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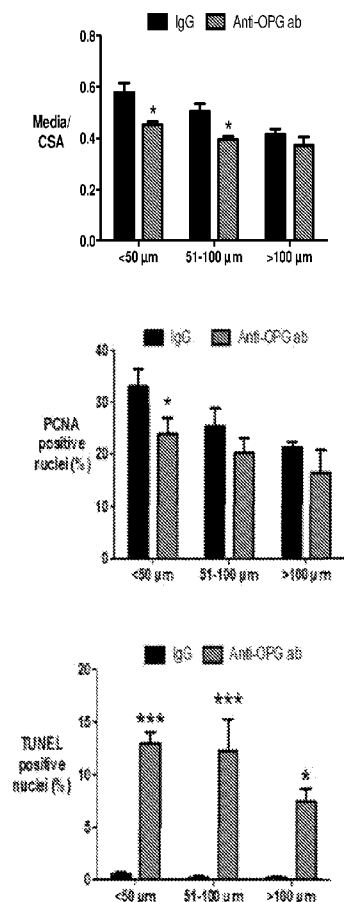
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(54) Title: COMBINATION THERAPY

Figure 1B



(57) Abstract: The disclosure relates to an agent or agents that inhibit the activity of both, Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] and osteoprotegerin (OPG) and their use in the treatment of pulmonary hypertension and including agents that bind OPG ligands/receptors



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Combination Therapy

Field of the Invention

5 The disclosure relates to an agent or agents that inhibit the activity of both, Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] and osteoprotegerin (OPG) and their use in the treatment of pulmonary hypertension [PH]. The disclosure also relates to receptors/ligands that bind OPG and agents, in particular antibodies, that bind these
10 receptors/ligands and their use in the treatment of PH either alone or in combination with each other and/or OPG/TRAIL agents.

Background to the Invention

PH covers a variety of conditions that result in abnormally high blood pressure in the lungs. PH can be in the form of pulmonary arterial hypertension (PAH) occurring in either
15 its idiopathic (IPAH) or hereditary (hPAH) form and also in association with other diseases, for example, connective tissue disease. PH can also result from left heart disease, lung diseases (particularly Congestive Obstructive Disease [COPD] and pulmonary fibrosis), thromboembolism as well as many other multifactorial conditions such as portal hypertension, sickle cell disease and HIV. The prognosis for patients suffering
20 from PH is poor and varies between disease groups. Current management of the disease includes the use of calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanylate cyclase and phosphodiesterase inhibitors. The side effect profiles of these treatments can result in further reduced quality of life and unsatisfactory disease control. Lung transplantation is the only curative treatment but is
25 very rarely done. Therefore there is a continuing need to identify new treatments and agents that are effective at slowing progression and/or reversing PH and which do not have the problems associated with current treatments.

TRAIL is a transmembrane protein and is homologous to members of the tumour
30 necrosis factor family. TRAIL associates in a homotrimer which similarly binds a trimer of TRAIL receptors. There are 5 known TRAIL receptors, two TRAIL receptors, TRAIL RI and TRAIL RII which are referred to as "death receptors" because once activated by TRAIL, apoptosis is induced resulting in programmed cell death. Some cancer cells are sensitive to TRAIL whereas normal cells are insensitive. TRAIL also binds so called
35 decoy receptors TRAIL RIII, TRAIL RIV and osteoprotegerin [OPG] which block the apoptotic activity of TRAIL thereby inhibiting apoptosis. Agonists that modulate TRAIL activity are known in the art and are generally used to enhance apoptosis in cancer cells.

Osteoprotegerin (OPG) is a protein of the Tumour Necrosis Factor (TNF) receptor family and binds at least two ligands; TRAIL and receptor activator of NFkB ligand [RANKL] which is expressed on osteoclast precursors, dendritic cells, T-cells and haematopoietic precursors. RANKL interacts with RANK on cell surfaces to stimulate the production and activity of osteoclasts, the principal cells involved in bone turnover. The interaction of OPG with RANKL inhibits RANKL's ability to bind to RANK and stimulate osteoclasts, and it is this activity of OPG that confers its ability to reduce bone loss.

In our pending applications PCT/GB2012/052629 and PCT/GB2012/052628, currently unpublished, we disclose TRAIL and OPG antagonists for the treatment of PH. TRAIL mRNA was found to be significantly increased in pulmonary artery smooth muscle cells (PASMC) isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) compared to those isolated from control lung. Using antagonistic antibodies directed to TRAIL, rats were protected from developing PAH in response to monocrotaline. Similarly, OPG mRNA levels were found to be increased in PASMC explanted and grown from transplanted lungs of patients with IPAH. As for TRAIL anti-OPG antibody treatment was shown to prevent the development of PAH in rat, and moreover, treatment of established PAH with an anti-OG antibody induced disease reversal.

This disclosure relates to one or more inhibitory agents directed towards both TRAIL and OPG and their use in the treatment and/or reversal of PH in a combined treatment regimen. The disclosure also relates to OPG receptors and antibodies that bind said receptors and their use in the treatment and/or reversal of PH. Antibody therapy in treating or reversing PH may include combination of OPG/TRAIL antibody therapy with one or more OPG receptor antibodies.

Statements of Invention

According to an aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] and osteoprotegerin [OPG].

In a preferred embodiment of the invention said agent is an antagonistic antibody, or active binding fragment thereof.

In a preferred embodiment of the invention said antibody, or active binding fragment, binds and inhibits the activity of a polypeptide comprising or consisting of the amino acid sequence in SEQ ID NO: 1 [TRAIL] and SEQ ID NO: 2 [OPG].

5 In a preferred embodiment of the invention said antibody is a polyclonal antibody.

In an alternative preferred embodiment of the invention said antibody is a monoclonal antibody.

10 In a preferred embodiment of the invention said antibody is a chimeric antibody.

In an alternative preferred embodiment of the invention said antibody is a humanized or human antibody.

15 In a preferred embodiment of the invention said composition comprises a first antibody, or active binding fragment, that binds and inhibits the activity of TRAIL and a second antibody, or active binding fragment that inhibits the activity of OPG.

20 In a preferred embodiment of the invention said agent is a bi-specific antibody comprising one immunoglobulin heavy and light chain that binds and inhibits TRAIL and a second immunoglobulin heavy and light chain that binds and inhibits OPG.

In an alternative preferred embodiment of the invention said agent is a single chain antibody fragment.

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In a preferred embodiment of the invention said agent is a bivalent single chain antibody fragment that binds and inhibits the activity of both TRAIL and OPG.

30 In an alternative preferred embodiment of the invention said agent is a diabody comprising two single chain antibody fragments that bind and inhibit the activity of both TRAIL and OPG.

In a preferred embodiment of the invention said antibody is pegylated.

35 In a preferred embodiment of the invention there is provided an antibody fragment selected from the group consisting of: a single chain antibody fragment, a bivalent single

chain antibody fragment, a diabody, a Fab fragment, Fab₂ fragment, F(ab')₂ fragment, Fv fragment, Fc fragment or a Fd fragment and is pegylated.

Because of their reduced size, antibody fragments usually penetrate tissues much more rapidly and efficiently than full antibodies but this benefit is counterbalanced by a very short serum half-life that decreases the overall tissue uptake of these small molecules. The most promising approach to increase serum half-life is chemical addition of polyethylene glycol (PEG) residues, which considerably increase the size of the fragments thereby reducing renal clearance. Pegylation increases molecular mass without affecting activity thereby reducing the number of doses administered to the patient. In addition pegylation reduces the immune reaction to the administered drug thereby increasing the effective period during which the drug is administered. An example is the certolizumab pegol a recently approved anti-TNF α PEGylated Fab fragment that has a 14 day serum half-life. PEG linkage (PEGylation) is very efficient for increasing the half-life and scFv stability, conferring improved activity and apparently also reducing immunogenicity. Improved circulation time and accumulation in tissues has been demonstrated with PEGylated scFv fragments, tandem scFv (two scFvs linked with a flexible linker) and diabodies.

When administered the agents of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers and supplementary therapeutic agents. The agents of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, transdermal or transepithelial.

The agents of the invention are administered in effective amounts. An "effective amount" is that amount of an agent that alone, or together with further doses, produces the desired response. In the case of treating pulmonary hypertension, the desired response is inhibiting the progression of the disease. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and

expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The agents used in the foregoing methods preferably are sterile and contain an effective amount of an agent according to the invention for producing the desired response in a unit of weight or volume suitable for administration to a patient. The doses of agents according to the invention administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

Other protocols for the administration of compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration and the like vary from the foregoing. The administration of compositions to mammals other than humans, (e.g. for testing purposes or veterinary therapeutic purposes), is carried out under substantially the same conditions as described above. A subject, as used herein, is a mammal, preferably a human, and including a non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent.

When administered, the agent preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents' (e.g. those typically used in the treatment of pulmonary hypertension). When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids:

hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

5

Agents may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" in this context denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application, (e.g. liposome or immuno-liposome). The components of the pharmaceutical compositions also are capable of being co-mingled with the agents of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

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The pharmaceutical compositions containing agents according to the invention may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt. The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

20

The agents may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. Compositions containing agents according to the invention may be administered as aerosols and inhaled. Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of agent, which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1, 3-butane diol. Among the acceptable solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous,

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intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

5 In a preferred embodiment of the invention said composition includes an effective amount of at least one additional agent effective in the treatment of pulmonary hypertension.

10 In a preferred embodiment of the invention said agent is selected from the group: calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanylate cyclase and phosphodiesterase inhibitors.

15 According to a further aspect of the invention there is provided a pharmaceutical composition according to the invention for use in the treatment and/or reversal of pulmonary hypertension.

20 According to a further aspect of the invention there is provided a method to treat and/or reverse pulmonary hypertension comprising administering an effective amount of an agent that inhibits the activity of OPG and an agent that inhibits the activity of TRAIL to a human subject in need of treatment.

25 In a preferred method of the invention said method administers a first agent that inhibits the activity of TRAIL and a second agent that inhibits the activity of OPG wherein the administration of first and second agents is temporally separated.

30 In an alternative preferred method of the invention said method administers a first agent that inhibits the activity of OPG and a second agent that inhibits the activity of TRAIL wherein the administration of first and second agents is temporally separated.

35 In a further alternative method of invention the first and second agents are administered substantially simultaneously.

In a preferred embodiment of the invention pulmonary hypertension is pulmonary arterial hypertension.

40 In an alternative preferred embodiment of the invention pulmonary hypertension is associated with lung disease.

According to a further aspect of the invention there is provided a combined diagnostic and method of treatment or reversal of pulmonary hypertension in a subject comprising:

- i) determining the level of expression of TRAIL and OPG in an isolated biological sample when compared to a control; and
- 5 ii) determining, based on the level of expression of both TRAIL and OPG, whether said subject would benefit from administration of an agent according to the invention to prevent or reverse pulmonary hypertension in said subject.

In a preferred method of the invention said method comprises:

- 10 i) forming a preparation comprising said sample and an oligonucleotide primer pair adapted to anneal to a nucleic acid molecule comprising a nucleic acid sequence as represented in SEQ ID NO: 3 or 4; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;
- 15 ii) providing polymerase chain reaction conditions sufficient to amplify said nucleic acid molecule;
- iii) analysing the amplified products of said polymerase chain reaction for the presence or absence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 3 and 4; and optionally
- 20 iv) comparing the amplified product with a normal matched control.

In a preferred method of the invention said method is a real time PCR method.

In an alternative preferred method of the invention said method comprises:

- 25 i) providing an isolated biological sample to be tested;
- ii) forming a preparation comprising said sample and an antibody or antibodies that specifically binds one or more polypeptide[s] in said sample as represented by the amino acid sequences presented in SEQ ID NO: 1 and 2 to form an antibody/polypeptide complex;
- 30 iii) detecting the complex or complexes so formed; and
- iv) comparing the expression of said polypeptide[s] with a normal matched control.

In a preferred method of the invention ratio of TRAIL: OPG is compared to the ratio of

- 35 TRAIL: OPG in a control sample.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of FAS receptor polypeptide [SEQ ID NO: 5, 6, 7, 8, 9, 10 or 11] for use in the treatment of pulmonary hypertension.

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According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of TNFSF11 polypeptide [SEQ ID NO: 12 or 13] for use in the treatment and/or reversal of pulmonary hypertension.

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According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of SDC1 polypeptide [SEQ ID NO: 14, 15 or 16] for use in the treatment and/or reversal of pulmonary hypertension.

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According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of GAP43 polypeptide [SEQ ID NO: 17 or 18] for use in the treatment and/or reversal of pulmonary hypertension.

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According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of ILRAPcP polypeptide [SEQ ID NO: 19] for use in the treatment and/or reversal of pulmonary hypertension.

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According to a further aspect of the invention there is provided a pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of transmembrane protease 11D polypeptide [SEQ ID NO: 20 or 21] for use in the treatment and/or reversal of pulmonary hypertension.

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In a preferred embodiment of the invention said composition comprises:

- i) an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, GAP43, ILRAPcP or transmembrane protease 11D; combined with
- 35 ii) an agent that inhibits OPG and/or TRAIL.

In an alternative preferred embodiment of the invention the treatment and/or reversal of pulmonary hypertension comprises the administration of an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, GAP43, ILRAPcP or transmembrane protease 11D and administration of an agent that
5 inhibits OPG and/or TRAIL wherein the said administrations are temporally separated.

In an alternative preferred embodiment of the invention the treatment and/or reversal of pulmonary hypertension comprises the administration of an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11,
10 GAP43, ILRAPcP or transmembrane protease 11D and administration of an agent that inhibits OPG and/or TRAIL wherein the said administrations are simultaneous or sequential.

In a preferred embodiment of the invention said agent is an antibody or active binding
15 fragment.

In a preferred embodiment of the invention said agent[s] bind the extracellular domain[s] of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, GAP43, ILRAPcP or transmembrane protease 11D.
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In a preferred embodiment of the invention pulmonary hypertension is pulmonary arterial hypertension.

In an alternative preferred embodiment of the invention pulmonary hypertension is
25 associated with lung disease.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other
30 moieties, additives, components, integers or steps. "Consisting essentially" means having the essential integers but including integers which do not materially affect the function of the essential integers.

Throughout the description and claims of this specification, the singular encompasses
35 the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described
 5 herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following figures:

10 Figure 1A: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-TRAIL antibody or IgG for 4 weeks. Mice that received the anti-TRAIL antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small pulmonary arteries/arterioles. This was associated with decrease in proliferating (PCNA) cells and
 15 an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries.

Figure 1B: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-OPG antibody or IgG for 4 weeks. Mice that received the anti-OPG antibody displayed a significant reduction in pulmonary vascular
 20 remodeling as shown by the reduction in media/CSA of the small pulmonary arteries/arterioles. This was associated with decrease in proliferating (PCNA) cells and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries.

Figure 2: OPG binds to FAS receptor which is increased in IPAH, mediates OPG-
 25 induced proliferation in PASMCs *in vitro* and regulates PAH associated genes. Confirmed protein interactions between OPG and syndecan-1 (SDC1), RANKL (TNFSF11), Growth Associated Protein 43 (GAP43), Fas, IL1-receptor accessory protein (IL1RAcP) and transmembrane protease, serine 11D (a). Co-Immunoprecipitation of OPG with FAS and IL-1RAcP in primary human PASMC (b). OPG, FAS and IL-1RAcP
 30 are expressed within remodelled pulmonary arteries and right ventricle of patients with IPAH (c). TaqMan expression of OPG in explanted PASMC from patients with IPAH normalised using $\Delta\Delta CT$ with 18S rRNA as the endogenous control gene (d). Proliferation of PA-SMCs with recombinant OPG (50 ng/ml) in the presence of anti-FAS neutralising antibody or IgG control (e). TaqMan expression of OPG regulated genes the presence
 35 of anti-FAS neutralising antibody or IgG control (f)

Preferred Embodiments

Antibodies, also known as immunoglobulins, are protein molecules which have specificity
5 for foreign molecules (antigens). Immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ , α , μ , δ and ϵ), all four linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule to another. In
10 addition, H and L chains contain regions that are non-variable or constant. The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region.

15 The H chains of Ig molecules are of several classes, α , μ , σ , α , and γ (of which there are several sub-classes). An assembled Ig molecule consisting of one or more units of two identical H and L chains, derives its name from the H chain that it possesses. Thus, there are five Ig isotypes: IgA, IgM, IgD, IgE and IgG (with four sub-classes based on
20 the differences in the H chains, i.e., IgG1, IgG2, IgG3 and IgG4). Further detail regarding antibody structure and their various functions can be found in, Using Antibodies: A laboratory manual, Cold Spring Harbour Laboratory Press.

Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse
25 or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complementarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complementarity determining regions (CDRs) are the regions within the N-terminal domain of both the
30 heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.

Antibodies from non-human animals provoke an immune response to the foreign
35 antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the

human antibody regions do not illicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are
5 therefore thought to be less immunogenic than chimeric antibodies.

Various fragments of antibodies are known in the art, [e.g. Fab, Fab₂, F(ab')₂, Fv, Fc, Fd, scFvs]. A Fab fragment is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light
10 chain variable region, covalently coupled together and capable of specifically binding to an antigen. Fab fragments are generated via proteolytic cleavage (with, for example, papain) of an intact immunoglobulin molecule. A Fab₂ fragment comprises two joined Fab fragments. When these two fragments are joined by the immunoglobulin hinge region, a F(ab')₂ fragment results. An Fv fragment is multimeric protein consisting of the
15 immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically binding to an antigen. A fragment could also be a single chain polypeptide containing only one light chain variable region, or a fragment thereof that contains the three CDRs of the light chain variable region, without an associated heavy chain variable
20 region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multi specific antibodies formed from antibody fragments, this has for example been described in US patent No 6,248,516. Fv fragments or single region (domain) fragments are typically generated by expression in host cell lines of the relevant identified regions. These and other
25 immunoglobulin or antibody fragments are within the scope of the invention and are described in standard immunology textbooks such as Paul, *Fundamental Immunology* or Janeway et al. *Immunobiology*. Molecular biology now allows direct synthesis (via expression in cells or chemically) of these fragments, as well as synthesis of combinations thereof. A fragment of an antibody or immunoglobulin can also have
30 bispecific function as described above.

Antibodies have emerged as an important class of therapeutics and typically have specificity for one antigen. More recently developed bi-specific antibodies (bsAb),
35 however, are able to recognise more than one epitope on the same or a different antigen simultaneously.

There are a variety of different types of bi-specific antibodies and methods for the production of such. Quadromas have two different antigen-binding arms and are produced by the somatic fusion of two different hybridoma cells. Although, this method typically produces just low yields of the bispecific antibody, increase of the desired
5 combination can be achieved by using particular IgG subclasses preferentially pairing with each other, or through pairing of two different antibody heavy chains by certain mutations in the CH3-domain of human IgG1. Apart from the antigen binding region, antibodies comprise an Fc region which can interact with Fc receptors or other immune molecules. This may be an undesired effect and can be avoided by enzymatic removal of
10 the Fc part. Similarly, diabodies, a subclass of bispecific antibodies, are solely made up from two different antigen-binding sites with minimal additional protein sequences acting as linker sequences.

Other methods for the production of bi-specific antibodies include chemical coupling of
15 two different monoclonal antibodies, or antibody fragments, with a hetero-bifunctional crosslinker or site-directed crosslinking of two different monovalent antigen binding arms through their hinge cysteine residue. Using advanced antibody engineering, new recombinant formats have been designed as for example tandem scFv, diabodies, tandem diabodies, dual variable domain antibodies and heterodimerization using a motif
20 such as CH1/Ck domain. Methods of producing bi-specific antibodies are known in the art and are disclosed in, for example, US2013/078182 and is herein incorporated by reference in its entirety.

The development of bispecific antibodies for the treatment of diseases is an active
25 research area. Removab® is approved for the treatment of malignant ascites in patients with EpCAM-positive cancer if a standard therapy is not available. Others include, bi-specific antibodies such as Ertumaxomab® or Rexomun® are designed to target HER2, a well-characterized breast tumour marker, possessing the same hybrid Fc portion as Removab®. Bi20 (Lymphomun™ or fBTA05) is targeting CD20 and CD3, and bispecific
30 antibodies targeting melanoma-associated proteoglycans or melanoma-associated gangliosides (GD2 and GD3) are developed for the potential treatment of malignant melanoma.

In general, doses of antibodies (or fragments thereof) of between 10 µg/ml and 500
35 µg/ml generally will be formulated and administered according to standard procedures. Exemplary doses can range from 10 µg/ml to 250 µg/ml, 30 µg/ml to 250 µg/ml, 50 µg/ml

to 250 µg/ml, 30 µg/ml to 100 µg/ml, or 50 µg/ml to 100 µg/ml, such as 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 µg/ml, 80 µg/ml, 90 µg/ml, 100 µg/ml, 250 µg/ml, 400 µg/ml or 500 µg/ml. Other protocols for the administration of compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration and the like vary from the foregoing.

Materials and Methods

Animals

Rats

Outbred male, albino Sprague Dawley rats (Charles River or Harlan, U.K) (starting weight approx. 200g) were used for experiments. A single subcutaneous injection of monocrotaline (MCT) (see below) into the left thigh was used to induce pulmonary arterial hypertension. We used a well-established dose of 60mg/kg that leads to the development severe PAH and which is fatal within 5-6 weeks.

200mg of Monocrotaline (MCT) (#C2041-500, Sigma Aldrich, UK) was first fully dissolved in 0.6ml of 1M Hydrochloric Acid and vortexed for 40min. Sterile water was added to make the volume to 5ml and the pH adjusted to 7.0 with sterile NaOH. A final solution to 10ml was made with sterile water.

Mice

All inbred mice were on a C57BL/6 background and were deficient for Apolipoprotein-E (ApoE^{-/-}), originally sourced from Jackson laboratories (Bar Harbor, ME, USA). Male mice aged between 8-14 weeks were used for all *in vivo* experiments.

Diets and husbandry

Rodents were fed standard laboratory chow (4.3% fat, 0.02% cholesterol, and 0.28% sodium, Harlan, UK). Where indicated, experimental mice were fed a high fat atherogenic diet (referred to here on as Paigen) which consisted of 18.5% fat, 0.9% cholesterol, 0.5% cholate, and 0.259% sodium for either 8 or 12 weeks (special Diet services, UK)

All animals had access to drinking water and fed *ad-libitum*. They were housed in dedicated laboratories with controlled temperature, humidity and a 12h day-night cycle.

Animal care and investigation conformed to the University's ethical policy statement and the UK Home office guidance in the operation of Animal Scientific Procedures Act 1986 on H.O. project license PPL 40/2952 (Held by Lawrie)..

5 Interventions

Where stated polyclonal goat anti-mouse TRAIL (Anti-TRAIL), polyclonal goat anti-mouse OPG (Anti-OPG) or control goat IgG isotype antibodies (R&D systems, UK) were delivered to rodents through subcutaneously implanted osmotic pumps (Durect Corp., CA, USA). Interventions were delivered via an Alzet® 1004 micro pump (100µl reservoir, 0.1µl/hour for 4 weeks) in mice and via an Alzet® 2002 mini-pump (200µl reservoir, 0.5µl/hr, 85ng/hr for 2 weeks) in rats.

Pump Implantation protocol

Each pump was filled with the appropriate intervention under sterile conditions in a class II laminar flow hood and placed in sterile 0.9% saline at 37°C 24 hours prior to implantation. Under isoflurane gas anaesthesia (2-3%, IsoFlo® 100% w/w inhalation vapour liquid, Abbot laboratories Ltd, Kent, UK) through 100% oxygen (flow rate 1.5L/min) overlying fur was clipped, the skin cleaned and sterilised prior to making a 1-1.5cm cutaneous incision over the left posterolateral thoracic wall, inferior to the lower costal margin. Under sterile surgical conditions, pre-filled pumps were implanted into a subcutaneous pocket created with blunt dissection. The wound was subsequently cleaned and closed using interrupted 2-0 Vicryl absorbable sutures (B-Braun, Sheffield, UK). Implantation for mice was identical except pumps were primed for 48 hours, implanted posterior to the cervical spine (scruff line) and wounds closed with interrupted Silk sutures (Silkam®, B-Braun Sheffield, UK).

Experimental Protocol

30 Mice

ApoE^{-/-}, TRAIL^{-/-} and ApoE^{-/-}/TRAIL^{-/-} knockout mice (10-16 weeks of age, n=4-7/group) were fed either chow or Paigen for 8 weeks before disease phenotyping (see below). In separate experiments ApoE^{-/-}/TRAIL^{-/-} mice (8-12 weeks of age, n=4-6/group) were treated with either rmTRAIL (10ng/hr) or placebo (PBS) by osmotic pumps for 4 weeks that coincided with the onset of feeding Paigen diet.

To determine the efficacy of inhibiting TRAIL in mice with established disease, ApoE^{-/-} mice (8-10 weeks of age, n=6-7/group) were fed a Paigen diet for 8 weeks and then received an anti-TRAIL antibody (20ng/hr) or isotype control with phenotyping performed at week 12.

5

ApoE^{-/-} knockout mice (10-16 weeks of age, n=4-7/group) were fed either chow or Paigen for 8 weeks before disease phenotyping (see below). To determine the efficacy of inhibiting OPG in mice with established disease, ApoE^{-/-} mice (8-10 weeks of age, n=6-7/group) were fed a Paigen diet for 8 weeks and then received an anti-OPG
10 antibody (20ng/hr) or isotype control with phenotyping performed at week 12.

Rats

To investigate if TRAIL or OPG was required for the development of disease (Prevention study) rats (200-240g, n=4/group) were treated with an anti-TRAIL antibody (84ng/hr) or
15 isotype control delivered for 2 weeks by osmotic pumps, commencing at baseline with MCT injection. Disease phenotyping was undertaken one week later, i.e.: 21 days after MCT injection.

To determine the efficacy of inhibiting TRAIL or OPG in established disease (Survival study), rats (200-240g, n=6/group) with MCT induced PAH (day 21 after MCT) received an anti-TRAIL antibody (84ng/hr) or isotype control for 2 weeks. We compared survival in the two groups and rats were sacrificed at day 35 post MCT (1-14 days following intervention) or sooner if they displayed morbidity and evidence of right heart failure. The latter were defined by outward illness (breathlessness, lethargy, ruffled fur) AND
20 significant weight loss (defined as >5% weight loss in 24 hours or a total of 10% over 48 hours). Where possible, echocardiographic and haemodynamic studies were performed immediately prior to sacrifice.

Disease Phenotyping

30

Each rodent underwent echocardiography (where indicated) before cardiac catheterisation and was then sacrificed whilst still under anaesthesia. Blood was collected by cardiac puncture for serum and isolation of RNA (where indicated). The abdominal aorta was cut and lungs were perfused with PBS via a needle in the right
35 ventricle until the lungs became visibly white. The heart and lungs were removed en-bloc. The right lung was quickly separated before immediately being snap frozen in liquid nitrogen for subsequent biochemical analyses. The left lung was perfusion fixed, via the

trachea with 10% (v/v) formalin at an inflation pressure of 20cm H₂O and then placed with the heart in 10% formalin overnight at 4°C. The left lung was subsequently used for histological and immunohistochemical analyses.

5 Rodent Echocardiography

Transthoracic echocardiography was performed with a preclinical high frequency ultrasound imaging system (Vevo 770®, Visual Sonics, Toronto, Canada) using either a RMV707B (mouse) or RMV710B (rat) scan head. Rodents were anaesthetised with isoflurane via oxygen before being placed supine on a heated platform and covered to minimise heat loss. Maintenance Isoflurane (0.5-1.5%) with oxygen was delivered via a nose cone and adjusted to achieve maximal heart rate (approx. 500bpm for mice and 350bpm for rats) which was continuously recorded along with respiration rate and rectal temperature. The chest of the mouse was depilated and preheated ultrasound gel was applied (Aquasonics 100 Gel, Parker Labs Inc. New Jersey, U.S) for subsequent image acquisition.

Study Protocol

Left Ventricle: Standard parameters of the left ventricle were measured in the short axis view at the mid-papillary muscle level. Manual tracing of the LV end diastolic and systolic areas were made to derive the fractional area change (FAC) as the primary index of contractility. M-Mode measurements were made for the LV wall and cavity dimensions (LVIDd), from which the ejection fraction (EF%), fractional shortening (FS%) and corrected LV mass were determined by standard automated analysis. Pulse wave tissue doppler (TDI) velocities were manually recorded from the endocardial aspect of the posterior wall of the left ventricle and represented another independent index of contractility. Stroke volume was derived from measuring the Velocity Timed integral (VTi) of flow and diameter at the level of aortic valve annulus and multiplied by heart rate to obtain the cardiac output.

Right Ventricle and Pulmonary Artery: From the right parasternal long axis view, right ventricle free wall measurements were recorded with M-Mode function. From the short axis view, doppler flow was recorded from the proximal pulmonary artery (just after the pulmonary valve). From the spectral Doppler tracing the time from onset of flow to peak velocity (PA acceleration time; PAAT), the duration of ejection (PA ejection time; PAET) and stroke work (PA VTI) were measured.

Analysis was performed offline using the accompanying software (Vevo 770, V3.0). Measurements were taken during the relevant phase of the cardiac cycle that did not coincide with inspiration artefact. To minimise inter-observer variability all image acquisition and analyses were performed by a single, experienced operator (AGH) blind to the status experimental subjects.

Rodent cardiac catheterisation

Following echocardiography, left and right ventricular catheterisation was performed using a closed chest method via the right internal carotid artery and right external jugular vein under isoflurane induced anaesthesia. Data was collected using a Millar ultra-miniature pressure-volume PVR-1045 1F catheter (mouse), SPR-838 (rat) (Millar Instruments Inc., Texas, USA) coupled to a Millar MPVS 300 and a PowerLab 8/30 data acquisition system (AD Instruments, Oxfordshire, UK) and recorded using Chart v7 software (AD Instruments). Pressure volume analysis was performed using PVAN v2.3 (Millar Instruments Inc).

HARVESTING AND PROCESSING OF TISSUE

BLOOD

Blood was allowed to coagulate on the bench and subsequently centrifuged at 1200rpm for 15min. The serum was collected, aliquoted, labelled and frozen at -80°C until subsequent analyses. Tubes containing whole blood for RNA (PAXgene®, Qiagen/BD U.K or Tempus®, Applied Biosystems, UK) were frozen at -20°C until subsequent isolation of RNA.

LUNG TISSUE

Protocol

After cardiac puncture the rodent was overdosed with anaesthetic followed by cervical dislocation. An incision in the upper abdominal wall was made to expose the liver. Whilst applying upward traction on the xiphoid process of the sternum, the diaphragm was carefully cut with fine scissors. The sternum and chest wall were resected away. The abdominal aorta was identified and cut (to exsanguinate). Using a 25G orange needle and syringe the right ventricle was identified and flushed with PBS until the lungs became pale. The trachea was identified and freed between the medial clavicular borders. Whilst applying firm upward traction on the trachea, the heart and lungs were

removed en-bloc from the posterior wall of thoracic cavity. Care was taken to avoid inadvertent lung puncture.

5 The right lung was secured tightly at the hilum using 5-0 silk sutures and separated away before being snap frozen in liquid nitrogen for subsequent isolation and determination of whole lung protein and RNA expression.

Polyethylene tubing was inserted into the trachea and secured tightly with a suture. The left lung was gently inflated manually with a syringe containing 10% phosphate buffered
10 formalin (0.4% w/v $\text{NaH}_2\text{PO}_4 \cdot 2(\text{H}_2\text{O})$, 0.65% w/v $\text{Na}_2\text{HPO}_4 \cdot 2(\text{H}_2\text{O})$ and 4% v/v formaldehyde in water) and then both heart and left lung were fixed in formalin for 24hours before transfer into PBS. From the rat prevention study onwards lungs were inflated using 20cm H_2O clamp set up to standardise inflation. The left lung was separated from the heart for subsequent histology.

15

Heart weights and right ventricle hypertrophy (RVH)

RVH was defined as the weight of the RV divided by the weight of the left ventricle/septum (RV/LV+S) as first described by Fulton et al.

20 Protocol

Using a small pair of fine scissors surrounding fat, tissue and great vessels were removed from around the heart. The atria were excised, cleared of any thrombus and weighed. The right ventricle was separated from the left ventricle and septum by the use of anatomical landmarks.

25

Starting from the right ventricular outflow tract (RVOT) the septal margin of the RV was dissected away to ensure no ridges of tissue were left. An incision was also made from the RVOT adjacent to and encircling the aortic root towards the medial tricuspid valve annulus to separate the base of the RV. From the lateral tricuspid annulus the RV free
30 wall was cut away ensuring again no ridges of RV tissue remained. The incision continued towards the apex and back up-towards the RVOT.

Finally the left ventricle was cut and any clot removed from it before all chambers were padded dry and weighed.

35

Tissue Processing and Histology

The left lung was divided in the sagittal (rats) or transverse (mice) plane. Lungs were processed by first dehydrating them in graded alcohols (50% up to 100%). They were then placed in Xylene before being embedded in molten paraffin wax. 5µm thick paraffin embedded sections were cut and mounted onto slides for subsequent histology, immunohistochemical staining and morphometric analyses.

All slides were initially dewaxed by placing in Xylene for 10mins and then repeating for 2mins. Slides were then rehydrated in graded alcohols (1min in each of 100%, 100%, 90%, 70%, 50% and then finally water). Following any staining as a final common step, all slides were dehydrated in an identical but reverse order and mounted in DPX (Dibutyl Phthalate Xylene) and allowed to dry overnight.

Alcian Blue Elastic Van Gieson (ABEVG)

Dewaxed and rehydrated slides were oxidised in 0.25% potassium permanganate for 3min and rinsed in distilled water before being bleached with 1% Oxalic acid for 3min. Following rinsing, slides were stained with Carazzi's Haematoxylin for 2min and differentiated in acid alcohol (1% v/v HCl in 70% IMS) for a few seconds prior to being submerged in hot running tap water for 5min. Slides were then stained with Alcian Blue (1% w/v in 3% aqueous acetic acid, pH2.5) for 5 min. Slides were rinsed again with water and soaked rapidly in 95% IMS before being dipped into Miller's elastin stain for 30min. Slides were then rinsed, placed in 95% IMS for a few seconds and rinsed in water again. They were then stained with Curtis' modified Van Gieson reagent for 6min. Slides were then dehydrated in identical but reverse order to those for rehydration above before mounting in DPX.

Immunohistochemistry

Paraffin embedded 5µm lung sections underwent immunohistochemical staining α-SMA for vascular smooth muscle cells, vWF to localise endothelial cells and PCNA for proliferating cells. Immunostaining for TRAIL/OPG was performed to identify any expression within pulmonary vascular lesions. Levels of apoptosis were determined with a colorimetric assay to detect DNA fragmentation (FRAGEL®, Calbiochem, UK) as specified by the manufacturer's instructions. A positive control was generated with DNase treatment of a control slide.

Protocol

Following dewaxing and rehydration of slides, endogenous tissue peroxidases were blocked by incubating slides in 3% (v/v) hydrogen peroxide for 10mins before being rinsed in tap water. Antigen retrieval (Slide permeabilisation) was done by incubating
5 slides in either:

- a) citrate buffer, pH 6.0 preheated to 95°C for 20min. before cooling for 20min at RT. Tissue was then permeabilised by incubation in 0.5% (v/v) tritonX100 for 10mins at RT (IHC for TRAIL)
- 10 b) 0.1% (w/v) Trypsin/TBS, pH7.8, preheated to 37C for 10minutes before stopping reaction by immersing in water (IHC for vWF)
- c) For SMA staining an antigen retrieval step was not performed

Slides were then blocked (to prevent non-specific binding of secondary antibody) in 1%
15 (w/v) skimmed milk/PBS for 30mins at RT. Milk was tipped off and excess blotted away. The relevant primary antibody diluted in PBS was added and incubated as follows:

- a) Monoclonal mouse anti-human aSMA 1:150, (#m081, Dako) for 1 hour at RT
- b) Polyclonal rabbit anti-human vWF 1:300 (#A0082, Dako) for 1hour at RT.
- 20 c) Polyclonal rabbit anti-human TRAIL 1:100 (#ab2435, Abcam) overnight at 4C

Slides were washed in PBS three times for 5mins before adding a species specific biotinylated secondary antibody (1:200 dilution in PBS) for 30minutes at RT. Slides were washed again in PBS three times for 5mins and an avidin biotinylated enzyme complex
25 added (Vectastain ABC Kit, Vector laboratories Inc. CA, US). Following a further PBS washing step, diaminobenzidine (DAB) substrate was added for 5-10min. After optimum development the colour reaction was stopped by washing slides in tap water. Slides were then counterstained with Carazzi's haematoxylin for 1 minute before a final wash in water. Slides were dehydrated as described and mounted with DPX mountant. Slides
30 were allowed to dry overnight before being examined under light microscopy

Morphometric lung and image analysis

The degree of pulmonary vascular remodelling was quantified in arterioles by two methods and categorised according to vessel size (20-50µm, 50-100µm and
35 >100µm)(Schermuly, Dony et al. 2005). Vessels were scored blind to the experimental status of rodents.

Medial to Cross Sectional Area (Media/CSA) ratio

Medial area/CSA represented the proportion of the total vessel area was taken up by muscularisation of the medial layer, as determined from α -SMA stained slides. Six vessels of each size group were analysed at a 40X objective (18 vessels/section and 1 section/rodent). Cross sectional Area was the total area defined by the outer vessel circumference with the media defined as the area between the internal and external elastic lamina of the vessel.

10 Percentage of vessels thickened

Percentage of vessels thickened was determined using slides stained with ABEVG. For each slide 3-4 random fields of view were sampled using a 10x Objective (100x mag). The number of vessels that were fully occluded, partly occluded and non-thickened per size group were counted and expressed as a percentage of the total number of vessels in each view.

Quantification of vascular proliferation and apoptosis levels

20 To determine the levels of proliferation within remodelled vessels, the number of PCNA positive stained nuclei were counted and expressed as a percentage of total nuclei within the vessel. Where relevant, nuclei in the adventitia or perivascular area were also counted when in direct extension from the vessel of interest. Six vessels of each size were scored from each section (one section/animal) at a 40X objective.

25 In an identical manner the percentage of apoptosis positive nuclei (as determined from a colorimetric assay for levels of DNA fragmentation) were quantified for six vessels of each size per lung section (one section/animal)

30 Slides were viewed with a light microscope (Nikon eclipse E600) connected to a digital camera (Nikon digital site DSRI1) and NIS basic elements software (Nikon Inc.).

Isolation and purification from Protein and RNA from lung tissue

35 Lung segments frozen in liquid nitrogen were ground using a pestle and mortar containing liquid nitrogen to a fine powder and weighed. Precautions were taken to minimise contamination by RNAase. Total protein and RNA were isolated using a

commercial RNA/Protein purification Kit (#23000, Norgen Biotek, Ontario, Canada) according to the protocol supplied by the manufacturer. The purification kit employed a spin column chromatography technique and allowed elution of proteins and RNA from the same sample within 30 minutes.

5

Protocol

Briefly lysis solution was added to the lung tissue and then ethanol added. This was loaded on to a spin-column. After centrifugation at 14000rpm, all nucleic acids within the solution were bound by a resin whilst the proteins were removed in the flow through. The bound RNA was washed, spun again and then purified RNA was eluted. The concentration of RNA in the elution was determined using a spectrophotometer (NanoDrop®, Thermo Scientific) and frozen at -80C.

Following pH adjustment the protein flow through was reloaded on to the original spin column, centrifuged, washed and eluted.

Finally protein concentrations were determined using a commercial assay (DC™ protein assay #500-0116, BioRAD Life Sciences, UK) according to the protocol provided by the manufacturer. Briefly it is a colorimetric assay that utilises a reaction between the protein and an alkaline copper tartrate solution. This is followed by a reduction step using Folin reagent. Absorbance was read at 750nm. The quantity of protein was determined from absorbance data generated from a protein standard curve (Albumin, BSA #23209, Pierce, Thermo Scientific Fisher, UK.) Protein samples were stored at -80C.

25

Protocol

Proteins were separated by SDS-Polyacrylamide gel electrophoresis using a commercial electrophoresis kit (NuPAGE® Kit, Invitrogen). All buffers and reagents were part of the NuPAGE range unless otherwise stated. A volume containing 35 µg of protein purified from the lungs of rats from the time course experiment, sample buffer and a reducing agent made to a final volume of 30 µl (in deionised water) was heated to 70°C for 10 min. Samples and a pre-stained marker ladder were then loaded onto 10 well pre-cast SDS polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Mini gels, Invitrogen). In addition a sample of mixed experimental lung tissue was also loaded onto every gel as an additional control to allow for subsequent quantitative analysis.

Immediately prior to placing the loaded gels into an electrophoresis cell (XCell SureLock® Mini cell, Invitrogen) that already contained SDS running buffer, 500 µl of antioxidant was added. The Gel was run at 200V for 35 min.

- 5 Gels were transferred onto a nitrocellulose membrane (membrane and blotting pads had been pre-soaked in the transfer buffer and air bubbles removed) in transfer buffer (containing antioxidant and 10% methanol v/v) and ran at 30V for 60min. Ponceau S staining was used to confirm adequate transfer.
- 10 The membranes were then blocked for 1h in 10ml of PBS with 5% milk (w/v) and 0.1% Tween-20 (v/v) on a shaking platform. Blots were rinsed in PBS/0.1% tween-20 three times before adding the relevant primary antibody in 5% milk/PBS/0.1% Tween-20 on a shaking platform overnight at 4°C. (Mouse anti-human TRAIL 1:50, Novo Castro Laboratories, Co Durham, UK and anti Mouse Beta Actin 1:2000, #c56 Santa Cruz, CA, USA).
- 15

- Blots were rinsed three times for 10min. before adding an appropriate, species specific peroxidase labelled secondary antibody diluted in PBS (polyclonal goat Anti-mouse immunoglobulin/HRP 1:2000, #p0447, Dako, Ely, UK). Following a further rinse step as
- 20 described enhanced chemoluminescence was performed by adding 1ml of a commercial assay on to the blots for 5min. in the dark (#34075 West Dura Super Signal, Thermo scientific Fisher). Blots were developed in a dark room using autoradiography film (#28906836, HyperFilm™ GE Amersham, UK) and developer/fixer solutions. Blots were stripped (#2502, Reblot Plus Mild Chemicon solution, Millipore) and re-probed for actin
- 25 as described above.

- The developed blots were dried and the ladder marked. The quantity of TRAIL in the bands was determined by normalising to actin and control samples using the densitometry function on commercial software (Syngene SNAP software, Chemigenius2
- 30 bioimaging system, SynGene).

Quantitative real time Polymerase Chain Reaction

Reverse transcription of RNA for first strand synthesis

- 35 This step was performed using components provided in a SuperScript™ III first strand synthesis system (#18080-051 and #18080-044, Invitrogen™ Life technologies, UK). A volume containing 3ug of total RNA isolated from the lungs (and whole blood using PAX-

gene tubes) of experimental rodents was made to 10µl using molecular grade water. 1µl of random hexamer primers (50ng) and 1µl of a 10mM dNTP were added to this and heated to 65°C for 5 minutes as a denature step. Samples were put on ice until 10µl of a cDNA synthesis mix [containing 10xRT buffer (2µl), 25mM MgCl₂ (4µl), 0.1M DTT (2µl),
 5 RNaseOUT™ (1µl) and SuperScript™ III reverse transcriptase (1µl)] was added to this solution and mixed. Samples were heated in a thermal cycler (G Storm GS1, GRI Ltd, Essex, UK) with parameters set as follows i) 25°C for 10min (annealing step), ii) 50°C for 50min. (cDNA synthesis) and finally iii) 85°C for 5min before being held at -4°C (to terminate the reaction). 1µl of RNaseH was added to each tube before a final incubation
 10 step at 37°C for 20min.

Alternatively (for all mouse and rat interventions) 2µg of RNA was reverse transcribed using a commercial high capacity RNA to cDNA kit (Applied Biosystems). Briefly RNA was added to PCR tubes containing 10µl of 2x RT buffer and 1µl of an RT enzyme mix.
 15 Samples were heated in a thermal cycler (G Storm GS1, GRI Ltd, Essex, UK) with parameters set as follows i) 37°C for 60min ii) 95°C for 5min and then held at 4°C to terminate the reaction.

Real Time quantitative PCR

20 Amplification of the target lung cDNA derived from the RT step above was then next performed. A volume containing 50ng of each cDNA was diluted to a volume of 4.5µl using sterile water. 5µl of a TaqMan® gene expression master mix-2X (#4369016, Applied Biosystems™ Life Technologies, UK) along with 0.5µl of the relevant target gene
 25 primers (10X) were added to the cDNA in the relevant well of a 384-well plate. Target genes were tested are listed in table 1 (all from Applied Biosystems™) 18s and ATP5B were selected as endogenous control genes having been determined in prior testing (GeNORM assay). Samples (in duplicate) for each gene were loaded on the same plate. The plate was centrifuged at 1000rpm for 1 min and the reaction was run on a 7900HT
 30 fast real time PCR system (Applied Biosystems™) with the following recommended settings. Relative expression for each gene was quantified by comparing the test gene with the housekeeping control gene and comparing this ratio between an experimental and control subject (delta, delta CT method) for each gene using SDS software (v2.2.1, Applied Biosystems™).

35

Statistical analysis

Data were plotted and analysed using Prism® v6.0 (Graphpad, US) software. Data are expressed as Mean [standard error] unless indicated otherwise. Two groups were compared with Student's unpaired t test and three or more groups by ANOVA with Bonferroni post comparison testing (where indicated). Statistical significance was defined by a p value of ≤ 0.05 .

Retrogenix Cell Microarray

In order to identify potential OPG binding partners, a Retrogenix cell microarray was performed under contract by Retrogenix Ltd (Sheffield, UK). Briefly, OPG was first screened against HEK cells expressing syndecan-1 (positive control) or the membrane protein TREM1 (negative control) with varying concentrations of recombinant human (rh) OPG (2 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$) and the anti-OPG antibody (2 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$) to determine levels of OPG binding and background.

Human embryonic kidney 293 (HEK293) cells were grown over microarray slides consisting of duplicate vectors of each full-length human plasma membrane protein. The expression vector pIRES-hEGFR-IRES-ZsGreen1 was spotted onto each slide in quadruplicate to ensure a minimum level of transfection is reached (mean signal from the expression vector of 1.5 previously defined). Cells were reverse transfected, fixed and treated with 0.5 $\mu\text{g/ml}$ rhOPG (Peprotech, London, UK), 0.5 $\mu\text{g/ml}$ anti-OPG (Peprotech, London, UK) followed by Alexafluor647 anti-goat antibody. Two replicate screens were performed and fluorescent images were analysed and quantified using ImageQuant software. Hits identified in one or both primary screens were then confirmed and analysed following the same methodology as for the primary screen. Vectors encoding hits were then sequenced to confirm protein identity

Co-Immunoprecipitation

Co-immunoprecipitation was used to pull down OPG binding partners and confirm the interaction between OPG and the binding partners identified by the Retrogenix cell microarray. HPASMCs (P5) were grown in fully supplemented SmBm growth media, until around 80% confluent. Cells were then stimulated with rhOPG (500 ng/ml) in SmBm, without synchronization, for 30 minutes. Cells were then lysed in CHAPS lysis buffer and protein concentration measured by Pierce 660nm protein assay.

Co-IP reactions were then set up to pull down OPG with Fas antibodies, from both HPASMC lysates and recombinant proteins. Co-IP reactions were incubated at 4°C, overnight, to allow immune complexes to form. nProteinG sepharose 4 Fast Flow beads
5 were then added to each Co-IP reaction and immune complexes were precipitated for 1 hour at 4°C. Immune complexes were then dissociated in NuPAGE reducing agent and 5% SDS v/v by heating at 95 degrees Celsius and the supernatants were then analysed by western blotting.

10 Immunohistochemistry

Histological slides of the pulmonary artery and right ventricle from donors with idiopathic PAH were obtained (Professor Nick Morrell, University of Cambridge, UK). Immunohistochemical analysis of the sections was then performed. Tissue sections were
15 de-waxed and rehydrated through graded alcohols to water. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide and antigen retrieval performed by incubating the slides in citrate buffer. Slides were permeabilised in 0.5% Triton X-100 for 10 minutes and blocked with 1% low fat milk for 30 minutes at room temperature. Primary antibodies, Fas monoclonal antibody (Enzo Life Sciences, ADI-AMM-227-E)
20 (1:100 dilution) or IL1RAcP polyclonal antibody (Abcam, Ab8110) (1:1000 dilution), were added and incubated overnight at 4°C. Slides were incubated with ABC complex for 30 minutes at room temperature and DAB substrate was then added for 10 minutes. Slides were counterstained with Carazzi's haematoxylin for 1 minute, washed in tap water and dehydrated through graded alcohols to xylene. Slides were mounted using DPX
25 mountant and images were then captured using a Zeiss LSM 510 NLO inverted confocal microscope.

30 Example 1

Figure 1A shows that treatment of established PAH with an anti-TRAIL antibody induces reverse remodelling of disease: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-TRAIL antibody or IgG for 4
35 weeks. Mice that received the anti-TRAIL antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small

pulmonary arteries/arterioles. This was associated with decrease in proliferating (PCNA) cells and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries.

Figure 1B shows that treatment of established PAH with an anti-OPG antibody induces reverse remodelling of disease: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-OPG antibody or IgG for 4 weeks. Mice that received the anti-OPG antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small pulmonary arteries/arterioles. This was associated with decrease in proliferating (PCNA) cells and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries.

The Anti-OPG treatment demonstrates a more pronounced pro-apoptotic response than anti-TRAIL, which was associated with a greater anti-proliferative response.

Example 2

Pre-Screen: Development of the detection system

In order to identify potential OPG binding partners, a cell microarray was performed by Retrogenix (Sheffield, UK). Pre-screening of rhOPG and antibody concentrations was performed to determine optimum binding conditions of rhOPG and the anti-OPG antibody. The detection system was optimised using the known interaction between rhOPG and Syndecan-1, and the negative control membrane protein, TREM1. Based on the pre-screen, optimum conditions were determined to be 0.5 µg/ml rhOPG and 0.5 µg/ml anti-OPG antibody. This condition showed successful binding of OPG to syndecan-1, with the lowest levels of background detected (2.1 fold over the glass slide alone), in the absence of non-specific binding to TREM1. The other conditions tested generated high background and non-specific binding to TREM1 or specific binding to syndecan-1 but with high background. For the primary and confirmation screens, 0.5 µg/ml OPG and 0.5 µg/ml anti-OPG antibody was then used during experimentation.

Example 3

Primary Screen

Two replicate primary screens were performed. In the primary screen, 2505 expression vectors, encoding each full-length human membrane protein, were arrayed across 7 slides. Each slide was arrayed in duplicate and the slides were screened with rhOPG, followed by anti-OPG primary antibody and AlexaFluor647 rabbit anti-goat secondary

antibody. A total of 16 hits were identified (Table 1). "Hits" were defined as an increase in fluorescence compared to background, observed in duplicate spots in one or both of the replicate primary screens. The minimum threshold for transfection efficiency was exceeded for each slide.

5

Table 1 List of Primary Hits In the primary screen, 2505 expression vectors, each encoding one full-length human membrane protein, were arrayed across 7 slides. Each slide was arrayed in duplicate and the slides were screened with 0.5 µg/ml OPG, followed by addition of 0.5 µg/ml anti-OPG primary antibody and secondary

10 AlexaFluor647 rabbit anti-goat secondary antibody. A total of 16 hits, duplicate spots identified as a "hit" in one or both of the replicate slides, were identified and the intensity of the duplicate spots were quantified using the ImageQuant software (GE).

Hit Number	Gene Id	Intensity of Duplicate Spots
1	FCGR1A	Weak/medium
2	SLC13A3	Inverse
3	TLR7/CDC42	Weak
4	FGFR1	Weak
5	GAP43	Very weak
6	FCGR2B	Medium
7	CXCR7	Inverse
8	TNFSF11	Strong
9	TAAR9	Medium
10	FAS	Strong
11	IL1RAcP	Strong
12	PTPRN/LYD6B	Medium
13	SDC1	Medium
14	TMPRSS11D	Medium
15	FGF6	Diffuse
16	FCGR2A	Medium

15

Inverse hits, CXCR7 and SLC13A3, detected were visible as white spots caused by the fluorescence level detected being less than background levels. Binding of OPG to the FC-gamma receptors FCGR1A, FCGR2A and FCGR2A was also observed, however,

this indicates indirect binding of the anti-OPG primary antibody to these proteins, which may be an artefact of the detection system. FGF6 was also identified as a diffuse hit.

The identity of each vector for the 16 primary hits (Table 1) was confirmed by sequencing
 5 and the vectors were then re-spotted onto the slide for the confirmation screen.

Example 4

10 Confirmation Screen

Each of the 16 hits identified in the primary screen were re-spotted on the slides and probed with rhOPG, anti-OPG primary antibody and AlexaFluor647 rabbit anti-goat secondary antibody. No rhOPG (anti-OPG primary antibody and AlexaFluor647 rabbit
 15 anti-goat secondary antibody) and no anti-OPG primary antibody (rhOPG and AlexaFluor647 rabbit anti-goat secondary antibody) negative controls were also included in the confirmation screen to determine non-specific binding of OPG and the anti-OPG primary antibody to the human membrane proteins.

20 Out of the 16 hits identified in the primary screen, binding of OPG to the FC-gamma receptors was found to be present in all conditions, even in the negative controls where rhOPG or anti-OPG antibody had not been added to the cells (Table 2). These "hits" were therefore regarded as non-specific and thus were discounted. The inverse SLC13A3 hit and diffuse FGF6 binding was again observed in the confirmation (Table 2)
 25 screen and therefore these "hits" were also discounted. After discounting the non-specific, inverse and diffuse hits, 6 confirmed interactions between OPG and syndecan-1 (SDC1), RANKL (TNFSF11), Growth Associated Protein 43 (GAP43), Fas, IL1-receptor accessory protein (IL1RAcP) and transmembrane protease, serine 11D (TMPRSS11D) (Table 2) were identified. Images of the spot intensities and binding controls for the 6
 30 confirmed interactions described in Table 2 are represented visually in Figure 2 a.

Table 2 Results of the Confirmation Screen. Each of the 16 hits identified in the primary screen were re-spotted and probed with 0.5 µg/ml OPG and 0.5 µg/ml anti-OPG antibody and AlexaFluor647 rabbit anti-goat secondary antibody. Binding to FC gamma receptors
 35 was present in all conditions, even in the negative controls where no OPG or anti-OPG primary antibody was added. SLC13A3 was identified as an inverse hit (white spots detected where fluorescence levels are less than background levels) and FGF6 was

- 5 identified as a diffuse hit. 6 OPG specific hits were identified as GAP43, TNFSF11, Fas, IL1RAP, SDC1 and TMPRSS11D. Background levels detected in the presence of OPG, anti-OPG and secondary antibodies were classified as medium/high and low in the negative controls.

Hit No:		1	2	3	4	5	6	7
Gene Id:		FCGR1A	SLC13A3	TLR7	CDC42	FGFR1	GAP43	FCGR2B
Condition	Rep. No							
OPG + anti-OPG + secondary	1	Strong	Inverse				Medium	Strong
OPG + anti-OPG + secondary	2	Strong	Inverse				Medium	Strong
Anti-OPG + secondary (no OPG)	1	Strong						Strong
Anti-OPG + secondary (no OPG)	2	Strong						Strong
OPG + secondary (no anti-OPG)	1	Strong						Strong
OPG + secondary (no anti-OPG)	2	Strong						Strong

Hit No:		8	9	10	11	12	13	14
Gene Id:		CXCR7	TNFSF11	TAAR9	FAS	IL1RAP	PTPRN	LYPD6B
Condition	Rep. No							
OPG + anti-OPG + secondary	1		Strong		Weak	Medium		
OPG + anti-OPG + secondary	2		Strong		Weak	Medium		
Anti-OPG + secondary (no OPG)	1							
Anti-OPG + secondary (no OPG)	2							
OPG + secondary (no anti-OPG)	1							
OPG + secondary (no anti-OPG)	2							

10

Hit No:		15	16	17	18	Background
Gene Id:		SDC1	TMPRSS11D	FGF6	FCGR2A	
Condition	Rep. No					
OPG + anti-OPG + secondary	1	Weak	Weak	Diffuse	Strong	Med/high
OPG + anti-OPG + secondary	2	Weak	Weak	Diffuse	Strong	Med/high
Anti-OPG + secondary (no OPG)	1				Strong	Low
Anti-OPG + secondary (no OPG)	2				Strong	Low
OPG + secondary (no anti-OPG)	1				Strong	Low
OPG + secondary (no anti-OPG)	2				Strong	Low



Binding of antibodies to FC-gamma receptors
OPG specific hits

15

The interactions between OPG and syndecan-1 and RANKL were already identified prior to the cell microarray, leaving 4 potential novel interactions between OPG and GAP43, Fas, IL1RAP and TMPRSS11D. After reviewing the literature and considering the role of IL-1 and Fas in cardiovascular disease and the involvement of IL-1 and another TNF family member, TRAIL, in PAH, binding of OPG to IL1RAcP and Fas was then confirmed in PA-SMCs.

Example 5

10 OPG binds Fas in HPASMC cell lysates

The interactions between OPG and syndecan-1 and RANKL were already identified prior to the Retrogenix cell microarray, leaving 4 potential novel interactions between OPG and GAP43, Fas, IL1RAP and TMPRSS11D. After reviewing the literature and considering the role of IL-1 and Fas in cardiovascular disease and the involvement of IL-1 and another TNF family member, TRAIL, in PAH, binding of OPG to IL1RAcP and Fas was then confirmed in PA-SMCs.

In order to validate the OPG-Fas and OPG-IL1RAcP interactions, Fas and IL1RAcP antibodies were used to pull down OPG from OPG-stimulated HPASMC lysates and from a mix of rhOPG, rhFas and rhIL1RAcP. After the complexes were precipitated, the supernatants were analysed by western blotting and probed for human OPG.

Fas antibody was found to pull down OPG from HPA-SMC lysates (Figure 2 (b)). This can be seen by a band in lane 2 at ~50 kDa after probing the membrane with anti-OPG primary antibody followed by secondary IRDye 680LT donkey anti-goat OPG was also co-immunoprecipitated from an in vitro mixture of recombinant human Fas and recombinant human OPG, shown by a band in lane 3 at ~50 kDa Furthermore, no bands were detected at ~50 kDa in the no antibody negative controls (Lanes 3 and 5). IL1RAcP antibody was also shown to pull down OPG from HPASMC lysates, shown by a band at ~50 kDa in lane 6. OPG was also co-immunoprecipitated from an in vitro mixture of recombinant human IL1RAcP and recombinant human OPG, shown by a band in lane 8 at ~50 kDa Furthermore, no bands were detected at ~50 kDa in the no antibody negative controls (Lanes 7 and 9),

Example 6**OPG and Fas expression is increased in the pulmonary artery and right ventricle of IPAH patients**

- 5 After identifying that OPG successfully binds to Fas and IL1RAcP, expression of Fas, IL1RAP and OPG in the pulmonary artery and right ventricle of patients with IPAH was investigated. Sections of the pulmonary artery and right ventricle were provided by for immunohistochemical analysis.
- 10 Sections were stained for Fas and IL1RAcP expression and Fas expression was found to be more extensive in the pulmonary artery from patients with IPAH compared to the healthy control pulmonary artery, and Fas expression was observed in the right ventricle of both the control and IPAH patient sections (Figure 2 c). IL1RAcP expression was not
- 15 observed in the pulmonary artery or right ventricle of the healthy control or IPAH patient sections (Figure 2 c).

Fas RNA expression was also measured in lungs from IPAH patients by TaqMan RT-qPCR. IPAH patients were found to have significantly higher Fas RNA expression compared to healthy control lungs (Figure 2 d).

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Claims

- 5 1. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] and osteoprotegerin [OPG].
2. The pharmaceutical composition according to claim 1 wherein said agent is an
10 antagonistic antibody, or active binding fragment thereof.
3. The pharmaceutical composition according to claim 2 wherein said antibody, or active binding fragment, binds and inhibits the activity of a polypeptide comprising or consisting of the amino acid sequence in SEQ ID NO: 1 [TRAIL] and SEQ ID NO: 2
15 [OPG].
4. The pharmaceutical composition according to claim 2 or 3 wherein said antibody is a polyclonal antibody.
- 20 5. The pharmaceutical composition according to claim 2 or 3 wherein said antibody is a monoclonal antibody.
6. The pharmaceutical composition according to claim 2 or 3 wherein said antibody is a chimeric antibody.
25
7. The pharmaceutical composition according to claim 2 or 3 wherein said antibody is a humanized or human antibody.
8. The pharmaceutical composition according to any one of claims 2 to 7 wherein
30 said composition comprises a first antibody, or active binding fragment, that binds and inhibits the activity of TRAIL and a second antibody, or active binding fragment, that binds and inhibits the activity of OPG.
9. The pharmaceutical composition according to any one of claims 2 to 7 wherein
35 said agent is a bi-specific antibody comprising one immunoglobulin heavy and light chain that binds and inhibits TRAIL and a second immunoglobulin heavy and light chain that binds and inhibits OPG.

10. The pharmaceutical composition according to any one of claims 2 to 8 wherein said agent is a single chain antibody fragment.
11. The pharmaceutical composition according to claim 10 wherein said agent is a bivalent single chain antibody fragment that binds and inhibits the activity of both TRAIL and OPG.
12. The pharmaceutical composition according to claim 11 wherein said agent is a diabody comprising two single chain antibody fragments that bind and inhibit the activity of both TRAIL and OPG.
13. The pharmaceutical composition according to any one of claims 1 to 12 wherein said antibody is pegylated.
14. The pharmaceutical composition according to any one of claims 1 to 12 wherein there is provided an antibody fragment selected from the group consisting of: a single chain antibody fragment, a bivalent single chain antibody fragment, a diabody, a Fab fragment, Fab₂ fragment, F(ab')₂ fragment, Fv fragment, Fc fragment or a Fd fragment and is pegylated.
15. The pharmaceutical composition according to any one of claims 1 to 14 wherein said composition includes an effective amount of at least one additional agent effective in the treatment of pulmonary hypertension.
16. The pharmaceutical composition according to claim 15 wherein said additional agent is selected from the group: calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanylate cyclase and phosphodiesterase inhibitors.
17. A pharmaceutical composition according to any one of claims 1 to 16 for use in the treatment and/or reversal of pulmonary hypertension.
18. A method to treat and/or reverse pulmonary hypertension comprising administering an effective amount an agent that inhibits the activity of OPG and an agent that inhibits the activity of TRAIL to a human subject in need of treatment.
19. The method according to claim 18 wherein said method administers a first agent that inhibits the activity of TRAIL and a second agent that inhibits the activity of OPG wherein the administration of first and second agents is temporally separated.

20. The method according to claim 18 wherein said method administers a first agent that inhibits the activity of OPG and a second agent that inhibits the activity of TRAIL wherein the administration of first and second agents is temporally separated.

5

21 The method according to claim 18 wherein the first and second agents are administered substantially simultaneously.

22. The composition or method according to any one of claims 18 to 21 wherein
10 pulmonary hypertension is pulmonary arterial hypertension.

23. The composition or method according to any one of claims 18 to 21 pulmonary hypertension is associated with lung disease.

15 24. A combined diagnostic and method of treatment or reversal of pulmonary hypertension in a subject comprising:

i) determining the level of expression of TRAIL and OPG in an isolated biological sample when compared to a control; and

20 ii) determining, based on the level of expression of both TRAIL and OPG, whether said subject would benefit from administration of an agent according to any one of claims 1 to 16 to prevent or reverse pulmonary hypertension in said subject.

25. The method according to claim 24 wherein said method comprises:

25 i) forming a preparation comprising said sample and an oligonucleotide primer pair adapted to anneal to a nucleic acid molecule comprising a nucleic acid sequence as represented in SEQ ID NO: 3 or 4; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;

30 ii) providing polymerase chain reaction conditions sufficient to amplify said nucleic acid molecule;

iii) analysing the amplified products of said polymerase chain reaction for the presence or absence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 3 and 4; and optionally

35 v) comparing the amplified product with a normal matched control.

26. The method according to claim 25 wherein said method is a real time PCR method.

27. The method according to claim 24 wherein said method comprises:
- i) providing an isolated biological sample to be tested;
 - ii) forming a preparation comprising said sample and an antibody or antibodies that specifically binds one or more polypeptide[s] in said sample as represented by the amino acid sequences presented in SEQ ID NO: 1 and 2 to form an antibody/polypeptide complex;
 - iii) detecting the complex or complexes so formed; and
 - iv) comparing the expression of said polypeptide[s] with a normal matched control.
28. The method according to any one of claims 24 to 27 wherein ratio of TRAIL: OPG is compared to the ratio of TRAIL: OPG in a control sample.
29. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of FAS receptor polypeptide [SEQ ID NO: 5, 6, 7, 8, 9, 10 or 11] for use in the treatment of pulmonary hypertension.
30. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of TNFSF11 polypeptide [SEQ ID NO: 12 or 13] for use in the treatment and/or reversal of pulmonary hypertension.
31. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of SDC1 polypeptide [SEQ ID NO: 14, 15 or 16] for use in the treatment and/or reversal of pulmonary hypertension.
32. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of GAP43 polypeptide [SEQ ID NO: 17 or 18] for use in the treatment and/or reversal of pulmonary hypertension.
33. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of ILRAPcP polypeptide [SEQ ID NO: 19] for use in the treatment and/or reversal of pulmonary hypertension.

34. A pharmaceutical composition comprising one or more inhibitory agent[s] wherein said agent[s] inhibit the activity of transmembrane protease 11D polypeptide [SEQ ID NO: 20 or 21] for use in the treatment and/or reversal of pulmonary hypertension.

5 35. The composition according to any one of claims 29 to 34 wherein said composition comprises:

- i) an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, GAP43, ILRAPcP or transmembrane protease 11; combined with
- 10 ii) an agent that inhibits OPG and/or TRAIL.

36. The composition according to claim 35 wherein the treatment and/or reversal of pulmonary hypertension comprises the administration of an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, 15 GAP43, ILRAPcP or transmembrane protease 11D and administration of an agent that inhibits OPG and/or TRAIL wherein the said administrations are temporally separated.

37. The composition according to claim 35 wherein the treatment and/or reversal of pulmonary hypertension comprises the administration of an agent that inhibits the activity of a polypeptide selected from the group consisting of: FAS receptor, SDC1, TNFSF11, 20 GAP43, ILRAPcP or transmembrane protease 11D and administration of an agent that inhibits OPG and/or TRAIL wherein the said administrations are simultaneous or sequential.

25 38. The composition according to any one of claims 29 to 37 wherein said agent is an antibody or active binding fragment.

39. The composition according to any one of claims 29 to 38 wherein said agent[s] binds the extracellular domain[s] of said polypeptide.

30

40. The composition according to any one of claims 29 to 39 wherein pulmonary hypertension is pulmonary arterial hypertension.

41. The composition according to any one of claims 29 to 39 wherein pulmonary 35 hypertension is associated with lung disease.

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Figure 1A

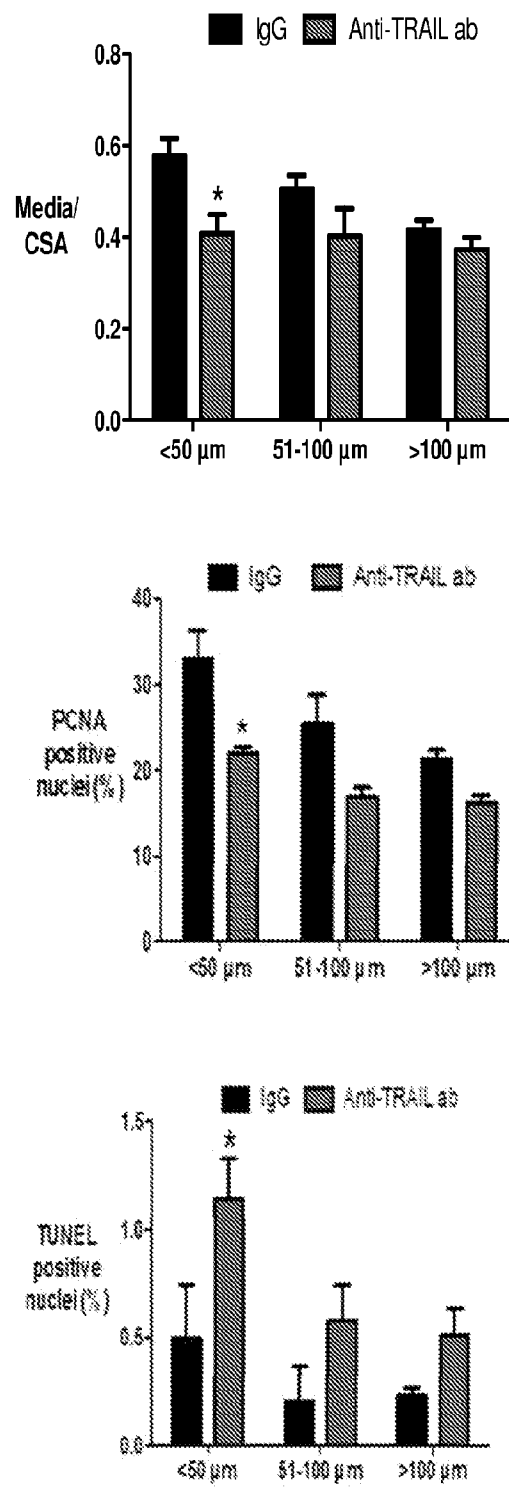
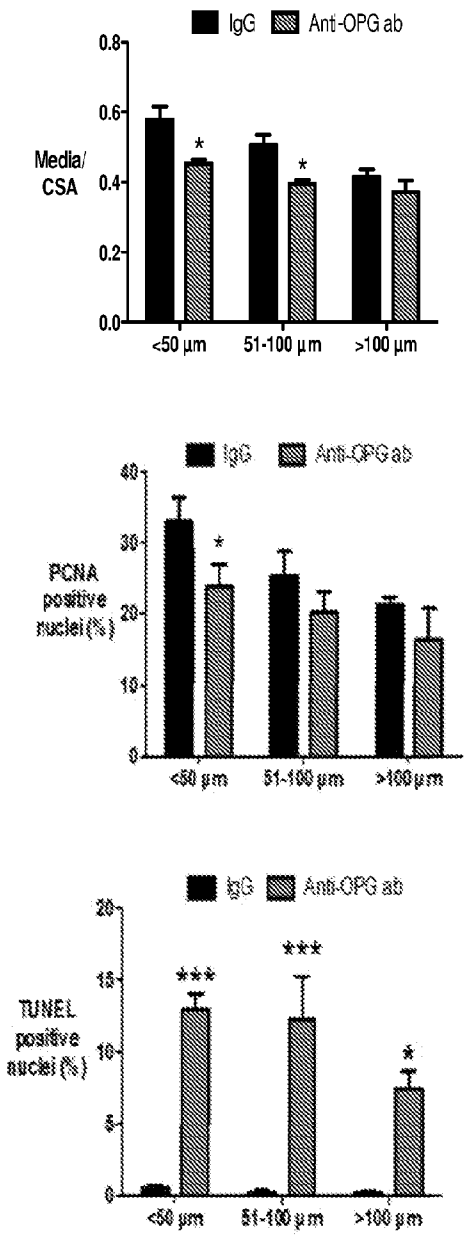


Figure 1B



TRAIL**3/6****SEQ ID NO: 1**

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PCWQVQVQLRQLVRKMILRTSEETISTVQEKQONISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRK
INSESSRSRSGHSFSLNLHLRNGELVIHEKGFYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKS
ARNSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG*

SEQ ID NO: 3

ATGGCTATGATGGAGGTCCAGGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGATCGTGATCTTCACAGTGCTC
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ATAAACTCCTGGGAATCATCAAGGAGTGGGCATTTCCTGAGCAACTTGCACTTGAGGAATGGTGAACCTGGTC
ATCCATGAAAAAGGTTTTACTACATCTATTCCCAAACATACTTTCGATTTAGAGGAGAAATAAAAGAAAACACA
AAGAACGACAAACAAAATGGTCCAATATATTTACAAATACACAAGTTATCCTGACCCTATATTTGTTGATGAAAAGT
GCTAGAAATAGTTGTTGGTCTAAAGATGCAGAATATGGACTCTATTCCATCTATCAAGGGGGAATATTTGAGCTT
AAGGAAAATGACAGAATTTTTGTTTCTGTAACAAATGAGCACTTGATAGACATGGACCATGAAGCCAGTTTTTTT
GGGGCCTTTTTAGTTGGCTAA

OPG**SEQ ID NO: 2**

MNNLLCCALVFLDISIKWTTQETFPFKYLHYDEETSHQLLCDKCPPGYLKQHCTAKWKTVCAPCPDHYYTDSWH
TSDECLYCSPVCKELQYVVKQECNRTHNRVCECKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNVTCKRCPDGF
SNETSSKAPCRKHTNCSVFLGLLLTQKGNATHDNICSGNSESTQKCGIDVTLCEEAFRRFAVPTKFTPNWLSVLVD
NLPGTQVNAESVERIKRQHSSQEQTQQLKLWKHQNKDQDIVKKIIQDIDLCEVQVRHIGHANLTFEQLRSLME
SLPGKKVGAEDIEKTIKACKPSDQILKLLSLWRIKNGDQDTLGLMHALKHKSITYHFPKTVTQSLKKTIRFLHSF
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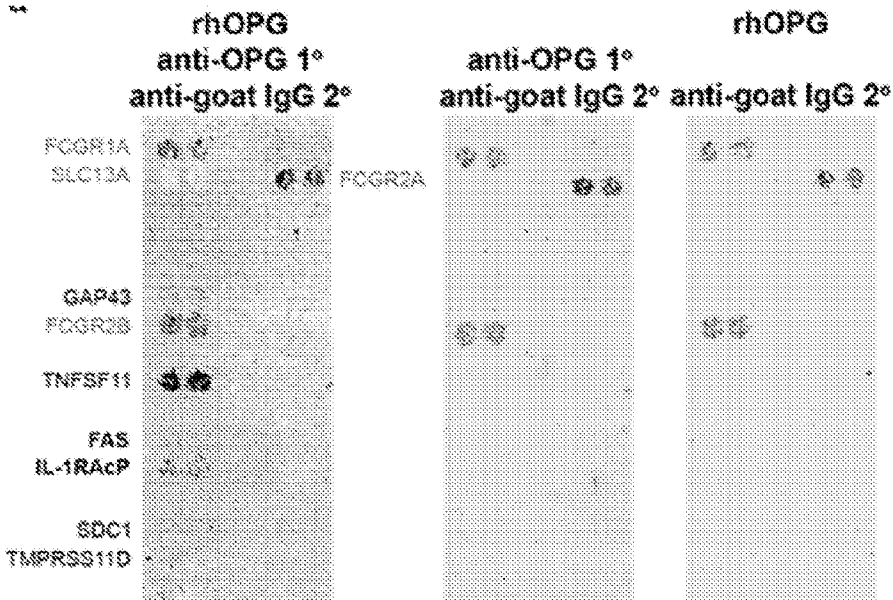
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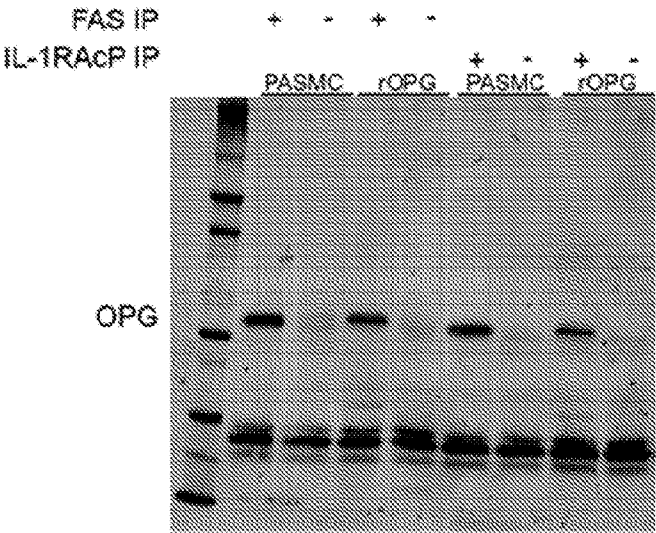
Figure 2

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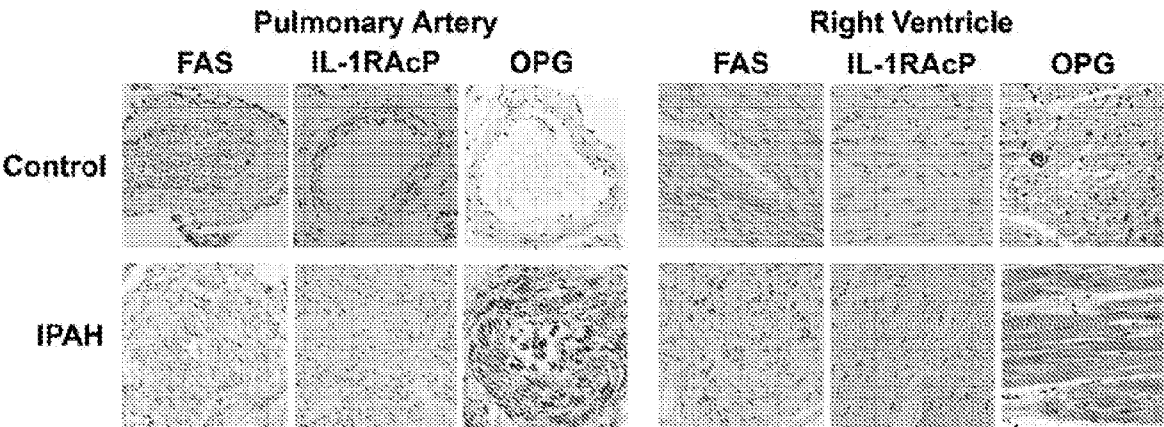
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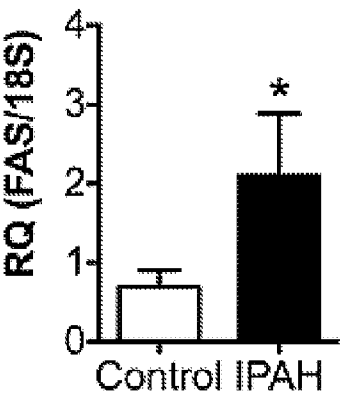
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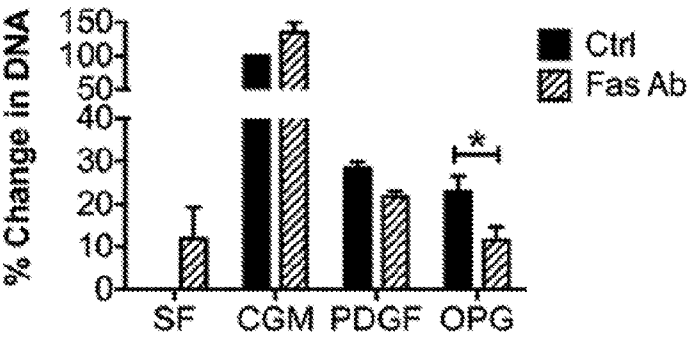
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(d)



(e)



(f)

