with one of six primary antibodies (IGF-1R, SHP-2, SHPS-1, PY99, B6H12 or FLAG, 1:500 dilution) overnight at 4°C and washed three times in TBST. Binding of the peroxidase labeled secondary antibody was visualized using enhanced chemiluminescence following the manufacturer's instructions (Pierce, Rockford IL, USA) and the immune complexes were detected by exposure to autoradiographic film or using the GeneGnome CCD imaging system (Syngene Cambridge, UK Ltd).

Chemiluminescent images obtained were scanned using a DuoScan T1200 (AGFA Brussels, Belgium) and band intensities of the scanned images were analyzed using NIH Image, version 1.61. The Student's t test was used to compare differences between treatments. The results that are shown are representative of at least three separate experiments.

Cell wounding and migration assay. Cells were plated in six-well plates and grown to confluency over seven days with one medium change. Wounding was performed as previously described (J. Jones et al. *Proc Natl Acad Sci U S A* **93:** 2482-7 (1996)). Briefly, a razor blade was used to scrape an area of cells leaving a denuded area and a sharp visible wound line. Six 1 mm areas along the wound edge were selected and recorded for each treatment. The wounded monolayers were then incubated with SFM (plus 0.2% FBS) with or without 100 ng/ml IGF-1 or PDGF (10 ng/ml). The cells were then fixed and stained (Diff Quick, Dade Behring, Inc., Newark, DE) and the number of cells migrating into the wound area was counted. At least five of the previously selected 1 mm areas at the edge of the wound were counted for each data point.

Assessment of cell proliferation. Cells were plated at 5000 cells/cm^2 on 24 well plates in DMEM-H with 2% FBS and allowed to attach and spread for 24 hours before changing medium to DMEM-H plus 0.2% human platelet poor plasma. Following a further 24-hour incubation, cells were pre-incubated in the presence or absence of B6H12 or an irrelevant control monoclonal antibody (4 μg/ml) for 2 hours prior to the addition of IGF-1 (100 ng/ml). Each treatment was set up in triplicate. Cells were then incubated for 48 hours and final cell number in each well determined. The Student's t test was used to compare differences between treatments. The results that are shown represent the mean (±SEM) from three separate experiments.

Results

IAP associates with SHPS-1 in stably attached pSMCs via its extracellular domain. Figure 1A shows that in stably attached quiescent SMCs there is detectable association between IAP and SHPS-1 as determined by co-immunoprecipitation experiments using both anti IAP and anti SHPS-1 antibodies for immunoprecipitation.

In order to investigate the role of IAP association with SHPS-1 in IGF-1R signaling two experimental models were developed in which the association between IAP and SHPS-1 was disrupted. The first approach was to use an anti-IAP monoclonal antibody, B6H12, to interfere with the binding of the two proteins. **Figure 1B** shows that following incubation of quiescent pSMCs with the anti IAP monoclonal antibody (B6H12) the interaction between IAP and SHPS-1 is reduced (a

75 ± 7.5 % reduction (mean ± S.E.M n = 3)). Preincubation with an irrelevant control monoclonal antibody has no effect on the association between the two proteins.

The binding between IAP and SHPS-1 specifically requires an intact disulfide bond in IAP between cysteine 33 in the extracellular domain and cysteine 261 within the putative transmembrane domain (R. Rebres et al., *J Biol Chem* **276:**7672-80 (2001)) If this bond is disrupted by mutagenesis, the interaction of IAP with αVß3 is preserved but binding to SHPS-1 is eliminated. Two mutant forms of IAP were generated and expressed in which the association between IAP and SHPS-1 would be expected to be disrupted. **Figure 1C** (top panel) shows the level of expression of three forms of IAP that were used in subsequent experiments. These included a) the FLAG tagged mutant form of IAP in which the complete extracellular domain has been deleted at amino acid residue 135 (IAPcyto), b) the FLAG tagged mutant form of IAP in which the two cysteine residues 33 and 261 had been substituted with serines (IAPc-s) and c) the FLAG tagged full length IAP (IAPfl).

A representative experiment shown in **Figure 1C** (lower panels) shows that disruption of the extracellular domain of IAP alters its ability to associate with SHPS-1. Expression of IAPcyto results in a 88 ± 6.4 % (mean ± SEM n=3) reduction in IAP association with SHPS-1 compared with association in cells expressing IAPfl. Since truncation of the extracellular domain of IAP also disrupts its association with αVß3 the SHPS-1/IAP interaction was analyzed in cells expressing the IAPc-s mutation. In cells expressing IAPc-s there is an 81 ± 4.5 % (mean ± SEM n=3) reduction in IAP association with SHPS-1 compared with cells expressing IAPfl. The control immunoblots show that similar levels of SHPS-1 were immunoprecipitated.

Blocking IAP-SHPS-1 association inhibits IGF-1 stimulated SHPS-1 phosphorylation and SHP-2 recruitment. To determine the functional consequences of loss of physical association between IAP and SHPS-1, studies were conducted to examine SHPS-1 phosphorylation in response to IGF-1 in wild type cells pretreated with the anti IAP monoclonal antibody B6H12. A representative experiment is shown in **Figure 2A** and it can be seen that in contrast to the 4.1 ± 0.9 (mean ± SEM n = 3) fold increase in SHPS-1 phosphorylation in response to IGF-1 in controls, cells pretreated with B6H12 show a significant decrease (0.93 ± 0.12 (mean ± SEM n = 3 $p < 0.05$) in the IGF-1 stimulated increase in SHPS-1 phosphorylation. In cells preincubated with an irrelevant control monoclonal antibody IGF-1 stimulated

SHPS-1 phosphorylation did not differ significantly from control cells. As can also been seen in **Figure 2A** this reduction in SHPS-1 phosphorylation in the presence of B6H12 is associated with a significant decrease in IGF-1 stimulated recruitment of SHP-2 to SHPS-1 (a 1.8 ± 1.1 fold increase in SHP-2 association in the presence of B6H12 compared with a 14 ± 1.5 fold increase in control cells (mean ± SEM n = 3 $p < 0.05$). Again there was no significant effect on IGF-1 stimulated recruitment of SHP-2 to SHPS-1 in cells preincubated with an irrelevant control monoclonal antibody.

The extracellular domain of IAP is required for IGF-1 stimulated SHPS-1 phosphorylation and SHP-2 recruitment. In order to confirm the previous observation that suggested that blocking IAP binding to SHPS-1 inhibited IGF-1 stimulated SHPS-1 phosphorylation, the ability of IGF-1 to stimulate SHPS-1 phoshorylation in cells expressing the mutant forms of IAP was compared with cells expressing wild type IAP. The results from a representative experiment are shown in **Figure 2B** and it can be seen that in contrast to the 3.6 ± 0.8 (mean ± SEM n = 3) increase in SHPS-1 phosphorylation in response to IGF-1 in cells expressing IAPfl, in cells expressing the IAPcyto mutant or IAPc-s mutant no significant increase in SHPS-1 phosphorylation in response to IGF-1 can be detected.

Consistent with the results obtained using B6H12, the lack of SHPS-1 phosphorylation observed in the cells expressing the mutant forms of IAP is associated with an inhibition in SHP-2 recruitment to SHPS-1 in response to IGF-1 (**Figure 2B**).

Since SHPS-1 has been shown to be phosphorylated in response to several growth factors, studies were done to investigate the specificity of the requirement of IAP binding to SHPS-1. **Figure 2C** shows that PDGF induces a marked increase in SHPS-1 phosphorylation following 5 minutes exposure in cells expressing IAPfl. However, in contrast to IGF-1, PDGF also stimulated SHPS-1 phosphorylation in the IAPc-s cells.

The association between the extracellular domain of IAP and SHPS-1 regulates the duration of IGF-1R phosphorylation via its modulation of SHP-2 recruitment. Phosphorylation of SHPS-1 is required for SHP-2 transfer to the IGF-1R and thereby regulates the duration of IGF-1R phosphorylation (T. Noguchi et al. *J Biol Chem* **271**:27652-8 (1996)); therefore studies were carried out to examine IGF-1R recruitment of SHP-2 and the duration of IGF-1R phosphorylation in cells pre

treated with B6H12 and cells expressing the mutant forms of IAP. In control cells, IGF-1 stimulates a 3.3 ± 0.4 (mean ± SEM n = 3) fold increase in SHP-2 recruitment to the IGF-1 receptor following 10 minutes treatment with IGF-1. However in cells pretreated with B6H12 recruitment of SHP-2 to the IGF-1R there is no significant increase seen in SHP-2 recruitment to the IGF-1R (**Figure 3A**). Consistent with previous results (L. Maile and D. Clemmons, *J Biol Chem* **277**:8955-60 (2002)) the recruitment of SHP-2 to the IGF-1R precedes a reduction in receptor phosphorylation observed following 20 minutes IGF-1 stimulation. However, in cells preincubated with B6H12 consistent with the lack of SHP-2 recruitment no reduction in IGF-1R phosphorylation is detectable at the 20-minute time point. To confirm that the lack of SHP-2 recruitment to the IGF-1R in the cells pretreated with B6H12 was due to the specific disruption between IAP/SHPS-1 IGF-1R phosphorylation was examined in cells expressing IAPc-s. **Figure 3B** shows that in these cells there is no increase in the recruitment of SHP-2 to the IGF-1R in response to IGF-1 and again this is associated with is a decrease in the amount of IGF-1R dephosphorylation observed following 20 minutes stimulation with IGF-1 in cells expressing full length IAP.

IGF-1 stimulated MAPK activity is inhibited following disruption of SHP-2 transfer. Previous studies have shown that expression of an inactive form of SHP-2 results in an inhibition of IGF-1 stimulated MAPK (S. Manes et al. *Mol Cell Biol* **4**:3125-35 (1999)). To examine the consequence of the lack of SHP-2 transfer following the disruption of IAP-SHPS-1 binding, the activation of MAPK in response to IGF-1 in the presence of B6H12 was analyzed.

Figure 4A shows that 10 minutes of IGF-1 treatment stimulates a marked increase in the activation of MAPK as determined by the assessment of the dual phosphorylation of p42/p44 MAPK (70 ± 5 % S.E.M n = 4). However, when cells were preincubated with B6H12, IGF-1 was unable to stimulate a sustained increase in p42/p44 MAPK phosphorylation. MAPK is required for IGF-1 to stimulate cell proliferation.

To examine the consequence of the disruption in IAP-SHPS-1 association on IGF-1 action in SMCs, the effect of B6H12 on IGF-1 stimulated cell proliferation was determined. **Figure 4B** shows that IGF-1 stimulates a 2.2 ± 0.2 (mean ± SEM n = 3) fold increase in cell proliferation. However when cells are incubated with B6H12 there is a significant reduction in IGF-1 stimulated cell proliferation (1.03 ± 0.01

mean ± SEM n = 3 $p < 0.05$ compared with cells incubated in the absence of B6H12. The inhibition in cell proliferation is consistent with the inhibition of IGF-1 stimulated MAPK activation.

Disruption of the IAP interaction with SHPS-1 inhibits IGF-1 stimulated cell migration. Preincubation of pSMCs with B6H12 inhibits IGF-1 stimulated migration in part by altering the interaction between IAP and αVβ3 (L. Maile et al. *J Biol Chem* **277:**1800-5 (2002)). To determine whether at least part of the effect of B6H12 was also due to the inhibition of IAP binding to SHPS-1 cell migration in response to IGF-1 was compared in cells expressing IAPfl and the IAPc-s mutant. In **Figure 5** it can be seen that IGF-1 stimulated a significant increase in pSMC migration in cells expressing IAPfl. However, in cells expressing the IAPc-s mutant IGF-1 stimulated migration is significantly reduced. In contrast, PDGF stimulated cell migration of the IAPc-s cells is not significantly different to cells expressing full length IAP.

Discussion

The role of SHPS-1 in intracellular signaling has largely been attributed to the recruitment of SHP-2 to the phosphorylated tyrosines contained within ITIM motifs in the cytoplasmic tail of SHPS-1 and the subsequent activation of SHP-2 phosphatase activity (L. Maile et al. *J Biol Chem* **277:**1800-5 (2002); T. Takada et al. *J Biol Chem* **273:**9234-42 (1998); J. Timms et al., *Curr Biol* **9:**927-30 (1999)). The requirement for transfer of activated SHP-2 to downstream signaling molecules for growth factors such as IGF-1 to stimulate their physiologic actions has been strongly suggested by studies showing that expression of dominant negative forms of SHP-2 result in failure to properly activate growth factor stimulated increases in MAP kinase (T. Noguchi et al. *Mol Cell Biol* **14:**6674-82 (1994); K. Milarski and A. Saltiel *J Biol Chem* **269:**21239-43 (1994); S. Xiao et al. *J Biol Chem* **269:**21244-8 (1994); K. Yamauchi et al. *Proc Natl Acad Sci* **92:**664-8 (1995); G. Pronk et al. *Mol Cell Biol* **14:**1575-81 (1994); T. Sasaoka et al. *J Biol Chem* **269:**10734-8 (1994)) and PI-3 kinase (C. Wu et al. *Oncogene* **20:**6018-25 (2001); S. Ugi et al. *J Biol Chem* **271:**12595-602 (1996); S. Zhang et al. *Mol Cell Biol* **22:**4062-72 (2002)) as well as failure to recruit SHP-2 to downstream signaling molecules. For IGF-1 it was specifically shown that expression of a dominant negative SHP-2 mutant resulted in a failure to activate MAP kinase or

cell migration in response to IGF-1 (S. Manes et al. *Mol Cell Biol* **4**:3125-35 (1999)). The results from this study have demonstrated that the interaction between the IAP and SHPS-1 is a key regulator of IGF-1 signaling since these data have shown that the interaction is necessary for SHP-2 recruitment and transfer. Disruption of the interaction between the two proteins using two independent approaches resulted in a loss of SHP-2 recruitment to SHPS-1 and subsequent transfer to the IGF-1R which was reflected in prolonged IGF-1R phosphorylation. The consequence of lack of SHP-2 recruitment and transfer was evident in the inability of IGF-1 to stimulate MAPK activation and subsequently cell proliferation or cell migration.

The interaction between SHPS-1 and IAP was first suggested by experiments that demonstrated that anti IAP monoclonal antibodies blocked the attachment of cerebellar neurons, erthyrocytes and thymocytes to a substratum containing P84 (a brain homolog of SHPS-1) (P. Jiang et al. *J Biol Chem* **274**:559-62 (1999); M. Seiffert et al., *Blood* **94**:3633-43 (1999)). That this interaction might play a role in cell-to-cell attachment was substantiated in experiments which demonstrated that the expression of the extracellular domain of SIRPα in SIRP negative cells supported adhesion of primary hematopoietic cells and this interaction was again inhibited by anti IAP monoclonal antibodies (E. Vernon-Wilson et al. *Eur J Immunol* **30**:2130-2137 (2000)).

Cell adhesion molecules mediating either cell attachment to the extracellular matrix, for example integrins and cell to cell adhesion molecules, for example cadherins, are important not only for cell attachment but also for the regulation of cell proliferation, survival and differentiation. The regulation of growth factor signaling by integrin receptors has been well documented. It has been previously reported that ligand occupancy of αVß3 is necessary for IGF-1 stimulated receptor signaling and a similar cooperative relationship between αVß3 and the PDGF receptor has also been described (S. Miyamoto et al. *J. Cell. Biol.* **135**:16633-1642 (1996)). IGF-1 has been shown to be a regulator of various homophilic cell to cell adhesion molecules. Guvakova et al. reported that the IGF-1R colocalizes with E-cadherin and increases cell adhesion of MCF-7 cells by increasing expression of ZO-1 which binds to E-cadherin and stabilizes its interaction with the cytoskeleton (L. Mauro et al. *J. Biol. Chem.* **276:** 3982-39897). Conversely, it has also been shown in human colonic tumor cells that IGF-1 via its ability to stimulate E-cadherin phosphorylation results in reduced membrane levels of E-cadherin and associated reduction in cell

adhesion. IGF-1 has also been reported to downregulate T-cadherin expression again this was associated with a decrease in cell adhesion. Despite the apparent role of cell to cell adhesion receptors in regulating cell function there is little data regarding their ability to regulate growth factor action. It has been shown previously that the interaction of neuronal cell adhesion molecules with the fibroblast growth factor receptor leads to receptor activation by autophosphorylation. VEGF has been shown to result in an increase in CEACAM expression and at least some of the effects of VEGF are mediated through CEACAM-1. The results from these experiments demonstrate that the interaction of the cell to cell adhesion molecules IAP and SHPS-1, in addition to mediating cell adhesion, also play an important regulatory role in growth factor signaling. Given the importance of cell to cell adhesion molecules in regulating cell function it is reasonable to conclude that the regulation of growth factor signaling by cell to cell adhesion molecules is a general mechanism for regulating growth factor action. PDGF signaling was not affected by disruption of the IAP-SHPS-1 interaction.

Since PDGF could still stimulate SHPS-1 phosphorylation in the absence of IAP binding to SHPS-1 this suggests that PDGF and IGF-1 may stimulate SHPS-1 phosphorylation via two different kinases. SHPS-1 has been shown to be phosphorylated directly by the insulin receptor kinase (Y. Fujioka et al., *Mol Cell Biol* **16,** 6887-99 (1996)). Given the homology between the tyrosine kinase domains in the insulin and IGF-1R (e.g., 84 %) it is possible that SHPS-1 is also a direct substrate for the IGF-1R kinase. IAP binding to SHPS-1 could modulate this process by localizing SHPS-1 in close proximity to the receptor kinase or alternatively IAP binding to SHPS-1 could alter the conformation of the SHPS-1 cytoplasmic domain making its tyrosines accessible to the IGF-1R kinase.

By virtue of its ability to stimulate SMC migration and proliferation, IGF-1 is likely to be an important contributor to the development of atherosclerosis (J. Jones et al. *Proc Natl Acad Sci USA* **93**:2482-7 (1996)); M. Khorsandi et al. *J.Clin,Invest.* **90** :1926-1931 (1992); B. Cerek et al. *Circ.Res.* **66** :1755-1760 (1990); P. Hayry et al., *FASEB J.* **9** :1336-1344 (1995)). In mice in which IGF-1 was overexpressed in SMCs there was an increase in the rate of neointimal formation after carotid injury that appeared to have resulted from increased SMC proliferation and migration. The effect was apparent despite equivalent levels of serum IGF-1 in plasma compared with control animals suggesting a paracrine effect of locally produced IGF-1 (B. Zhu

et al. *Endocrinology* **142**:3598-3666 (2001)). Given the apparent role of IGF-1 in the development of atherosclerosis and the effect of this interaction on IGF-1 signaling it is likely that this system may play a role in the development of atherosclerosis and disruption of the interaction may represent a novel therapeutic strategy to specifically inhibit IGF-1 action. Current approaches to target IGF-1 signaling have focused on blocking the activity of the receptor itself using antibodies or peptides. Disrupting cell to cell adhesion molecule interactions that specifically inhibit growth factor signaling offers a novel therapeutic strategy.

EXAMPLE 2

Treatment of diabetic retinopathy with a monoclonal antibody (NPG-1) that disrupts IAP binding to SHPS-1

***In vivo* measurement of vascular permeability**

Rats were injected with Nembutal (80 mg/kg) (Southern Anesthesia). Once deep anesthesia had been achieved, warmed Evans blue (45 mg/kg) (Fisher Scientific) solution was injected via the tail vein. After 2 hrs a lethal dose of anesthetic (100 mg/kg) was administered. The chest cavity was opened and a needle inserted into the left ventricle. The right atrium was clipped and blood was centrifuged at 12,000 x g for 5 min. The rats were perfused with 1% paraformaldehyde in citrate then the eyes were removed and placed in PBS. The retinas were removed, lyophilized, and then resuspended in formamide and incubated at 70°C. After 18 hrs the retina/formamide was centrifuged at 13,000 x g for 10 min.

A standard curve was generated using serial dilutions of Evans Blue (30 mg/ul). The absorbance of the standard curve as well as each retina was measured using a Nanodrop spectrophotometer (Thermo-Scientific) using an excitation and emission wavelength of 620 and 740 nm, respectively. The amount of Evans Blue permeation from each retina was calculated using this formula:

$$\frac{\text{Evans Blue (µg) / retina dry weight (g)}}{\text{Time-averaged Evans Blue concentration (µg) / plasma (µl) x circulation (h)}}$$

Diabetes induction protocol

Control (CON) rats received an injection of vehicle. Streptozotocin (STZ) was given by intraperitoneal injection (50 mg/kg; 100 ml). After 6 days rats with blood glucose >350 mg/dl were denoted as having diabetes. The STZ treated group was divided into two groups. At 20 days post-injection, the first group received an

injection of control, mouse IgG (5.0 mg/kg), every 72 hours for 30 days. The second received an injection of rat anti IAP antibody NPG-1 (5.0 mg/kg) every 72 hours for 30 days. The rats were weighed daily and if weight loss was apparent they received insulin (4-8 units/kg).

Cell lysis, immunoprecipitation and immunoblotting

Lysates were prepared from endothelial cell monolayers that had been exposed to various treatments. They were immunoprecipitated and immune complexes were separated by SDS-PAGE and transferred to Immobilon filters (Millipore) prior to immunoblotting to visualize proteins. Antibodies used for immunoblotting were anti-phosphotyrosine (PY99, Santa Cruz), anti-SHPS-1 (BD Biosources) and anti-occludin (Invitrogen).

In vitro permeability assay

Transwell inserts (24 well) were coated with collagen 10 ug/cm^2 (BD Biosciences) for 1 hr at 22°C. HUVECs were plated on the coated inserts at 5 x 10^4 cells/ml/insert in growth medium (15mM glucose). After 24 hrs, 500 µl of growth medium was placed in the lower chambers. After 24 hrs, medium was changed to SFM-199 (15mM glucose) containing IGF-1 (50 ng/ml) plus anti IAP antibody NPG-1 (1 µg/ml). After 14 hrs, fluorescently labeled dextran was added (Sigma) (0.5 mg/ml). After 1 hr, medium was removed from the lower chamber and the amount of FITC-dextran was measured in a fluorescence detecting microplate reader (Fluor Imager 595 Molecular Dynamics) (using excitation and emission wave lengths of 294 and 521 nm, respectively).

In vitro tube formation assay

Human umbilical vein endothelial cells (HUVECs) were grown to confluence and then changed to SF M-199 containing IGF-1 (50 ng/ml), **NPG-1** (1 µg/ml) for 14 hr. They were trypsinized and resuspended in SF M-199 (15mM glucose) and then plated on 24 well plates coated with 500 µl of growth factor reduced matrigel (BD Sciences) (1.5 x 10^5 cells/ml/well). After 4 hours the plates were photographed at 10x and the number of tubes/cm^2 area in 6 random areas of each well was determined. One tube is the area between two branch points (shown in **Figure 7C** as the area between two "x" markers on the image).

Endothelial cell culture

Primary human unbillical vein endothelial cells (HUVECs) (Lonza, Walkersville, MD, USA) were grown in M-199 (Life Technologies, Grand Island,

NY, USA) plus EGM-2 Endothelial Cell Growth Medium supplements (Lonza) containing 5 mmol/l glucose. HUVECs were switched to growth medium containing 15 mmol/l glucose for 3 days. Mannitol (10 mmol/l) was added to the medium containing 5 mmol/l glucose to control for the difference in osmolarity. Cultures were quiesced for 14 h in serum-free M-199 containing 5 or 15 mmol/l glucose, and exposed to IGF-1 (50 ng/ml; Genentech, San Francisco, CA, USA), with or without the anti-IAP antibody, NPG-1 (1 μg/ml). The use of human cells was approved by the University of North Carolina Ethics Committee.

Results

Two types of endothelial cells (rat and human) were used for the *in vitro* assays. The monoclonal antibody NPG-1 directed against amino acids 71 through 80 of the human IAP protein (amino acid numbering according to reference sequence provided herein) binds specifically to human IAP. **Figure 6A** shows a comparison between control and NPG-1 and provides a direct demonstration that this antibody can disrupt IAP binding to SHPS-1. The cells were pre-incubated with NPG-1 and then SHPS-1 was immunoprecipitated and the immunoprecipitate was immunoblotted for IAP. Because the disruption results in inhibition of IGF-1 signaling one would expect critical signal transduction elements that are activated in response to IGF-1 to be inhibited. **Figure 6B** shows that Shc, which has to bind to SHPS-1 to be activated in endothelial cells, does not bind normally if the cells are pre-incubated with this antibody. Most antibodies that react with IAP also disrupt IAP binding to $\alpha_v\beta_3$. **Figure 6C** shows that NPG-1 is specific in that it disrupts IAP binding to SHPS-1 without disrupting IAP binding to β_3. This is important because disrupting IAP binding to β3 could lead to side effects such as increased platelet aggregation. This is an important distinguishing feature of this antibody. SHPS-1 association with IAP was determined in the aorta homogenates from control (Con), diabetic (D) and diabetic rats treated with the anti-IAP antibody (R569) (D + AB) as shown in **Figure 6D**. The antibody was fully active in vivo and inhibited IAP/SHPS-1 association.

As a companion to this study, an experiment is shown wherein the ability of human endothelial cells to form tubes in an *in vitro* assay of capillary formation that occurs *in vivo* is demonstrated. Endothelial cell tube formation is shown in **Figure 7C**. **Figure 7A** shows cell permeability that is measured *in vitro* based on dextran blue permeation. The endothelial cells grow in monolayer much as they do in blood

vessels and the ability of this dye to penetrate the monolayer is measured. As can be seen from **Figure 7A**, IGF-1 stimulates permeation and this is inhibited in the presence of the IAP antibody. **Figure 7B** shows that the tight junction protein occludin, which allows endothelial cells to form a permeability barrier, is disrupted in the presence of IGF-1; i.e., occludin leaves the junctional complex that is normally formed and diffuses out into the cell. That is why there is a decrease in immunoblot intensity of the occludin band. In the presence of the antibody NPG-1, this effect of IGF-1 is completely inhibited. In **Figure 7C**, in IGF-1 treated cells tube formation can be seen, wherein the capillary cells are joining each other with capillary tubes. In the presence of the antibody NPG-1, this is completely disrupted as shown in the lower two panels of **Figure 7C** and on the bar graph, where the number of tubes per cm^2 is shown.

Studies described in **Figure 8** and **Figure 9** were conducted in rat endothelial cells. This is because the amino acid sequence 71 through 80 of the IAP protein is not conserved across species. Because the amino acids 71-80 are different in rat IAP (NKNSTTREQN, SEQ ID NO:8) as opposed to human IAP (ALNKSTVPTD, SEQ ID NO:6), studies were conducted to show that this amino acid sequence had the same functional significance. This was necessary because if the human antibody to IAP is administered to rats *in vivo* it would not work not because this amino acid sequence is not conserved across species therefore the human antibody would not be expected to disrupt rat IAP binding to rat SHPS-1. Therefore an anti-rat IAP antibody was prepared by immunizing rabbits with an immunogen that was comprised of a 10 amino acid sequence from rat IAP that was homologous to the human IAP sequence that was used to prepare the monoclonal antibody. As stated herein, this peptide was conjugated to KLH and rabbits were immunized. The IgG was then purified from rabbit serum using Protein A sepharose. This was the purified IgG that was injected into the rats to inhibit capillary permeability. This rat antibody was validated in the same way the human antibody was validated, to show that the rat antibody would inhibit IAP/SHPS-1 association in rat endothelial cells and that it would inhibit IGF-1 signaling. As shown in **Figure 8A**, the control antibody (Con) had no effect on IAP/SHPS-1 interaction whereas the anti-rat IAP antibody (AB) completely disrupted their association. **Figure 8B** shows that following IGF-1 stimulation there is tyrosine phosphorylation of SHPS-1, stimulation of AKT and MAPK activation and that these are inhibited in the presence of the rat anti-IAP antibody.

Figure 9 shows the results of an *in vivo* experiment in which the same antibody that disrupted SHPS1/IAP association in rat endothelial cells in vitro was injected into rats *in vivo*. This experiment was conducted over a three week period. The rats were made diabetic as described herein and then received injections of the purified anti-rat antibody twice a week. Evans blue dye permeation out of the retinal capillaries into the retina of the diabetic rats was then measured. As shown in **Figure 9**, there is a major increase in capillary permeability in diabetic rats (hyperglycemic), which is a hallmark of diabetic retinopathy. As also shown in **Figure 9**, injection of the rat anti-IAP antibody inhibited this increase in permeability. Vascular permeability is one of the first changes that occur in human diabetic retinopathy. This rat model demonstrates Evans blue leakage, which is a standard assay for measuring vascular permeability (see Kern "*In vivo* models of diabetic retinopathy" *Contemporary Diabetes* 2:137-151 (2008; Bhatt and Addepalli "Attenuation of diabetic retinopathy by enhanced inhibition of MMP-2 and MMP-9 using aspirin and minocycline in streptozotocin-diabetic rats" *Am. J. Transl. Res* 2(2):181-189 (2010)). Therefore it is known in the art as a surrogate animal model of these early changes that occur in human diabetic retinopathy. This leakage of vessels is directly linked to visual loss since it can result in fluid accumulation around the macula and macular damage is a known cause of severe visual loss in patients with diabetic retinopathy. Furthermore factors such as vascular endothelial growth factor inhibitors that inhibit this capillary leak have been shown to inhibit other changes that occur in diabetic retinopathy. In summary since the antibody directed against amino acids 71 through 80 of rat IAP (a region that is homologous to the same sequence in human IAP) inhibits retinal capillary leak it is expected that it will be an effective treatment for human diabetic retinopathy.

EXAMPLE 3

Rationale: Integrin associated protein, also known as IAP and CD 47, is a transmembrane protein that functions as a self recognition antigen on cells. This means that when it binds to a protein (termed SHPS-1) that is localized on macrophage surfaces, these cells do not secrete cytokines that activate cell killing and the target cells that express IAP remain viable. Many types of cells undergoing normal apoptosis fail to express IAP thus enabling this killing to take place efficiently. Several types of cancer cells in contrast either express IAP abnormally or

do not proteolytically cleave the SHPS-1 binding site from IAP and therefore are resistant to engulfment by activated macrophages. It has been postulated that disrupting IAP/SHPS-1 association would lead to increased killing of tumor cells. However, an amino acid sequence within IAP that possesses enough heterogeneity to be able to encode several types of different self recognition sequences has not been identified. The studies of this invention have established that the region within amino acids 71 to 80 contains this self recognition information and that it is involved in IAP binding to SHPS-1. In contrast to other types of antibodies that have been developed that disrupt the association of IAP/SHPS-1, the antibody of this invention targets this sequence within amino acids 71-80, thereby directly interfering with self recognition thus resulting in tumor cell engulfment. Importantly the antibody of this invention does not interrupt interactions between IAP and other cell surface proteins such as the beta 3 subunit of the alphaVbeta3 integrin. This is important because these other interactions mediate cell processes that are necessary for the maintenance of physiologic functions of normal cells.

As the antibody of this invention is directed against this specific site, experiments will be done to establish that disruption of the binding of this site to SHPS-1 on tumor cells results in inhibition of cancer cell growth. These experiments will be performed in two phases. In the first phase, the monoclonal antibody of this invention or control IgG will be added to cancer cells (*e.g.*, breast, prostate, colon, bladder, ovarian cancer cells, etc., as described herein) grown in culture under standard conditions and stimulated to grow with 10% fetal calf serum. Additional cultures would contain the growth factor insulin like growth factor I (IGF-I), since IAP/SHPS-1 association is known to be involved in IGF-I stimulated cell proliferation. Increasing concentrations of antibody between 0.05 and 5.0 ug/ml would be added to cultures and cell proliferation measured 48 hours later by direct counting. Control cultures will receive an equal concentration of IgG. IGF-I would be used in a concentration of 50 ng/mL. If the antibody is successful in inhibiting the proliferation of tumor cells then an *in vivo* experiment will be undertaken. In this experiment, immunocompromised mice will be injected with, e.g., 3 million tumor cells/ml subcutaneously. There will be, e.g., 12 mice/treatment. One group will receive the active antibody at a concentration that will be determined from the results of the *in vitro* experiment. For example if an antibody concentration of 100 ng /ml resulted in complete inhibition of cell growth, then to achieve that serum

concentration, 400 μg would be injected intraperitoneally into each mouse. All mice in the active treatment group would receive the same concentration of antibody that would be administered weekly for a period of six weeks to eight weeks. Control animals would receive an equal concentration of mouse IgG. The animals in both groups will be sacrificed and tumor volume and weight determined. Tumor metastases will be determined by analyzing histologic sections of lung, liver, kidney and brain. The tumor tissue will also be analyzed for the presence of intact IAP by SDS polyacrylamide gel electrophoresis and for IAP/SHPS-1 association by immunoprecipitation of SHPS-1 followed by immunoblotting for IAP. Further analyses of activation of the kinases involved in cell proliferation such as AKT and MAP kinase will also be undertaken using known techniques to determine their activated forms. The results of this experiment will establish that disrupting the binding of this specific site on IAP to SHPS-1 results in inhibition of tumor cell proliferation.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A monoclonal antibody that specifically binds an epitope within amino acids 71-80 of the human IAP protein and is an antagonist of IAP to SHPS-1 binding.

2. The antibody of claim 1, coupled to a detectable group.

3. The antibody of claim 1, coupled to a therapeutic group.

4. The antibody of claim 1, wherein the antibody does not disrupt IAP binding to a β_3 protein.

5. A pharmaceutical formulation comprising the antibody of claim 1 in a pharmaceutically acceptable carrier.

6. The antibody of claim 1, wherein the antibody is selected from the group consisting of (a) the monoclonal antibody produced by hybridoma NPG-1, and (b) a monoclonal antibody that competes for binding to the same epitope as the epitope bound by a monoclonal antibody produced by the hybridoma NPG-1.

7. The antibody of claim 6, coupled to a detectable group.

8. The antibody of claim 6, coupled to a therapeutic group.

9. A pharmaceutical formulation comprising the antibody of claim 6 in a pharmaceutically acceptable carrier.

10. A method of inhibiting IGF-1 actions in a subject in need thereof, comprising administering to the subject the antibody of claim 1.

11. A method of inhibiting IGF-1 actions in a subject in need thereof, comprising administering to the subject the antibody of claim 6.

12. A method of treating retinopathy in a subject, comprising administering to the subject an effective amount of the antibody of claim 1 or claim 6.

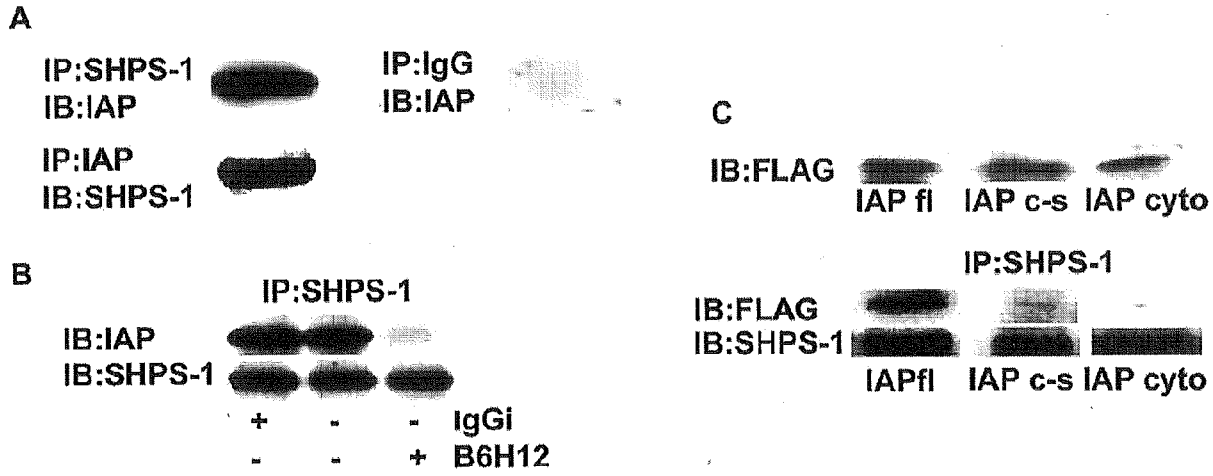
13. A method of treating atherosclerosis in a subject, comprising administering to the subject an effective amount of the antibody of claim 1 or claim 6.

14. A method of treating nephropathy in a subject, comprising administering to the subject an effective amount of the antibody of claim 1 or claim 6.

15. A method of treating coronary artery disease in a subject, comprising administering to the subject an effective amount of the antibody of claim 1 or claim 6.

16. A method of treating cancer in a subject, comprising administering to the subject an effective amount of the antibody of claim 1 or claim 6.

Fig. 1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- - + B6H12
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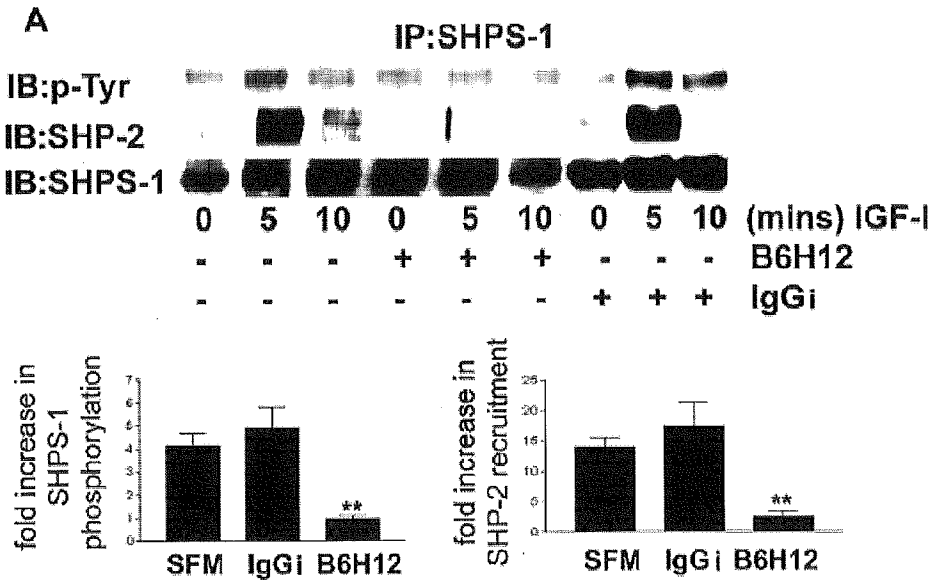
Fig. 2A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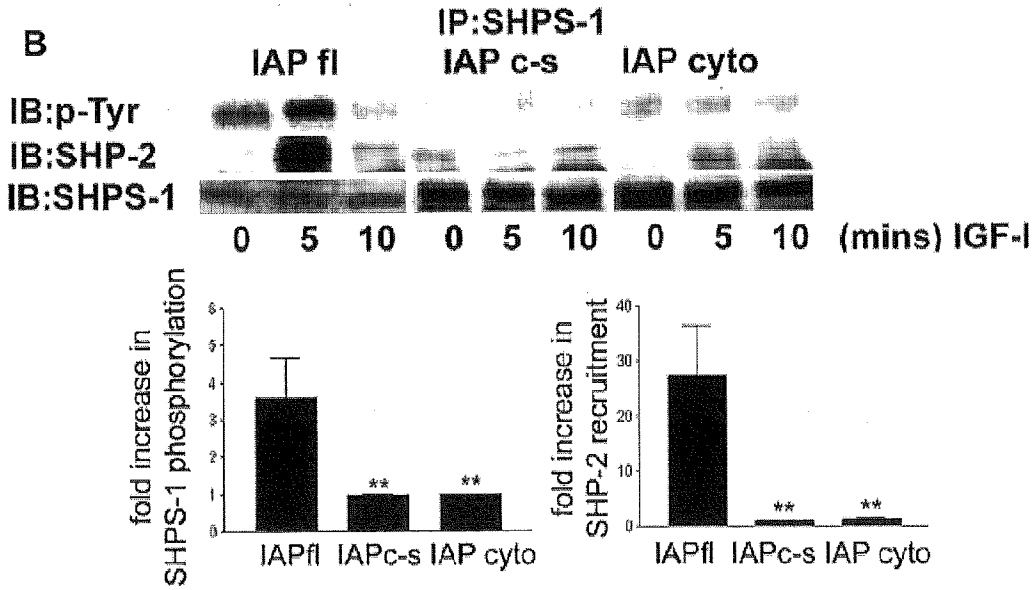
fold increase in SHP-2 recruitment
SFM IgGi B6H12

Fig. 2B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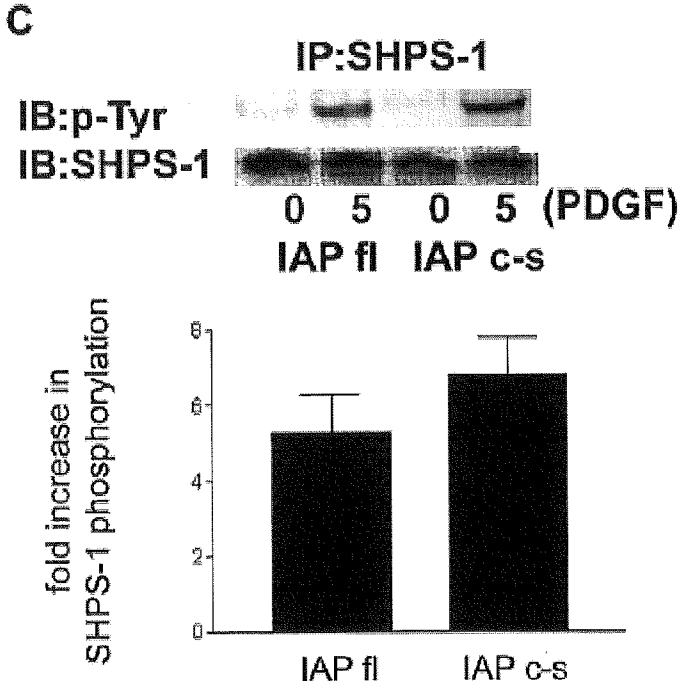
fold increase in SHP-2 recruitment
IAPfl IAPc-s IAP cyto

Fig. 2C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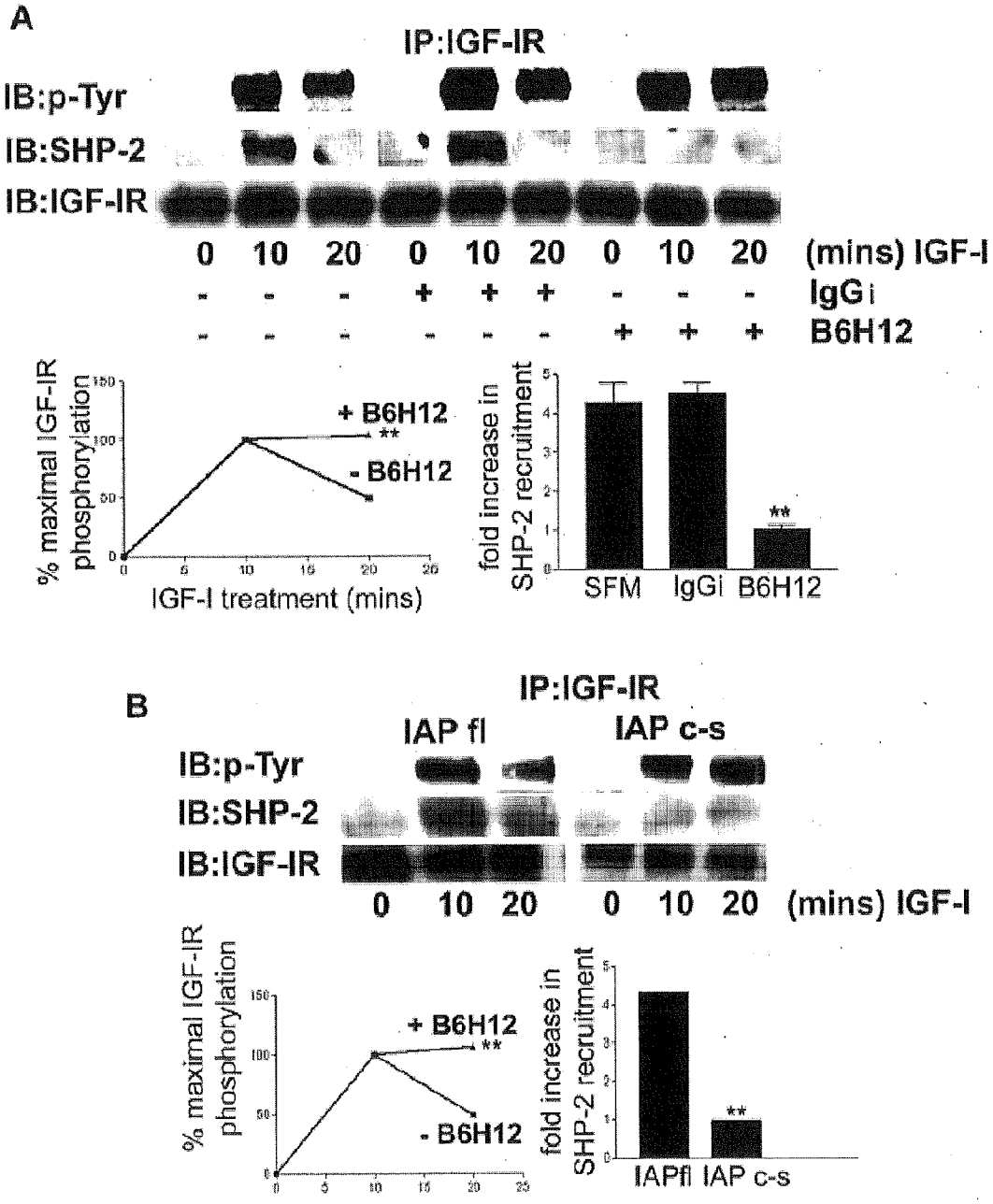
IAP c-s

Fig. 3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+ B6H12
**
- B6H12
IGF-I treatment (mins)
fold increase in SHP-2 recruitment
**
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IAP c-s
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IB:IGF-IR
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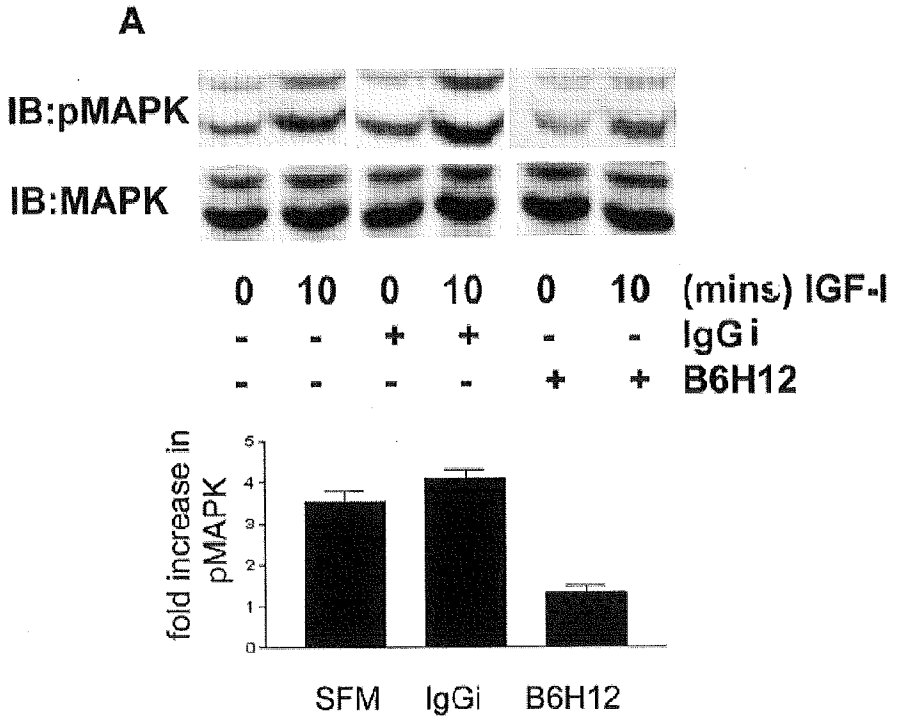
- B6H12
fold increase in SHP-2 recruitment
**
IAPfl IAP c-s

Fig. 4A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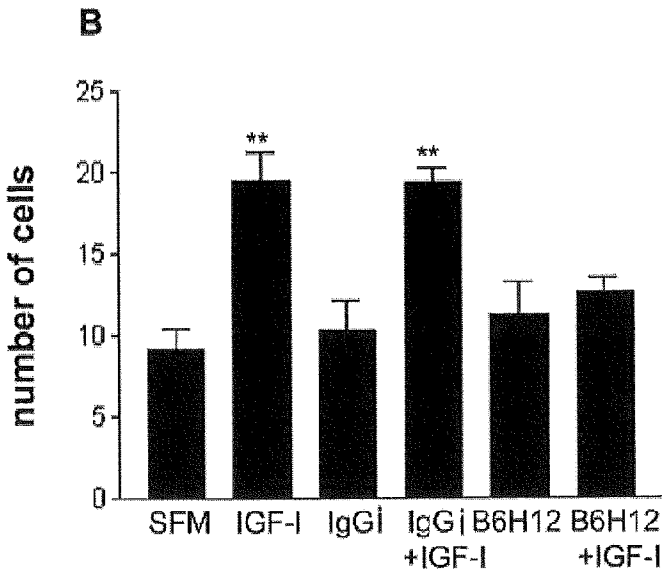
SFM
IgGi
B6H12

Fig. 4B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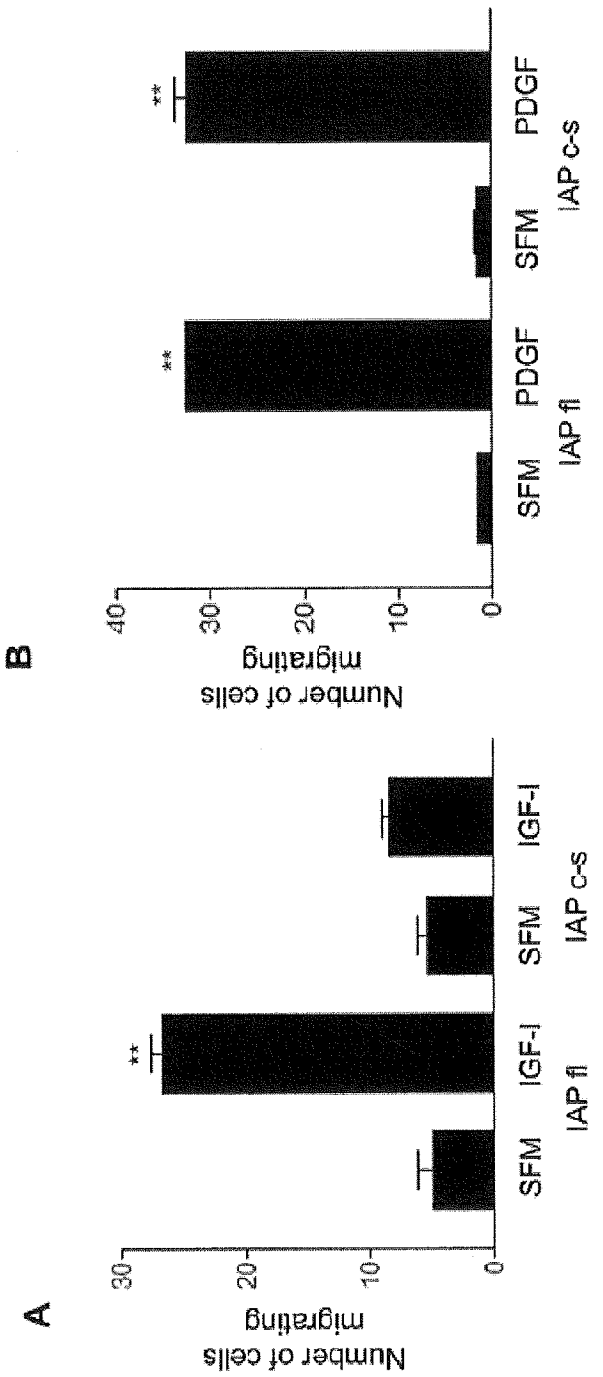
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30
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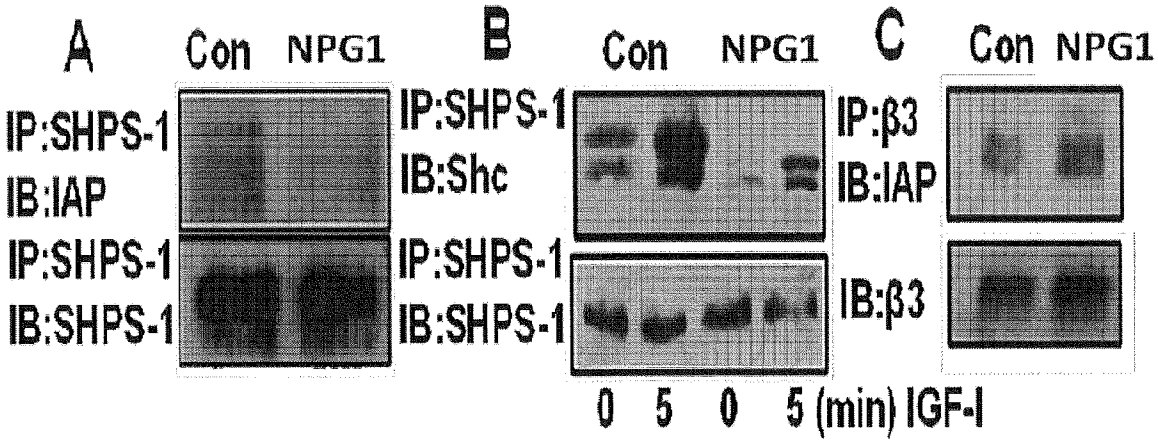
SFM
IGF-I
IAP fl
SFM
IGF-I
IAP c-s
B
Number of cells migrating
0
10
20
30
40
**
SFM
PDGF
IAP fl
SFM
PDGF
IAP c-s

Fig. 5

Fig. 6


A
Con NPG1
IP:SHPS-1
IB:IAP
IP:SHPS-1
IB:SHPS-1
B
Con NPG1
IP:SHPS-1
IB:Shc
IP:SHPS-1
IB:SHPS-1
0 5 0 5 (min) IGF-I
C
Con NPG1
IP:β3
IB:IAP
IB:β3

D

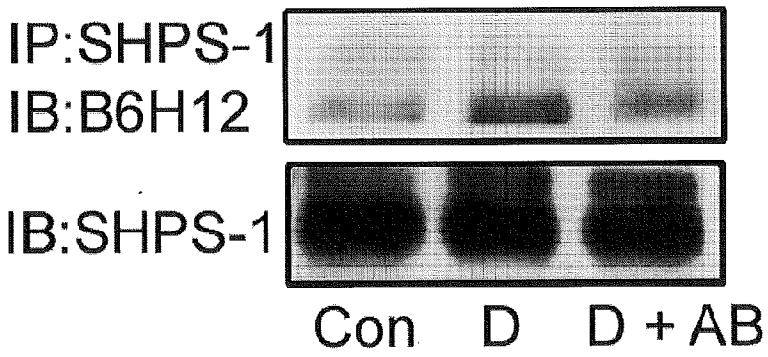

IP:SHPS-1
IB:B6H12
IB:SHPS-1
Con D D + AB

Fig. 7

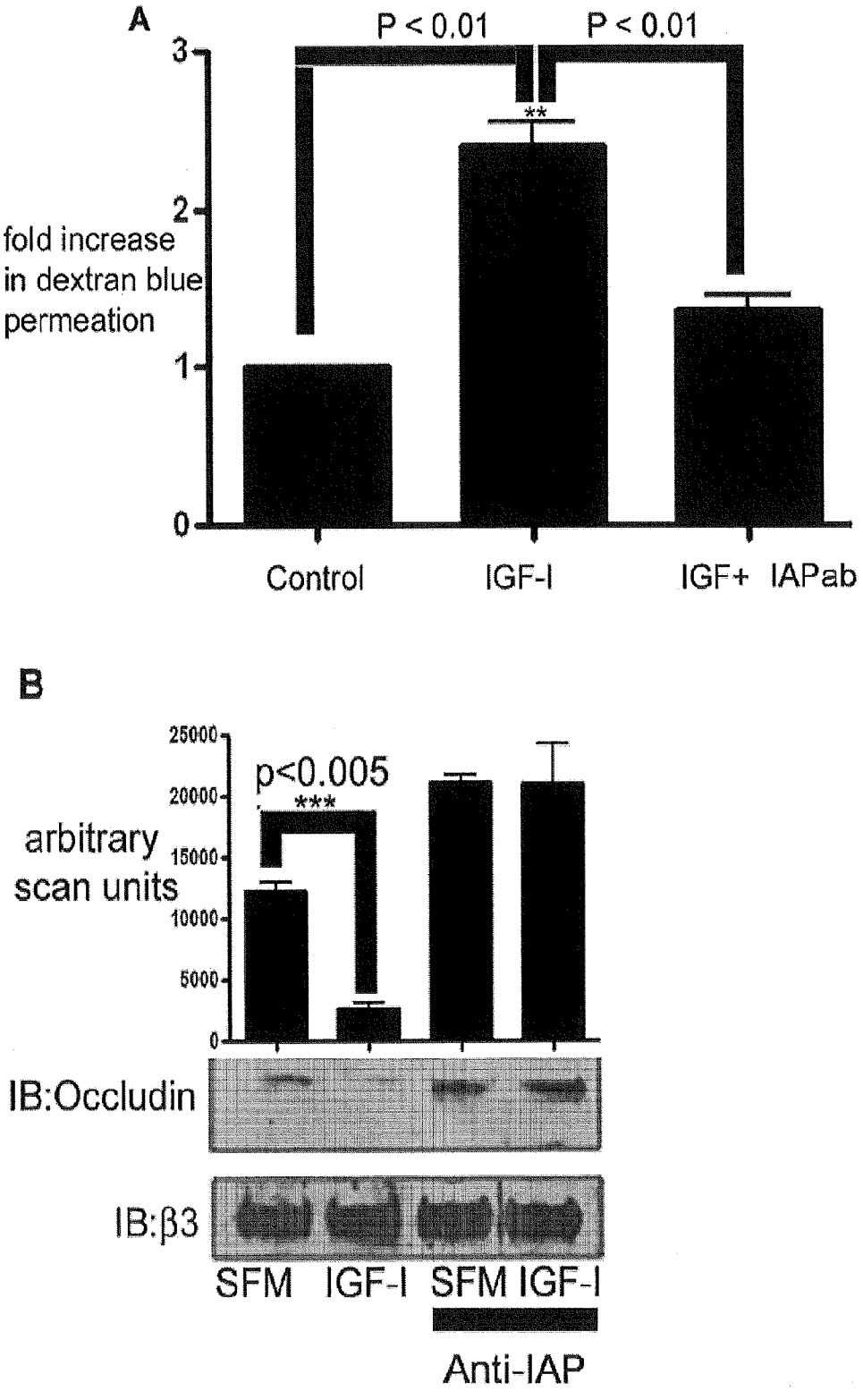

A
P < 0.01
P < 0.01
**
3
2
1
0
fold increase in dextran blue permeation
Control
IGF-I
IGF+ IAPab
B
25000
20000
15000
10000
5000
0
p<0.005
***
arbitrary scan units
IB:Occludin
IB:β3
SFM
IGF-I
SFM
IGF-I
Anti-IAP

Fig. 7

C

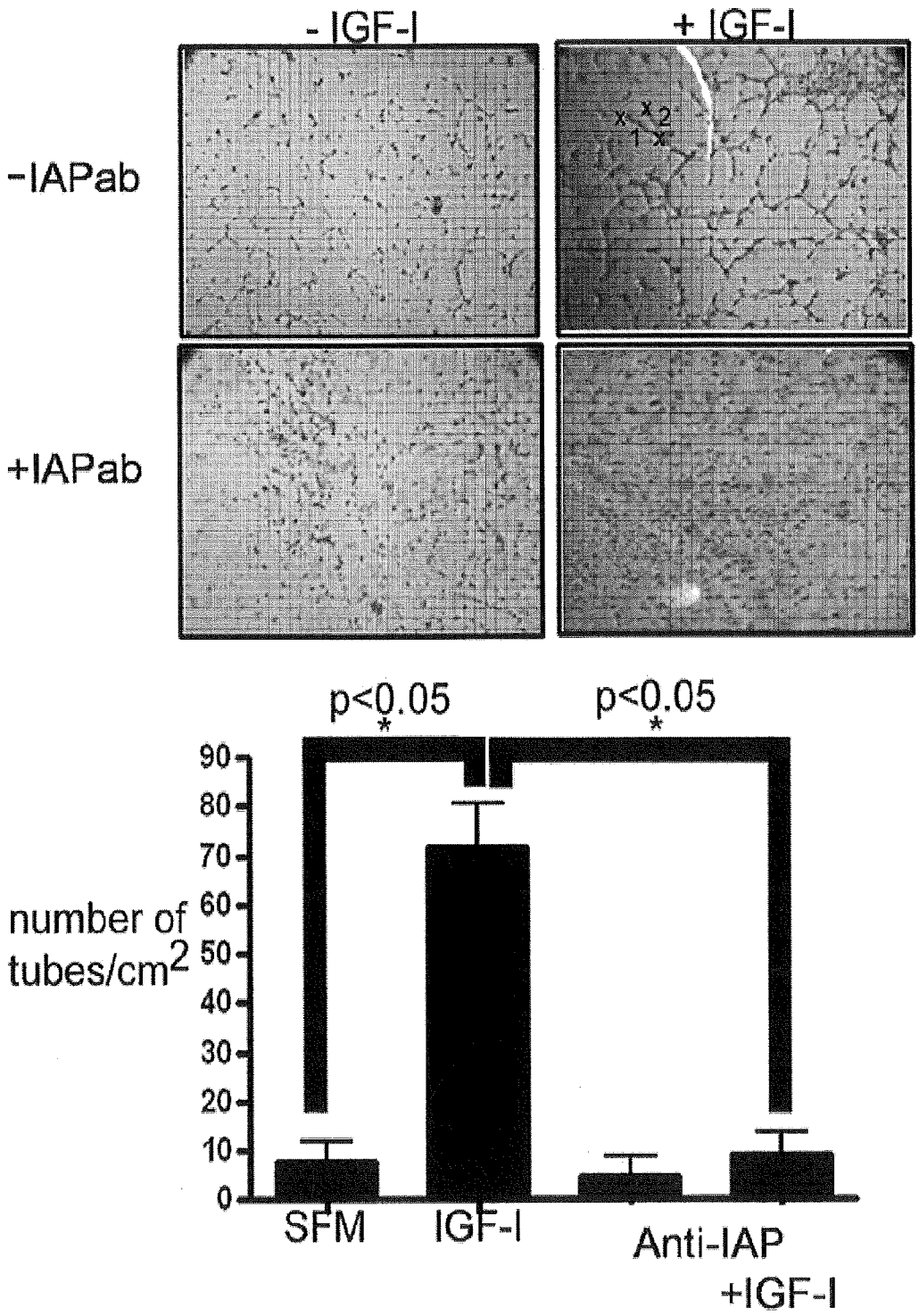
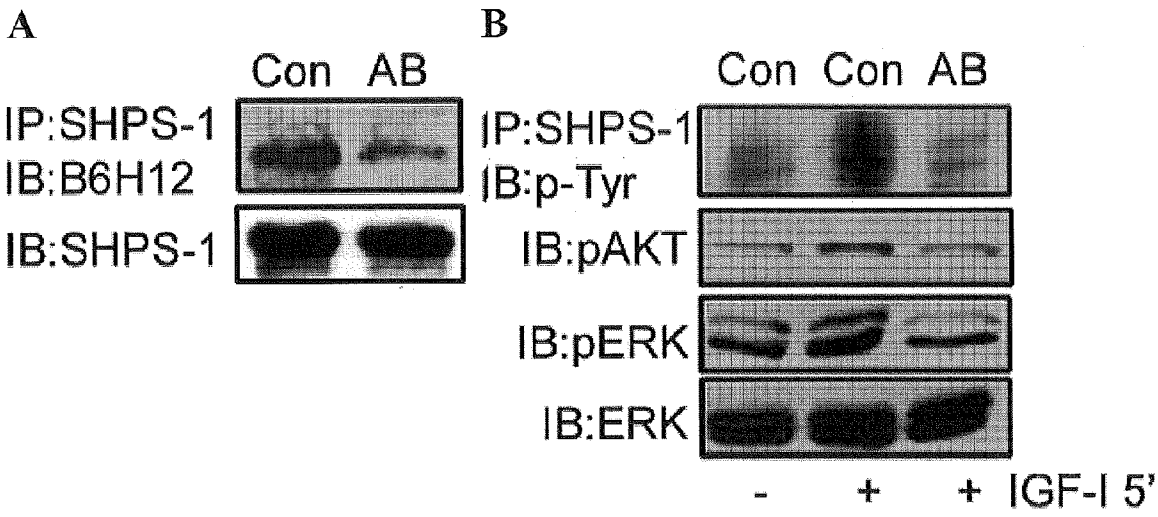

- IGF-I
+ IGF-I
-IAPab
+IAPab
p<0.05
*
p<0.05
*
number of tubes/cm2
90
80
70
60
50
40
30
20
10
0
SFM
IGF-I
Anti-IAP
+IGF-I

Fig. 8


A
Con AB
IP:SHPS-1
IB:B6H12
IB:SHPS-1
B
Con Con AB
IP:SHPS-1
IB:p-Tyr
IB:pAKT
IB:pERK
IB:ERK
- + + IGF-I 5'

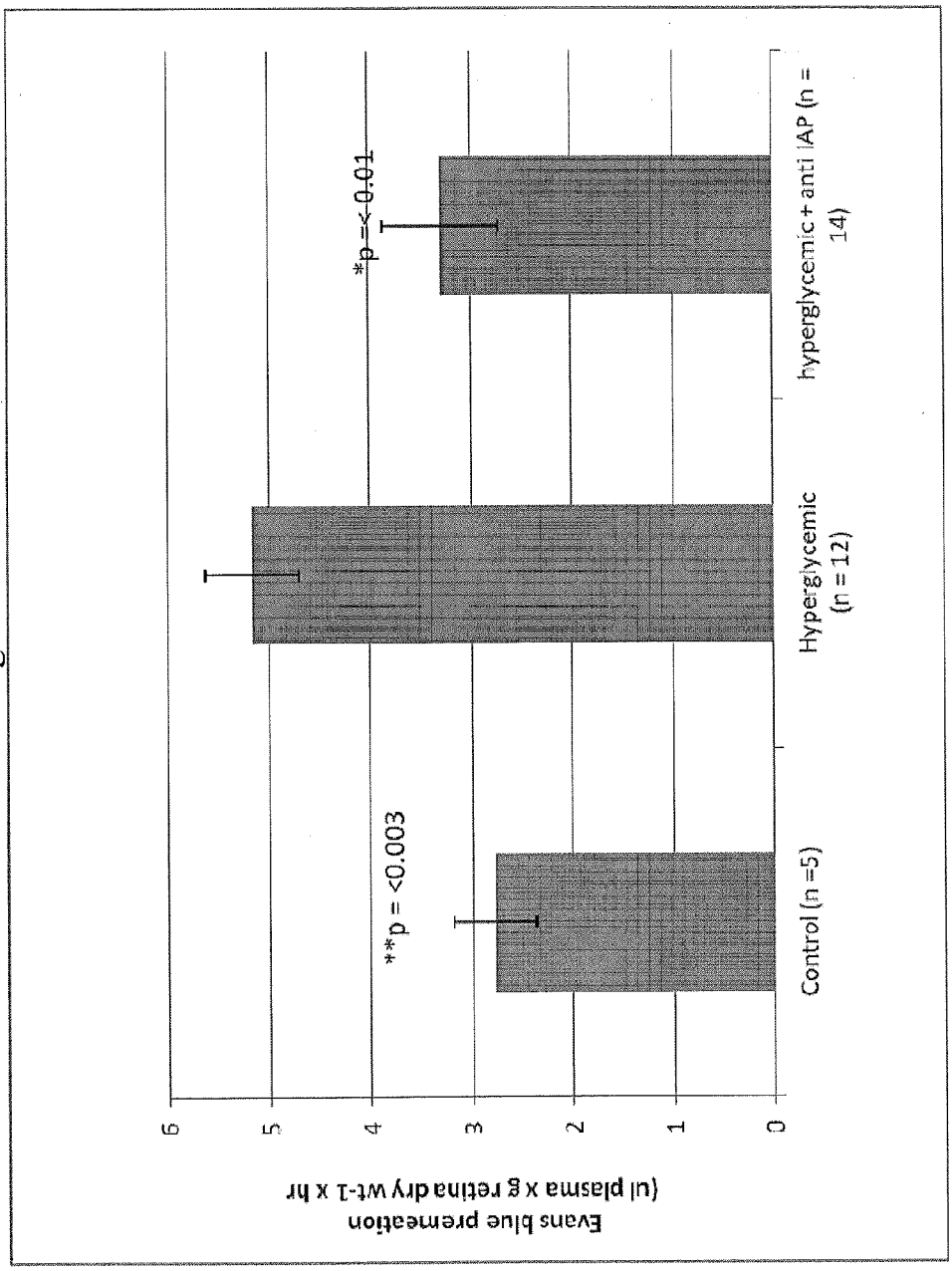

Retinal Vasculature Leakage in Diabetic Rats
Evans blue premeation
(ul plasma x g retina dry wt-1 x hr
0
1
2
3
4
5
6
**p = <0.003
*p =< 0.01
Control (n =5)
Hyperglycemic (n = 12)
hyperglycemic + anti IAP (n = 14)

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/052384

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i, A61K 38/30(2006.01)i, A61P 35/00(2006.01)i, A61P 29/00(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 39/395; C12P 21/04; A61K 38/16; C07K 16/30; G01N 33/574

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

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

eKOMPASS(KIPO internal) & Keywords: anti-IAP mAb, SHPS-1, antagonist

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010-0215640 A1 (CLEMMONS, D. R. et al.) 26 August 2010 See paragraphs [0008] and [0014].	1-9
A	MAILE, L. A. et al. "The association between integrin-associated protein and SHPS-1 regulates insulin-like growth factor-I receptor signaling in vascular smooth muscle cells" Mol. Biol. Cell., 2003, Vol. 14, pages 3519-3528. See page 3521.	1-9
A	US 2003-0157100 A1 (FUKUSHIMA, N. et al.) 21 August 2003 See paragraph [0013].	1-9
A	US 2003-0157577 A1 (FUKUSHIMA, N.) 21 August 2003 See claims 1, 3, 4, and 8.	1-9
A	US 2008-0107654 A1 (KIKUCHI, Y. et al.) 08 May 2008 See claims 1 and 2.	1-9

☐ 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	Date of mailing of the international search report
31 JANUARY 2013 (31.01.2013)	**01 FEBRUARY 2013 (01.02.2013)**
Name and mailing address of the ISA/KR Facsimile No. 82-42-472-7140	Authorized officer CHOI, Sung Hee Telephone No. 82-42-481-8740

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/052384

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 10-16
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 10-16 are directed to a treatment method of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2012/052384

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010-0215640 A1	26.08.2010	EP 1622565 A2	08.02.2006
		US 2004-0213792 A1	28.10.2004
		US 2008-0160013 A1	03.07.2008
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US 2003-0157100 A1	21.08.2003	AU 1998-90028 B2	01.11.2001
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		JP 11-155569 A	15.06.1999
		JP 3568398 B2	22.09.2004
		KR 10-2001-0023866 A	26.03.2001
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		JP 09-295999 A	18.11.1997
		JP 3725653 B2	14.12.2005
		US 6579692 B1	17.06.2003
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US 2008-0107654 A1	08.05.2008	AU 2004-287722 A1	19.05.2005
		CA 2545166 A1	19.05.2005
		CN 101133083 A0	27.02.2008
		EP 1693385 A1	23.08.2006
		JP 4637749 B2	23.02.2011
		KR 10-2006-0121150 A	28.11.2006
		US 2012-0156724 A1	21.06.2012
		US 8101719 B2	24.01.2012
		WO 2005-044857 A1	19.05.2005