Cell-based tissue engineering for lung regeneration

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Andrade CF, Wong AP, Waddell TK, Keshavjee S, Liu M. Cell-based tissue engineering for lung regeneration. Am J Physiol Lung Cell Mol Physiol 292: L510–L518, 2007. First published October 6, 2006; doi:10.1152/ajplung.00175.2006.—Emphysema is a chronic lung disease characterized by alveolar enlargement and tissue loss. Tissue engineering represents an attractive potential for regeneration of several organ systems. The complex three-dimensional architectural structure of lung parenchyma requiring connections of alveolar units to airways and the pulmonary circulation makes this strategy less optimistic. In the present study, we used Gelfoam sponge as a scaffold material, supplemented with fetal rat lung cells as progenitors, to explore the potential application of cell-based tissue engineering for lung regeneration in adult rats. After injection into lung parenchyma, the sponge showed porous structures similar to alveolar units. It did not induce severe local inflammatory response. Fetal lung cells in the sponge were able to survive in the adult lung for at least 35 days, determined by CMTMR [5-(and-6)-[[(4-chloromethyl)benzoyl]amino]tetramethylrhodamine] labeling. Proliferation of cells within the sponge was demonstrated in vivo by bromodeoxyuridine (BrdU) labeling. Cells formed “alveolar-like structures” at the border between the sponge and the surrounding lung tissue with positive immunohistochemical staining for epithelial and endothelial cells. Neovascularization of the sponge was demonstrated with India ink perfusion. The sponge degraded after several months. This study suggests that cell-based tissue engineering possesses the potential to regenerate alveolar-like structures, an important step towards our ultimate goal of lung regeneration.

Increased interest has been focused on the use of stem cells as a potential source for replacement of damaged pulmonary cells (32, 34). Stem cells likely play a key role in repair of lung injury (15, 17, 18, 33), but regeneration of lung tissue remains more challenging. Injecting cell suspensions directly into lung tissue or transfusion of cell suspensions into the pulmonary vasculature may lead cells to areas where the alveolar wall and pulmonary capillary vessels still exist, not necessarily to the areas where normal tissues have been lost. Other substances have also been studied for treatment of end-stage lung disease, such as retinoic acid, which is able to reverse anatomic and physiological signs of pulmonary emphysema induced by elastase instillation in a rat model (29). However, the role of retinoic acid as a therapeutic alternative for emphysema or its involvement in bone marrow mobilization in lung regeneration needs to be proven (9, 15, 26).

In the present study, we proposed to use a tissue engineering approach for lung regeneration. Tissue engineering is the creation of living, physiological three-dimensional tissues or organs utilizing specific combinations of cells, scaffolds, and cell signals (13). Tissue engineering has been explored in other organ systems, such as skin, blood vessels, heart valve leaflets, bone, cartilage, and bladder (11, 37). A common feature of tissue engineering in these organ systems is the production of a continuous sheet or mass of tissue before implantation. The complex three-dimensional architectural structure and the flexible nature of lung mechanics make tissue engineering especially challenging when it comes to the lung.

In previous studies, Gelfoam sponges have been used as a substratum for cell culture, to determine the mechanisms of fetal lung growth and differentiation (30, 31, 40, 41). Dispersed, fetal rat lung cells cultured on sponges formed “alveolar-like structures” within 7 days. Microvilli were found on the apical side of the polarized epithelial cell layer, and mesenchymal cells were found at the basolateral side (22, 36). In this three-dimensional culture, cells proliferated (22, 24) and retained their specific functions, such as producing lung surfactant (6) and extracellular matrix molecules (30, 31, 40, 41). In addition to fetal lung cells, pulmonary arterial endothelial cells developed a configuration similar in appearance to the endothelium in the small vessels on Gelfoam sponge (3). Endothelial and vascular smooth muscle cells from adult rat aorta also attach very well to sponges (20). Gelfoam has been used clinically as a hemostatic surgical material for decades. This soft and pliable material can be completely absorbed in soft tissue with little tissue reaction.

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In the present study, we used Gelfoam sponges as scaffold materials and fetal rat lung cells as the progenitor cells. Interestingly, when injected into the lung, the porous structure of sponge resembles alveolar units, and these structures ultimately became connected to the pulmonary circulation, suggesting that tissue engineering could become a new avenue for lung regeneration.

MATERIALS AND METHODS

All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23), and the Guide to the Care and Use of Experimental Animals formulated by the National Society for Laboratory Animal Care (National Institutes of Health publication no. 85-23, Revised 1996, US Government Printing Office, Washington, DC 20402-9325), and the Guide to the Care and Use of Laboratory Animals (8th edition, National Institutes of Health). All animals received care in compliance with all applicable federal, state, and local laws and regulations. The animals were housed in temperature-controlled rooms with a 12/12-hr light/dark cycle, provided food and water ad libitum, and were killed by an overdose of pentobarbital sodium. The in situ lung experiments were performed in compliance with the Guide to the Care and Use of Laboratory Animals formulary (National Institutes of Health Publication no. 85-23, Revised 1996, US Government Printing Office, Washington, DC 20402-9325), and the Guide to the Care and Use of Laboratory Animals (8th edition, National Institutes of Health). All animals received care in compliance with all applicable federal, state, and local laws and regulations. The animals were housed in temperature-controlled rooms with a 12/12-hr light/dark cycle, provided food and water ad libitum, and were killed by an overdose of pentobarbital sodium. The in situ lung experiments were performed in compliance with the Guide to the Care and Use of Laboratory Animals formulary (National Institutes of Health Publication no. 85-23, Revised 1996, US Government Printing Office, Washington, DC 20402-9325), and the Guide to the Care and Use of Laboratory Animals (8th edition, National Institutes of Health).

Fetal lung cell isolation. Fetal lung cells were isolated and cultured as previously described (25). Pregnant Fischer 344 rats (200–250 g; Charles River, Montreal, Canada) were anesthetized in an isoflurane chamber and intubated with a 14-gauge intravenous cannula. The lungs were ventilated with a volume-controlled ventilator (model 683; Harvard Rodent Ventilator, South Natick, MA), with a fraction of inspired oxygen of 1.0, isoflurane 1–3%, and a tidal volume of 10 ml/kg at 80 breaths/min. A midline laparotomy was performed, and the fetuses were aseptically removed at day 19 of gestation (term = 22 days). Fetal lungs were pooled from at least two litters for each cell preparation. Fetal lungs were dissected out in cold Hanks’ balanced salt solution without calcium or magnesium (HBSS(−)) and cleared of major airways and vessels. The lungs were washed twice in HBSS(−), minced, and suspended in HBSS(−). The lungs were digested with trypsin solution (0.125% trypsin and 0.002% of DNase) for 25 min. The tissue suspension was filtered through a 100-μm nylon mesh blotting cloth (Nitol HC-3-100; Tekto, Depew, NY). DMEM with 5% FBS was added, and the mixture was centrifuged. The pellet was resuspended in DMEM containing 0.1% collagenase (Worthington, Freehold, NJ). After 15-min incubation at 37°C, the collagenase activity was neutralized by adding DMEM plus 5% FBS. Twenty microliters of mixed fetal lung cells were inoculated onto Gelfoam sponges (5 × 5 × 2 mm3) at a density of 6 × 104 to 6 × 105 cells per sponge. After inoculation, cells were incubated semidry at 37°C for 2 h before the addition of 1 ml of DMEM plus 5% FBS. Medium was changed every other day.

Gelfoam implantation. Gelfoam sponges alone or sponges with fetal lung cells were implanted into the rat lungs for varying periods of time. Five animals were used for each time point. Male rats (200–250 g; Fischer 344, Charles River) were anesthetized and ventilated as described above. Preoperatively, the animals were given 15 mg/kg im cefazolin (Kefzol; Eli Lilly, Toronto, Canada). Local anesthesia was performed with 0.5 ml of 1% lidocaine (AstraZeneca, Mississauga, Canada) at the incision site. A left thoracotomy was performed as described above. This method reliably reveals vascular connections between the graft and the vasculature of the lung (7, 16). Black ink particles were identified in blood vessels within the sponge.

Immunohistochemistry. To determine cell proliferation in vivo, animals received 25 μg of bromodeoxyuridine (BrdU) subcutaneously 24 h before death. Immunohistochemistry was performed on 5-μm sections taken from the paraffin-embedded tissues. Briefly, after deparaffinization, antigen retrieval was performed (Digest All Pepsin; Zymed Laboratories, South San Francisco, CA) followed by blocking of endogenous peroxidase activity with 1.5% H2O2 in methanol. Non-specific sites were blocked with 5% serum for 1 h at room temperature. Sections were then incubated at 4°C overnight with primary antibodies against Clara cell secretory protein (CCSP; 1:2,000 dilution; Upstate Biotechnology, Lake Placid, NY) for bronchiolar epithelial cells, prosurfactant protein C (proSP-C; 1:500 dilution; Chemicon International, Temecula, CA) for alveolar epithelial cells, von Willebrand factor (vWF; 1:1,000 dilution, Chemicon) for endothelial cells, and CD45 (1:1,000 dilution; Abcam, Cambridge, MA) for monocytes/macrophages.
MA) for leukocytes. After an extensive wash, the appropriate secondary antibodies were applied (Vector Rabbit and Mouse Elite Kits; Vector Laboratories, Burlingame, CA). Immunostaining was visualized with 3–3’-diaminobenzidine (Vector Laboratories), and nuclei were counterstained with hematoxylin (Sigma). For negative controls, the primary antibodies were replaced with nonimmune serum or isotype-specific IgG. Sections were also incubated without primary or secondary antibodies as additional negative controls (data not shown). Photomicrographs were captured using conventional bright field microscopy.

Statistical analysis. Statistical analysis was performed using JMP5 Statistical Discovery Software (SAS Institute, Campus Drive, Cary, NC). All data are expressed as means ± SD. The differences between two groups was determined by unpaired Student’s t-test. Statistical significance is accepted at \( P < 0.05 \).

RESULTS

Responses of lung tissue to implanted Gelfoam sponge. Ideally, a scaffold material for tissue regeneration should induce minimal inflammatory response. In human emphysema, tissue loss leads to the formation of bullae, which could be greater than 1 cm in diameter. Therefore, the implants would need to be relatively large for tissue replacement. We prepared small cubic pieces of sponges of \( 5 \times 5 \times 2 \) mm\(^3\) and incubated them in vitro for 7 days to simulate the period required for in vitro cell culture and then implanted them into adult rat lungs. Some animals were killed on the same day to determine the morphology of the sponges before any potential remodeling and degradation. Because sponges were injected into normal lung tissue, a compressed border zone (thin arrows in Fig. 1) could be easily seen (Fig. 1A). Interestingly, the shape and size of the porous structures of the implanted sponge were very similar to that of the surrounding normal alveoli. Red blood cells (arrowheads in Fig. 1) were found inside the sponge (Fig. 1A), which became more obvious at day 1 (not shown) and day 3 (Fig. 1B), suggesting bleeding from the surrounding tissue. These red blood cells were completely cleared by day 7 (Fig. 1C).

Two weeks later, a small number of cells (open arrowheads in Fig. 1) infiltrated into the sponge along the scaffold (Fig. 1D). One month later, the infiltration of cells into the sponge was increased (Fig. 1E). At 2 mo, degradation of the sponges was seen, starting from the peripheral zone (Fig. 1F, asterisk). After 6 mo, the sponges were completely dissolved, and there were a few cells remaining in the area where the sponge had been implanted (data not shown). These results suggest that the

Fig. 1. Responses of lung tissue to implanted Gelfoam sponges. Sponges were incubated with cell culture medium for 7 days to simulate the period required for cell culture in vitro. A: day 0: the morphological structure of implanted sponge is similar to surrounding tissue. A clear border zone can be seen with the presence of red blood cells. B: day 3: hemorrhage is noted from day 0 to day 3 sections (arrowheads). C: day 7: the hemorrhage within the sponge is cleared. D: day 14: small numbers of cells are seen infiltrating the implanted sponges. E: day 30: the number of infiltrated cells is further increased. F: day 60: sponges started to degrade from the periphery towards the center (asterisk). For each time point, representative pictures photographed at \( \times 40 \) and \( \times 400 \) magnifications are shown. Thin arrows indicate the border zone between the implanted sponge and surrounding lung tissue, closed arrowheads show red blood cells, and open arrowheads show infiltration of cells into the sponge.
sponge does provide a satisfactory temporary scaffold for lung cells to migrate in; however, without progenitor cells, the sponge alone does not lead to the formation of normal lung structure.

Fetal lung cell-based tissue engineering induced formation of alveolar-like structures. We seeded sponges with mixed fetal rat lung cells, incubated for 7 days, and then implanted them into the adult rat lungs. Through a pilot study, we had noted that at the early stages of implantation, the morphology of the sponge in the lung tissues closely resembled what was seen in Fig. 1 when sponges were implanted alone (data not shown). We then focused on the later stages for further studies. After 40 days, many cells were found inside the sponges (Fig. 2A). Many vascular-like structures were also seen (thin arrows in Fig. 2), suggesting angiogenesis occurring in the sponge (Fig. 2B). After 50 days, the density of the sponge decreased (Fig. 2C). Cells inside the sponge resembled tissue cells along the porous structure (open arrowheads in Fig. 2) and along some of the vascular-like structures (Fig. 2D). After 60 days, more cells were present in the sponge with a very thin layer along the porous sponge structure (Fig. 2, E and F).

The density of the sponges decreased gradually (Fig. 2, A, C, and E). This trend continued over the longer periods (Fig. 3, A and D), likely indicating remodeling and degradation of the sponge. After 100 days, the border between the sponge and the surrounding area could not be clearly distinguished (Fig. 3, A–C). Four months after implantation, the scaffold structure of sponge became less clear (Fig. 3, D–F) compared with what was seen in earlier stages (Fig. 2). The porous structures formed inside the sponges were covered with very thin epithelial-like cells (open arrowheads, Fig. 3, C and F). Compared with the alveolar units in the surrounding area (Fig. 3F, inset), cells within the sponge formed alveolar-like structures with small vascular structures (thin arrows). The sponges were almost completely absorbed (Fig. 3, E and F). Most of these newly formed alveolar-like structures were found close to the border zone between the sponge and surrounding normal tissue.

Survival of fetal lung cells in vivo. To investigate whether the intrapulmonary injection procedure could wash out the fetal lung cells from the sponges and ascertain fetal lung cell viability in adult lung tissue, we labeled the cells with a CellTracker Orange CMTMR (red, thin arrows in Fig. 4), a fluorescent probe that is retained in living cells through several generations but not transferred to adjacent cells. Nuclei were counterstained with Hoechst dye (blue, arrowheads in Fig. 4). Sponges with labeled cells were either incubated in vitro or

![Fig. 2. Sponge with fetal rat lung cells in the adult rat lung. A and B, day 40; C and D, day 50; E and F, day 60. Cells formed small vessels lined with endothelial-like cells. Epithelial-like cells were surrounded by the sponge and connective tissue-like cells. The tissue density inside the sponge reduced gradually (comparing A, C, and E). For each time point, representative pictures photographed at ×40 and ×100 magnification are shown. Thin arrows indicate the vascular-like structures inside the sponge area, closed arrowheads indicate cells with paired nuclei that imply cell division, and open arrowheads indicate epithelial-like cells along the sponge.](http://ajplung.physiology.org/fig2.jpg)
implanted into the adult rat lungs. The CMTMR labeling efficiency was 51 ± 12% (means ± SD) (Fig. 4A). At day 10 (Fig. 4C), cell density and positively labeled cells in vitro appeared similar to that in day 0 (Fig. 4A). CMTMR positive cells were also found in vivo. At day 0 (Fig. 4B), the number of positively stained cells was 36 ± 15%, similar to that observed in vitro (Fig. 4A) \( P > 0.05 \). Thus the implantation procedure did not reduce the number of fetal lung cells in the sponges. More importantly, positively stained cells were found at day 35 (Fig. 4D), indicating that the implanted cells remained in the sponge. The morphology and cell density of the sponge before implantation is shown in Fig. 4E. Electron microscopy confirmed the attachment of fetal lung cells to the Gelfoam sponge (Fig. 4F).

Angiogenesis, cell proliferation, and differentiation inside sponges. Ultimately, for gas exchange purposes, newly generated lung tissues will need to be in close proximity to the pulmonary circulation. To determine whether the vascular-like structures identified inside the sponges were indeed connected to the pulmonary circulation, we perfused the pulmonary artery with India ink at 30 and 60 days after the sponge implantation and examined the sponges for the presence of ink particles within the vascular-like structures within the sponges (Fig. 5, A and B).

To examine cell proliferation, animals were given BrdU 24 h before death. We found BrdU positive cells within the sponges 60 days after implantation (Fig. 5C). Many of these cells were paired (Fig. 5C and Fig. 2), suggesting cell division.

Clara cells have been shown to be the progenitors of ciliated cells in the bronchioles (1). CCSP-expressing cells of the airway have been reported to be critical for epithelial renewal after progenitor cell depletion (14). We performed immunohistochemical staining with anti-CCSP antibody to identify positive cells within the sponges 30 days after the implantation (Fig. 5D). Positive immunostaining for proSP-C, a marker of type II alveolar epithelial cells, was also found in cells within the sponges (Fig. 5E). The vascular-like structures inside the sponge were positively stained with an anti-vWF antibody, a marker of endothelial cells (Fig. 5F). Using CD45 as a marker, we confirmed that few cells inside the sponge were infiltrating leukocytes (Fig. 5G). Positive controls and multiple negative controls were performed for each antibody as described in MATERIALS AND METHODS. A negative control staining for proSP-C in normal rat lung tissue is given as an example (Fig. 5H).

**DISCUSSION**

In the present study, using Gelfoam sponge material and fetal lung cells, we have demonstrated that cell-based tissue engineering strategies show significant potential for lung regeneration studies. Tissue engineering has been studied in other organ systems to develop biomaterials for in vivo applications (37). The challenge of applying this type of technology in the lung is the complex three-dimensional architectural structure of alveolar units. We cannot use...
simple tissue masses or sheets to replace lost tissues, as has been attempted in skin, cartilage, heart, and muscle (37). The implants ultimately have to achieve a highly organized three-dimensional structure with the potential to connect to the existing airways for ventilation and to blood vessels for gas exchange. We are excited with the serendipitous finding that the shape and pore size of Gelfoam sponges are so similar to alveolar structures in the adult rat lung. We have also demonstrated that the sponge is an excellent supporting material for lung cells to attach to. After the formation of alveolar-like structures, timely degradation of the scaffolds may leave the newly formed “alveoli” in place to perform the functions of the lung.

Currently, a number of synthetic scaffold materials have been produced for regenerative purposes. The general principles of designing and producing these materials have been the subject of several recent review articles (11–13). The present study has raised several specific issues related to the selection of scaffold material for lung regeneration. Scaffolds for lung tissue engineering need to be flexible, stretchable, and compressible during breathing. The pore size of scaffolds ideally should be similar to that of alveolar units. Finally, after formation of new lung tissue, the original scaffold should degrade, and the degradation products should not be toxic or proinflammatory.

Ideally, if we can avoid the use of cells from exogenous sources, we will be able to circumvent a number of technical and ethical issues. In the present study, when sponge alone was implanted into the adult lung, cell migration into the sponge was observed. The cells attached along the porous structural surface of the sponge. However, the number of cells and the kinetics of cell migration were not of sufficient magnitude to be useful for tissue regeneration. The addition of fetal rat lung cells to the sponges appeared to be important for several reasons. First, without cells, the sponges tended to become compressed and degraded after 1 or 2 mo. Supplementing the sponge with fetal lung cells altered the process of remodeling of the sponge within the lung. Second, the addition of fetal lung cells significantly improved the formation of alveolar-like structures within the sponge. It is well known that fetal cells have great potential for proliferation and further differentiation (21, 23). Using BrdU labeling, we indeed demonstrated cell proliferation within the sponges. CMTMR labeling demonstrated that the fetal lung cells survived in vivo and in vitro for at least 35 days. It is recognized that some of these CMTMR positive cells could be descendants of the original fetal lung...
cells. Fetal cardiomyocytes have been cultured on sponges for cell-based therapy in scar tissues of the heart (19). The potent proliferative and differentiating potential combined with the decreased antigenic properties of fetal cells make them a very attractive candidate for repair or regeneration of adult tissues.

We believe that the cells found within the sponge area include both implanted fetal lung cells and cells that had migrated from the surrounding tissues. However, at this point we do not have direct quantitative evidence of how many cells were descendents of the originally seeded cells and their direct contribution to tissue regeneration. This is a particularly vital question especially since we observed new vascular structures within the implants. Were these vessels formed by differentiation of the seeded fetal progenitors, or were they derived from surrounding lung tissue induced by angiogenic factors produced by the implanted fetal lung cells? The number of fetal lung cells seeded in the sponge was low (Fig. 4E), and only a few CMTMR positive cells were found at 35 days after implantation (Fig. 4D). Therefore, it is conceivable that most of cells within the sponge area were in fact derived primarily from the recipient. It is thus possible that the major function of these implanted cells is to perform as a source of growth factors and other soluble factors. A number of recent studies on cell transplants in the heart have suggested a paracrine mechanism of implanted cells in modulating endogenous cellular activity (10, 42). Further investigation is clearly required in this important area.

Fig. 5. Angiogenesis and immunohistochemistry staining in the implanted sponges. A and B: animals were perfused with India ink (Ink) through pulmonary artery, and ink pigment is found inside the sponges within small vascular-like structures (thin arrows). C: bromodeoxyuridine (BrdU) staining demonstrates cell proliferation inside the sponge (thin arrows). D: the sponge and surrounding lung tissue were immunostained with anti-Clara cell secretory protein antibody (CCSP) (brown color, thin arrows) to demonstrate bronchiolar epithelial cells. E: alveolar epithelial cells were revealed with an antibody for prosurfactant protein C (proSP-C; thin arrows). F: endothelial cells were stained with antibody for von Willebrand factor (VWF; thin arrows). G: anti-CD45 antibody was used to illustrate the lack of infiltrating leukocytes in the sponge. H: nonimmune serum or isotype-specific IgG were used as negative controls for each antibody. A photomicrograph of the negative control for SP-C is shown as an example. The immunohistochemistry staining was performed on samples collected at day 60 and photographed at ×100 magnification.
Most studies have focused on the use of stem cells for tissue regeneration (1, 2, 35). An attractive future application lies in the development of bioengineered implants where the recipient provides the cells that will be used to repair his or her own damaged tissues in a type of “auto-graft.” The three-dimensional structure of scaffolds could be used to foster the growth and development of bone marrow stem cells isolated from the recipient. With optimized cell culture conditions, the number of these cells could be amplified in vitro before the application in vivo. While some of the alveolar-like structures formed within the sponges are amazingly similar to alveolar units, the overall morphology of this “lung tissue” is still not perfected. Optimization of in vitro culture conditions may lead to better outcome. We have found that simply increasing the inoculation cell density increased the number of cells dying before and after implantation (data not shown). Additional biochemical and biophysical signals may be required to enhance cell survival in the sponge, both in vitro and in vivo. It is possible that these tissues need to be fostered in enriched culture conditions and biophysical signals may be required to enhance cell survival, proliferation, differentiation, and reorganization before implantation.

One of the unique features of the lung is that lung tissues are subjected to mechanical stretch during breathing. These mechanical forces are important for both fetal and adult lung cell proliferation and function (21, 23). Applying mechanical stretch to cultured cells to simulate breathing in vitro may facilitate cell-based tissue engineering. Another possible approach is to deliver necessary genes to cultured cells to genetically enhance the ability of the implanted cells to connect to recipient lung tissues in an effort to match the processes of alveolarization and angiogenesis.

In the present study, we implanted one piece of sponge in each animal in one lobe of the lung to examine the morphological changes of the sponge and implanted cells in the lung. After implantation, we found fresh hemorrhage in the sponge, likely related to the injection process. After several months, alveolar-like structures formed within the sponges and became less distinguishable from the surrounding lung tissue. In the center of the sponge, however, the structures were less cellular. Thus, for future studies, the size of sponge should be even smaller. The surgical technique for the delivery of engineered tissues and techniques to assess the effect of these therapeutic modalities on lung function as well as the long-term effects all need to be further developed. To further determine and realize the potential of tissue engineering in the lung, large animal models will ultimately need to be used. The elastase-induced emphysema model that has been reported in dogs, pigs, and rabbits (28) would appear to be very useful in this regard.

In summary, the present study demonstrates a useful strategy for cell-based tissue engineering for lung regeneration. The important factors revealed in this study have highlighted several important areas for future investigation that hopefully will make lung regeneration a reality.

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