COMPOSITION FOR TREATING DEVELOPMENTAL AND/OR CHRONIC LUNG DISEASES COMPRISING CELLS SEPARATED OR PROLIFERATED FROM UMBILICAL CORD BLOOD

Technical Field

The present invention relates to a composition for treating developmental and/or chronic lung diseases comprising cells separated or proliferated from umbilical cord blood.

Background Art

Developmental and/or chronic lung diseases include adult chronic obstructive lung disease (COPD) such as cystic fibrosis and emphysema, and bronchopulmonary dysplasia of infant or premature baby. The seriousness of these diseases is that there are no effective prevention and treatment methods for these diseases in spite of the severeness and chronicity thereof.

For example, the bronchopulmonary dysplasia is a chronic lung disease induced by respiratory failure in newborn- or premature babies kept in a ventilator. Recent data for treating premature babies show an increase in the incidence of said untreatable diseases (Avery ME et al., *Pediatrics* 79:26-30, 1987). Not only the disease is a major cause of death of newborn infants, particularly, premature babies, but also surviving babies have to be hospitalized for a long period of time and serious side effects such as pulmonary hypertension must be dealt with. Even after discharge from the hospital, the rate of re-hospitalization of infants suffering from bronchopulmonary dysplasia is usually more than 50% because of their bronchopulmonary dysplasia due to their susceptibility to viral acute bronchiolitis and pneumonia. It is also known that bronchopulmonary dysplasia may progress to bronchial asthma because of continuous bronchial hyper-sensitivity (Coalson JJ. *Semin Neonatol,* 8:73-81, 2003), and is further associated with a serious neurodevelopmental sequela such as cerebral palsy (Bregman J and Farrell EE, *Clin Perinatol,*

19:673-94, 1992).

An effective treatment method for bronchopulmonary dysplasia as well as other chronic lung disease of adults has not yet been developed. Studies have been focused on an approach for the treatment of bronchopulmonary dysplasia to reduce barotrauma and volutrauma caused by positive pressure ventilation or reduce oxygen concentration during the artificial ventilation treatment of newborn- and premature babies, besides the fact that steroids have been used to prevent and treat inflammation of the damaged lung. However, steroid treatment is now limited due to the recent reports suggesting that the use of steroid are associated with later abnormal neurodevelopmental prognosis, especially with the increase in cerebral palsy (Committee on Fetus and Newborn, *Pediatrics*, 109:330-8, 2002).

Recently anticipation has been rising for the treatment using stem cells having a potential to be differentiated into every organs. However, the transplantation of embryonic stem cells, which have excellent differentiation potential, has serious developmental problems caused by the generation of uncontrollable teratoma or genomic imprinting as well as ethical problems. Therefore, the application of embryonic stem cells has become limited, and instead, adult stem cells have recently attracted much interest for the treatment thereof.

Among adult stem cells, the stem cells of hematopoietic system have received much attention. The bone marrow-derived stem cells as a source for stem cells of hematopoietic system are generally grouped into two: hematopoietic stem cells and mesenchymal stem cells.

It has been known that the hematopoietic stem cells in bone marrow have plasticity, suggesting that they are differentiated into not only cells of hematopoietic system but also other various organ cells (Gussoni E et al. *Nature*., 401:390-394, 1999; Petersen BE et al., *Science*, 284:1168-1170, 1999; Mezey E et al., *Science*, 290:1779-1782, 2000; and Krause DS et al., *Cell*., 105:369-377, 2001). However, it is though not very common so that there is a doubt of biological usefulness. Some reports suggest that such phenomena might result from cell fusion (Wagers AJ et al., *Science*, 297:2256-2259, 2002).

On the other hand, mesenchymal stem cells separated from bone marrow of adult mice, named 'multipotent adult progenitor cells (MAPC)', are capable of differentiating into all three germ layers of ectoderm, mesoderm and endoderm, and in fact they have proven to differentiate into almost all organ cells when injected into the blastocyst of a mouse. These cells were reported to have embryonic stem cell markers such as OCT-4, Rex-1 and SSEA-1 (Jiang Y et al., *Nature*, 418:41-49, 2002).

It has been much noted that similar stem cells separated from human bone marrow can be used for cell therapy for various diseases and damages (Reyes M et al., *Blood*, 98:2615-2625, 2001; and Woodbury D et al., *J Neurosci Res.*, 61:364-370, 2000). However, the numbers of hematopoietic stem cells and mesenchymal stem cells in bone marrow decrease with aging (Geiger H et al., *Nat Immunol.*, 3:329-333, 2002), besides the problem that bone marrow extraction is distressing to the patient, which limits the actual clinical application. Thus, alternatives have been searched.

Umbilical cord is the line connecting a mother and the fetus through which nutrition is provided and wastes are excreted, and the blood inside thereof is so-called umbilical cord blood. The umbilical cord blood seems to be the most appropriate alternative of bone marrow in extracting the stem cells of hematopoietic system because it contains more primitive stem cells than those of bone marrow. In addition, such cell extraction is much easier.

The transplantation of hematopoietic stem cells extracted from umbilical cord blood has been clinically applied since 1980s, because of their advantages over bone marrow: higher hematopoietic proliferation activity which means more hematopoietic stem cells present per unit volume (Szilvassy SJ et al., *Blood*, 98:2108-2115, 2001); less HLA (human leukocyte antigen) incompatibility which means less graft versus host reactions (Rocha V et al., *N Engl J Med.*, 342:1846-1854, 2000); easier and less invasive extraction (Rubinstein P et al., *N Engl J Med.*, 339:1565-1577, 1998); and remarkably lower risks compared to those which may be caused by autologous bone marrow transplantation in case of various types of cancer or other diseases. In particular, umbilical cord blood bank has recently been in operation to provide

services of preservation and amplification of umbilical cord blood, which has triggered various clinical practices for transplantation of hematopoietic stem cells of umbilical cord blood.

The question has not been settled and public attention has been directed as to whether the mesenchymal stem cells, particularly, MAPC-like cells having excellent differentiation potential into various organ cells are present in umbilical cord blood. This is because it would be a break-through discovery in cell therapy, and cell and tissue regenerative medicine, if mass-production of such mesenchymal stem cells or MAPC-like cells from umbilical cord blood can be achieved. It has been predicted based on the primitiveness of stem cells of umbilical cord blood that MAPC-like cells exist more in umbilical cord blood than in the bone marrow. Recently mesenchymal stem cells were successfully separated from umbilical cord blood (Erices A et al., *Br J Haematol.*, 109:235-242, 2000) and it has been proven that the cells have MAPC cell-level multipotency enabling them to differentiate into osteoblasts, adipocytes and neuron-like cells *ex vivo* (Lee OK et al., *Blood.*, 103:1669-1675, 2004). Further, it was a common belief that the number of mesenchymal cells taken from umbilical cord blood at first was very small and the proliferation thereof was very impractical. But, according to recent reports, it has been proven that *ex vivo* amplification of the umbilical cord blood-derived mesenchymal stem cells is possible to obtain a large number of mesenchymal stem cell (Yang SE et al, *Cytotherapy*, 6:476-486, 2004; and Kern SH et al., *Stem Cells*, 24:1294-1301, 2006).

It has been reported that these cells still possess multipotency even after amplification, and can be differentiated into osteoblasts, chondroblasts, adipocytes and neuron-like cells *ex vivo,* while differentiating *in vivo* into nerve cells with the migrating ability, cartilage and bone cells, cells of hematopoietic system and liver cells (Kogler G et al., *J Exp Med.*, 200:123-135, 2004).

Methodologically, the umbilical cord blood extracted from the real placental tissue is an ideal source for autologous and allogeneic stem cells, and such stem cells obtained thereby can be used directly or after amplifying stage whenever and as many as required.

However, there has been no attempt to apply the umbilical cord blood-derived stem cell transplantation to the developmental and/or chronic lung diseases. There are a few experimental reports that the adult bone marrow stem cells transplanted into a mouse with pneumonia induced by irradiation were differentiated into bronchial cells and type II cells of lung parenchyma (Theise ND et al. *Exp Hematol.,* 30:1333-1338, 2002), and reduced the bleomycin-induced pulmonary fibrosis in adult animal models (Ortiz L et al. *Proc Natl Acad Sci USA,* 100:8407-8411, 2003; and Rojas M et al., *Am J Respir Cell Mol Biol,* 33:145-152, 2005).

There are some patents aimed for treatment of diseases using the umbilical cord blood-derived cells. For example, Korean Patent Publication No. 2003-0015160 describes a composition for treating articular cartilage damage comprising cell components separated, proliferated or differentiated from the umbilical cord blood and a medium containing thereof, and Korean Patent Publication No. 2005-0105467 describes a method for treating myelodysplastic syndrome and myelosclerosis by administrating a high dose of umbilical cord blood-derived stem cells. However, there have been no descriptions on the therapeutic effect of the transplantation of umbilical cord blood-derived stem cells in treating developmental and/or chronic lung diseases.

Thus, the present inventors established a bronchopulmonary dysplasia model by administrating highly concentrated oxygen continuously, and then administrating the composition of the present invention intratracheally. As a result, pulmonary alveoli were increased in their numbers and developed normally, and the administered cells were differentiated into lung parenchymal cells. Thus, the present inventors have completed this invention by confirming that the composition of the present invention can be effectively used for the treatment of developmental and/or chronic lung diseases.

Disclosure

Technical Problem

Accordingly, it is an object of the present invention to provide a

composition for treating developmental and/or chronic lung diseases.

It is another object of the present invention to provide a method for treating the developmental and/or chronic lung diseases by intratracheal administration of the inventive composition to a patient.

Technical Solution

In accordance with one aspect of the present invention, there is provided a composition for treating developmental and/or chronic lung diseases comprising cells separated or proliferated from umbilical cord blood as an active ingredient.

In accordance with the other aspect of the present invention, there is provided a method for treating the developmental and/or chronic lung diseases comprising administrating the inventive composition to a patient suffering from such diseases.

Description of Drawings

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

Figs. 1 to 3: photographs illustrating the improved pathological views of the lung tissues of neonatal rats induced with bronchopulmonary dysplasia, one of the developmental and/or chronic lung diseases, after treating with the inventive therapeutic composition.

NC: normal control group,

HC: hyperoxia-exposed (bronchopulmonary dysplasia) group, and

HT: hyperoxia-exposed and umbilical cord blood-derived mesenchymal stem cell intratracheal transplanting group (HT).

Fig. 4: a graph illustrating improved fibrosis of lung tissues of neonatal rats induced with bronchopulmonary dysplasia, one of the developmental and/or chronic lung diseases, after treating with the inventive therapeutic composition.

NC, HC and HT have the same meanings as defined in Figs. 1 to 3.

*significant difference from NC group ($p<0.05$)

#significant difference from HC group ($p<0.05$)

Figs. 5 to 7: graphs illustrating the improved alveolar development in the lung tissues of neonatal rats induced with bronchopulmonary dysplasia, one of the developmental and/or chronic lung diseases, which was represented by the values of radial alveolar count (RAC), mean linear intercept (MLI) and mean alveolar volume, respectively, after treating with the inventive therapeutic composition.

NC, HC, HT, * and # have the same meanings as defined in Figs. 1 to 4.

Fig. 8: a graph illustrating reduced myeloperoxidase (MPO) activity, which representing the accumulation of neutrophils as inflammatory cells, in the lung tissues of neonatal rats induced with bronchopulmonary dysplasia, one of the developmental and/or chronic lung diseases, after treating with the inventive therapeutic composition.

NC, HC, HT, * and # have the same meanings as defined in Figs. 1 to 4.

Fig. 9: a photograph illustrating that the transplanted therapeutic cellular components were safely located in the lung tissue of a neonatal rat induced with bronchopulmonary dysplasia, after treating with the inventive therapeutic composition.

Blue: cell nuclei

Red: therapeutic cellular component; and

Fig. 10: a photograph illustrating that the transplanted therapeutic cellular components were safely located in the lung tissue of a neonatal rat induced with bronchopulmonary dysplasia and differentiated into type II cells of lung parenchyma, after treating with the inventive therapeutic composition.

PKH26: transplanted therapeutic cells labeled with PKH26 (red fluorescence)

Pro SP-C: type II cells of lung parenchyma labeled with Pro SP-C (green fluorescence)

DAPI: cell nuclei labeled with DAPI (blue fluorescence)

Merge: co-label with PKH26, Pro SP-C and DAPI

Modes for Invention

Umbilical cord blood, the origin of therapeutic cells of the present invention, is the blood taken from umbilical vein connecting placenta and a fetus, and a natural by-product of childbirth. The umbilical cord blood is much easier to obtain than general mesenchymal tissues like bone marrow requiring several steps of operation, and it is also very easy to find a donor because umbilical cord blood deposit industry has been developing steadily and the related infrastructure has already been established. In addition, umbilical cord blood-originated cells do not express histocompatibility antigen HLA-DR (class II) which is the major cause of rejection after tissue- or organ transplantation (Le Blanc, KC, *Exp Hematol*, 31:890-896, 2003; and Tse WT et al., *Transplantation*, 75:389-397, 2003). Thus, these cells can minimize the immune response when transplantation is conducted, for example rejection against transplanted tissue or organ, suggesting that autologous as well as allogeneic umbilical cord blood can be used.

The procedure for the extraction and separation of umbilical cord blood is as follows.

In case of normal vaginal delivery, placenta is still in the uterus right after childbirth, and umbilical vein is expelled. Thus, umbilical cord blood is extracted from the exposed umbilical vein. In case of cesarean section, placenta is also expelled right after childbirth, and umbilical cord blood is extracted from the exposed umbilical vein.

When umbilical cord blood is taken from the exposed umbilical vein right after childbirth, the umbilical cord blood is extracted from the umbilical vein connecting the placenta and the fetus by aseptic manipulation. At this time, umbilical cord blood can be taken either before or after placental separation from uterus. In the case of cesarean section, the umbilical cord blood is extracted from the umbilical vein *ex vivo* after placental separation from uterus, and taken into an umbilical cord blood sampling bag containing anticoagulant using a sampling needle.

The composition of the present invention contains cells separated or

proliferated from the umbilical cord blood, preferably, one or more cells selected from the group consisting of monocytes containing hematopoietic stem cells and mesenchymal stem cells separated from the umbilical cord blood, mesenchymal stem cells separated from the umbilical cord blood, and mesenchymal stem cells amplified from the mesenchymal stem cells by sub-culture, and the mesenchymal stem cells separated from the umbilical cord blood or mesenchymal stem cells amplified from the mesenchymal stem cells by sub-culture are more preferred.

The mesenchymal stem cells separated from the umbilical cord blood are multipotent, unlike the typical stromal cells of bone marrow, suggesting that they can be differentiated into mesenchymal tissues such as bone, cartilage, adipose tissue, muscle, tendon, etc., under appropriate conditions. Further, umbilical cord blood-derived mesenchymal stem cells have self-renewal ability, suggesting that they are capable of proliferating under suitable conditions without differentiating into specific cells or tissues, and might exhibit anti-inflammation activity when transplanted. In addition, the cells are more primitive and have much better cell proliferation, differentiation and secretory capacity of the regulatory molecules or substances, as compared with those derived from the mesenchymal stem cells separated from general mesenchymal tissues such as bone marrow, muscle and skin.

To separate and culture the mesenchymal stem cells from the harvested umbilical cord blood, any of the methods described in Korean Patent Publication No. 2003-0069115 and published articles including [Pittinger MF et al. *Science*, 284:143-7, 1999; and Lazarus HM et al. *Bone Marrow Transplant*, 16:557-64, 1995] can be used, and one example is shown below.

First, the harvested umbilical cord blood is for example, centrifuged by using Ficoll-Hypaque gradient to separate monocytes comprising hematopoietic stem cells and mesenchymal stem cells, which are then washed several times to eliminate impurities. The washed monocytes are cultured in a culture vessel with proper density, and then, the cells are allowed to proliferate to form a single layer. Among the proliferating cells, those who are observed to be homogeneous and to form a colony of a spindle shape under phase contrast

microscopy, are the mesenchymal stem cells. When the cells are proliferated, sub-cultures are performed until the cells are amplified enough.

The cells contained in the inventive composition can be cyropreserved in accordance with the conventional method well-known to those in the art (Doyle et al., 1995). That is, the cells are suspended at the concentration of $1x10^6 \sim 5x10^6$ cells per 1 ㎖ in a medium for the cryopreservation comprising 10-20% FBS (fetal bovine serum) and 10% DMSO (dimethylsulfoxide).

The cell suspension is distributed into glass- or plastic ampoules for deep freezing, and then the ampoules are sealed and put in a deep freezer kept at a programmed temperature. At this time, it is preferred to use a freeze-program that controls the freezing rate at -1℃/min so that cell damage during thawing can be minimized. When the temperature of the ampoule reaches -90℃, it is transferred into a liquid nitrogen tank and maintained at less than -150℃.

To thaw the cells, the ampoule has to be transferred from the liquid nitrogen tank into a 37℃ water bath quickly. The thawed cells in the ampoule are placed in a culture vessel containing a culture medium quickly under an aseptic condition.

In the present invention, the medium used in the separation or proliferation of the mesenchymal stem cells may be any medium for general cell culture well-known to those in the art containing 10% to 30% FBS, for example, DMEM, MEM, α-MEM, McCoys 5A medium, Eagle's basal medium, CMRL medium, Glasgow minimum essential medium, Ham's F-12 medium, IMDM (Iscove's modified Dulbecco's medium), Liebovitz' L-15 medium, RPMI 1640 medium, and DMEM is preferred. The cells are suspended at the concentration of $5x10^3 \sim 2x10^4$ cells per 1 ㎖ of the medium.

Further, the cell culture medium of the present invention can additionally include one or more auxiliary components, for example, fetal bovine serum, horse serum or human serum; and antibiotics such as Penicillin G, streptomycin or gentamycin sulfate, antifungal agent such as amphotericin B or nystatin, and a mixture thereof to prevent microorganism contamination.

In one embodiment of the present invention, the obtained umbilical cord

blood was centrifuged to separate monocytes, and then the separated cells were cultured with an appropriate density in a culture vessel. When the cells were grown to an appropriate density, sub-cultures were performed. Further, a bronchopulmonary dysplasia model in neonatal rats was established by administrating highly concentrated oxygen continuously from the birth. As a result, rats with bronchopulmonary dysplasia thus obtained exhibited increased respiratory rate and poor weight gain. Then, the lungs were extracted from the rat and stained. As a result, rats with bronchopulmonary dysplasia (HC) exhibited chronic inflammatory reactions with increased monocytes and fibrosis with over-proliferated interstitial fibroblasts in the lung (*see* Figs. 2 and 4). In addition, in the lung tissue of the rat (HC), radial alveolar count (RAC, *see* Husain AN et al., *Pediatr Pathol.*, 13:475-484, 1993) representing the number of alveoli was significantly lowered, mean linear intercept (MLI, *see* Dunnill MS., Thorax 17:320-328, 1962) representing the size of alveoli and mean alveolar volume (*see* McGowan S et al., *Am J Respir Cell Mol Biol.*, 23:162-167, 2000) increased remarkably (*see* Figs. 5 to 7), and resultantly alveolar development was significantly abnormal, compared with that in the wild type normal rat (NC). However, in the lung tissue of the rat intratracheally administered with the mesenchymal stem cells (HT), the damage was moderated (*see* Figs. 3 and 4), RAC increased, and MLI and mean alveolar volume became lower (*see* Figs. 5 to 7).

Further, the result of measurement of myeloperoxidase (MPO) activity as the index of neutrophil accumulation shows that MPO activity in lung tissue of hyperoxia-exposed neonatal rat (HC) increased markedly as compared with that of room air-exposed rat, while MPO activity in lung tissue of rat Intratracheally administered with the inventive composition (HT) was markedly lower than that of hyperoxia-exposed neonatal rats (*see* Fig. 8), which means that the accumulation of neutrophils as inflammatory cells was significantly reduced.

In addition, the composition of the present invention comprising cells labeled with red fluorescent PKH26 was intratracheally administered and then the lung tissue of the rat was observed with a fluorescent microscope. As a

result, the cells included in the inventive composition were safely located in the lung (*see* Fig. 9), and the part of the cells safely located in the lung tissue were differentiated into lung parenchymal cells (*see* Fig. 10).

Accordingly, the cells of the present invention, which are separated and proliferated from umbilical cord blood, can be effectively used to treat adult chronic obstructive lung diseases (COPD) such as cystic fibrosis and emphysema, and developmental and/or chronic lung diseases such as bronchopulmonary dysplasia of a infant and a premature baby.

The inventive composition for treating developmental and/or chronic lung diseases comprising the cells separated or proliferated from umbilical cord blood as an active ingredient can additionally comprise one or more auxiliary components selected from the group consisting of a medium to suspend the cells, a gene effective in the treatment of lung disease (e.g., anti-inflammatory cytokine gene, siRNA or anti-sense primer against inflammatory cytokine) or an expression vector comprising thereof, a cytokine providing autocrine or paracrine effect (e.g., interleukin-10), growth factor (e.g., karatinocyte growth factor), and a mixture thereof.

At this time, the medium may be identical with those described above for the cell culture medium, except for not including any serum, antibiotics and antifungal agent.

The gene or the expression vector comprising thereof may be transferred by any of the conventional methods known to those in the art, for example, viral transfection or non-viral method, or simply combined with the cells. At this time, the introduction of the gene may be conducted in accordance with any of the methods known to those in the art including adenoviral transformation, gene gun, liposome-mediated transformation, and retrovirus or lentivirus-mediated transformation, plasmid or adeno-associated virus without limitation. Further, the cells may be transplanted together with carriers having gene delivery system, which can release or deliver a gene to the cells for long periods of time.

Further, the composition of the present invention, may include $1.0x10^5$ to $1.0x10^9$ cells/㎖, preferably $1.0x10^6$ to $1.0x10^8$ cells/㎖, more preferably $1.0x10^7$ cells/㎖.

The inventive composition can be used as not-frozen or can be frozen for later use. To freeze the composition, a standard cryopreservative agent (e.g., DMSO, glycerol, Epilife™ or cell freezing medium (Cascade Biologics)) may be added to the cells.

Further, the composition can be administered by formulating a unit dosage suitable for administrating to a patient by conventional methods in the pharmaceutical field, and the dosage contains an effective amount enough to induce alveolar development by a single dose or in divided doses. For this purpose, a formulation for parenteral administration include injection formulation such as injection ampoule, infusion formulation such as infusion bag, and spray formulation such as aerosol preferably. The injection ampoule may be mixed with injection solution such as saline solution, glucose, mannitol and ringer's solution just before use. Further, the cells can be carried by infusion bag textured by polyvinyl chloride or polyethylene, for example, a product of Baxter, Becton-Dickinson, Medcep, National Hospital Products or Terumo.

The pharmaceutical formulation of the present invention may additionally comprise one or more pharmaceutically acceptable inactive carriers except for the active ingredient, for example, preservative, analgesic controller, solubilizer or stabilizer for injection formulation, and base, excipient, lubricant or preservative for topical formulation.

The prepared composition or pharmaceutical formulation of the present invention can be administered in accordance with any conventional method in the art together with other stem cells used for transplantation and other purposes, or in the form of a mixture therewith. Direct engraftment or transplantation to the lesion of the lung, or transplantation or injection into airway is preferred, but not always limited thereto. Further, both non-surgical administration using catheter and surgical administration such as injection or transplantation after thoracotomy are possible, but non-surgical administration using catheter is more preferred. In addition, the composition or therapeutic agent can also be administered parenterally, for example, intravenous injection, one of the conventional methods for transplantation of stem cells of hematopoietic system,

besides direct administration to the lesion.

The cells separated or proliferated from umbilical cord blood of the present invention can be administered in an effective amount ranging from about $1.0x10^4$ to $1.0x10^{10}$ cells/kg (body weight), preferably $1.0x10^5$ to $1.0x10^9$ cells/kg (body weight) per day in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the disease to be treated, the condition to be treated, the severity of the patient's symptom, the chosen route of administration, and the body weight, age and sex of the individual patient; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

The present invention further provides a method for treating developmental and/or chronic lung diseases using a composition comprising cells separated or proliferated from umbilical cord blood as an active ingredient.

The method of the present invention includes a step of administrating the composition or pharmaceutical formulation to a patient by using the above various administration methods.

The inventive method for treating developmental and/or chronic lung diseases by intratracheally administrating the composition or pharmaceutical formulation as close to the lung tissue preferably can increase therapeutic effect by elevating accessibility, compared with the conventional cell transplantation method using intravenous injection. Further, umbilical cord blood is a natural by-product of childbirth. The umbilical cord blood is much easier to obtain than general mesenchymal tissue-like bone marrow requiring several steps of operation and it is also very easy to find a donor because umbilical cord blood deposit industry is developing steadily and the related infrastructure has already been established. In addition, umbilical cord blood-originated cells do not express histocompatibility antigen HLA-DR (class II) which is the major cause of rejection after tissue- or organ transplantation. Thus, these cells can minimize the immune response when transplantation is conducted, for example rejection against transplanted tissue or organ, suggesting that autologous as well as allogeneic umbilical cord blood can be used. From the above advantages,

the inventive method can effectively treat developmental and/or chronic lung diseases comprising cystic fibrosis, chronic obstructive pulmonary disease and broncopulmonary dysplasia.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Separation and culture of cells

The therapeutic cells of the present invention, mesenchymal stem cells, were separated from human umbilical cord blood and cultured as follows (*see* Yang SE et al., Cytotherapy, 6(5):476-86, 2004).

<1-1> Extraction of umbilical cord blood (UCB)

Umbilical cord blood sample was collected from the umbilical vein right after childbirth with the mother's approval. Specifically, the umbilical vein was pricked with a 16G needle connected to an UCB-collection bag containing 23 or 44.8 ㎖ of a CDPA-1 anticoagulant (Green cross corp., Korea) such that the UCB was collected into the collection bag by gravity. The UCB thus obtained was handled within 48 hours after collection, and the viability of the monocytes was more than 90%.

<1-2> Separation and amplification of mesenchymal stem cells

The UCB collected in <1-1> was centrifuged by using Ficoll-Hypaque gradient (density: 1.077 g/㎤, Sigma, USA) to obtain monocytes. The resulting cells were washed with a basal medium (α-MEM medium (Gibco BRL, USA) supplemented with 10% FBS (HyClone, USA)) several times. 5×10^6 cells/㎠ were inoculated into a basal medium, suspended, and incubated at 37℃ under a humid condition containing 5% CO_2, while replacing the medium with a fresh medium twice a week. As a result, adherent fibroblast-like cells were identified.

Within 3 weeks, 0.25% Trypsin (HyClone) was added to a monolayer colony of the mesenchymal stem cells, and the cells were washed. Then, 5×10^4 cells/㎠ were inoculated into a basal medium and repeated subcultivation was conducted so that the cells expanded *ex vivo*.

Example 2: Hyperoxia-exposed bronchopulmonary dysplasia model

All animal tests were approved by Research Aminal Laboratory Committee of Samsung Biomedical Research Institute (Korea), and conducted in accordance within their guideline.

First, in order to prepare a bronchopulmonary dysplasia model, timed-pregnant Sprague-Dawley rats (Daehan biolink Co. Ltd.) were purchased and raised in a research animal cage for at least 1 week before childbirth. Highly concentrated oxygen was administered into the neonatal rats right after their birth (within 10 hours from the birth) for 14 days.

Specifically, the rat dams and the neonates were put in a 69.5 x 50.0 x 32.0 ㎝ acryl box (sealed Plexiglas cage) which was controlled as at a humidity 40-60% and a temperature 23-26℃ under 1 atm, then the box was saturated with 100% oxygen at a rate of 10 ℓ/min for the first 10 minutes. When the oxygen saturation reached 95%, measured by an oxygen analyzer (572, Servomex, USA), 100% oxygen was refluxed at a rate of 2.5 ℓ/min, during which the oxygen saturation was measured continuously to keep the oxygen saturation at around 95%. To avoid pulmonary edema caused by oxygen toxicity, nursing rat dams were switched between room air and 95% O_2 every 24 hour for 14 days.

Example 3: Administration of UCB-derived mesenchymal stem cells

0.05 ㎖ of UCB-derived 5.83×10^5 mesenchymal stem cell suspension in α-MEM without FBS was intratracheally administered to the neonatal rat of Example 2 using a 26-gauge needle on the 5th day from birth after confirming

pup-up of air from trachea in the midline area of the neck of the rat.

Example 4: Tissue preparation after animal sacrifice

On the 14th day, the rats were anesthetized by intraperitoneal phentobarbital injection. After fixing the limbs, thoracotomy was performed to expose their heart and lung. A part of the sacrificed rats was subjected to transcardiac perfusion with ice cold PBS to extract the heart and the lung. A catheter was inserted intratracheally and fixed tightly. 4% formaldehyde as a fixative was instilled through the inserted catheter, and the lung was expanded uniformly under the pressure of 25 ㎝ H_2O followed by fixing with the fixative. Further, the lungs of the other rats were extracted and immediately frozen in liquid nitrogen.

Example 5: Measurement of grade of fibrosis, RAC, MLI and mean alveolar volume

The lung tissue section fixed with 4% formaldehyde for 24 hours in Example 4 was embedded in paraffin, which was then cut into a 4 ㎛ thick slices which was stained with hematoxylin eosin, followed by observation under optical microscope, to determine the number of neutrophils, the grade of fibrosis, the cell numbers and thickness of alveolar septa and pulmonary interstitium, while the presence or absence of pulmonary edema was investigated. Radial alveolar count (RAC) representing the newly-formed saccules and alveoli and mean linear intercept (MLI) representing the size of alveoli were measured, and mean alveolar volume was calculated.

The grade of fibrosis was assessed quantitatively according to a modification of the method described in [Stocker, J.T, Hum Pathol., 17:943-961, 1986]. Specifically, 6 non-overlapped lung tissue sections per rat were randomly selected, and 5 fields per section, totally to 30 fields, were observed at a magnification of x 10. The grade of fibrosis was scored as follows: Grade 0=normal lung; Grade 1=minimal fibrosis of alveolar septa; Grade 2=moderate

fibrosis of alveolar septa; and Grade 3=severe fibrosis of alveolar septa. The fibrosis score was expressed as a mean grade of fibrosis for each sample.

To measure RAC (*see* Husain AN et al., *Pediatr Pathol.*, 13:475-484, 1993), a vertical line was drawn from the terminal bronchiole to the nearest fibrovascular septum, and the number of saccules between the terminal bronchiole and the fibrovascular septum was counted.

MLI (*see* Dunnill MS., *Thorax* 17:320-328, 1962) was calculated using 1 ㎜ ruler, counting the number of septum while observing the tissue under the optical microscope (x 40).

To measure RAC and MLI, randomly selected areas were each observed at the 10 fields.

For the calculation of mean alveolar volume (*see* McGowan S et al., *Am J Respir Cell Mol Biol.*, 23:162-167, 2000), a grid that contained equally spaced crosses was placed on the photomicrograph of the distal lung magnified 200 times, and the diameters of the alveoli that were within each of the cross were measured. The cube of the alveolar diameter times π and divided by 3 was used to estimate the mean alveolar volume, and the resulting values obtained from 6 photos randomly selected for a test rat were averaged.

Each data was analyzed statistically by using corresponding mean value. The image of each section was taken by a digital camera of Olympus BX81 microscope. Data are expressed as mean±SD, and statistical analysis was performed by analysis of variance (ANOVA), followed by the Mann-Whitney test or Kruskal-Wallis test using an SAS (enterprise guide version of three, SAS Institute, USA) (Results were considered significant when the p value was less than 0.05).

As a result, the lung tissue of a bronchopulmonary dysplasia induced rat (HC) showed chronic inflammatory reaction accompanied by increased number of monocytes such as alveolar macrophages and lymphocytes, and fibrosis accompanied by over-proliferation of interstitial fibroblasts. However, the damage in pathology was significantly alleviated in the lung tissue of the rat intratracheally administered with the mesenchymal stem cells (HT) (Figs. 1 to 3), the quantitative analysis of grade of fibrosis was markedly reduced (Fig. 4).

Further, in the lung tissue of a bronchopulmonary dysplasia induced rat (HC), as compared with that of a normal rat (NC), RAC was significantly decreased, and MLI as well as mean alveolar volume were remarkably increased. As a result, impediment of alveolar development showing a reduced alveolar number and an abnormally enlarged alveolar size was obvious. However, in the lung tissue of the rat intratracheally administered with the mesenchymal stem cells (HT), as compared with that of a bronchopulmonary dysplasia induced rat (HC), RAC was increased, while MLI and mean alveolar volume became lower (Figs 5 to 7). The result suggests that the alveolar development has been improved by showing the increased alveolar number and the decreased alveolar size.

Example 6: MPO (myeloperoxidase) activity

Myeloperoxidase (MPO) activity, an index of neutrophil accumulation, was determined using a modification of the method described in [Gray KD et al., *Am J Surg.*, 186:526-530, 2003].

Specifically, a frozen lung tissue sample was homogenized in a phosphate buffer (pH 7.4), centrifuged at 30,000 x g for 30 min, and the resulting pellet was resuspended in a phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide. The resuspended pellet was allowed to react with 0.167 ㎎/㎖ O-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the rate of change in the absorbance was measured at 460 ㎚ to determine its MPO activity. 1 unit of MPO activity was defined as the quantity of enzyme degrading 1 μmol/min of peroxide. All data was expressed in mean±SD, and statistical analyses were conducted in accordance with the method of Example 5 (Results were considered significant when the p value was less than 0.05).

As assessed, the MPO activity in lung tissues of hyperoxia-exposed neonatal rat (HC) was higher than that of room air-exposed rat, which means that the accumulation of neutrophils as inflammatory cells has increased significantly. However, when the mesenchymal stem cells were administered

to rats intratracheally (HT), the MPO activity was markedly reduced than that of hyperoxia-exposed neonatal rats (Fig. 8), which means that the accumulation of neutrophils as inflammatory cells was significantly reduced.

Example 7: Double-labeled immunohistochemistry staining

The differentiation of the mesenchymal stem cells transplanted into the lung was observed with a microscope.

First, in order to check whether the mesenchymal stem cells were properly transplanted into the lung, the mesenchymal stem cells of Example 1, labeled with red fluorescent PKH26 (Sigma, USA), were intratracheally administered to each rat with bronchopulmonary dysplasia, and then, the lung tissue of the rat was observed with a fluorescent microscope.

The result showed that the mesenchymal stem cells of Example 1 were safely located in the lung (Fig. 9).

Further, in order to check the character of differentiation of the cells located in the lung, whether the transplanted cells labeled with PKH26 were stained with pro SP-C (pro surfactant protein C) as a specific marker of type II pneumonocyte, one of cells of lung parenchyma, was examined by subjecting a frozen tissue section to immunohistochemistry staining against the cell specific marker.

Specifically, a frozen lung section was fixed with 4% paraformaldehyde for 30 min, treated with a mixture of 1% bovine serum albumin and 0.1% Triton X-100 for 30 min, and blocked with PBS containing 0.1% Triton X-100 and 3% normal goat serum at room temperature for 1 hour. The tissue section was then subjected to a reaction with pro SP-C (rabbit polyclonal, Sterologicals Co., USA) as a 1st antibody at 1:100 dilution at 4℃ overnight. The tissue was washed with PBS, and treated with Alexa Fluor 488 goat anti-mouse (1:500, Molecular Probes, USA) as a 2nd antibody in blocking solution for 1 hour. The resulting tissue was counter stained with using DAPI (4'-6-diamidino-2-phenylindole, Molecular Probes, USA), mounted by using Vector shield (Vector Laboratories, USA), and observed with a confocal imaging microscopy

(Bio Rad, USA). A fluorescence photo was taken with an Olympus EX41 fluorescence microscope using Olympus MaganFire camera X100 and X 400 lens.

As a result, the lung tissue of the rats in the control group, that were not administered with the mesenchymal stem cells, showed minimum background staining, but the lung tissues of the rat intratracheally administered with the mesenchymal stem cells, showed many PKH26 labeled transplanted cells having red fluorescence. Further, as a result of co-staining with a specific marker of type II pneumonocyte, one of lung parenchymal cells, labeled with green fluorescence, a part of located cells were co-stained. Therefore, the part of the transplanted mesenchymal stem cells, which were safely located in the rat lung tissue, was differentiated into lung parenchymal cells (Fig. 10).

Accordingly, it has been confirmed that intratracheal administration of the therapeutic composition of the present invention is very effective for treating developmental and/or chronic lung diseases.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made and also fall within the scope of the invention as defined by the claims that follow.

What is claimed is:

1. A composition for treating developmental or chronic lung diseases comprising cells separated or proliferated from umbilical cord blood as an active ingredient.

2. The composition of claim 1, wherein the cells are selected from the group consisting of monocytes separated from the umbilical cord blood, mesenchymal stem cells separated from the umbilical cord blood, mesenchymal stem cells amplified from the mesenchymal stem cell by sub-culture, and a mixture thereof.

3. The composition of claim 2, wherein the monocytes comprise hematopoietic stem cells and mesenchymal stem cells.

4. The composition of any one of claims 1 to 3, which further comprises an auxiliary component selected from the group consisting of a culture medium, a gene effective in the treatment of lung disease or an expression vector comprising the same, a cytokine providing autocrine or paracrine effect, a growth factor, and a combination thereof.

5. The composition of claim 4, wherein the medium is selected from the group consisting of DMEM, MEM, α-MEM, McCoys 5A medium, Eagle's basal medium, CMRL medium, Glasgow minimum essential medium, Ham's F-12 medium, Iscove's modified Dulbecco's medium (IMDM), Liebovitz' L-15 medium and RPMI 1640 medium.

6. The composition of claim 1, which is intratracheally administered to a patient.

7. The composition of claim 1, wherein the developmental or chronic lung disease is cystic fibrosis, chronic obstructive pulmonary disease or bronchopulmonary dysplasia.

8. A therapeutic agent for treating developmental or chronic lung diseases prepared from the composition of claim 1.

9. The agent of claim 8, which is selected from the group consisting of injection formulation, infusion formulation and spray formulation.

10. A method for treating developmental or chronic lung diseases comprising intratracheally administrating the composition of claim 1, which comprises cells separated or proliferated from umbilical cord blood as an active ingredient, into a patient suffering such diseases.