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Intratracheal Instillation of Stem Cells in Term Neonatal Rats

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Dear Sir,

This is in regard to our manuscript JoVE61117 entitled "Intratracheal Instillation of Stem Cells in Term Neonatal Rats". Thanks very much for Editor’s suggestions and the corrections of our writing in the manuscript. We have address specific comments marked in the attached manuscript.

The corrected parts were marked in red font.

Sincerely

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TITLE:

Intratracheal Instillation of Stem Cells in Term Neonatal Rats

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KEYWORDS:

intratracheal instillation, stem cells, bronchopulmonary dysplasia, respiratory disease, hyperoxia, neonatal rats

SUMMARY:

Described is a protocol for performing intratracheal transplantation of mesenchymal stromal cells (MSCs) through intratracheal injection in term neonatal rats. This technique is a clinically viable option for delivery of stem cells and drugs into neonatal rat lungs to evaluate their efficacy.

ABSTRACT:

Prolonged exposure to high concentrations of oxygen leads to inflammation and acute lung injury, which is similar to human bronchopulmonary dysplasia (BPD). In premature infants, BPD is a major complication despite early use of surfactant therapy, optimal ventilation strategies, and noninvasive positive pressure ventilation. Because pulmonary inflammation plays a crucial role in the pathogenesis of BPD, corticosteroid use is one potential treatment to prevent it. Nevertheless, systemic corticosteroid treatment is not usually recommended for preterm infants due to long-term adverse effects. Preclinical studies and human phase I clinical trials demonstrated that use of mesenchymal stromal cells (MSCs) in hyperoxia-induced lung injuries and in preterm infants is safe and feasible. Intratracheal and intravenous MSC transplantation has been shown to protect against neonatal hyperoxic lung injury. Therefore, intratracheal administration of stem cells and combined surfactant and glucocorticoid treatment has emerged as a new strategy to treat newborns with respiratory disorders. The developmental stage of rat lungs at birth is equivalent to that in human lungs at 26−28 week of gestation. Hence, newborn rats are appropriate for studying intratracheal administration to preterm infants with respiratory distress to evaluate its efficacy. This intratracheal instillation technique is a clinically viable option for delivery of stem cells and drugs into the lungs.

INTRODUCTION:

Supplemental oxygen is often required to treat newborn infants with respiratory distress[1]. However, hyperoxia therapy in infants has adverse long-term effects. Prolonged exposure to high concentrations of oxygen leads to inflammation and acute lung injury, which is similar to human bronchopulmonary dysplasia (BPD)[2]. BPD is a major complication of hyperoxia treatment that can occur in spite of early surfactant therapy, optimal ventilation procedures, and increased use of noninvasive positive pressure ventilation in premature infants. While many treatment strategies have been reported for BPD[3], no known therapy can reduce this complication.

Corticosteroid use is one potential treatment to prevent BPD, because pulmonary inflammation plays a crucial role in its pathogenesis. However, systemic corticosteroid therapy is not usually recommended for preterm infants due to long-term adverse effects[4,5].

Mesenchymal stromal cells (MSCs) have pluripotent characteristics and can differentiate into various cell types, including bone, cartilage, adipose tissue, muscle, and tendons[6]. MSCs have immunomodulatory, anti-inflammatory, and regenerative effects[7], and animal studies show the therapeutic benefits of MSCs and their secreted components in hyperoxia-induced lung injury in rodents[8,9]. Intratracheal and intravenous MSC transplantation has been shown to protect against neonatal hyperoxic lung injury. Therefore, intratracheal administration of stem cells and combined surfactant and corticosteroid therapy might be a potential treatment strategy to treat newborns with respiratory disorders. Preclinical studies have used intratracheal administration of stem cells and adeno-associated virus in newborn rats[10-12]. However, a step-by-step presentation of the technique and in vivo tracking of the transplanted stem cells is not available. The newborn rat is appropriate for studying the effects of intratracheal administration on preterm infants with respiratory distress because the saccular stage of the rat lung at birth is equivalent to that of the human lung at 26−28 week of gestation[13]. An effective method for administration into the rat trachea is crucial for successful pulmonary distribution. The technique presented here allows for the study of intratracheal administration of cells and/or drugs for treatment of neonatal pulmonary diseases using rats as a model for humans.

PROTOCOL:

This procedure was approved by the Animal Care and Use Committee at Taipei Medical University.

NOTE: Human MSCs stably transfected with green fluorescent protein (GFP) and firefly luciferase genes (Fluc) were obtained from a commercial company (**Table of Materials**).

1. Characterization of human MSCs with firefly luciferase and green fluorescent protein

1.1. Maintain human MSCs transfected with GFP and Fluc in complete media (minimum essential medium eagle-alpha modification [αMEM], supplemented with 10−15% fetal bovine serum [FBS], 2 mM L-glutamine, 1 ng/mL basic FGF, and PSF) at 37 °C with saturated humidity and 5% CO_2. Passage cells at ~70−90% confluence.

1.2. Observe MSCs under a fluorescence phase contrast microscope (**Figure 1A**) and analyze the expression levels of Fluc and GFP[14].

1.3. Characterize the MSCs by analyzing the expression of CD markers including CD44, CD73, CD90, CD105 using flow cytometry (**Figure 1B**). Induce trilineage differentiation of stem cells to adipocytes, chondrocytes, and osteocytes, and confirm trilineage differentiation (**Figure 1C**) by von Kossa, oil red O, and Alcian blue staining following a commercial protocol[15,16].

2. Anesthetization of rat pups

2.1. Allow time-dated pregnant Sprague–Dawley rats to deliver vaginally at term.

2.2. Remove the rat pups from the cage on postnatal day 5.

2.3. Anesthetize the rat pups using gas anesthesia (i.e., 2% isoflurane) in an anesthesia chamber.

2.4. Confirm adequate anesthesia by checking breathing and reflexes.

NOTE: Breathing should become shallow and reflexes should diminish. Rat pups remain unconscious for at least ~10 min with this isoflurane concentration.

3. Intratracheal instillation

3.1. Once anesthetized, restrain the rat pups on an intubation stand angled at ~60° and hold pups in place with laboratory labeling tape on all four limbs.

3.2. Apply tape below the nose to fix the head and pinpoint the neck for puncture tracheotomy.

3.3. Disinfect the incision area (i.e., neck) with a 75% alcohol prep pad.

3.4. Make a 0.3 cm vertical midline neck incision above the trachea with microscissors to avoid damaging the carotid arteries.

3.5. Dissect the fat and muscle layers away to locate the trachea with curved tip tapered tweezers without a hook.

3.6. Hold the trachea with the curved tip tapered tweezers.

3.7. Hold a 100 µL syringe upright and slowly inject 30 µL of normal saline (i.e., control) or 30 µL of Fluc-GFP labeled MSCs (1 x 10^5 cells) into the trachea through a 30 G needle syringe during inspiratory phase.

3.8. Close the incision with one 6-0 silk stitch, tie the knot as small as possible, and cut the ends

as short as possible.

3.9. Place the rats under infrared light or on a heating pad to keep warm and allow them to recover from anesthesia.

3.10. Confirm the rats are warm, pink, and capable of spontaneous movement before returning the rats to the cage.

4. Monitoring the pulmonary distribution of MSCs

4.1. To track the transplanted human MSCs, intraperitoneally inject the rats with luciferin potassium salt in phosphate buffered saline (PBS) at a dose of 125 mg/kg of body weight 15 min after MSC injection.

4.2. Anesthetize the rats using 2% isoflurane through nose cones.

4.3. Acquire sequential images at 5–15 s intervals 10 min after luciferin administration with medium binning, 1 f/stop, and a 26 cm field of view using a small-animal imaging system (**Table of Materials**).

4.4. Quantify the luminescence activity from the lungs based on the automatic regions of interest using imaging software (**Table of Materials**)[17].

REPRESENTATIVE RESULTS:

The pulmonary distribution of intratracheal instillation of stem cells in the term neonatal rats was determined by firefly luciferase (Fluc)-labeled stem cells. MSCs were labeled with Fluc and tagged with green fluorescent protein through lentiviral transduction. **Figure 1A** demonstrates a high level of GFP expression in human MSCs, and 93.7% of the population showed GFP positive expression detected by flow cytometry. MSCs were characterized by analyzing the expression of CD markers (i.e., CD 44, CD73, CD90, and CD105) and the capability of trilineage differentiation into osteocytes, chondrocytes, and adipocytes (**Figure 1B,C**). To track the transplanted human MSCs in vivo, luminescence imaging of the rats was performed using a small-animal imaging system. The measurements were taken ventrally. The rats were fixed with adhesive tape and subsequently an intraperitoneal injection of 30 mg/mL of luciferin potassium salt in PBS at a dose of 125 mg/kg of body weight was administered. Luciferase combines with luciferin, oxygen, and ATP, and generates light through a chemical reaction, resulting in bioluminescence[18]. During the imaging procedure, the rats were anesthetized using 2% isoflurane administered through nose cones. Rat images were acquired 10 min after luciferin administration. Sequential images were acquired at 5–15 s intervals (no time delay) for at least 1 min, with medium binning, 1 f/stop, and a 26 cm field of view. Using measurement data from a sequence of spectral images filtered at different wavelengths (560–660 nm), the depth and location of the bioluminescent reporter was determined. Luminescence signals from the lungs were calculated based on the automatic regions of interest in the circle selection mode. The average luminescence intensity in normal saline-treated animals was assigned a value of one, and data for each MSC treated animal were

normalized to those of normal saline-treated animals.

Figure 2A shows a representative image of luminescence in the rat lungs. No luminescence was observed in the lung regions of the rats treated with normal saline. The rats treated with MSCs exhibited luminescence in the trachea and central lung regions. Quantification of the luminescence intensity revealed that the rats treated with MSCs exhibited an approximately 13 fold increase in luminescence activity, compared with the rats treated with normal saline alone (**Figure 2B**).

FIGURE LEGENDS:

Figure 1: Characterization of human MSCs. (**A**) GFP expression in human MSCs after lentivirus transduction. (**B**) Expression of human MSC-specific CD markers (i.e., CD 44, CD73, CD90, CD105). (**C**) Trilineage differentiation of human MSCs.

Figure 2: Monitoring of pulmonary distribution of luminescence using a small-animal imaging system. (**A**) A representative image of the pulmonary distribution of the labeled MSCs in rats. No luminescence was observed in the lung region of rats treated with normal saline. Rats treated with human MSCs exhibited luminescence in the tracheal and lung regions. (**B**) Quantification of luminescence activity in the rat lungs (n = 4). The error bars represent standard deviation. The scale is photons/s/cm^2/sr in Y axis. **P < 0.01.

DISCUSSION:

Newborn infants with respiratory distress commonly require intratracheal surfactant and/or corticosteroid treatment[19]. Human phase I clinical trials have demonstrated the safety of intratracheal MSCs in preterm infants[8]. These studies suggest that intratracheal administration of drugs is an important option for newborn infants with respiratory distress. Animal model studies are most helpful if the model features are directly pertinent to humans. Term newborn rats are useful models for preterm lung injury and development studies. However, the upper airway of neonatal rats is too small to permit direct tracheal intubation as performed in adult rats[20]. Intratracheal instillation through tracheotomy is a feasible alternative technique for intratracheal administration of stem cells or drugs to the neonatal rat lungs.

In vivo bioluminescence imaging is a valuable tool for in vitro and in vivo monitoring of the fate of the transplanted stem cell, accomplished by labeling cells with the constitutive expression of a luciferase reporter protein[21]. Luciferase enzymes catalyze the oxidation of a substrate (luciferin), and release light as a product of the reaction. Visual imaging through bioluminescence allows a noninvasive and real-time analysis of disease processes in living organisms. Bioluminescence imaging was used for in vivo monitoring of the migration, survival, and morphological differentiation of the MSCs[22]. This study evaluated the distribution of the transplanted stem cells in the lungs using an in vivo imaging system. In vivo bioluminescence imaging relies on the monitoring of cell-contained particles. Because these may be phagocytosed upon cell death, it could lead to the tracking of host macrophages. Therefore, luminescence was measured less than 10 min after luciferin administration.

The limitation of this study is that interanimal variation was perceived in the IVIS images. Hence, the luminescence signals from the lungs were calculated based on the automatic regions of interest and normalizing the average luminescence intensity to one in normal saline-treated animals.

Correct and effective intratracheal instillation is essential for the evaluation of MSC efficacy in the neonatal rats, but it may be useful for testing other medicinal treatments as well. Hence, this rat model technique may be adjustable to a variety of pulmonary applications. Intratracheal instillation of stem cells or medicines represents a relatively easy and cost-effective treatment of pulmonary diseases.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

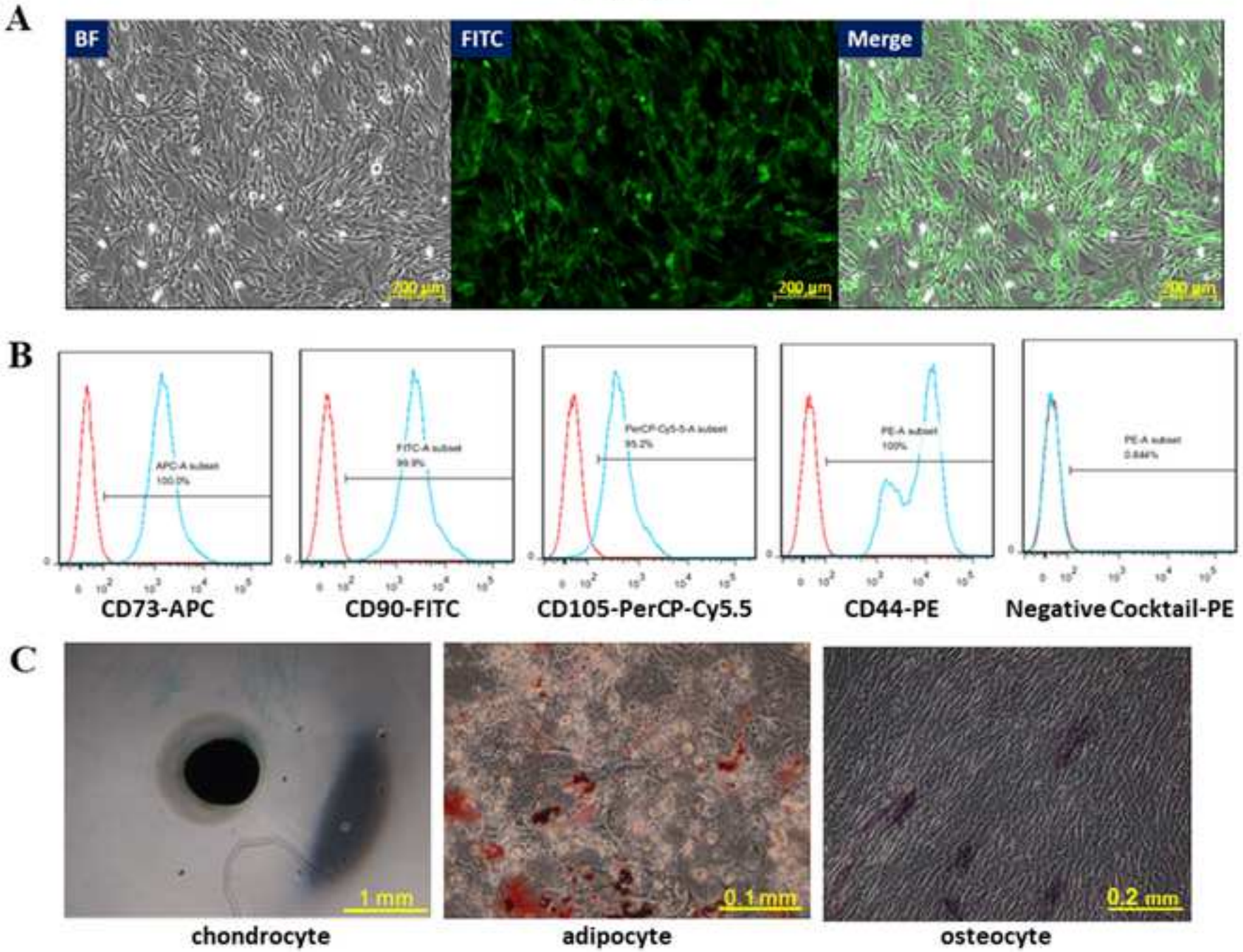
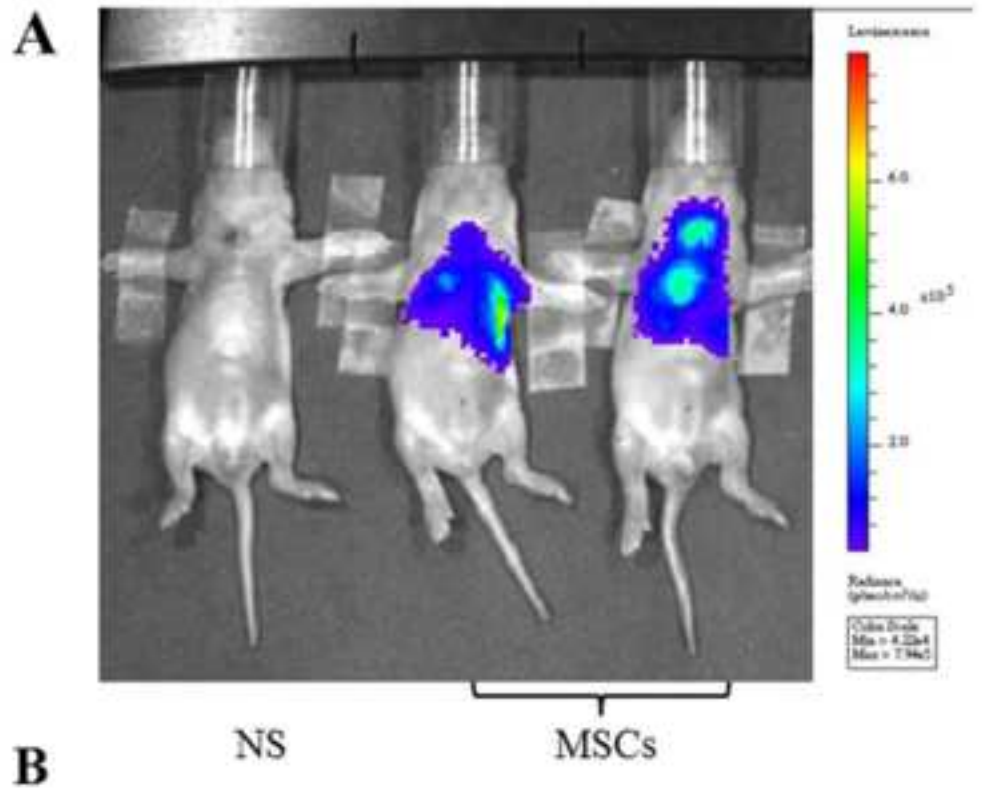


Figure 2



Name of Material/Equipment	Company	Catalog Number	Comments/Description
6-0 silk	Ethicon	1916G	
Alcohol Prep Pad	CSD	3032	
BD Stemflow hMSC Analysis Kit	BD Biosciences	562245	CD markers
CMV-Luciferase-EF1α-copGFP BLIV 2.0 Lentivector for In Vivo Imaging	SBI	BLIV511PA-1	
CryoStor10	BioLife Solutions	640222	
Human MSCs	Meridigen Biotech Co., Ltd. Taipei, Taiwan		
Infrared light	JING SHANG	JS300T	
Isoflurane	Halocarbon	26675-46-7	
IVIS-200 small animal imaging system	Caliper LifeSciences, Hopkinton, MA		
Luciferin potassium salt	Promega, Madison, WI		
Micro-scissors, straight	Vannas	H4240	
Normal saline	TAIWAN BIOTECH CO., LTD.	113531	Isotonic Sodium Chloride Solution
Small Hub RN Needle, 30 gauge	Hamilton Company, Reno, NV	7799-06	
Syringe (100 µl)	Hamilton Company, Reno, NV	81065	
Xenogen Living Image 2.5 software	Caliper LifeSciences, Hopkinton, MA	N/A	

Response to Editorial comments:

This is in regard to our manuscript JoVE61117 entitled "Intratracheal Instillation of Stem Cells in Term Neonatal Rats". Thanks very much for Editor's suggestions and the corrections of our writing in the manuscript. We have address specific comments marked in the attached manuscript.