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(54) Title: DETECTING, PREVENTING, AND TREATING BRONCHOPULMONARY DYSPLASIA

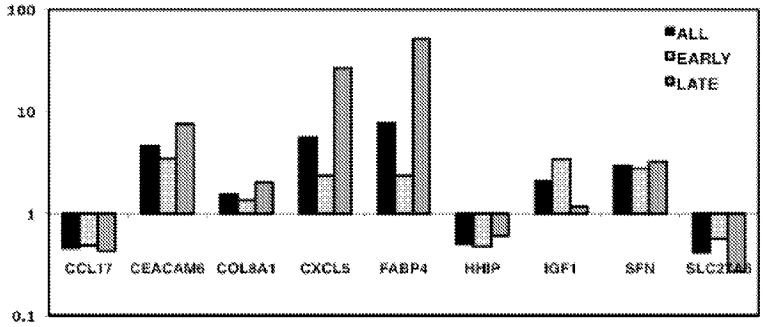

Figure 2

(57) Abstract: Provided herein are methods for preventing or treating bronchopulmonary dysplasia (BPD) in a subject. The methods comprise identifying connective tissue mast cells (CTMCs) in a subject and administering an agent that blocks an activity of, blocks an increase in a level of, or reduces a level of the CTMCs in the subject. Also provided are methods of identifying a subject with or at risk for developing BPD. Also provided are methods for determining the effectiveness of a treatment regime for BPD in a subject.

WO 2012/158763 A1

Detecting, preventing, and treating bronchopulmonary dysplasia

CROSS-REFERENCE TO PRIORITY APPLICATION

This application claims priority to U.S. Provisional Application No. 61/486,613, filed May 16, 2011, which is incorporated herein by reference in its entirety.

BACKGROUND

Bronchopulmonary Dysplasia (BPD) is a lung disease of the premature infant defined by dependence on supplemental oxygen for more than 28 days post-partum and/or at 36 weeks corrected gestational age. In the United States, more than 500,000 babies are born prematurely each year, with approximately 60,000 at high risk for BPD and 10,000 diagnosed with this disease (Chronic Lung Disease after Premature Birth Eugenio Baraldi, M.D., and Marco Filippone, M.D. N Engl J Med 2007; 357:1946-1955). The overall costs of treating infants with BPD in the United States are estimated to be $2.5 billion, second only for pediatric lung disease to the costs for treating asthma and far exceeding the cost of treating cystic fibrosis. The incidence of BPD has increased over the last 30 years, largely due to higher survival rates of premature babies.

SUMMARY

Provided herein are methods for preventing or treating bronchopulmonary dysplasia (BPD) in a subject. The methods comprise directly or indirectly identifying a subset of mast cells, chymase-expressing connective-tissue mast cells (CTMCs), in a biological sample from the subject and administering to the subject an agent that blocks an activity of, blocks an increase in, reduces activation of, or reduces the level of chymase-expressing CTMCs in the subject.

Also provided are methods of identifying a subject with or at risk of developing BPD. The methods comprise directly or indirectly detecting chymase-expressing CTMCs in a biological sample from the subject. The presence of chymase-expressing CTMCs, as compared to a control, indicates the subject has BPD.

Also provided are methods of determining the effectiveness of a treatment regime for BPD in a subject. The methods comprise identifying a first level of chymase-expressing CTMCs, directly or indirectly, in a first biological sample from the subject before administration of the treatment regime, identifying a second level of chymase-expressing CTMCs in a second biological sample from the subject after administration of the treatment regime, and adjusting the treatment regime if the second level of chymase-expressing CTMCs is the same or higher than

the first level. The method optionally comprises a third or subsequent identification step or steps.

Further provided are assay systems comprising, for example, a microarray or a gene chip with at least two selective binding agents. Each selective binding agent is specific for a biomarker selected from the group consisting of chymase and/or carboxypeptidase A3 (CPA3), optionally with a binding agent specific for tryptase beta 2 (TPSB2), tryptase alpha/beta 1 (TPSAB1), cathepsin G, and/or prostaglandin D2 (PGD2).

The details of one or more embodiments are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 shows canonical pathways affected in bronchopulmonary dysplasia (BPD) lungs. In order to estimate general biological mechanisms that are altered during BPD pathogenesis, pathways analysis using Ingenuity software (Ingenuity Systems; Redwood City, CA) was performed. 159 genes whose expression was significantly altered in BPD lung tissue were identified and over-representation of this gene set was assessed in known pathways. Shown are "canonical" pathways, ranked by Fisher's-exact T-test p-value, listing the percentage of the BPD-associated gene set in each pathway (dark gray bar), versus the percentage of all genes in that pathway (light gray bar).

Figure 2 shows a graph demonstrating validation of gene expression. Quantitative real-time PCR (qPCR) was used to confirm expression patterns predicted by microarray analysis. Nine genes were selected based upon their magnitude of change and biological interest. Shown are fold-differences in expression (log scale) for each gene, comparing all samples, or samples divided by gestational age. All genes demonstrated evidence for replication, with 7 of 9 displaying significant differences between BPD and controls.

Figure 3 shows a graph showing increased mast cell marker expression in an animal model of BPD using qPCR to assess gene expression for the mast cell markers CPA3, TPSAB1 and TPDB2 in whole lung tissue obtained from wild type (WT, $n \geq 3$) orFGFR3/4 compound deficient (KO, $n \geq 3$) mice at 1 month of age. Shown are mean relative gene expression values for each group and for each gene (*, p-value < 0.05).

Figure 4 shows an increased mast cell accumulation in BPD lungs. Immunohistochemistry was used to identify tryptase-expressing cells, a general mast cell-specific marker. Figure 4A (top left) shows quantitative analysis of the number of tryptase-

expressing cells in BPD and non-BPD control lungs. Shown are the average numbers of stained cells/field for each individual subject (dots), the group means (bar) and interquartile range (box). Figures 4B and 4C (top and middle right) show examples of staining patterns in age-matched control subjects. Control staining is shown as Non-Immune Serum. Figures 4D and 4E (bottom left and right) are bar graphs showing examples of staining patterns in BPD subjects. Distribution of stained cells within the alveolar, peribronchiolar or perivascular region of BPD and control tissues. Data are presented as the average absolute number of cells per subject in each region (bottom left) or the frequency (proportion) of stained cells found in each region (bottom right). Magnification is as indicated on scale bars. A significant approximate 5-fold increase in tryptase positive cells was observed in BPD lungs.

Figure 5 shows an increased connective tissue-type mast cell accumulation in BPD lungs. Immunohistochemistry was used to identify chymase-expressing cells, a specific marker for connective tissue-type mast cells. Quantitative analysis of the number of chymase-expressing cells in BPD and non-BPD control lungs is indicated at the top left (5A). Shown are the average numbers of stained cells/field for each individual subject (dots), the group means (bar) and interquartile range (box). Figures 5B-5C (top and middle right) show individual images of staining patterns in corrected gestational age matched control and BPD subjects (subject ID indicated). Panels are arranged according to gestational age at death. Also shown is an absence of staining in non-immune serum controls. Bar graphs at bottom show distribution of stained cells within the alveolar, peribronchiolar or perivascular region of BPD and control tissues. Data are presented as the average absolute number of cells per subject in each region (bottom left) or the frequency (proportion) of stained cells found in each region (bottom right). Bar graphs at bottom show distribution of stained cells within the alveolar, peribronchiolar or perivascular region of BPD and control tissues. Data are presented as the average absolute number of cells in each region (bottom left) or the frequency of stained cells found in each region (bottom right).

Figure 6 shows the amount of chymase detected in infants relative to the day after birth on which the sample was collected. Serial tracheal aspirates were collected from a set of quadruplets born at less than 29 weeks. Chymase was detected by ELISA in each sample and is presented relative to the day after birth on which the sample was collected. Unique patterns of tracheal aspirate chymase content were detected in each infant. Figure 6A shows the amount of chymase detected in subject 48 relative to the day after birth on which the sample was collected. Figure 6B shows the amount of chymase detected in subject 49 relative to the day after birth on which the sample was collected. Figure 6C shows the amount of chymase detected in subject 50

relative to the day after birth on which the sample was collected. Figure 6D shows the amount of chymase detected in subject 51 relative to the day after birth on which the sample was collected.

DETAILED DESCRIPTION

Bronchopulmonary dysplasia (BPD) is a major complication of premature birth. Thus, premature infants, particularly infants born at less than 32 weeks of gestation or weighing less than 1500 grams (about 3.3 pounds) are at a risk for developing BPD. In addition to weight and gestational age at birth, risk factors for BPD are complex and include prenatal or perinatal infection, O_2 toxicity, and ventilation-associated injury. Thus, infection, O_2 administration, and ventilators also indicate an infant at risk for BPD. BPD pathology is complex and characterized by inflammation, dysmorphic airspaces and vasculature, and aberrant extracellular matrix accumulation.

Provided herein are methods for preventing or treating bronchopulmonary dysplasia (BPD) in a subject. The methods comprise identifying connective tissue mast cells (CTMCs) in a biological sample from the subject and administering to the subject an agent that blocks an activity of, blocks an increase in a level of, or reduces a level of CTMCs in the subject. The CTMCs are a subset of mast cells. They can be positive for such biomarkers as chymase, carboxypeptidase A3 (CPA3), tryptase beta 2 (TBSP2), tryptase alpha/beta 1 (TBSAB1), cathepsin G (CTSG), and prostaglandin D2 (PGD2). Blocking an activity of, blocking an increase in a level of, or reducing a level of CTMCs results in the prevention or treatment of BPD in the subject.

Blocking an activity of a CTMC (e.g., a chymase-expressing CTMC) can, for example, include blocking the activation of the CTMC. Blocking the activation of a CTMC can, for example, result in blocking the release of granules (e.g., histamine granules) and various hormonal mediators into the interstitium. Blocking activation of CTMCs reduces the inflammatory process. Blocking an increase in the level of or reducing a level of a CTMC as used herein, means the number of CTMCs stays the same or decreases as compared to a control (e.g., a known number or level or a control without BPD).

As used herein a control can be a treated or untreated, non-BPD diseased sample from a different subject. Optionally, a control can be an untreated sample from the same subject. Optionally, a control can be a reference value previously obtained for a treated or untreated, non-BPD diseased sample or for a treated or untreated, diseased sample. In the case of a non-BPD diseased sample, as described herein, there are no CTMCs or a very low level of CTMCs; however, there is a baseline level of tryptase-expressing mast cells (MC_T).

The CTMCs can, for example, express an increased level of chymase protein or mRNA and/or an increased level of carboxypeptidase A3 (CPA3) protein or mRNA as compared to a non-connective tissue mast cell. A non-connective tissue mast cell can, for example, include any type of mast cell that is not a connective tissue mast cell. A non-connective tissue mast cell is positive for tryptase or other general mast cell biomarkers, but is negative for connective-tissue mast cell biomarkers such as chymase.

Optionally, the agent used to treat or prevent BPD blocks expression or activity of CPA3 or chymase. Blocking expression of CPA3 or chymase, as used herein, means the agent decreases the level of expression or inhibits expression of CPA3 or chymase. Blocking the activity of CPA3 or chymase, as used herein, means that CPA3 or chymase cannot perform their natural protease function.

The agent can, for example, be a neutralizing antibody to CPA3 or chymase. The term antibody is used herein in a broad sense and includes both polyclonal and monoclonal antibodies. The term can also refer to a human antibody and/or a humanized antibody. Examples of techniques for human monoclonal antibody production include those described by Cole et al. (Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985) and by Boerner et al. (J. Immunol. 147(1):86-95 (1991)). Human antibodies (and fragments thereof) can also be produced using phage display libraries (Hoogenboom et al., J. Mol. Biol. 227:381 (1991); Marks et al., J. Mol. Biol. 222:581 (1991)). The disclosed human antibodies can also be obtained from transgenic animals. For example, transgenic, mutant mice that are capable of producing a full repertoire of human antibodies, in response to immunization, have been described (see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA 90:2551-5 (1993); Jakobovits et al., Nature 362:255-8 (1993); Bruggermann et al., Year in Immunol. 7:33 (1993)).

Functional antibody fragments or single chain antibodies can also be used. The term antibody or fragments thereof can also encompass chimeric antibodies and hybrid antibodies, with dual or multiple antigen or epitope specificities, and fragments, such as F(ab')2, Fab', Fab and the like, including hybrid fragments. Thus, fragments of the antibodies that retain the ability to bind their specific antigens are provided. For example, fragments of antibodies which maintain CPA3 or chymase binding activity are included within the meaning of the term antibody or fragment thereof. Such antibodies and fragments can be made by techniques known in the art and can be screened for specificity and activity according to general methods for producing antibodies and screening antibodies for specificity and activity (See Harlow and Lane. Antibodies, A Laboratory Manual. Cold Spring Harbor Publications, New York (1988)).

Also included within the meaning of antibody or fragments thereof are conjugates of antibody fragments and antigen binding proteins (single chain antibodies) as described, for example, in U.S. Pat. No. 4,704,692, the contents of which are hereby incorporated by reference in their entirety.

Optionally, the CTMCs express an increased level of one or more biomarkers selected from the group consisting of chymase, carboxypeptidase A3 (CPA3), tryptase beta 2 (TBSP2), tryptase alpha/beta 1 (TBSAB1), cathepsin G (CTSG), and prostaglandin D2 (PGD2).

Optionally, the agent can be selected from the group consisting of a small molecule, a polypeptide, a nucleic acid, or a peptidomimetic. The nucleic acid can, for example, be selected from the group consisting of a small interfering RNA (siRNA), a microRNA (miRNA), or an antisense nucleic acid.

As used herein, an inhibitory nucleic acid sequence can be a short-interfering RNA (siRNA) sequence or a micro-RNA (miRNA) sequence that is specific for the mRNA of one or more of the biomarkers (e.g., carboxypeptidase A3, chymase, tryptase beta 2 (TPSB2), and tryptase alpha/beta 1 (TPSAB1)). A 21-25 nucleotide siRNA or miRNA sequence can, for example, be produced from an expression vector by transcription of a short-hairpin RNA (shRNA) sequence, a 60-80 nucleotide precursor sequence, which is subsequently processed by the cellular RNAi machinery to produce either a siRNA or miRNA sequence. Alternatively, a 21-25 nucleotide siRNA or miRNA sequence can, for example, be synthesized chemically. Chemical synthesis of siRNA or miRNA seuquences is commercially available from such corporations as Dharmacon, Inc. (Lafayette, CO), Qiagen (Valencia, CA), and Ambion (Austin, TX). A siRNA sequence preferably binds a unique sequence within the mRNA with exact complementarity and results in the degradation of the mRNA molecule. A siRNA sequence can bind anywhere within the mRNA molecule. A miRNA sequence preferably binds a unique sequence within the mRNA with exact or less than exact complementarity and results in the translational repression of the mRNA molecule. A miRNA sequence can bind anywhere within the mRNA sequence, but preferably binds within the 3' untranslated region of the mRNA molecule. Methods of delivering siRNA or miRNA molecules are known in the art. See, e.g., Oh and Park, Adv. Drug. Deliv. Rev. 61(10):850-62 (2009); Gondi and Rao, J. Cell Physiol. 220(2):285-91 (2009); and Whitehead et al., Nat. Rev. Drug. Discov. 8(2):129-38 (2009).

As used herein, an inhibitory nucleic acid sequence can be an antisense nucleic acid sequence. Antisense nucleic acid sequences can, for example, be transcribed from an expression vector to produce an RNA which is complementary to at least a unique portion of the biomarker mRNA and/or the endogenous gene which encodes the biomarker. Hybridization of an antisense

nucleic acid under specific cellular conditions results in inhibition of the biomarker protein expression by inhibiting transcription and/or translation.

Also provided are methods for determining the effectiveness of a treatment regime for BPD in a subject. The methods comprise identifying a first level of CTMCs in a first biological sample from the subject before administration of a treatment regime to the subject, identifying a second level of CTMCs in a second biological sample from the subject after administration of a treatment regime to the subject, comparing the first and second level of CTMCs, and adjusting the treatment regime if the second level of CTMCs is the same or higher than the first level. Optionally, for a treatment regime that blocks the activity of a CTMC (e.g., blocks the activation of the chymase-expressing CTMC), the methods comprise determining a first and second level of activation of the CTMCs, comparing the first and second level of activation, and adjusting the treatment regime if the second level of activation is the same or higher than the first level of activation. The determining, comparing, and adjusting steps can be repeated, as needed, over the course of the treatment regime or over the course of various treatment regimes.

Also provided are methods of identifying a subject with or at risk for developing BPD. The methods comprise detecting CTMCs in a biological sample from the subject, wherein the presence of CTMCs as compared to a control indicates the subject has BPD.

As used herein, a biological sample can include, a sample selected from the group consisting of blood, an airway aspirate, a tracheal aspirate, a bronchoalveolar lavage (BAL), urine, and lung tissue. An airway aspirate can include a lower airway washing (e.g., a deep lung washing) or an upper airway washing (e.g., a nasal washing or nasal scraping). A biological sample can also be a biopsy of lung tissue.

The CTMCs can be detected directly or indirectly. Direct detection can, for example, include histological detection of CTMC in the biological sample. Indirect detection can, for example, include detection of one or more secreted CTMC biomarkers. Thus, optionally, detection of CTMCs comprises detecting expression of one or more biomarkers selected from the group consisting of chymase, CPA3, TPSB2, TPSAB1, CTSG, and PGD2.

The level of expression of the one or more biomarkers is detected by the level of RNA or polypeptide in the biological sample. Optionally, the level of RNA is determined using an assay selected from the group consisting of a microarray analysis, a gene chip, a Northern blot, an *in situ* hybridization assay, a RT-PCR assay, a one step PCR assay, and a quantitative real time (qRT)-PCR assay. Optionally, the level of polypeptide is determined using an assay selected from the group consisting of a Western blot, an enzyme-immunosorbent assay (ELISA), an enzyme immunoassay (EIA), a radioimmunoassay (RIA), an immunohistochemistry (IHC)

assay, and a protein array. The analytical techniques to determine RNA or polypeptide expression are known. See, e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (2001).

Further provided are assay systems comprising a microarray or gene chip with at least two selective binding agents specific for one or more CTMC biomarkers. Each selective binding agent is specific for a biomarker of a chymase-expressing CTMC selected from the group consisting of chymase, CPA3, TPSB2, TPSAB1, CTSG, and PGD2. The assays systems can, for example, be a DNA microarray or gene chip, a RNA microarray or gene chip, or a protein array. Arrays and gene chips are known in the art. See, e.g., Dufva, Methods Mol. Biol. 529:1-22 (2009); Plomin and Schalkwyk, Dev. Sci. 10:19-23 (2007); Kopf and Zharhary, Int. J. Biochem. Cell Biol. 39(7-8):1305-17 (2007); Haab, Curr. Opin. Biotechnol. 17(4):415-21 (2006); US Patent No. 5,445,934; US Patent No. 5,800,992; and US Patent No. 5,807,552. Optionally, the assay system is limited to binding agents specific for CTMCs but may include one or more control markers as well.

Provided herein are compositions containing the provided small molecules, polypeptides (including, e.g., antibodies or antibody fragments), nucleic acid molecules, and/or peptidomimetics and a pharmaceutically acceptable carrier described herein. The herein provided compositions are suitable for administration *in vivo*. By pharmaceutically acceptable carrier is meant a material that is not biologically or otherwise undesirable, i.e., the material is administered to a subject without causing undesirable biological effects or interacting in a deleterious manner with the other components of the pharmaceutical composition in which it is contained. The carrier is selected to minimize degradation of the active ingredient and to minimize adverse side effects in the subject.

Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy, 21st Edition*, David B. Troy, ed., Lippicott Williams & Wilkins (2005). Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carriers include, but are not limited to, sterile water, saline, buffered solutions like Ringer's solution, and dextrose solution. The pH of the solution is generally about 5 to about 8 or from about 7 to 7.5. Other carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the immunogenic polypeptides. Matrices are in the form of shaped articles, e.g., films, liposomes, or microparticles. Certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being

administered. Carriers are those suitable for administration of the agent, e.g., the small molecule, polypeptide, nucleic acid molecule, and/or peptidomimetic, to humans or other subjects.

The compositions are administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. The compositions are administered via any of several routes of administration, including topically, orally, parenterally, intravenously, intra-articularly, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, intrahepatically, intracranially, nebulization/inhalation, or by instillation via bronchoscopy. Optionally, the composition is administered by oral inhalation, nasal inhalation, or intranasal mucosal administration. Administration of the compositions by inhalant can be through the nose or mouth via delivery by spraying or droplet mechanism, for example, in the form of an aerosol.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives are optionally present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids, and powders. Conventional pharmaceutical carriers, aqueous, powder, or oily bases, thickeners and the like are optionally necessary or desirable.

Compositions for oral administration include powders or granules, suspension or solutions in water or non-aqueous media, capsules, sachets, or tables. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders are optionally desirable.

Optionally, the nucleic acid molecule or polypeptide is administered by a vector comprising the nucleic acid molecule or a nucleic acid sequence encoding the polypeptide. There are a number of compositions and methods which can be used to deliver the nucleic acid molecules and/or polypeptides to cells, either *in vitro* or *in vivo* via, for example, expression vectors. These methods and compositions can largely be broken down into two classes: viral based delivery systems and non-viral based deliver systems. Such methods are well known in the art and readily adaptable for use with the compositions and methods described herein.

As used herein, plasmid or viral vectors are agents that transport the disclosed nucleic acids into the cell without degradation and include a promoter yielding expression of the nucleic acid molecule and/or polypeptide in the cells into which it is delivered. Viral vectors are, for example, Adenovirus, Adeno-associated virus, herpes virus, Vaccinia virus, Polio virus, Sindbis, and other RNA viruses, including these viruses with the HIV backbone. Also preferred are any viral families which share the properties of these viruses which make them suitable for use as vectors. Retroviral vectors, in general are described by Coffin et al., *Retorviruses*, Cold Spring Harbor Laboratory Press (1997), which is incorporated by reference herein for the vectors and methods of making them. The construction of replication-defective adenoviruses has been described (Berkner et al., J. Virol. 61:1213-20 (1987); Massie et al., Mol. Cell. Biol. 6:2872-83 (1986); Haj-Ahmad et al., J. Virol. 57:267-74 (1986); Davidson et al., J. Virol. 61:1226-39 (1987); Zhang et al., BioTechniques 15:868-72 (1993)). The benefit and the use of these viruses as vectors is that they are limited in the extent to which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infections viral particles. Recombinant adenoviruses have been shown to achieve high efficiency after direct, *in vivo* delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma, and a number of other tissue sites. Other useful systems include, for example, replicating and host-restricted non-replicating vaccinia virus vectors.

The provided polypeptides and/or nucleic acid molecules can be delivered via virus like particles. Virus like particles (VLPs) consist of viral protein(s) derived from the structural proteins of a virus. Methods for making and using virus like particles are described in, for example, Garcea and Gissmann, Current Opinion in Biotechnology 15:513-7 (2004).

The provided polypeptides can be delivered by subviral dense bodies (DBs). DBs transport proteins into target cells by membrane fusion. Methods for making and using DBs are described in, for example, Pepperl-Klindworth et al., Gene Therapy 10:278-84 (2003).

The provided polypeptides can be delivered by tegument aggregates. Methods for making and using tegument aggregates are described in International Publication No. WO 2006/110728.

Non-viral based delivery methods can include expression vectors comprising nucleic acid molecules and nucleic acid sequences encoding polypeptides, wherein the nucleic acids are operably linked to an expression control sequence. Suitable vector backbones include, for example, those routinely used in the art such as plasmids, artificial chromosomes, BACs, YACs, or PACs. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, WI), Clonetech (Palo Alto, CA), Stratagene (La Jolla, CA),

and Invitrogen/Life Technologies (Carlsbad, CA). Vectors typically contain one or more regulatory regions. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, protein binding sequences, 5' and 3' untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, and introns.

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis B virus, and most preferably cytomegalovirus (CMV), or from heterologous mammalian promoters, e.g. β-actin promoter or EF1α promoter, or from hybrid or chimeric promoters (e.g., CMV promoter fused to the β-actin promoter). Of course, promoters from the host cell or related species are also useful herein.

Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' or 3' to the transcription unit. Furthermore, enhancers can be within an intron as well as within the coding sequence itself. They are usually between 10 and 300 base pairs (bp) in length, and they function in *cis*. Enhancers usually function to increase transcription from nearby promoters. Enhancers can also contain response elements that mediate the regulation of transcription. While many enhancer sequences are known from mammalian genes (globin, elastase, albumin, fetoprotein, and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The promoter and/or the enhancer can be inducible (e.g. chemically or physically regulated). A chemically regulated promoter and/or enhancer can, for example, be regulated by the presence of alcohol, tetracycline, a steroid, or a metal. A physically regulated promoter and/or enhancer can, for example, be regulated by environmental factors, such as temperature and light. Optionally, the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize the expression of the region of the transcription unit to be transcribed. In certain vectors, the promoter and/or enhancer region can be active in a cell type specific manner. Optionally, in certain vectors, the promoter and/or enhancer region can be active in all eukaryotic cells, independent of cell type. Preferred promoters of this type are the CMV promoter, the SV40 promoter, the β-actin promoter, the EF1α promoter, and the retroviral long terminal repeat (LTR).

The vectors also can include, for example, origins of replication and/or markers. A marker gene can confer a selectable phenotype, e.g., antibiotic resistance, on a cell. The marker product is used to determine if the vector has been delivered to the cell and once delivered is being expressed. Examples of selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hygromycin, puromycin, and blasticidin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. Examples of other markers include, for example, the *E. coli* lacZ gene, green fluorescent protein (GFP), and luciferase. In addition, an expression vector can include a tag sequence designed to facilitate manipulation or detection (e.g., purification or localization) of the expressed polypeptide. Tag sequences, such as GFP, glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or FLAG™ tag (Kodak; New Haven, CT) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino terminus.

As used herein, the terms peptide, polypeptide, or protein are used broadly to mean two or more amino acids linked by a peptide bond. Protein, peptide, and polypeptide are also used herein interchangeably to refer to amino acid sequences. It should be recognized that the term polypeptide is not used herein to suggest a particular size or number of amino acids comprising the molecule and that a peptide of the invention can contain up to several amino acid residues or more.

As used throughout, subject can be a vertebrate, more specifically a mammal (e.g., a human). The term does not denote a particular sex. The subjects are typically newborns. Thus, newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject with a disease or disorder (e.g., bronchopulmonary dysplasia). The term patient or subject includes human and veterinary subjects.

A subject at risk of developing a disease or disorder can be born prematurely (e.g., about 10 weeks before the due date), have breathing problems, low birth weight, prolonged O_2 administration, use of a ventilator, and/or have an infection before, during, or shortly after birth. All of these factors place a neonate at risk for BPD. A subject at risk of developing a disease or disorder can be genetically predisposed to the disease or disorder, e.g., have a family history or have a mutation in a gene that causes the disease or disorder, or show early signs or symptoms of the disease or disorder. A subject currently with a disease or disorder has one or more than one symptom of the disease or disorder and may have been diagnosed with the disease or disorder.

The methods and agents as described herein are useful for both prophylactic and therapeutic treatment. For prophylactic use, a therapeutically effective amount of the agents described herein are administered to a subject prior to onset (e.g., before obvious signs of bronchopulmonary dysplasia) or during early onset (e.g., upon initial signs and/or symptoms of bronchopulmonary dysplasia). Prophylactic administration can occur for several days to a week prior to the manifestation of symptoms of bronchopulmonary dysplasia. Prophylactic administration can be used, for example, in the preventative treatment of subjects at risk of developing bronchopulmonary dysplasia (e.g., a subject diagnosed with a genetic predisposition to bronchopulmonary dysplasia). Therapeutic treatment involves administering to a subject a therapeutically effective amount of the agents described herein after diagnosis or development of bronchopulmonary dysplasia.

According to the methods taught herein, the subject is administered an effective amount of the agent. The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary to produce a desired physiologic response. Effective amounts and schedules for administering the agent may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for administration are those large enough to produce the desired effect in which one or more symptoms of the disease or disorder are affected (e.g., reduced or delayed). The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease, the extent of the disease or disorder, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosages can vary, and can be administered in one or more dose administrations daily, for one or more days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products.

As used herein the terms treatment, treat, or treating refers to a method of reducing or delaying the effects of a disease or condition or symptom of the disease or condition. Thus, for example, treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of an established disease or condition or symptom of the disease or condition. For example, a method for treating a disease is considered to be a treatment if there is a 10% reduction in one or more symptoms of the disease in a subject as compared to a control. Thus the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to control levels (e.g., in the absence

of treatment). Treatment can also cause a delay in the onset of new symptoms or further progression of existing symptoms. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disease, condition, or symptoms of the disease or condition.

As used herein, the terms prevent, preventing, and prevention of a disease or disorder refers to an action, for example, administration of a therapeutic agent, that occurs before or at about the same time a subject begins to show one or more symptoms of the disease or disorder, which inhibits or delays onset or exacerbation of one or more symptoms of the disease or disorder. As used herein, references to decreasing, reducing, or inhibiting include a change of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater as compared to a control level. Such terms can include but do not necessarily include complete elimination.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications to the agents or steps of the methods are discussed, each and every combination and permutation of the agents and method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

EXAMPLE

Example 1: Genome Wide Transcriptional Profiling in Brochopulmonary Dysplasia

Materials and Methods

Human tissue samples and RNA isolation. Over the past decade, under an IRB-approved protocol, a unique biorepository of autopsy tissue samples from human premature babies

diagnosed with BPD and non-BPD controls was established. These lung samples were harvested within 6 hours and snap frozen in liquid nitrogen. Estimated gestational age was based on obstetrical dating of the last menstrual period and early trimester ultrasound fetal measurements, confirmed by physical assessment at birth. In order to discover novel BPD biomarkers, 28 of these samples were selected for genome-wide expression profiling, including 11 BPD and 9 control (non-BPD) cases, which were matched for gestational age at birth and death, as well as 4 cases with culture-positive, acute overwhelming sepsis and 4 cases of early postnatal cardiovascular collapse secondary to immaturity or necrotizing enterocolitis (Table 1). Histopathological sections from 4 slides with a diagnosis of BPD, obtained at the Cincinnati Children's Hospital Medical Center were used for replication.

Table 1. Subject demographics, including age, and pathological diagnosis.

Sample ID	Phenotype	Diagnosis	GA at Birth (wks)	GA at Death (wks)
Group 1: Controls				
Early Gestation				
35	Control	Extreme prematurity, RDS	23	23.9
52	Control	HIE, NLD to very mild RDS	24.5	26.1
53	Control	RDS, Pulmoary hemorrhage	24.4	25.0
56	Control	NLD to very mild RDS; Central Line Event	26.6	28.5
Late Gestation				
8	Control	Neuromuscular Abnormality, NLD	40	40.6
30	Control	HIE, NLD	38.5	38.6
46	Control	HIE, Acute Meconium Aspiration	40.9	41.0
50	Control	HIE, NLD	41	41.4
51	Control	NLD to very mild RDS, Central Line Event	32.3	32.7
Group 2: Bronchopulmonary Dysplasia (BPD)				
Early BPD				
10	BPD	BPD	26	27.7
11	BPD	BPD, Necrotizing Enterocolitis	26	34.1
14	BPD	BPD, Necrotizing Enterocolitis	26	28.4
17	BPD	BPD, CMV	25	34.1
49	BPD	BPD, Rhinovirus	24.7	31.8
58	BPD	BPD, Acute Pneumonia	25.4	34.0
Late BPD				
18	BPD	BPD	27	40.7
19	BPD	Healed BPD, Recurrent Necrotizing Enterocolitis	28	50.6
33	BPD	BPD, CMV	29.2	43.1
44	BPD	BPD	28	45.0
47	BPD	BPD, Cor Pulmonale	29.1	46.5
Group 3: Non-BPD lung disease				
Acute Inflammation, minimal prior lung disease				
13	Others	Chorioamnionitis; Probable Sepsis; RDS	27	27.1
29	Others	Chorioamnionitis; RDS; Pulmonary Hemorrhage	28	28.1
39	Others	Resolved Mild RDS; Necrotizing Enterocolitis	32	33.1
40	Others	Resolved Mild RDS; Necrotizing Enterocolitis	31.2	32.3
Severe Sepsis/ Pneumonia				
3	Others	HIE; Necrotizing Pneumonia; Probable Aspiration	42	43.3

5	Others	Necrotizing Enterocolitis; *E. coli* Sepsis	28	33.0
20	Others	*E coli* Sepsis	31	31.6
24	Others	Herpes Simplex Viral Sepsis and Pneumonia	27.7	28.4

GA: Gestational Age; BPD: Bronchopulmonary Dysplasia; RDS: Respiratory Distress Syndrome; NLD: no lung disease; HIE: Hypoxic Ischemic Encephalopathy

Frozen tissue was homogenized in Trizol regent (Invitrogen; Carlsbad, CA), and total RNA was purified using a two step protocol using the Agilent MiniPrep kit (Agilent Technologies; Santa Clara, CA) including an on–column DNase I treatment. The quality of purified RNA was assessed by microcapillary electrophoresis using Bio-Rad Experion® (Bio-Rad; Hercules, CA). RNA concentration was determined by spectrophotometry using a NanoDrop ND-300 (Thermo Scientific; Wilmington, DE). Only RNA samples with an RNA concentration > 100 ng/μl and a RNA Integrity Number (RIN) > 6 were used for microarray analysis.

Microarray profiling. RNA samples were analyzed using the Affymetrix Human Genome GeneChip U133 Plus 2.0 microarray (Affymetrix; Santa Clara, CA), containing 54,675 probes corresponding to 19,501 unique NCBI Entrez genes. RNA from individual samples was transcribed into a labeled target and hybridized to an array. The arrays were washed and scanned according to manufacturer's recommendations. Expression values were extracted from .CEL files using Robust Multi–array Average (RMA) as implemented in BioConductor (Fred Hutchinson Cancer Research Center; Seattle, WA).

Data Analysis. Ingenuity Pathway Analysis (IPA) was used for gene selection and pathway analysis. Significance in gene expression difference between 11 BPD cases and 9 non-BPD controls was defined using multiple criteria of T-Test $p < 0.05$ and Fold Change ≥ 2. Genes meeting these criteria were used for further pathway analysis.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qPCR). qPCR was performed on a Stratagene MX3000P using pre-developed commercial or non-commercial assays. Gene expression levels were calculated relative to the measured Ct value of PPIA (peptidyl prolyl isomerase A or cyclophilin A) as an internal, endogenous control, according to the ddCT method. See Table 2.

Table 2: Primer sequences and assay information for qPCR validation performed in this study. Shown are gene ID, forward and reverse primer sequences, or assay ID for commercial assays.

Gene	*Forward Primer*	*Reverse Primer*
SYBR Assay		
Human		
PPIA	CCCACCGTGTTCTTCGACATT (SEQ ID NO:1)	GGACCCGTATGCTTTAGGATGA (SEQ ID NO:2)
IGF1	ATGCTCTTCAGTTCGTGTGTG (SEQ ID NO:3)	GGGCTGATACTTCTGGGTCTT (SEQ ID NO:4)
CCL17	CTTCTCTGCAGCACATCCAC (SEQ ID NO:5)	CTGCCCTGCACAGTTACAAA (SEQ ID NO:6)
CEACAM5	CCCAGACTCGTCTTACCTTGC (SEQ ID NO:7)	TTGGCGATAAAGAGAACTTGTGT (SEQ ID NO:8)
CEACAM6	TCCCCCTCAAAGGCCAATTAC (SEQ ID NO:9)	TGGAACGTCCCATTGATAAACC (SEQ ID NO:10)
SLC27A6	ACTCCGTATGCCATTTCCAA (SEQ ID NO:11)	TTGGAGAACTTTGTCGCTACC (SEQ ID NO:12)
FABP4	AGCACCATAACCTTAGATGGGG (SEQ ID NO:13)	CGTGGAAGTGACGCCTTTCA (SEQ ID NO:14)
SFN	AGGCCGAACGCTATGAGGA (SEQ ID NO:15)	GGTTTCGCTCTTCGCAGGA (SEQ ID NO:16)
COL8A1	GCTGCCACCTCAAATTCCTC (SEQ ID NO:17)	CAGGGGCTGGTATTGTGGA (SEQ ID NO:18)
HHIP	CTTTGGCCCTGACGGCTTT (SEQ ID NO:19)	GTAGCACTGAGCCTGTGAAATC (SEQ ID NO:20)
TEK	TGCCACCCTGGTTTTTACGG (SEQ ID NO:21)	TTGGAAGCGATCACACATCTC (SEQ ID NO:22)
CPA3	GGGTTTGATTGCTACCACTCTT (SEQ ID NO:23)	GCCAAGTCCTTTATGATGTCTGC (SEQ ID NO:24)
TPSB2	CCGCGACCGATACTGGATG (SEQ ID NO:25)	GATCTGGGCGGTGTAGAACT (SEQ ID NO:26)
TPSAB1	GTGACGCAAAATACCACCTTGGC (SEQ ID NO:27)	CCATTCACCTTGCACACCAGGG (SEQ ID NO:28)
Mouse		
CPA3	AATTGCTCCTGTCCACTTTGAC (SEQ ID NO:29)	TCACTAACTCGGAAATCCACAGT (SEQ ID NO:30)
TPSB2	CTGGCTAGTCTGGTGTACTCG (SEQ ID NO:31)	ATGCCCACTCGCTGATTGG (SEQ ID NO:32)
TPSAB1	GCCAATGACACCTACTGGATG (SEQ ID NO:33)	GAGCTGTACTCTGACCTTGTTG (SEQ ID NO:34)

Taqman Assay	*ABI Assay ID*
PPIA	Hs04194521_s1
CXCL5	Hs00171085_m1

Immunohistochemistry (IHC). Immunostaining was performed on formalin-fixed, paraffin-embedded lung tissue sections from human samples. Total mast cells numbers were identified by expression of tryptase using a tryptase antibody (mouse, anti-human antibody M7052; 1:1000; Dako; Carpinteria, CA) and the connective tissue mast cell sub-population (MC_{TC}) was identified by chymase expression using a chymase antibody (mouse, anti-human antibody MCA1930; 1:500; ABD Serotec; Raleigh, NC). The number of mast cells per field (200X) was defined in 10 random fields and was summarized as the total number of cells/field for each

subject. At least eight non-BPD and 11 BPD samples were studied. Control slides were stained with either secondary antibody alone, purified IgG or pre-immune serum. For some analyses, the anatomical location of the cell (parenchyma, mucosal, perivascular, peribronchoiolar) was further defined. In this case, data were normalized for the number of specific fields containing that anatomical feature. For mouse samples, mast cell numbers were identified by chymase (Cma1;Mcp5) expression (MCA1930, 1:200, ABD Serotec), using the Mouse-on-Mouse kit (Vector Labs, Burlingame, CA). Generation and analysis of mice deficient in expression of both FGFR3 and FGFR4 (FGFR3/4) was performed. Whole lung tissue RNA was isolated from FGFR3/4 mutant and wild type controls at 1 month of age and subjected to qPCR analysis for CPA3, TPSAB1 and TPSB2 using gene-specific primers.

Statistical Analysis. Statistical analysis of microarray data was performed using standard approaches as described above. For qPCR and IHC, data were summarized independently for each subject. Group means and group variation were used to calculate significance according to the non-parametric Mann-Whitney U-test.

Results

Subject Demographics

From the biorepository collection, lung tissue samples from a total of 28 subjects were studied (Table 1). Eleven of these subjects died with a clinical and pathological diagnosis of BPD. Nine control subjects, matched for age at birth and death, displayed evidence of mild lung pathology (pneumonia, respiratory distress syndrome (RDS) or alveolar hemorrhage). Another 8 subjects with significant, non-BPD pulmonary pathology, primarily consisting of inflammation associated with sepsis, were used as an additional comparison group. The primary analysis was to distinguish all the BPD (n=11) subjects from all mild lung pathology controls (n=9). Sub-analyses examined gene expression patterns stratified by age, as defined by "early" (< 27 weeks estimated gestational age (EGA) at birth; < 35 weeks EGA at death) or "late" (> 27 weeks EGA at birth; > 35 weeks EGA at death) gestation. When matched for age at birth or death, only one sample was re-classified; sample 51 was defined as "late" at birth, but "early" at death.

Pathways Affected in BPD

RNA was isolated from grossly dissected distal lung tissue and processed as described above. All RNA samples studied passed quality control assessments and were interrogated for genome-wide expression using the Affymetrix Hu133 Plus 2.0 array, essentially as previously

described (Bhattacharya et al., Am. J. Respir. Cell Mol. Biol. 40:359-367 (2009); Kho et al., Am. J. Respir. Crit. Care Med. 181:54-63 (2010)). Normalized and background corrected data were extracted using RMA.

Significant diminution of vessel formation occurs in BPD lungs. Therefore, the microarray-based expression patterns of vascular molecules including cell surface proteins (PECAM, TIE2, FLT1, ENG), growth factors (VEGF, ANG1) and signaling molecules (HIF1A, HIF3A) were examined in this data set. Seven of nine genes examined (and 19 of 26 probe sets examined) demonstrated evidence for reduced expression (fold change < 2) in BPD when compared to controls (Table 3). Among the 7 vascular marker genes demonstrating reduced expression, HIF3A and TIE2 were significantly reduced ($p<0.05$) in BPD tissue. Additionally, expression of NOS genes was examined, since NO has been implicated in BPD pathogenesis. All three NOS genes (NOS1, NOS2, NOS3) showed some evidence for decreased expression, and NOS2 was significantly reduced ($p<0.02$) in BPD. These data validate that the approach was able to reliably capture known molecular alterations associated with BPD pathology.

This study further focused on a set of 159 genes with significant differences in expression between BPD and controls ($p<0.05$; > 2-fold difference) as defined by the microarray data set. A complete list of these genes is presented in Table 4. Canonical pathways over-represented in these 159 genes were tested for using Ingenuity Pathways Analysis software (Figure 1) (Ingenuity Systems; Redwood City, CA). Discovered biological processes significantly associated with BPD included cell cycle regulation (DNA damage-related, Polo-like kinase) and immune cell regulation (immunodeficiency signaling, B cell development). Additionally, gene expression in BPD was associated with specific developmental and biochemical pathways, including sonic hedgehog signaling and retinol metabolism. These results demonstrated that BPD has a complex disease pathology associated with changes in cell proliferation, oxidant stress-related DNA damage-repair, inflammatory cell infiltration and ongoing developmental processes.

Table 3: Gene-based expression patterns of vascular molecules in BPD.

Gene Symbol	Probeset ID	P-value	Fold Change
HIF3A	222123_s_at	0.051517864	0.499235075
HIF3A	219319_at	0.046117395	0.516916465
HIF3A	222124_at	0.081431338	0.54159644
HIF3A	233517_s_at	0.093358093	0.542803877
HIF3A	232669_at	0.103914538	0.554755041
HIF3A	1555318_at	0.008245153	0.554952475
HIF3A	1556069_s_at	0.12641404	0.563880546
FLT1	204406_at	0.09223114	0.771862157
NOS1	1560974_s_at	0.21261651	0.786716109

NOS2	210037_s_at	0.017367679	0.817104988
TIE2	206702_at	0.023569983	0.81958691
NOS3	205581_s_at	0.41164593	0.88274014
NOS1	207309_at	0.149091481	0.892412662
NOS1	239132_at	0.630650867	0.901441679
PECAM1	208983_s_at	0.307257654	0.905981347
PECAM1	208982_at	0.07038192	0.909589048
PECAM1	1559921_at	0.471753205	0.922816889
PECAM1	208981_at	0.200078132	0.929374932
TIE2	217711_at	0.232586453	0.929629703
HIF1AN	226648_at	0.455298294	0.931352423
ENG	201808_s_at	0.680594812	0.940850606
VEGF	212171_x_at	0.665922235	0.941991647
ENG	201809_s_at	0.742002371	0.946332992
VEGF	210512_s_at	0.581133153	0.947641054
NOS1	207310_s_at	0.299146087	0.952411042
VEGF	59999_at	0.278196965	0.95664521
FLT1	218525_s_at	0.469167701	0.965453072
HIGD2A	218030_at	0.667760675	0.975167336
HIGD2A	232809_s_at	0.885289397	0.989030128
HIGD1A	240911_at	0.945131937	0.995488305
ANG1	205608_s_at	0.59	1.08
HIGD1A	210287_s_at	0.73949446	1.047586412
HIGD1A	209328_x_at	0.106842365	1.06248233
ANG1	209329_x_at	0.338908305	1.06289106
ANG1	217845_x_at	0.172427453	1.073999065
HIF1A	200989__at	0.06	1.13
VEGF	242317_at	0.308540229	1.102899948

Table 4: Complete list of 159 gene-based expression patterns

Rank	Probeset ID	Symbol	P-value	Fold Change	Location	Type
1	205624_at	CPA3	2.27E-03	7.250095506	ECS	peptidase
2	32128_at	CCL18	1.95E-03	6.727171322	ECS	cytokine
3	217022_s_at	IGHA1	1.94E-02	6.685335671	ECS	other
4	207134_x_at	TPSB2	5.65E-03	5.248845018	ECS	peptidase
5	205683_x_at	TPSAB1	4.88E-03	5.151526518	ECS	peptidase
6	203180_at	ALDH1A3	2.25E-03	4.540968409	Cytoplasm	enzyme
7	211430_s_at	IGHM	8.02E-02	4.37414183	PM	TMR
8	220542_s_at	PLUNC	3.47E-02	4.172754374	ECS	other
9	203980_at	FABP4	8.77E-03	4.158317741	Cytoplasm	transporter
10	232798_at	DNAJC5B	8.24E-03	4.0278222	unknown	transporter
11	1553155_x_at	ATP6V0D2	8.54E-03	3.721798631	unknown	transporter
12	205242_at	CXCL13	3.60E-02	3.637633007	ECS	cytokine
13	205767_at	EREG	9.83E-03	3.282966435	ECS	growth factor
14	201884_at	CEACAM5	1.66E-02	3.168939244	PM	other
15	205713_s_at	COMP	9.43E-03	3.018851937	ECS	other
16	226067_at	C20ORF114	9.41E-02	2.97729051	ECS	other
17	212592_at	IGJ	1.81E-02	2.950582914	ECS	other
18	206932_at	CH25H	1.84E-02	2.844154821	Cytoplasm	enzyme
19	1557197_a_at	LGALS3	9.13E-03	2.834314793	ECS	other
20	33323_r_at	SFN	2.96E-02	2.785622961	Cytoplasm	other
21	215214_at	IGL@	6.29E-02	2.783692784	Nucleus	other
22	214587_at	COL8A1	1.97E-04	2.718856484	ECS	other
23	222049_s_at	RBP4	5.51E-02	2.71508996	ECS	transporter
24	231702_at	TDO2	2.15E-02	2.670296607	Cytoplasm	enzyme
25	207067_s_at	HDC	8.44E-03	2.575763259	Cytoplasm	enzyme
26	207430_s_at	MSMB	1.39E-01	2.559744828	ECS	other
27	210511_s_at	INHBA	2.22E-02	2.549121255	ECS	growth factor
28	219918_s_at	ASPM	4.88E-03	2.498392251	Nucleus	other

29	205542_at	STEAP1	9.45E-03	2.486299338	PM	transporter
30	202238_s_at	NNMT	4.02E-03	2.465704651	Cytoplasm	enzyme
31	219295_s_at	PCOLCE2	8.37E-02	2.46058269	ECS	other
32	215217_at	IGKC	1.75E-01	2.45206972	ECS	other
33	219148_at	PBK	2.11E-02	2.438510188	Cytoplasm	kinase
34	224942_at	PAPPA	4.72E-02	2.421666168	ECS	peptidase
35	211657_at	CEACAM6	5.82E-04	2.401606855	PM	other
36	205758_at	CD8A	1.16E-01	2.386671486	PM	other
37	219502_at	NEIL3	3.92E-03	2.383365149	Nucleus	enzyme
38	231562_at	APOC2	3.09E-02	2.352182501	ECS	transporter
39	206519_x_at	SIGLEC6	1.99E-02	2.339175328	ECS	other
40	209773_s_at	RRM2	5.62E-03	2.337554497	Nucleus	enzyme
41	206632_s_at	APOBEC3B	1.06E-02	2.316585612	Cytoplasm	enzyme
42	229070_at	C6ORF105	3.13E-02	2.316585612	unknown	other
43	1560011_at	PSCA	1.10E-03	2.314980434	PM	other
44	238125_at	ADAMTS16	2.20E-02	2.300583787	ECS	other
45	218469_at	GREM1	8.91E-02	2.292624371	ECS	Other
46	242809_at	IL1RL1	2.05E-01	2.289448321	PM	TMR
47	214844_s_at	DOK5	2.82E-03	2.286276671	PM	Other
48	215101_s_at	CXCL5	6.16E-02	2.286276671	ECS	cytokine
49	204602_at	DKK1	4.06E-02	2.283109414	ECS	growth factor
50	231534_at	CDK1	2.91E-03	2.281527432	Nucleus	Kinase
51	204637_at	CGA	6.86E-02	2.279946545	ECS	Other
52	222958_s_at	DEPDC1	9.66E-03	2.261061134	unknown	Other
53	206211_at	SELE	1.58E-01	2.261061134	PM	Other
54	205220_at	GPR109B	3.74E-02	2.250116969	PM	GPCR
55	208168_s_at	CHIT1	5.80E-03	2.233025924	ECS	Enzyme
56	228703_at	P4HA3	1.33E-02	2.223758315	unknown	Enzyme
57	203700_s_at	DIO2	2.69E-03	2.212994706	Cytoplasm	Enzyme
58	221651_x_at	IGK@	1.47E-01	2.212994706	ECS	Other
59	230318_at	SERPINA1	5.78E-02	2.203810232	ECS	Other
60	204638_at	ACP5	7.05E-02	2.200757219	Cytoplasm	phosphatase
61	203649_s_at	PLA2G2A	1.07E-01	2.199232299	ECS	Enzyme
62	228636_at	BHLHE22	1.61E-01	2.196185628	Nucleus	TR
63	204822_at	TTK	8.40E-03	2.188587403	Nucleus	kinase
64	1554452_a_at	C7ORF68	2.15E-02	2.185555478	unknown	other
65	205857_at	SLC18A2	1.38E-02	2.184041091	PM	transporter
66	224397_s_at	TMTC1	2.21E-02	2.184041091	unknown	other
67	236641_at	KIF14	3.11E-03	2.177994031	Cytoplasm	other
68	222039_at	KIF18B	1.93E-02	2.177994031	unknown	other
69	203963_at	CA12	1.66E-02	2.176484883	PM	enzyme
70	223122_s_at	SFRP2	1.42E-01	2.173469725	PM	TMR
71	205157_s_at	KRT17	1.49E-01	2.164449289	Cytoplasm	other
72	223660_at	ADORA3	7.77E-02	2.15248025	PM	GPCR
73	211144_x_at	TARP	5.10E-02	2.146520573	Cytoplasm	other
74	217640_x_at	SKA1	8.40E-03	2.145033234	unknown	other
75	206680_at	CD5L	3.23E-02	2.143546925	PM	TMR
76	209596_at	MXRA5	8.70E-02	2.143546925	unknown	other
77	203764_at	DLGAP5	1.16E-02	2.142061646	Nucleus	phosphatase
78	218451_at	CDCP1	2.31E-02	2.142061646	PM	other
79	209642_at	BUB1	2.74E-03	2.139094176	Nucleus	kinase
80	1552619_a_at	ANLN	1.05E-02	2.137611982	Cytoplasm	other
81	202953_at	C1QB	3.46E-02	2.137611982	ECS	other
82	202503_s_at	KIAA0101	7.27E-03	2.125791349	Nucleus	other
83	205306_x_at	KMO	3.22E-02	2.125791349	Cytoplasm	enzyme
84	219181_at	LIPG	1.48E-01	2.124318373	ECS	enzyme
85	205167_s_at	CDC25C	1.18E-02	2.119905567	Nucleus	phosphatase
86	207496_at	MS4A2	3.05E-02	2.118436669	PM	TMR
87	220655_at	TNIP3	1.38E-02	2.112571251	unknown	other
88	218232_at	C1QA	2.81E-02	2.099433367	ECS	other
89	224156_x_at	IL17RB	1.20E-02	2.097978655	PM	TMR

90	1558972_s_at	THEMIS	1.29E-01	2.096524951	unknown	other
91	219669_at	CD177	1.94E-01	2.096524951	Cytoplasm	other
92	204962_s_at	CENPA	1.40E-02	2.09216988	Nucleus	other
93	212023_s_at	MKI67	1.23E-02	2.0907202	Nucleus	other
94	244427_at	KIF23	2.23E-03	2.087823855	Cytoplasm	other
95	219697_at	HS3ST2	2.48E-02	2.087823855	Cytoplasm	enzyme
96	223700_at	MND1	5.08E-03	2.084931522	Nucleus	other
97	209182_s_at	C10ORF10	5.14E-02	2.080600533	Cytoplasm	other
98	1555778_a_at	POSTN	3.37E-02	2.07915887	ECS	other
99	209728_at	HLA-DRB4	4.81E-01	2.07915887	PM	TMR
100	230237_at	ADCYAP1	1.18E-01	2.077718207	ECS	other
101	220301_at	CCDC102B	1.22E-02	2.064797071	unknown	other
102	220658_s_at	ARNTL2	2.86E-02	2.057653416	Nucleus	TR
103	219519_s_at	SIGLEC1	4.98E-02	2.054802879	PM	other
104	202094_at	BIRC5	1.76E-02	2.051956291	Cytoplasm	other
105	229538_s_at	IQGAP3	4.34E-02	2.051956291	unknown	other
106	210052_s_at	TPX2	6.15E-03	2.047693801	Nucleus	other
107	209542_x_at	IGF1	8.90E-03	2.034959384	ECS	growth factor
108	209891_at	SPC25	3.00E-02	2.025109615	unknown	other
109	209714_s_at	CDKN3	6.98E-03	2.022304162	Nucleus	phosphatase
110	219799_s_at	DHRS9	1.91E-02	2.020902893	Cytoplasm	enzyme
111	206504_at	CYP24A1	3.11E-02	2.020902893	Cytoplasm	enzyme
112	201291_s_at	TOP2A	1.82E-02	2.018103268	Nucleus	enzyme
113	205266_at	LIF	1.16E-01	2.018103268	ECS	cytokine
114	228892_at	SH3RF2	7.57E-03	2.01670491	unknown	other
115	213338_at	TMEM158	2.15E-02	2.01670491	PM	other
116	220997_s_at	DIAPH3	7.49E-03	2.011121161	Cytoplasm	enzyme
117	204641_at	NEK2	8.27E-03	2.008335086	Nucleus	kinase
118	205819_at	MARCO	1.17E-01	2.001386775	PM	TMR
119	1569675_at	POU2AF1	9.41E-02	0.499653546	Nucleus	TR
120	226690_at	ADCYAP1R1	6.13E-02	0.497235084	PM	GPCR
121	206209_s_at	CA4	6.51E-04	0.496202187	PM	enzyme
122	213909_at	LRRC15	1.37E-01	0.493116352	PM	other
123	228806_at	RORC	6.18E-03	0.486664687	Nucleus	LDNR
124	210081_at	AGER	1.28E-02	0.484309095	PM	TMR
125	213992_at	COL4A6	7.68E-03	0.479964631	ECS	other
126	207245_at	UGT2B17	9.48E-02	0.477310507	Cytoplasm	enzyme
127	230469_at	RTKN2	9.16E-02	0.473356816	PM	other
128	221728_x_at	XIST	5.58E-01	0.473028823	Nucleus	other
129	204913_s_at	SOX11	5.48E-02	0.47237352	Nucleus	TR
130	219564_at	KCNJ16	4.91E-02	0.471065637	PM	ion channel
131	1556037_s_at	HHIP	3.44E-02	0.469761375	PM	other
132	204664_at	ALPP	1.03E-02	0.468136124	ECS	phosphatase
133	210445_at	FABP6	9.35E-03	0.465870215	Cytoplasm	transporter
134	229125_at	KANK4	3.42E-04	0.464258426	unknown	other
135	219932_at	SLC27A6	1.02E-03	0.461691155	PM	transporter
136	222106_at	PRND	2.65E-01	0.449066186	PM	other
137	211734_s_at	FCER1A	1.78E-02	0.433168428	PM	TMR
138	230924_at	TTLL6	3.36E-04	0.42720487	unknown	enzyme
139	223862_at	GHRL	1.26E-01	0.415235012	ECS	growth factor
140	231223_at	CSMD1	1.22E-02	0.414372452	PM	other
141	207861_at	CCL22	2.28E-02	0.413511684	ECS	cytokine
142	205969_at	AADAC	4.54E-02	0.412081042	Cytoplasm	enzyme
143	207900_at	CCL17	4.50E-04	0.40528256	ECS	cytokine
144	205216_s_at	APOH	2.54E-02	0.404440674	ECS	transporter
145	238062_at	GPIHBP1	7.14E-02	0.393653988	unknown	other
146	219949_at	LRRC2	2.16E-02	0.393108646	unknown	other
147	232193_at	GSTT1	1.06E-02	0.389852421	Cytoplasm	enzyme
148	219732_at	LPPR1	3.34E-02	0.385820044	unknown	other
149	235763_at	SLC44A5	9.88E-03	0.385552706	unknown	other
150	213456_at	SOSTDC1	1.39E-02	0.37579037	ECS	other

151	206027_at	S100A3	6.59E-03	0.371645746	unknown	transporter
152	206658_at	UPK3B	9.10E-03	0.358737384	PM	other
153	211480_s_at	SLCO1A2	3.67E-02	0.333555792	PM	transporter
154	1554524_a_at	OLFM3	1.43E-02	0.323536414	Cytoplasm	other
155	203074_at	ANXA8L2	1.51E-02	0.312949115	unknown	other
156	215729_s_at	VGLL1	6.71E-03	0.306508715	Nucleus	TR
157	208399_s_at	EDN3	4.52E-02	0.282436804	ECS	other
158	223597_at	ITLN1	4.14E-02	0.282241101	PM	other
159	205048_s_at	PSPH	8.37E-02	0.261340025	unknown	phosphatase

ECS: extracellular space; PM: plasma membrane; GPCR: G-protein coupled receptor; TMR: transmembrane receptor; LDNR: ligand-dependent nuclear receptor; TR: transcriptional regulator

BPD-Associated Gene Expression

Global expression patterns for the 159 genes in all samples were analyzed in order to assess age-related changes in expression and disease-specificity. Significant variability in expression of individual genes within subject groups was apparent, as expected, given the diverse pathology associated with this disease. Even so, genes displaying consistent increases or decreases in expression in BPD, as compared to controls, were apparent. Interestingly, a majority of these genes did not display evidence for age-dependent changes in expression in controls. However, a small set of genes showed a trend for reduced expression over time in controls, but persistent expression over time in BPD tissues. The specificity of gene expression changes identified in BPD was also assessed. While most gene expression changes identified in BPD lungs were specific, a subset of genes induced in BPD tissues showed similar changes in non-BPD lung disease tissues. Another set of genes displayed similar expression patterns restricted to BPD and sepsis only.

In order to further assess the reliability of the microarray data set and the methods for identifying disease gene expression biomarkers, the expression of selected genes were validated by qPCR (Figures 2 and 3). Genes were chosen for validation studies based upon their inclusion in the set of 159 differentially expressed genes, their magnitude of change in expression, and interest in their potential biological function. In addition to comparing all BPD and all controls, the analysis was stratified by estimated gestational age (EGA) to compare disease-related patterns in subjects earlier in age (< 27 weeks EGA at birth; < 35 weeks EGA at death) or later in age (> 27 weeks EGA at birth; > 35 weeks EGA at death)

A significant difference ($P<0.05$) in expression between BPD and controls was confirmed for 8 out of a total of 13 genes tested including CCL17, CEACAM6, COL8A1, CXCL5, FABP4, HHIP, IGF1, SFN, SLC27A6. Other genes showed some evidence for differential expression by qPCR, consistent with the microarray results. CXCL5 showed a 5-fold increase by qPCR and was significantly increased in "late" stage BPD samples. CPA3 showed a ~3-fold increase and was significantly increased in "late" stage BPD samples (GAB, trend GAD). CCL17 was

decrease 2-fold and demonstrated a trend for significance in all samples (P=0.06). TPSB2 was increased 3.5-fold in "late" stage BPD samples. HHIP showed a 2-fold reduction in all samples.

Table 5: Genes significantly affected in BPD lung tissue.

Genes	*Probeset*	*P Value*	*Fold Change*
CPA3	205624_at	2.27E-03	7.25
CCL18	32128_at	1.95E-03	6.73
IGHA1	217022_s_at	1.94E-02	6.69
TPSB2	207134_x_at	5.65E-03	5.25
TPSAB1	205683_x_at	4.88E-03	5.15
ALDH1A3	203180_at	2.25E-03	4.54
IGHM	211430_s_at	8.02E-02	4.37
PLUNC	220542_s_at	3.47E-02	4.17
FABP4	203980_at	8.77E-03	4.16
DNAJC5B	232798_at	8.24E-03	4.03
ATP6V0D2	1553155_x_at	8.54E-03	3.72
CXCL13	205242_at	3.60E-02	3.64
EREG	205767_at	9.83E-03	3.28
CEACAM5	201884_at	1.66E-02	3.17
COMP	205713_s_at	9.43E-03	3.02
C20ORF114	226067_at	9.41E-02	2.98
IGJ	212592_at	1.81E-02	2.95
CH25H	206932_at	1.84E-02	2.84
LGALS3	1557197_a_at	9.13E-03	2.83
SFN	33323_r_at	2.96E-02	2.79
IGL@	215214_at	6.29E-02	2.78
COL8A1	214587_at	1.97E-04	2.72
RBP4	222049_s_at	5.51E-02	2.72
TDO2	231702_at	2.15E-02	2.67
HDC	207067_s_at	8.44E-03	2.58
MSMB	207430_s_at	1.39E-01	2.56
INHBA	210511_s_at	2.22E-02	2.55
ASPM	219918_s_at	4.88E-03	2.50
STEAP1	205542_at	9.45E-03	2.49
NNMT	202238_s_at	4.02E-03	2.47
PCOLCE2	219295_s_at	8.37E-02	2.46
IGKC	215217_at	1.75E-01	2.45
PBK	219148_at	2.11E-02	2.44
PAPPA	224942_at	4.72E-02	2.42
CEACAM6	211657_at	5.82E-04	2.40
CD8A	205758_at	1.16E-01	2.39
NEIL3	219502_at	3.92E-03	2.38
APOC2	231562_at	3.09E-02	2.35
SIGLEC6	206519_x_at	1.99E-02	2.34
RRM2	209773_s_at	5.62E-03	2.34
APOBEC3B	206632_s_at	1.06E-02	2.32
C6ORF105	229070_at	3.13E-02	2.32
PSCA	1560011_at	1.10E-03	2.31

ADAMTS16	238125_at	2.20E-02	2.30
GREM1	218469_at	8.91E-02	2.29
IL1RL1	242809_at	2.05E-01	2.29
DOK5	214844_s_at	2.82E-03	2.29
CXCL5	215101_s_at	6.16E-02	2.29
DKK1	204602_at	4.06E-02	2.28
CDK1	231534_at	2.91E-03	2.28
CGA	204637_at	6.86E-02	2.28
DEPDC1	222958_s_at	9.66E-03	2.26
SELE	206211_at	1.58E-01	2.26
GPR109B	205220_at	3.74E-02	2.25
CHIT1	208168_s_at	5.80E-03	2.23
P4HA3	228703_at	1.33E-02	2.22
DIO2	203700_s_at	2.69E-03	2.21
IGK@	221651_x_at	1.47E-01	2.21
SERPINA1	230318_at	5.78E-02	2.20
ACP5	204638_at	7.05E-02	2.20
PLA2G2A	203649_s_at	1.07E-01	2.20
BHLHE22	228636_at	1.61E-01	2.20
TTK	204822_at	8.40E-03	2.19
C7ORF68	1554452_a_at	2.15E-02	2.19
SLC18A2	205857_at	1.38E-02	2.18
TMTC1	224397_s_at	2.21E-02	2.18
KIF14	236641_at	3.11E-03	2.18
KIF18B	222039_at	1.93E-02	2.18
CA12	203963_at	1.66E-02	2.18
SFRP2	223122_s_at	1.42E-01	2.17
KRT17	205157_s_at	1.49E-01	2.16
ADORA3	223660_at	7.77E-02	2.15
TARP	211144_x_at	5.10E-02	2.15
SKA1	217640_x_at	8.40E-03	2.15
CD5L	206680_at	3.23E-02	2.14
MXRA5	209596_at	8.70E-02	2.14
DLGAP5	203764_at	1.16E-02	2.14
CDCP1	218451_at	2.31E-02	2.14
BUB1	209642_at	2.74E-03	2.14
ANLN	1552619_a_at	1.05E-02	2.14
C1QB	202953_at	3.46E-02	2.14
KIAA0101	202503_s_at	7.27E-03	2.13
KMO	205306_x_at	3.22E-02	2.13
LIPG	219181_at	1.48E-01	2.12
CDC25C	205167_s_at	1.18E-02	2.12
MS4A2	207496_at	3.05E-02	2.12
TNIP3	220655_at	1.38E-02	2.11
C1QA	218232_at	2.81E-02	2.10
IL17RB	224156_x_at	1.20E-02	2.10
THEMIS	1558972_s_at	1.29E-01	2.10
CD177	219669_at	1.94E-01	2.10
CENPA	204962_s_at	1.40E-02	2.09
MKI67	212023_s_at	1.23E-02	2.09

KIF23	244427_at	2.23E-03	2.09
HS3ST2	219697_at	2.48E-02	2.09
MND1	223700_at	5.08E-03	2.08
C10ORF10	209182_s_at	5.14E-02	2.08
POSTN	1555778_a_at	3.37E-02	2.08
HLA-DRB4	209728_at	4.81E-01	2.08
ADCYAP1	230237_at	1.18E-01	2.08
CCDC102B	220301_at	1.22E-02	2.06
ARNTL2	220658_s_at	2.86E-02	2.06
SIGLEC1	219519_s_at	4.98E-02	2.05
BIRC5	202094_at	1.76E-02	2.05
IQGAP3	229538_s_at	4.34E-02	2.05
TPX2	210052_s_at	6.15E-03	2.05
IGF1	209542_x_at	8.90E-03	2.03
SPC25	209891_at	3.00E-02	2.03
CDKN3	209714_s_at	6.98E-03	2.02
DHRS9	219799_s_at	1.91E-02	2.02
CYP24A1	206504_at	3.11E-02	2.02
TOP2A	201291_s_at	1.82E-02	2.02
LIF	205266_at	1.16E-01	2.02
SH3RF2	228892_at	7.57E-03	2.02
TMEM158	213338_at	2.15E-02	2.02
DIAPH3	220997_s_at	7.49E-03	2.01
NEK2	204641_at	8.27E-03	2.01
MARCO	205819_at	1.17E-01	2.00
POU2AF1	1569675_at	9.41E-02	0.50
ADCYAP1R1	226690_at	6.13E-02	0.50
CA4	206209_s_at	6.51E-04	0.50
LRRC15	213909_at	1.37E-01	0.49
RORC	228806_at	6.18E-03	0.49
AGER	210081_at	1.28E-02	0.48
COL4A6	213992_at	7.68E-03	0.48
UGT2B17	207245_at	9.48E-02	0.48
RTKN2	230469_at	9.16E-02	0.47
XIST	221728_x_at	5.58E-01	0.47
SOX11	204913_s_at	5.48E-02	0.47
KCNJ16	219564_at	4.91E-02	0.47
HHIP	1556037_s_at	3.44E-02	0.47
ALPP	204664_at	1.03E-02	0.47
FABP6	210445_at	9.35E-03	0.47
KANK4	229125_at	3.42E-04	0.46
SLC27A6	219932_at	1.02E-03	0.46
PRND	222106_at	2.65E-01	0.45
FCER1A	211734_s_at	1.78E-02	0.43
TTLL6	230924_at	3.36E-04	0.43
GHRL	223862_at	1.26E-01	0.42
CSMD1	231223_at	1.22E-02	0.41
CCL22	207861_at	2.28E-02	0.41
AADAC	205969_at	4.54E-02	0.41
CCL17	207900_at	4.50E-04	0.41

APOH	205216_s_at	2.54E-02	0.40
GPIHBP1	238062_at	7.14E-02	0.39
LRRC2	219949_at	2.16E-02	0.39
GSTT1	232193_at	1.06E-02	0.39
LPPR1	219732_at	3.34E-02	0.39
SLC44A5	235763_at	9.88E-03	0.39
SOSTDC1	213456_at	1.39E-02	0.38
S100A3	206027_at	6.59E-03	0.37
UPK3B	206658_at	9.10E-03	0.36
SLCO1A2	211480_s_at	3.67E-02	0.33
OLFM3	1554524_a_at	1.43E-02	0.32
ANXA8L2	203074_at	1.51E-02	0.31
VGLL1	215729_s_at	6.71E-03	0.31
EDN3	208399_s_at	4.52E-02	0.28
ITLN1	223597_at	4.14E-02	0.28
PSPH	205048_s_at	8.37E-02	0.26

Shown are gene symbol, probe set ID, fold-change (BPD/control) and p-value as defined by student's t-test, for 159 genes.

The expression of KITLG, BLP and IL4 was also tested, as these have previously been reported to be altered in BPD. The results are shown in Table 5. No difference was observed in the expression of these genes in the microarray data set. IL4 expression was virtually undetectable in lung tissue samples by qPCR (CT>35). KITLG and BLP showed appreciable expression levels but did not show any significant changes in their expression by qPCR.

Table 5: qPCR validation of IL4, GRP and KITLG expression in human lung tissue

	Control	***BPD***	
IL4	ND	ND	not detectable
GRP	1.26 (±0.96)	1.70 (±1.28)	p>>0.05
KITLG	1.21 (±0.67)	1.06 (±0.55)	p>>0.05

Shown are mean fold-change (± standard deviation) in each group, non-BPD control (CTL) or BPD, relative to the control population. IL4 expression was not detectable in lung tissue from either group. GRP and KITLG expression were detectable in all lung tissues, but no differences in BPD subjects were observed.

Identification of Mast Cell Gene Signature

Among this list of 159 BPD-associated genes, those genes with the greatest magnitude of change were examined (Table 6). Three of the top 5 genes, and 9 of the top 26 probe sets, were mast cell specific markers, suggesting an increase in mast cells in BPD tissues. Interestingly, the most highly induced gene encodes CPA3, a marker specific for connective tissue-type mast cells (MC_{TC}), a sub-population of mast cells that are not frequently found within the lung.

qPCR for mast cell specific marker expression, for both mucosal-type mast cells (MC_T) (TPSAB1, TPSB2) and MC_{TC} (CPA3) populations, was consistent with increases in these cells, particularly in "late" stage BPD samples (Figure 3). CPA3 showed a ~3-fold increase and was significantly increased in "late" stage BPD samples. TPSB2 was increased 3.5-fold in "late" stage BPD samples.

In order to confirm the gene expression data and more thoroughly to assess the predicted increase in mast cells in BPD lungs, immunohistochemistry was performed for mast cell markers (Figure 4). Immunostaining for tryptase, a marker for all mast cell sub-types, confirmed a significant increase in total mast cell number in our BPD subjects (6.8 vs. 1.5 cells/field, p=0.005).

Table 6: Ranked list (based upon fold-change between BPD and control) of 25 most affected genes among 159 significantly dysregulated in BPD.

Gene Symbol	Gene Title	Probeset ID	P-value	Fold Change
CPA3	carboxypeptidase A3 (mast cell)	205624_at	0.00	7.14
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	32128_at	0.00	6.74
IGHA1	immunoglobulin heavy constant alpha 1	217022_s_at	0.02	6.90
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	209924_at	0.00	6.27
TPSAB1	tryptase alpha/beta 1	216474_x_at	0.01	6.16
TPSAB1	tryptase alpha/beta 1	207134_x_at	0.01	5.15
TPSAB1	tryptase alpha/beta 1	205683_x_at	0.00	5.03
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	203180_at	0.00	4.60
TPSAB1	tryptase alpha/beta 1	217023_x_at	0.01	4.38
TPSAB1	tryptase alpha/beta 1	210084_x_at	0.01	4.34
FABP4	Fatty acid binding protein 4, adipocyte	235978_at	0.01	3.87
PLUNC	palate, lung and nasal epithelium carcinoma associated	220542_s_at	0.03	4.28
FABP4	fatty acid binding protein 4, adipocyte	203980_at	0.01	4.28
DNAJC5B	DnaJ (Hsp40) homolog, subfamily C, member 5 beta	232798_at	0.01	4.14
TPSB2	tryptase alpha/beta 1 /// tryptase beta 2	207741_x_at	0.01	4.10
ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	1553153_at	0.01	3.82
ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	1553155_x_at	0.01	3.78
CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	205242_at	0.04	3.66
C1orf186	chromosome 1 open reading frame 186	230381_at	0.00	3.82
MEG8	Maternally expressed (in Callipyge) 8	240083_at	0.00	3.45
TPSAB1	tryptase alpha/beta 1	215382_x_at	0.01	3.64
VSIG1	V-set and immunoglobulin domain containing 1	243764_at	0.02	3.45
DNAJC5B	DnaJ (Hsp40) homolog, subfamily C, member 5 beta	1554258_a_at	0.01	3.36
EREG	epiregulin	205767_at	0.01	3.30
TPSAB1	tryptase alpha/beta 1	216485_s_at	0.01	3.29

Increased Connective Tissue Mast Cells are associated with BPD

As mentioned above, mast cell sub-types in the lung have been appreciated, each with different anatomical distributions and characteristic secretory products. Therefore, the distribution of tryptase expressing cells in 1) the parenchymal region of the lung, 2) adjacent to the airways and 3) adjacent to the large/intermediate vasculature was assessed. Region-specific differences between BPD and control tissues were tested. A significant increase in tryptase staining cells in BPD in the parenchymal region (6.5 vs. 1.3 cells/field, p=0.004) only, with no differences in the airway or vascular regions was observed. Interestingly, at this early developmental stage, a majority of tryptase-staining cells was observed in the parenchymal region, and not the airway, for both groups. However, these data are confounded by the proportion of tissue occupied by each region, even though the number of fields in each subject were normalized with each morphological component (e.g., parenchyma, airway, vessel).

Gene expression profiling identified both mucosal mast cell (tryptase) and MC_{TC}-specific (CPA3) markers increased in BPD tissue. Furthermore, excessive tryptase-expressing cell accumulation was observed predominantly in the parenchymal region of the lungs in BPD, a location where connective tissue mast cells are typically found. Therefore, immunostaining for chymase, a MC_{TC}-specific marker, was performed (Figure 5). A >50-fold increase in chymase-expressing cells in BPD was observed (0.1 vs. 5.9 cells/field, p=0.0003). As expected, the chymase expressing MC_{TC} mast cells were highly restricted to the parenchymal region (97% of all positive cells in BPD, 100% of all positive cells in non-BPD).

Using an independent cohort of subjects obtained from Cincinnati Children's Hospital Medical Center, we validated that increases in MCTC accumulation were not restricted to the original study cohort. Histopathological specimens from four subjects with a diagnosis of BPD were analyzed, each of which showed a frequency of MCTC (1.3, 1.9, 2.1, 2.4 chymase-expressing cells/field) substantially above controls (0.0-0.5 chymase-expressing cells/field). These data demonstrate that the specific accumulation of MCTC represents a general, and previously unappreciated, aspect of BPD pathology.

Increased expression of MCTC markers in a mouse model of BPD-like pathology.

Lung development abnormalities are often present in mice lacking expression of both Fibroblast Growth Factor Receptor (FGF)-3 and -4. The FGFR3/4 mutant mouse phenocopies certain aspects of human BPD pathology including chronic lung disease characterized by structural changes in distal lung architecture consistent with developmental arrest, dysplastic elastin fiber formation and alveolar enlargement. Interestingly, under-appreciated proximal airway phenotypes in this model were detected that are reminiscent of pathology observed in

individuals with BPD (squamous changes to conducting airway epithelium, bronchiolar exudates). Critically, mild monocytic infiltrates were detected in both the proximal and distal airways. Given the observations demonstrating robust accumulation of MCTC in human BPD lung samples and the presence of BPD-like pathologies in the FGFR3/4 mutant mouse lungs, the samples were tested for the presence of mast cells. Similar to the observations in human lung tissue from subjects with BPD, significant increases were noted in the expression of both general mast cell (tryptase) markers TPSAB1 (4-fold, $p<0.05$) and TPSB2 (3.5-fold, $p=0.05$), and the connective tissue-type mast cell marker CPA3 (4-fold increase, $p<0.05$), at 1 month of age in the FGFR3/4 mice (Figure 3). Immunostaining for chymase supported these data, showing immunoreactive cells in airway walls and in distal airspaces of mutant mice. Dramatically fewer total inflammatory cells and chymase staining cells were observed in control mice.

Example 2: Identification of Chymase in Tracheal aspirates of Prematurely Born Infants

Materials and Methods

Sample Acquisition: Samples were obtained following parents' informed, written, permission. Tracheal aspirate sample was collected by sterile technique during routine suctioning by the bedside nurse. Mechanical ventilation lungs continued through the suctioning procedure as per NICU protocol. Normal saline (0.5 ml) was instilled into the endotracheal tube through the side port of an in-line Ballard suction device. Two to five breaths later, the effluent was suctioned from the endotracheal tube into a Leukens trap. Suction catheter tip did not extend beyond tip of endotracheal tube. Infant stability was assured and steps 3-5 were repeated. The suction catheter was rinsed into the Leukens trap with an additional 1.0 mL saline. The resulting material in the Leukens trap constituted a sample. Optionally, suctioning was done without saline instillation. In this case, the suction catheter was rinsed with 2 mL saline after the suctioning procedure. The Leukens trap was labeled and capped, removing the suction tubing. The trap was placed in a refrigerator (40 C) until the sample was processed.

Sample Processing: Samples were processed within 24 hrs of collection. Contents of Leuken's trap were decanted into a 15 ml conical tube. Sample was centrifuged at 3000 rpm for 5 minutes. The supernatant was collected by pipetting into cryovials. Each cryovial aliquot was between 0.5-1 mL supernatant. The cryovials were labeled with printed subject specific study ID number and date. The supernatant aliquots were maintained in -800C freezer until assayed. Pellets from the centrifugation were processed onto slides by cytospin technique for future histology or immunohistochemistry.

Chymase Assay: The gene product was a chymotryptic serine proteinase that belongs to the peptidase family S1. It is expressed in mast cells and thought to function in the degradation of the extracellular matrix, the regulation of submucosal gland secretion, and the generation of vasoactive peptides. In the heart and blood vessels, this protein, rather than angiotensin converting enzyme, is largely responsible for converting angiotensin I to the vasoactive peptide angiotensin II. Angiotensin II has been implicated in blood pressure control and in the pathogenesis of hypertension, cardiac hypertrophy, and heart failure. Thus, this gene product is a target for cardiovascular disease therapies. This gene maps to 14q11.2 in a cluster of genes encoding other proteases.

An aliquot of the tracheal aspirate sample supernatant was removed from the freezer, thawed to room temperature and further aliquoted and assayed utilizing the commercial Human Mast Cell Chymase ELISA kit purchased from MyBioSource.com, catalog # MBS704628 (MyBioSource. com; San Diego, CA). The assay was performed as directed by the supplier. In order to bring samples into the linear range of the assay, they were diluted 10 – 50 fold with Sample Diluent.

Results

Serial tracheal aspirates were collected from a set of quadruplets born at less than 29 weeks. Chymase was detected by ELISA in each sample and is presented relative to the day after birth on which the sample was collected. Unique patterns of tracheal aspirate chymase content were detected in each infant (Figures 6A-D).

WHAT IS CLAIMED IS:

1. A method of identifying a subject with or at risk for developing bronchopulmoary dysplasia (BPD), the method comprising detecting chymase-expressing connective tissue mast cells (CTMCs) in a biological sample from the subject, wherein the presence of chymase-expressing CTMCs as compared to a control indicates the subject has BPD.

2. The method of claim 2, wherein the biological sample is selected from the group consisting of blood, airway aspirate, airway scrapping, brochoalveolar lavage (BAL), urine, and lung tissue.

3. The method of claim 1 or 2, wherein detecting a level of chymase-expressing CTMCs comprises detecting expression of one or more biomarkers consisting of chymase or carboxypeptidase A3.

4. The method of claim 3 further comprising detecting expression of one or more biomarkers selected from the group consisting of tryptase beta 2 (TPSB2), tryptase alpha/beta 1 (TPSAB1), cathepsin G (CTSG), and prostaglandin D2 (PGD2).

5. The method of any one of claims 3-4, wherein the level of expression is detected by the level of RNA or polypeptide.

6. The method of claim 5, wherein the level of RNA is determined using an assay selected from the group consisting of a microarray analysis, a gene chip, a Northern blot, an *in situ* hybridization assay, a RT-PCR assay, a one step PCR assay, and a quantitative real time (qRT)-PCR assay.

7. The method of claim 5, wherein the level of polypeptide is determined using an assay selected from the group consisting of a Western blot, an enzyme-linked immunosorbent assay (ELISA), an enzyme immunoassay (EIA), a radioimmunoassay (RIA), an immunohistochemistry (IHC) assay, and a protein array.

8. A method for determining the effectiveness of a treatment regime for bronchopulmonary dysplasia (BPD) in a subject, the method comprising:

(a) identifying a first level of chymase-expressing connective tissue mast cells (CTMCs) in a first biological sample from the subject before administration of a treatment regime to the subject;

(b) identifying a second level of chymase-expressing CTMCs in a second biological sample from the subject after administration of a treatment regime to the subject;

(c) comparing the first and second level of chymase-expressing CTMCs; and

(d) adjusting the treatment regime if the second level of chymase-expressing connective tissue mast cells is the same or higher than the first level.

9. An assay system comprising a microarray or a gene chip with at least two selective binding agent, wherein each selective binding agent is specific for a biomarker selected from the group consisting of chymase, carboxypeptidase A3 (CPA3), tryptase beta 2 (TPSB2), tryptase alpha/beta 1 (TPSAB1), cathepsin G (CTSG), and prostaglandin D2 (PGD2).

10. A method for preventing or treating bronchopulmonary dysplasia (BPD) in a subject, the method comprising:

(a) identifying chymase-expressing connective tissue mast cells (CTMCs) in a biological sample from the subject; and

(b) administering to the subject an agent that blocks an activity of, blocks an increase in a level of, or reduces a level of chymase-expressing CTMCs in the subject.

11. The method of claim 10, wherein the chymase-expressing CTMCs express an increased level of carboxypeptidase A3 (CPA3) mRNA as compared to a control mast cell.

12. The method of claim 10 or 11, wherein the agent blocks expression or activity of CPA3 or chymase.

13. The method of claim 12, wherein the agent is an antibody to CPA3 or chymase.

14. The method of claim 12, wherein the agent is selected from the group consisting of a small molecule, a polypeptide, a nucleic acid, or a peptidomimetic.

15. The method of claim 14, wherein the nucleic acid is selected from the group consisting of a small interfering RNA (siRNA), a microRNA (miRNA), or an antisense nucleic acid.

16. The method of any one of claims 10-15, wherein the chymase-expressing CTMCs express an increased level of one or more biomarkers selected from the group consisting of carboxypeptidase A3 (CPA3), tryptase beta 2 (TPSB2), tryptase alpha/beta 1 (TPSAB1), cathepsin G (CTSG), and prostaglandin D2 (PGD2).

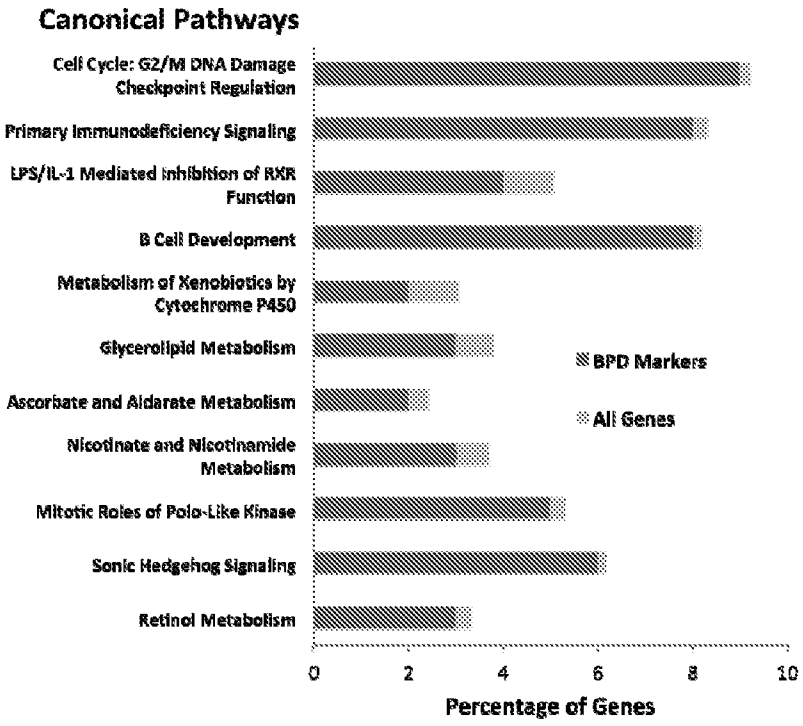

Figure 1

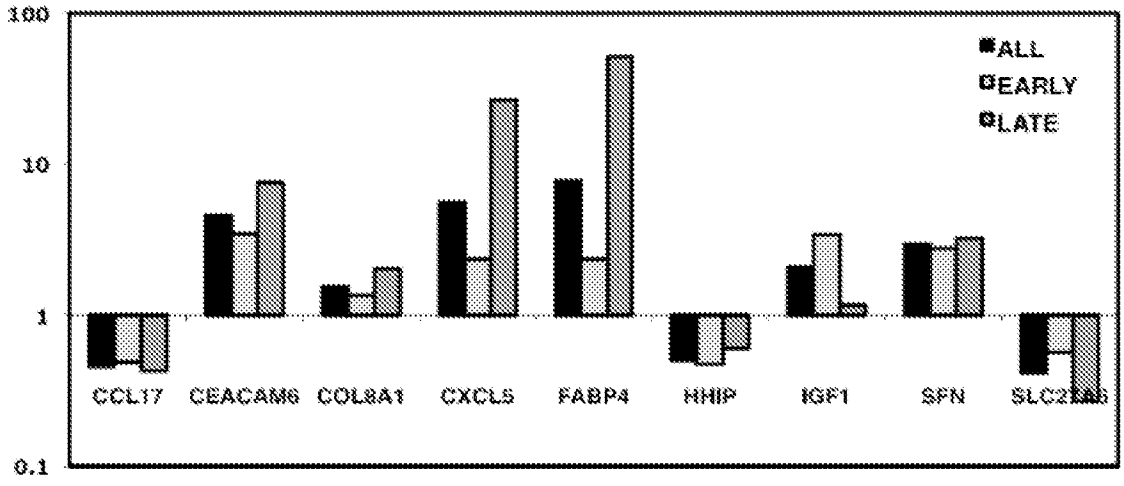

Figure 2

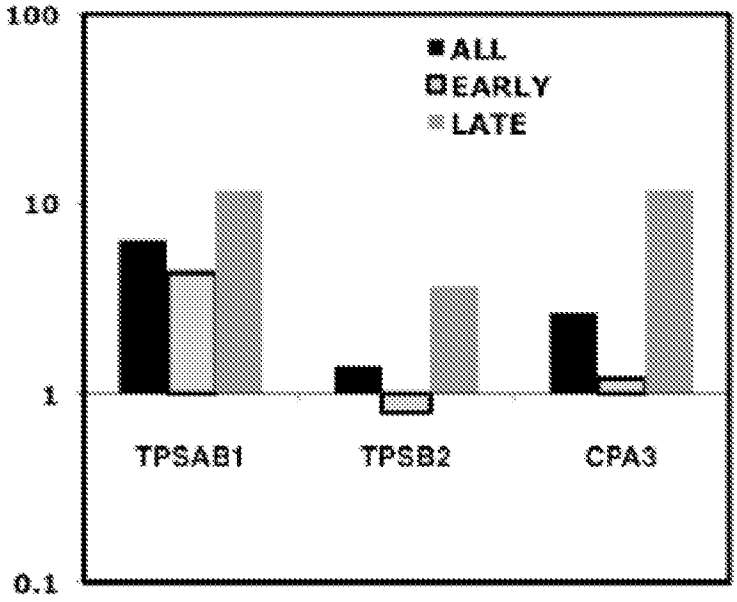

Figure 3

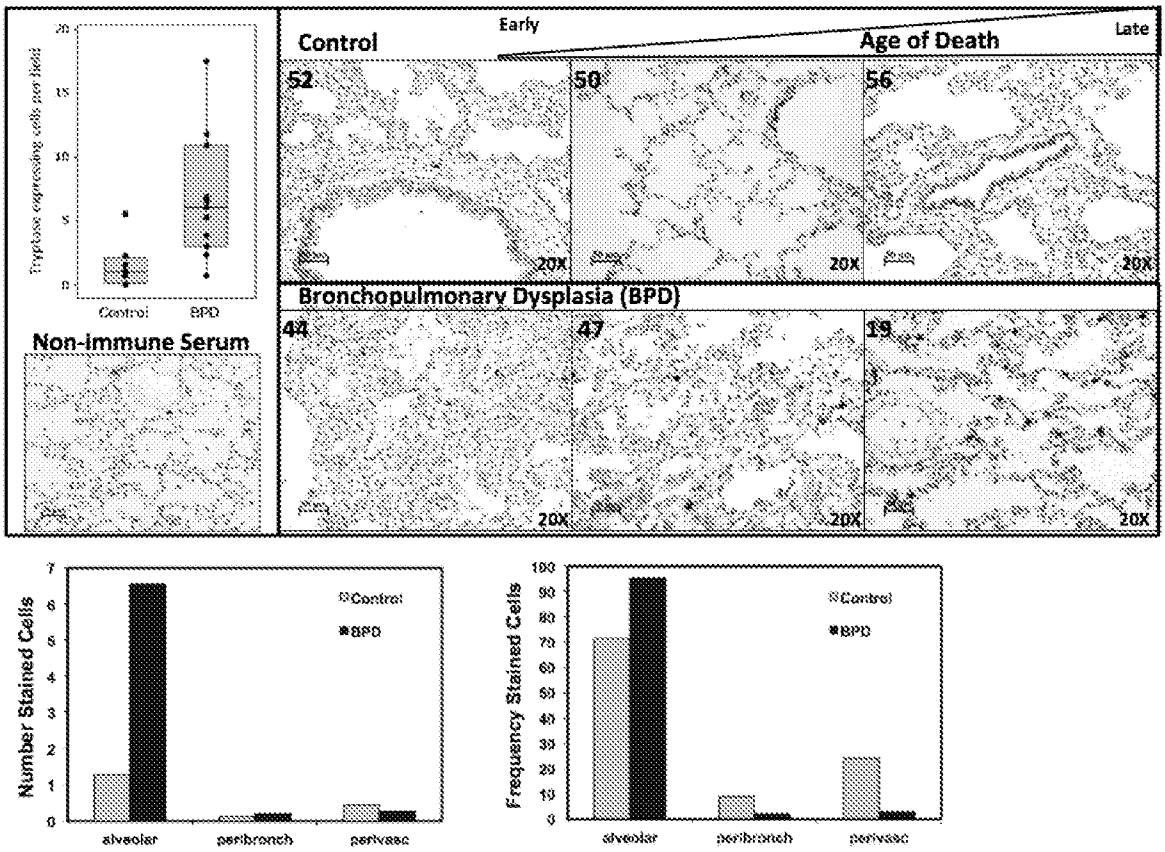

Figure 4

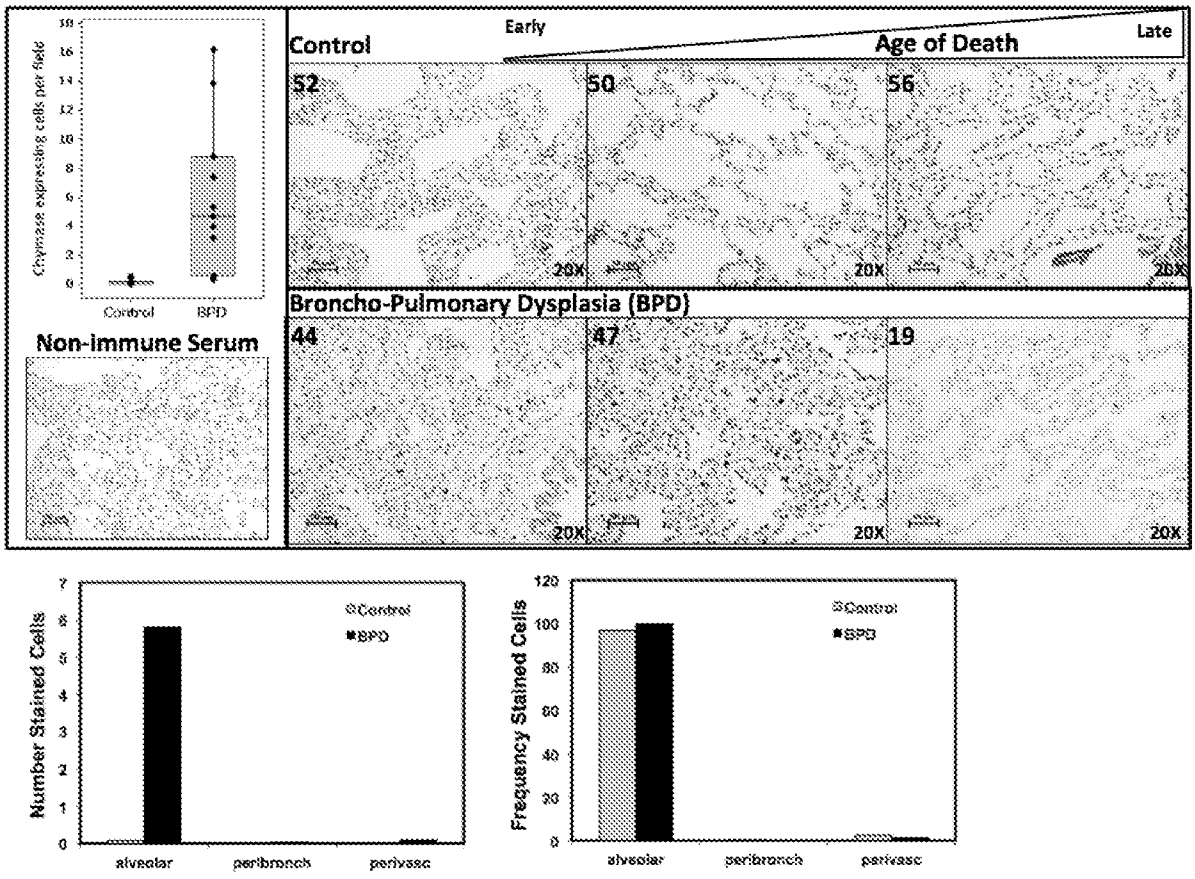
Chymase expressing cells per field
Control
BPD
Non-immune Serum
Control
Early
Age of Death
Late
52
50
56
20X
Broncho-Pulmonary Dysplasia (BPD)
44
47
19
Number Stained Cells
Control
BPD
alveolar
peribronch
perivasc
Frequency Stained Cells
Control
BPD
alveolar
peribronch
perivasc

Figure 5

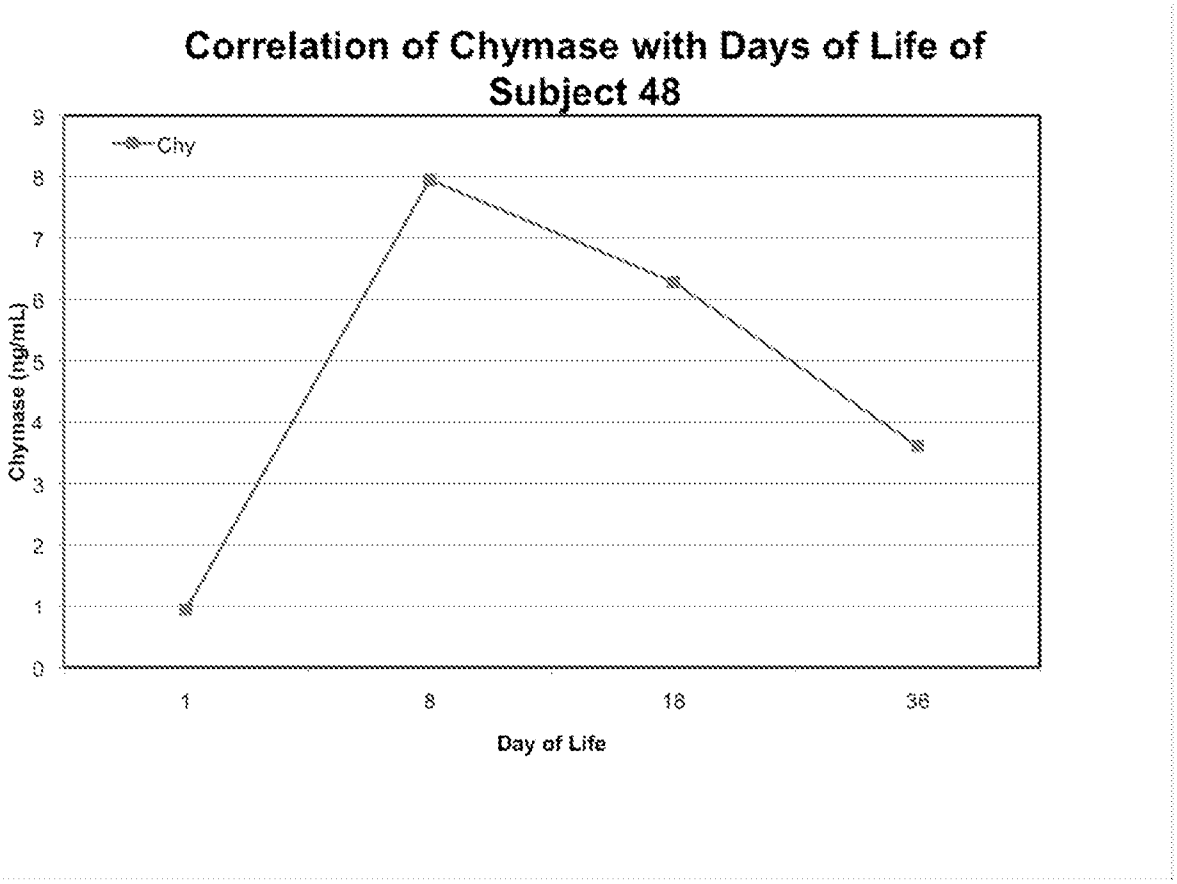
Correlation of Chymase with Days of Life of Subject 48
Chy
Chymase (ng/mL)
0
1
2
3
4
5
6
7
8
9
1
8
18
36
Day of Life

Figure 6A

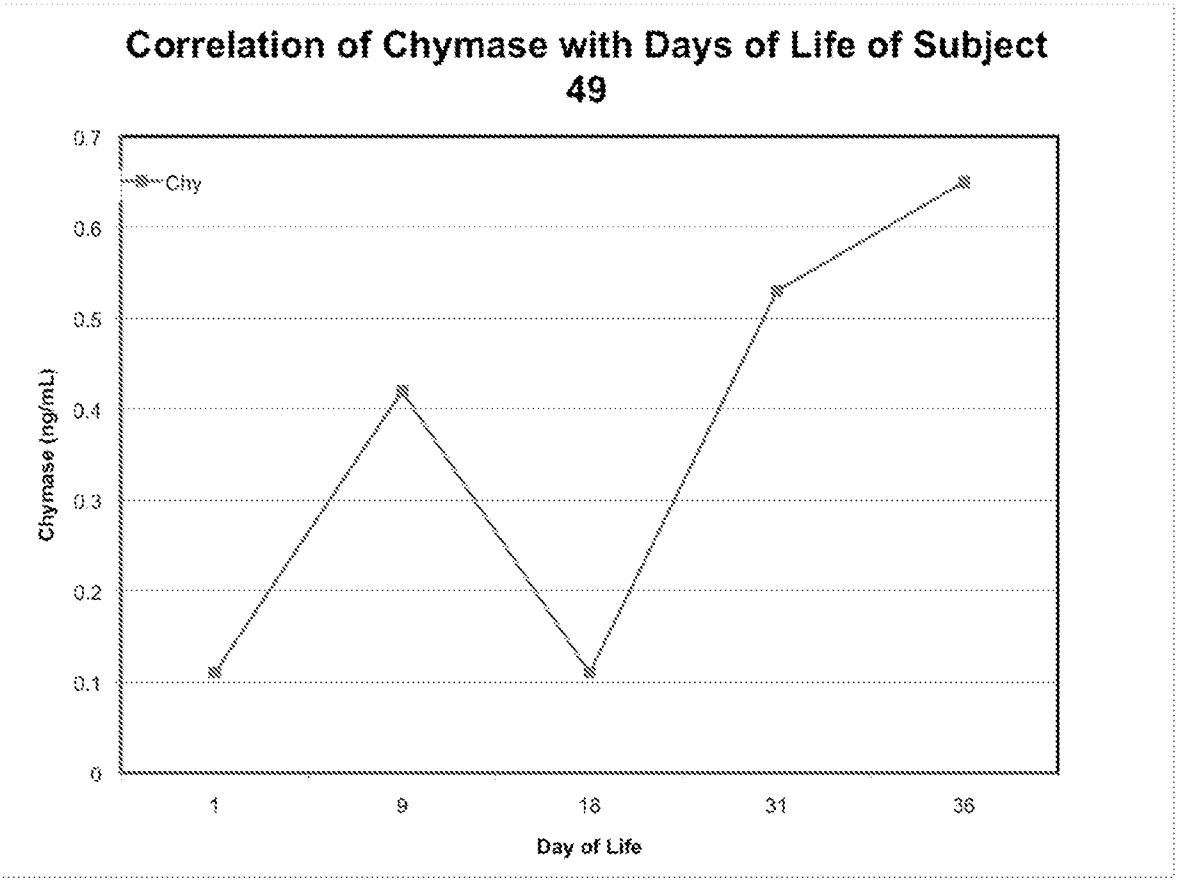
Correlation of Chymase with Days of Life of Subject 49
Chy
Chymase (ng/mL)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
9
18
31
36
Day of Life

Figure 6B

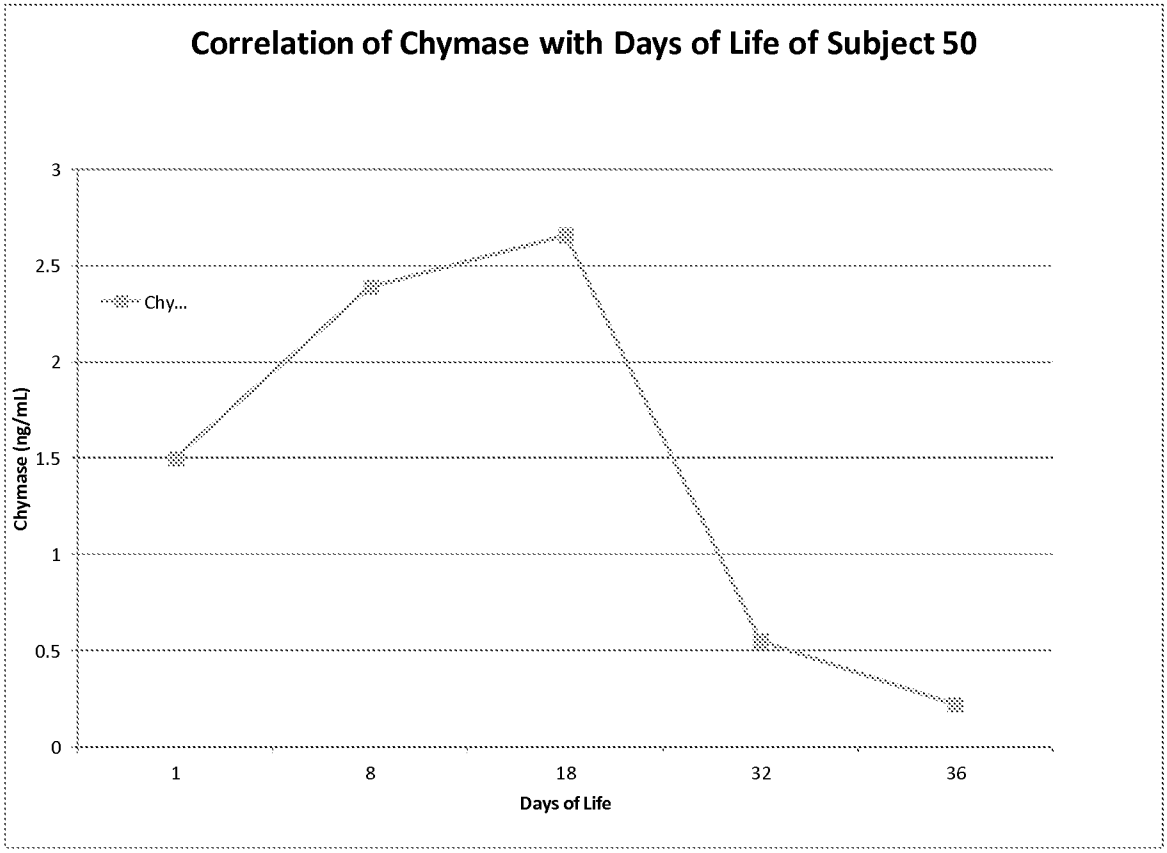
Correlation of Chymase with Days of Life of Subject 50
Chymase (ng/mL)
3
2.5
2
1.5
1
0.5
0
Chy...
1
8
18
32
36
Days of Life

Figure 6C

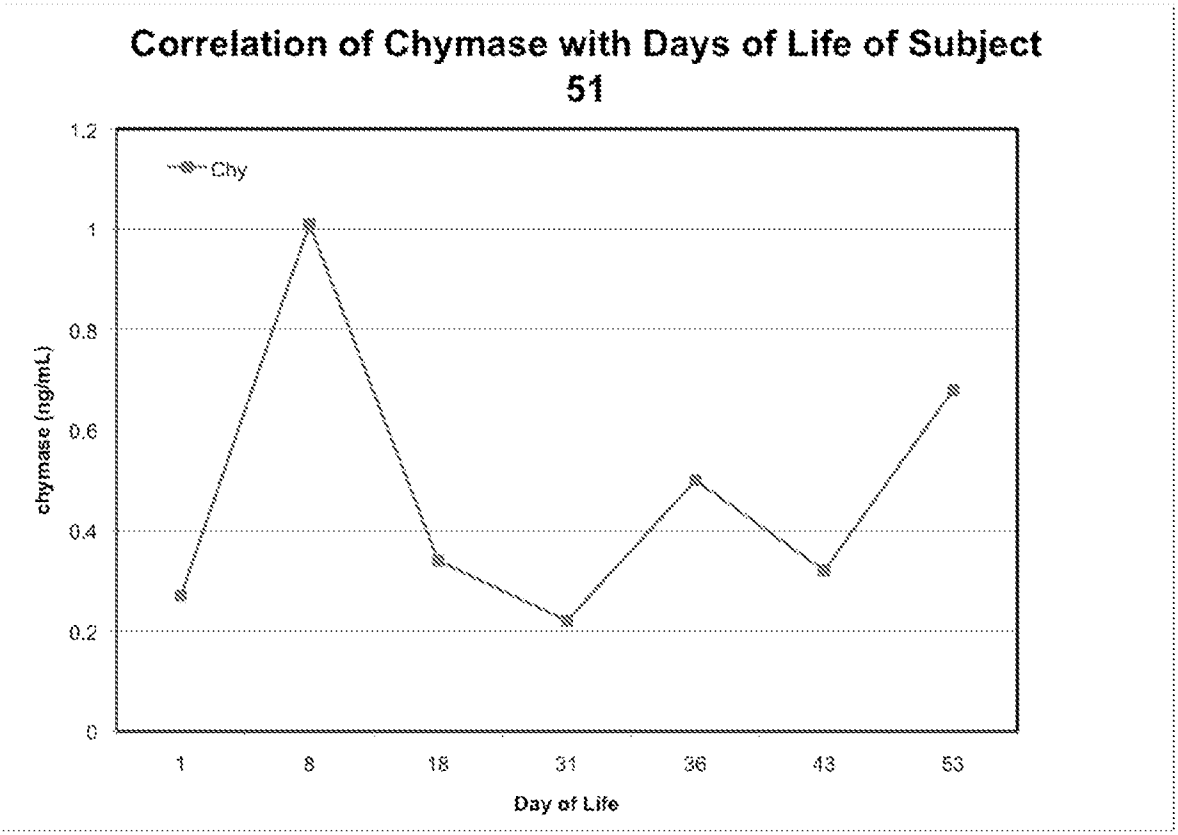
Correlation of Chymase with Days of Life of Subject 51
Chy
chymase (ng/mL)
1.2
1
0.8
0.6
0.4
0.2
0
1
8
18
31
36
43
53
Day of Life

Figure 6D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/038090

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/14 *(2006.01)* ***G01N 33/53*** *(2006.01)*
A61K 35/42 *(2006.01)* ***A61P 11/00*** *(2006.01)*

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 35/14, 35/42, G01N 33/53, A61P 11/00

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)

PATSEARCH, ESP@CENET, RUPAT, USPTO, PUBMED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Bose CL et al. " Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate". Arch Dis Child Fetal Neonatal Ed. 2008, Nov.; 93(6): F455-61, (abstract) [online] [Retrieved 2012-08-27] Retrieved from PubMed, PMID:18676410	1-7
Y	Hiromichi Hamada et al. "Increased Expression of Mast Cell Chymase in the Lungs of Patients with Congenital Heart Disease Associated with Early Pulmonary Vascular Disease." Am.J. Respir. crit. Care Med." October 1, 1999, vol.160, no 4, 1303-1308, [online] [Retrieved 2012-08-28] Retrieved from the Internet: <URL: http//ajrccm.atsournals.org/content/160/4/1303.full>, p. 1304, p.1306	1-7, 8, 9, 10-16
Y	Miller HR, Pemberton AD. "Tissue-specific expression of mast cell granule serin proteinases and their role in inflammation in the lung and gut". Immunology, 2002, Apr.:105(4): 375-90, [online] [Retrieved 2012-08-28] Retrieved from the Internet: <URL: http//www.ncbi.nlm.gov/pmc/articles/PMC1782685/?tool= =pubmed>, p.5, paragraph 4	4, 8, 10, 11-16

☐ 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 document 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 another 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
28 August 2012 (28.08.2012)	04 October 2012 (04.10.2012)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer N. Litvinenko Telephone No. (495) 531-64-81

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2012/038090

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gerry T.M. et al. Gene expression profile and histopatology of experimental bronchopulmonary dysplasia induced by prolonged oxidative stress, FreeRadical Biology &Medicine, vol.36, No 6, pp.782-801, 2004, p.786	6, 9

专利名称(译)	检测，预防和治疗支气管肺发育不良		
公开(公告)号	EP2709635A1	公开(公告)日	2014-03-26
申请号	EP2012785586	申请日	2012-05-16
[标]申请(专利权)人(译)	罗彻斯特大学		
申请(专利权)人(译)	罗切斯特大学		
当前申请(专利权)人(译)	罗切斯特大学		
[标]发明人	MARIANI THOMAS J PRYHUBER GLORIA S		
发明人	MARIANI, THOMAS J. PRYHUBER, GLORIA S.		
IPC分类号	A61K35/14 G01N33/53 A61K35/42 A61P11/00		
CPC分类号	A61K38/482 A61P11/00 C12Y304/21039 G01N33/6884 G01N33/573		
代理机构(译)	HOEFER & PARTNER		
优先权	61/486613 2011-05-16 US		
外部链接	Espacenet		

摘要(译)

本文提供了预防或治疗受试者的支气管肺发育不良（BPD）的方法。该方法包括鉴定受试者中的结缔组织肥大细胞（CTMC）并施用阻断受试者中CTMC活性，阻断其水平或降低其水平的试剂。还提供了鉴定患有BPD或有发展BPD风险的受试者的方法。还提供了用于确定受试者中BPD治疗方案的有效性的方法。