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Novel Methods for Delivery of Cell-Based Therapies

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Background. **Pulmonary hypoplasia (PH) is found in 15% to 20% of all neonatal autopsies, accounting for 2850 deaths yearly. Development of engineered tissue substitutes that could functionally restore damaged tissue remains a unique opportunity for biotechnology. Recently, we isolated and characterized murine fetal pulmonary cells (FPC) and engineered 3-D pulmonary tissue constructs *in vitro*. Our goal is to devise a reliable and reproducible method for delivering FPC into a live animal model of PH.**

Materials and methods. **Three methods of delivery were explored: intraoral, intratracheal, and intrapulmonary injection. Adult Swiss Webster mice were anesthetized and fluorescent labeled microspheres (20 μm diameter) were delivered by intraoral and intratracheal injection. Subsequently, labeled FPC (Cell Tracker, CMTPX; Molecular Probes, Eugene, OR) were delivered by the same methods. In addition, direct transpleural intrapulmonary injection of FPC was performed. Outcome analysis included survival, reproducibility, diffuse *versus* confined location of the injected substance, and adequacy of delivery. Routine histological examination, fluorescent microscopy, and immunostaining were performed.**

Results. **Microspheres: We demonstrated reproducible, diffuse instillation via tracheotomy into the distal alveoli. Intraoral delivery appeared less reliable compared to direct intratracheal injection. FPC: Intratracheal injection was a reliable method of delivery. Labeled FPC showed transepithelial migration after 7 d of *in vivo* culture. Intrapulmonary injection led to local accumulation of cells in sites of injection.**

Conclusions. **We demonstrate that delivery of FPC is feasible with intratracheal injection giving the most reliable, diffuse delivery throughout the lung. This represents the first step toward translational research with site-specific delivery for a cell-based therapeutic approach toward PH and similar pulmonary diseases.**

Key Words: **cell-based therapy; pulmonary hypoplasia; murine fetal pulmonary cells; intratracheal injection; intraoral injection; intrapulmonary injection; transepithelial migration.**

INTRODUCTION

Preterm delivery with resultant developmental lung abnormalities (i.e., pulmonary hypoplasia [PH]) is a major problem in neonatology and accounts for more than 70% of perinatal mortality [1]. The pathology of PH includes reduced lung mass, insufficient surfactant production, poorly differentiated alveolar epithelium, and a reduction of alveolar gas exchange [2].

The loss or failure of lung tissue is one of the most frequent, devastating, and costly problems in health care. The most frequently used and successful method of therapy is transplantation. However, the severe scarcity of donor organs, especially in the pediatric population, is a major limitation and has thus stimulated investigation into selective cell transplantation and other molecular-based therapies [3–8].

Because of advances in tissue engineering, novel cell-based therapies are emerging as available treatment modalities for damaged tissues [7, 8]. Delivery of engineered cells into hypoplastic lung tissue has the potential to improve underdeveloped lung tissue and aid in restoring the process of natural tissue development. Drug delivery alone does not have the ability to achieve both of these effects.

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We have recently been able to demonstrate histiotypic tissue construct formation from a mixed population of fetal pulmonary cell culture *in vitro* [9, 10]. After characterization of the cells, we further examined methods of introduction into the lung parenchyma. We began by investigating three different delivery methods for the *in vivo* administration of fetal pulmonary cells (FPC) into the pulmonary system.

MATERIALS AND METHODS

The Institutional Animal Care and Usage Committee (IACUC no.16150) at Drexel University approved all animal protocols in compliance with the Guide for the Care and Use of Laboratory Animals. Adult Swiss Webster female mice (Charles River Laboratories, Wilmington, VA) were injected with different substrates between March 2006 and December 2006.

Methods of Injection (Fig. 1)

Intraoral Injection

Adult Swiss Webster mice were anesthetized with isofluorane. Direct laryngoscopy with a small spatula was performed and a 24 gauge angiocathether was inserted into the oropharynx to deliver either microspheres (100 μL suspension, microsphere solution diluted 1:1 in 10X phosphate-buffered saline [PBS]) or fluorescently labeled fetal pulmonary cells (10 million CMTPX CellTracker labeled FPC suspended in 100 μL of medium as described below).

Intratracheal Injection

Adult Swiss Webster mice were anesthetized with isofluorane. A small incision was made over the anterior neck in a transverse fashion. Blunt dissection was used to identify the trachea and a 27 gauge needle was inserted between the tracheal rings. Microspheres or labeled FPC (10 million labeled FPC suspended in 100 μL of medium) were delivered as described below. Adequate injection was evidenced by visualization of the suspension through the tracheal tissue with minimal reflux of suspension back through the nose. Pain relief was obtained with buprenorphine (0.2 mL Buprenex diluted 1:100 in 10X PBS) injected subcutaneously before termination of the procedure. The incision site was closed with 4-0 silk sutures.

Intrapulmonary Injection

Adult Swiss Webster mice were anesthetized with isofluorane and a skin incision was made over the right chest. The muscle layers were dissected sharply until the lung was visualized through the intercostal spaces. With a 27 gauge needle 10 million labeled FPC in 100 μL of 1 mg/mL collagen Type 1 solution (BD Biosciences, San Jose, CA) were injected through the intercostal space directly into the lung parenchyma. The collagen solution was used as a delivery vehicle to localize the distribution of the engrafted cells, as the collagen solution gels rapidly at 37°C. The skin was closed in an interrupted fashion with 4-0 silk sutures. Buprenorphine was administered subcutaneously for pain relief as described above.

Spheres and Labeled FPC (Fig. 1)

Sphere Delivery

One hundred μL of a solution containing fluorescent microspheres (microspheres diluted 1:1 in 10X PBS, yellow; 20 μm diameter, Polysciences, Warrington, PA) were administered with a 24 gauge angiocatheter intraorally or with a 27 gauge needle intratracheally. After the procedure, the animals were housed overnight. Lungs were harvested on the next day, washed in 10X PBS and fixed in paraformaldehyde overnight. Tissue was embedded in OCT compound (Triangle Biomedical Sciences, Durham, NC), snap-frozen at −80°C and stored at −80°C. Thirty micron sections were prepared with a cryostat. Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlington, CA) for nuclear counterstaining.

Cellular Delivery

Labeled FPC were administered intraorally, intratracheally or intrapulmonary, as described above.

FPC Isolation

Murine FPC were obtained from the lungs of timed pregnant Swiss Webster mouse fetuses at gestational day 18 (Charles River Laboratories) as previously described [9–11]. Briefly, fetal lungs were surgically removed, rinsed in Hanks balanced salt solution (HBSS; Cellgro, Herndon, VA) and minced. Following mincing, the tissue was first triturated in and then digested with 0.5% trypsin in PBS for 5 and 20 min, respectively. Following quenching of the trypsin with Dulbecco's modified Eagle medium containing FBS (Cambrex, East Rutherford, NJ) and filtration through a 70 μ filter (BD Falcon, San Jose, CA), the cell suspension was pelleted for 5 min at 800 rpm. The pellet was resuspended for 30 s in 900 μL distilled water to remove the RBCs by hypotonic lysis, followed by the addition of 100 μL of 10 × PBS (Cellgro). The cells were washed once more in 1 × Ca^{2+}/Mg^{2+} containing PBS, and resuspended in complete medium (DMEM +10% fetal bovine serum + antibiotics). A suspension of 10 million CMTPX CellTracker labeled FPC in 100 μL of medium or 100 μL of 1 mg/mL collagen Type 1 solution was injected.

Labeling

To identify the injected FPC *in vivo*, the cells were labeled *in vitro* with Cell Tracker (CMTPX; Molecular Probes, Eugene, OR), a fluo-

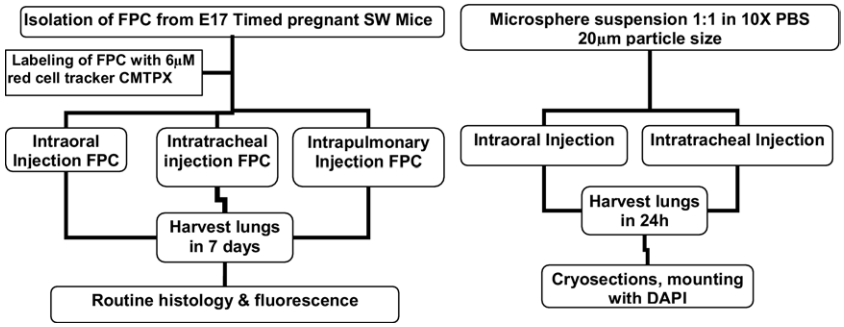

FIG. 1. Schematic depicting the delivery of fluorescent microspheres and labeled FPC, harvesting and sample processing times.

rescent probe that is retained in living cells through several generations. It is not transferred to adjacent cells. The labeling procedure, performed according to the manufacturer's instructions, consisted of incubating the cells with 25 μM CMTPX for 30 min in serum-free medium, followed by three 5 min washing steps in 1X PBS. The CMTPX CellTracker passes freely through cell membranes and, unlike some of the other CellTracker dyes, it does not require enzymatic activity once in the cell to activate fluorescence.

Housing, Harvesting and Sample Processing (Fig. 1)

After the surgical procedure, the animals were housed for up to 1 wk, depending on the protocol when the lungs were harvested (Fig. 1). Following harvesting, the lungs were washed in 10X PBS and fixed in paraformaldehyde overnight. Routine histology and immunostaining for surfactant protein C (ProSpC, Type 2 alveolar epithelial cells) were performed. Identification of alveolar epithelial cells was carried out by immunofluorescence as previously described [9, 10, 12].

Statistical Analysis

Comparison of intraoral (IO) versus intratracheal (IT) injection was performed in both the microsphere group and the labeled FPC group (Fig. 4). Examination of serial micrographs was performed and the percentage of animals with adequate delivery was calculated as the adequate delivery/inadequate delivery $\times$ 100. The adequate delivery percentage was then compared between groups (IO *versus* IT) and analyzed statistically using Fisher's exact test, with $P < 0.05$ considered statistically significant. Error bars represent variance.

RESULTS

Intraoral and Intratracheal Administration of Microspheres

Intraoral Route

The first route explored for delivery of substances into the pulmonary parenchyma was the intraoral, which used cell-sized fluorescent microspheres as cell surrogates. The quality of the injection was defined as "adequate delivery" if more than 10 microspheres were identified per 400$\times$ high power field. These results were assessed in randomly acquired images from harvested lungs of eight mice. Three of the injections were identified as adequate (37.5%) and five as poor (62.5%). Figure 2 depicts adequate injections of microspheres in the intratracheal and intraoral groups, showing more than 10 microspheres per high power field in the proximal and distal airways.

Intratracheal Route

In search of a more reliable and reproducible delivery route, the intratracheal route was explored, which ensured direct placement of the needle into the trachea. Microspheres were administered intratracheally to 23 mice. Two animals were excluded from the analysis due to intraoperative death. One animal died due to excessive dissection and pneumothorax and the sec-

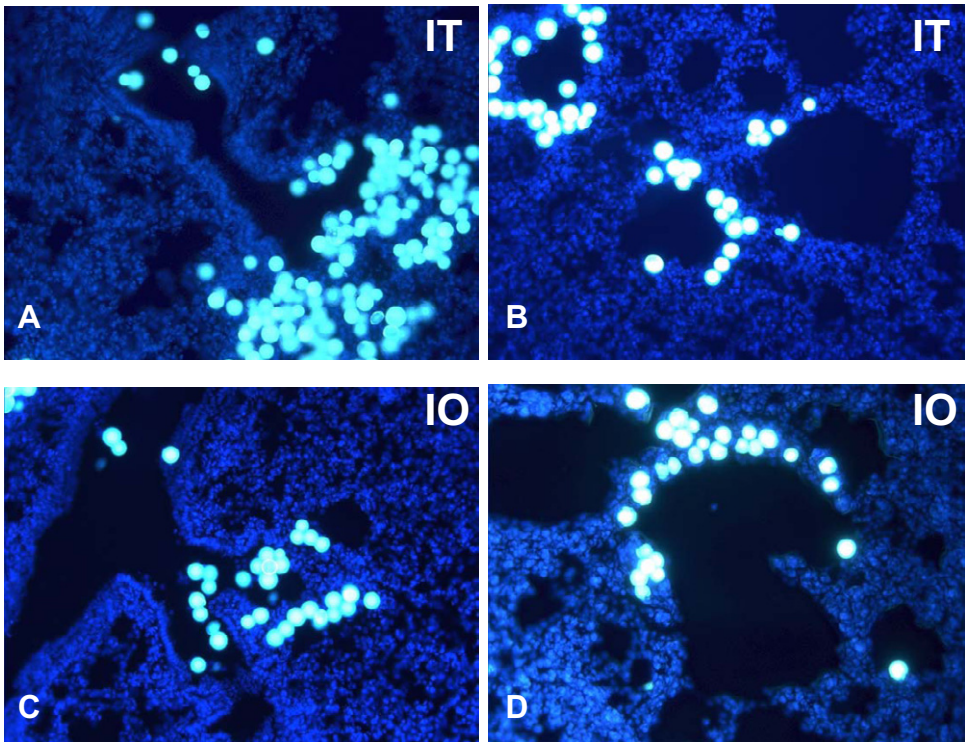

FIG. 2. (A)–(B) Microspheres: fluorescent microscopy of IT injected microspheres (yellow, particle size 20 μm) evident in proximal (Fig. 1A) (200$\times$) and distal (Fig. 1B) (400$\times$) airway of adult mice. (C) and (D) Fluorescent microscopy of IO injected microspheres evident in proximal (Fig. 1C) and distal (Fig. 1D) airway of adult mice; with DAPI (blue) nuclei counterstaining.

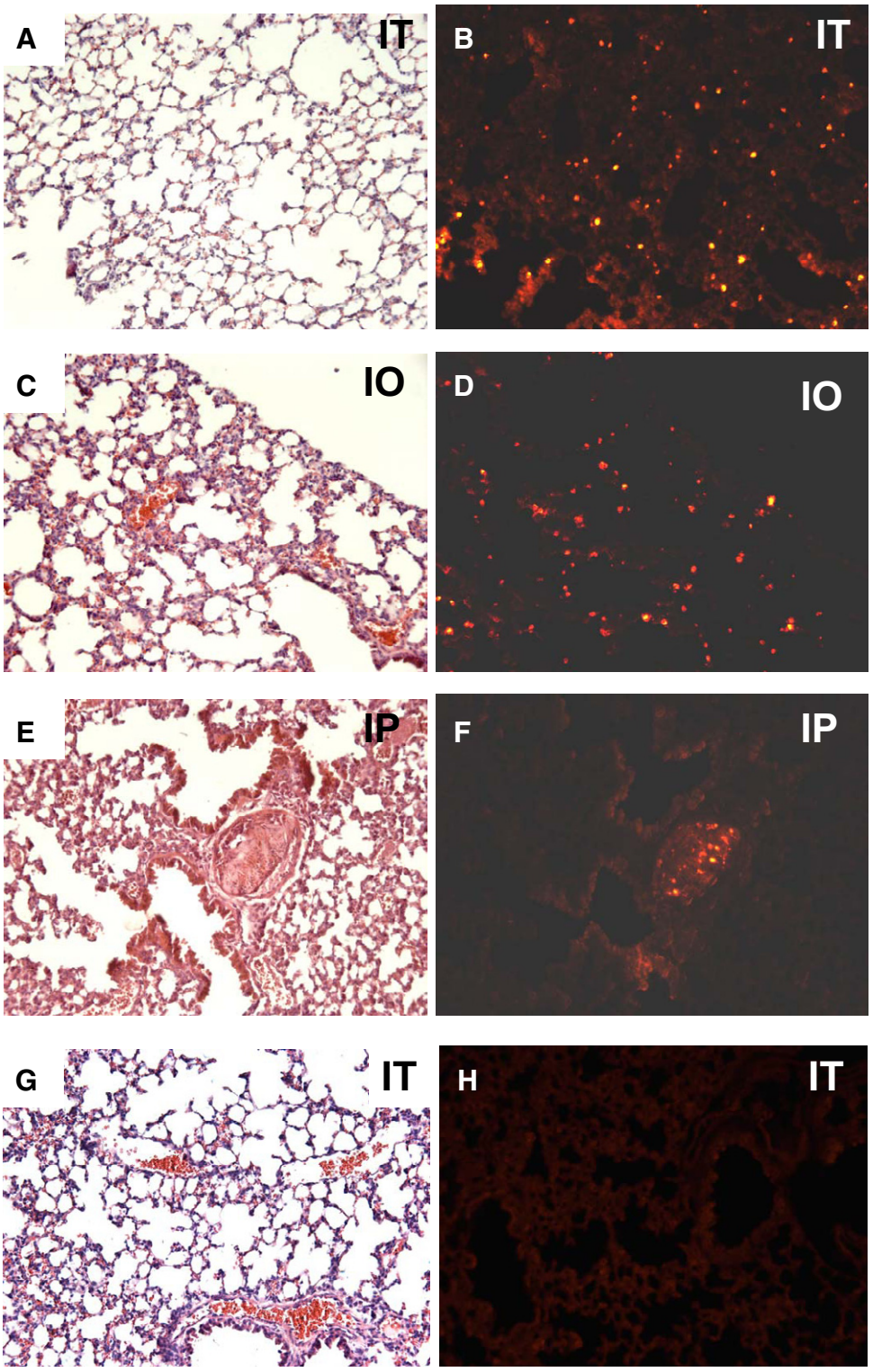

FIG. 3. Representative distribution of engrafted fetal pulmonary cells in successful experiments using the three injection methods, as described in the Materials and Methods section. Shown are serial sections stained with H and E and the subsequent section photographed for the distribution of the CellTracker CMTPX-labeled FPC in lungs instilled via intratracheal injection (A), (B), intraoral injection (C), (D), and intrapulmonary injection (E), (F). (G) and (H) illustrate a control animal injected intratracheally with unlabeled FPC, (no CellTracker CMTPX-labeling used), depicting minimal tissue autofluorescence; all micrographs are 200× magnification.

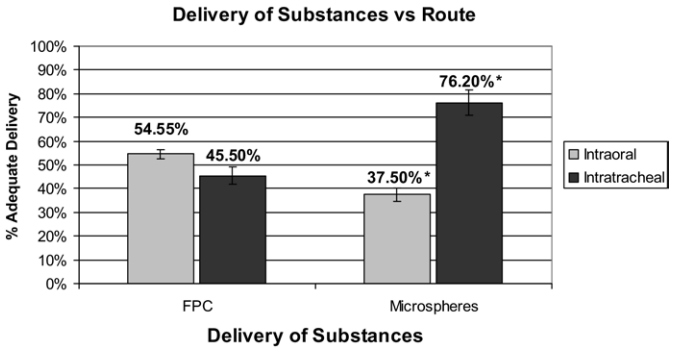

FIG. 4. Labeled FPC and microspheres delivery outcomes according to different delivery route (IO *versus* IT). Results for adequate delivery expressed in percentage value (*values are statistically significant with $P < 0.05$). Error bars represent variance.

ond due to buprenorphine overdose. Of the 21 mice included, 16 were identified as adequate injections (76.2%) (Fig. 2A, B) and five as poor (23.8%) ($P < 0.05$).

Intraoral and Intratracheal Administration of Fetal Pulmonary Cells

Figure 3 depicts routine H and E and fluorescent micrographs of serial lung sections after intratracheal, intraoral, and intrapulmonary delivery of labeled FPC. The percentage of animals with adequate delivery in the intratracheal and intraoral groups are shown in Fig. 4.

Intraoral Route

Of a total of 11 mice administered labeled FPC intraorally, six were identified as adequate (54.5%) and five as poor (45.5%) (Fig. 3C, D). Comparing the microsphere delivery intraorally with the labeled FPC delivery intraorally, there was a trend for an improvement in the number of adequate injections from 37.5% to 54.5% (P = NS) (Fig. 4).

Intratracheal Route

A total of 23 mice were administered labeled FPC by intratracheal injection. One animal was excluded due to excessive dissection leading to pneumothorax and immediate intraoperative death. Of the 22 mice included, 10 were identified as adequate injections (45.5%) (Fig. 3A, B; Fig. 4), and 12 as poor injections (54.5%). Given the high rate of poor injections (54.5%), further intratracheal experiments with microspheres were carried out to improve the technique and limit amount of animal usage. To allow for comparison and standardization of tissue autofluorescence, an intratracheal experiment with unlabeled FPC was included (Fig. 3G, H).

Transepithelial Migration of FPC Administered Intratracheally

The intratracheally delivered labeled FPC showed evidence of transepithelial migration after 7 d of *in vivo* culture. Figure 5A and B show fluorescent micrographs in which labeled FPC are seen not only in the alveolar lumen but also within the lung parenchyma. In addition, Fig. 5C illustrates co-localization (yellow) of Type 2 alveolar epithelial cells with prosurfactant protein C (green) and labeled FPC (red), which provides initial evidence that alveolar Type 2 cells of donor origin may integrate into the lung epithelium/parenchyma.

Intrapulmonary Route

Experiments with microspheres were not carried out in this experimental subset since the intrapulmonary route delivers the substance directly into the lung parenchyma. Labeled FPC were administered intrapulmonary to 16 mice. The mortality rate associated with this procedure was 6.25%. As evidenced in Fig. 3E and F, the intrapulmonary injection of labeled FPC resulted in the formation of pockets of cells that did not appear to disperse throughout the tissue. These cells did not form any discernable structures within the pockets. In addition, the pockets formed by intrapulmonary injection did not interface with the surrounding tissue and incited an apparent local fibrotic response (fibrous tissue in H and E section, Fig. 3E). In these "successful" intrapulmonary injections, no hemorrhage on H and E staining occurred in the host lungs. However, due to the inability to visualize the path of the needle, 6.25% of intrapulmonary injections resulted in severe hemorrhage (Fig. 6, RBCs evident in H and E sections) and lung injury.

DISCUSSION

In this study, we explored potential methods for delivering *in vitro* engineered fetal pulmonary cells as a first step toward translational research. Initially, we investigated the distribution of microspheres, chosen as particles that model cells; administered via intraoral and intratracheal injection into the airways as a means of testing the feasibility of the approach for distributing cells/particles into the respiratory tree. Second, we used fluorescently labeled isolated FPC and investigated intraoral and intratracheal injection, attempting to achieve diffuse, intrapulmonary distribution. Furthermore, we examined direct intrapulmonary injection to achieve localized delivery of labeled FPC. We evaluated our results based on animal survival, diffuse *versus* local dissemination of the injected substance, and reproducibility as well as adequacy of delivery. The main results of this study indicate that (1) delivery of cells into live animals via all three routes is feasible, and (2) the intratracheal route appears to

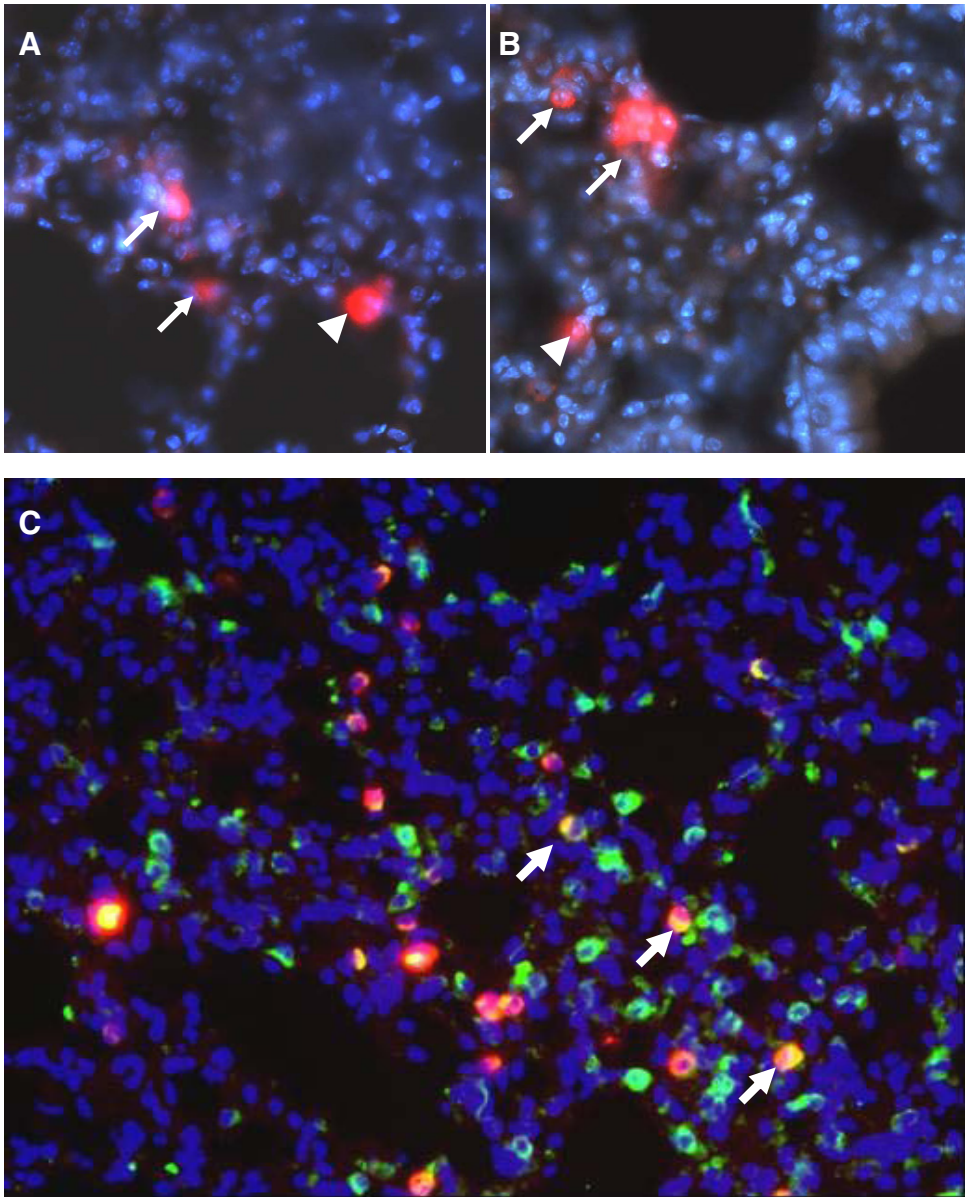

FIG. 5. (A) and (B) Fluorescent microscopy of intratracheally injected CellTracker CMTPX-labeled FPC (red) with evidence of transepithelial migration. Labeled FPC are seen not only in the alveolar lumen (arrowheads), but also within the lung parenchyma (arrows); all micrographs are 400× magnification. (C) Fluorescent microscopy of intratracheally injected CellTracker CMTPX-labeled FPC (red). Immunoreactivity for Pro-Surfactant protein C is displayed in green (fluorescent stain for Type 2 alveolar epithelial cells), while nuclei appear blue (DAPI blue nuclei staining). Yellow stains represent confluence of ProSpC and cell tracker stains (arrows); micrograph 200× magnification.

be the most reliable and reproducible route for diffuse delivery of cells into the distal airways.

Not surprisingly, a clear learning curve was found with each of the experimental procedures. The intratracheal method appeared to deliver the cells diffusely and reliably into the alveolar spaces. We believe that the direct access to the trachea close to the carina lessens the distance the cells need to travel when compared with the intraoral delivery route. However, the intratracheal group resulted in a higher mortality rate than the intraoral group. Overall, the intraoral group mortality was 0%, whereas the mortality rate in the intratracheal group was 6.5%. Mortality cases occurred predominantly at the beginning of experimental procedures, suggesting they were related to the precise surgical technique. This indicates that intratracheal administration is more technically demanding, and follows a learning curve, with adequate delivery increasing from 45.5% (FPC group) to 76.2% (microsphere group), $P < 0.05$, (Fig. 4).

As shown in Fig. 3, administration of labeled FPC into the distal airways via direct intratracheal (tracheotomy) or intraoral injection resulted in a relatively uniform distribution of engrafted FPC in the periph-

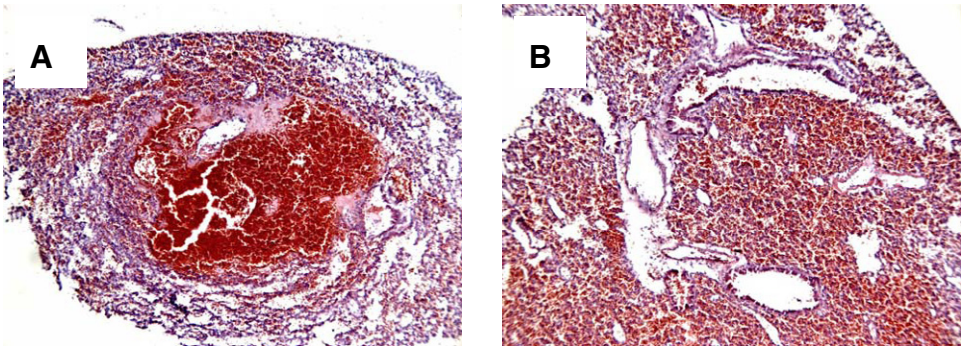

FIG. 6. (A) and (B) H and E micrographs showing intrapulmonary injection of CellTracker CMTPX-labeled FPC with noticeable hemorrhage. (A) 100× magnification; B, 200× magnification.

eral airway tissue in adequate injections. This suggests that administration of cells via the airways is a viable method of distributing cells widely within the lung parenchyma. In addition, we demonstrate with the intratracheal delivery of labeled FPC, evidence of transepithelial migration of cells 7 d after injection (Fig. 5A and B, arrows). This finding is significant since it indicates that it is possible for cells delivered intratracheally to intercalate into the distal airways and enter the lung parenchyma. This phenomenon would be a requirement for cell-based therapy of PH to be successful. Initial phenotypic characterization of the labeled FPC indicated that a subpopulation of the injected FPC maintained Type 2 alveolar cell phenotype. This suggests that the intratracheal method may be a useful means of instilling "engineered, optimized" cells in future cell-based pulmonary therapeutics, although the integration and functionality of specific pulmonary cell lineages in normal or diseased lung are unknown. We are currently studying the fate of engrafted cells in normal and diseased lungs in our laboratories.

In our study, we aimed to optimize the method of delivery and compare previously published methods of delivering cells into pulmonary tissue. Different types of cells have been delivered intratracheally or intrapulmonary [13–15], with no clear consensus regarding adequacy of delivery that would translate into potential successful therapeutic results. The intratracheal administration of mesenchymal stem cells as a means of treating endothelial dysfunction in pulmonary hypertension was described by Baber *et al.* [13]. In their experiments, a similar concentration of cell suspension was injected intratracheally with successful results toward attenuation of pulmonary hypertension. In addition, the injected cells were followed for a period of 21 d. Similarly, Yang *et al.* [14] reported the intratracheal delivery of engineered dendritic cells as a means of treating lung epithelial cancers. The authors described the delivery of fewer amounts of unlabeled dendritic cells intratracheally resulting in increased local leukocyte infiltration, decreased tumor progression, and increased survival due to dendritic cells' effects within the cancerous lung tissue. Andrade *et al.* [15], in work most closely related to ours, delivered rat FPC into the pulmonary cavity as a potential method for the treatment of emphysema. They demonstrated adequate delivery of cells directly into the lung parenchyma, without any local inflammatory reaction. They followed *in vivo* implanted labeled rat FPC for a period of 35 d. However, the number of cells and the kinetics of cell migration were not of sufficient magnitude to be useful for tissue regeneration. Our results presented here suggest that differences among reported studies will be secondary to the fact that the technique requires a learning curve, and illustrates the fact that much work needs to be done before such procedures can be standardized for clinical practice.

In summary, the intratracheal route of delivery of substances was the most reliable and reproducible route for delivery of fetal pulmonary cells into the distal airway. In addition, the intratracheally delivered FPC showed transepithelial migration through the alveoli after 7 d of *in vivo* culture. This finding suggests incorporation of intratracheally injected fetal pulmonary cells into the recipient lung parenchyma, with initial evidence of alveolar Type 2 cell integration. Further research in this innovative area on the fate and functionality of engrafted cells will hopefully lead to the realization of cell-based pulmonary therapeutics for both pediatric and adult lung pathologies.

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