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RESILIENCE OF THE HUMAN FETAL LUNG FOLLOWING STILLBIRTH:

Potential relevance for pulmonary regenerative medicine.

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Abstract

Recent advances in pulmonary regenerative medicine have increased the demand for alveolar epithelial progenitor cells. Fetal lung tissues from spontaneous pregnancy losses may represent a neglected, yet ethically and societally acceptable source of alveolar epithelial cells. The aim of this study was to determine the regenerative capacity of fetal lungs obtained from second trimester stillbirths. Lung tissues were harvested from 11 stillborn fetuses (13–22 weeks' gestation) at post-delivery intervals ranging from 10 to 41 hours and grafted to the renal subcapsular space of immune suppressed rats to provide optimal growth conditions. Histology, epithelial and alveolar type II cell proliferation, and surfactant protein-C mRNA expression were studied in preimplantation lung tissues and in xenografts at post-transplantation week 2. All xenografts displayed advanced architectural maturation compared with their respective preimplantation tissues, regardless of gestational age and post-delivery interval. The proliferative activity of the grafts was significantly higher than that of the preimplantation tissues (mean Ki-67 labeling index $26.7 \pm 7.7\%$ versus $14.7 \pm 10.5\%$, $P < 0.01$). The proliferative activity of grafts obtained after a long (> 36 h) post-delivery interval was significantly higher than that of the corresponding preimplantation tissue, and equivalent to that of grafts obtained after a short post-delivery interval (< 14 h). The regenerative capacity of fetal lung tissue was greater at younger (13–17 weeks) than at older (19–22 weeks) gestational ages. The presence of inflammation/chorioamnionitis did not appear to affect graft regeneration. All grafts studied displayed robust surfactant protein-C mRNA expression. In conclusion, fetal lung tissues from second trimester stillbirths can regain their inherent high regenerative potential following short-term culture, even if harvested more than 36 hours after delivery.

Keywords

alveolar type II cell; cell therapy; miscarriage; tissue engineering; xenograft

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DECLARATION OF INTEREST STATEMENT

None of the authors have any financial or personal relationships with other people or organizations that could influence (bias) their work.

INTRODUCTION

Adult lung tissue has only limited regenerative capacity. At present, lung transplantation is the only treatment option available for many patients with progressive and incurable lung diseases, such as end stage chronic obstructive lung disease/emphysema, interstitial fibrosis, cystic fibrosis and the acute respiratory distress syndrome (ARDS). The use of lung transplantation is limited by the ever-widening disparity between available donor lungs and the demand of patients on waiting lists, as well as the open problem of chronic allograft rejection. The quest for alternative, more effective strategies has led to increased experimental and preclinical interest in cell-based therapeutic approaches for severe lung diseases.

Multiple studies over the past decade have demonstrated that bone marrow or cord blood-derived stem or progenitor cells can structurally engraft as mature differentiated airway and alveolar epithelial cells¹⁻⁶. The physiologic significance of stem cell-based approaches and their clinical potential to improve histologic or clinical outcomes remain uncertain, which may be due, in part, to the relatively low engraftment rate of stem or progenitor cells. Serrano-Mollar et al.⁷ recently studied the effects of direct delivery of alveolar type II cells, rather than stem or progenitor cells, to injured lungs. Intratracheal delivery of alveolar type II cells, freshly isolated from adult rats, resulted in integration of donor-derived type II cells in the alveolar wall and attenuation of the fibrotic process in a rat model of bleomycin-induced lung fibrosis⁷.

In other exciting developments, two separate groups recently reported successful generation of functional bioartificial lungs by tissue engineering approaches^{8,9}. In both studies, adult rat lungs were decellularized with preservation of the structural characteristics of the lung. Reseeding of this 'scaffold' with endothelial cells and transformed or freshly isolated alveolar type II cells produced artificial lung tissue that resembled native lung tissue and was capable of gas exchange *in vitro* and – albeit short term – *in vivo*^{8,9}.

Regenerative pulmonary medicine, whether by cell therapy or tissue engineering strategies, will benefit greatly from a wider availability of alveolar epithelial type II cells, which act as the progenitor cells of the alveolar epithelium of the distal respiratory unit¹⁰. At present, the main sources of alveolar type II cells are lung biopsies or lung resections from adult patients. In view of the modest regenerative capacity of the adult lung, these limited sources are unlikely to meet the prospected high demand for alveolar type II cells created by the rapid expansion of pulmonary regenerative medicine. Fetal lung tissue from spontaneous miscarriages may represent an abundant and powerful alternative source of lung epithelial cells, if it can be demonstrated that these tissues retain the striking regenerative capacity inherent in fetal tissues *in situ*.

The aim of the present study was to determine the regenerative capacity of human fetal lung tissue derived from early second trimester (12 to 22 weeks' gestation) pregnancy losses. To test the regenerative potential of fetal lung tissues under ideal culture conditions, we opted for the human-to-rodent xenograft model. The renal subcapsular space of immune suppressed rodents has been utilized for more than four decades as a near-physiological "incubator" milieu that supports optimal growth and development of adult and fetal human lung tissue^{11,12}. Previous studies have demonstrated that human embryonic lung tissue derived from legal abortions can be successfully differentiated in immune suppressed mice¹². Although the duration of the interval between induced abortion and implantation of the lung tissues was not explicitly mentioned, it seems fair to assume that the post-delivery interval may have been relatively short after these planned abortions.

Any future clinical use of fetal tissues from spontaneous miscarriages, rather than from induced abortions, will need to take into account the effects of the larger post-delivery interval needed for the parents to process their loss, to receive detailed information about the tissue donation procedure, and to discuss and sign a full informed consent. To ensure the clinical relevance of this study, the fetal lung tissues were harvested after varying post-delivery intervals and obtained from pregnancies complicated by clinical conditions that are frequently associated with second trimester stillbirths, such as infection/chorioamnionitis and abruption.

METHODS

Patients

Lung samples were obtained from previable second trimester stillbirths delivered at Women and Infants Hospital (Providence, RI). Previability (gestational age 22 weeks or less) was determined by the obstetrician. The study protocol was approved by the institutional review board. Full informed written consent was obtained in compliance with institutional guidelines. Consent was obtained following delivery by a dedicated research coordinator who was specifically trained to recognize the potential vulnerability of the patients and to understand the sensitivities related to this particular type of research. This study was limited to fetuses delivered following spontaneous (non-induced) stillbirth. Fetuses delivered by elective or medical abortions and fetuses with congenital, chromosomal, pulmonary, or cardiac anomalies were excluded. In addition, fetuses with evidence of maceration, reflecting a prolonged interval between fetal demise and delivery, were excluded. Records were reviewed for postmenstrual age (PMA) at delivery and likely cause of death and/or preterm delivery. In all cases, the post-delivery interval (i.e., time between delivery and specimen harvesting) was recorded.

Harvesting of the fetal lung

The fetal examinations were performed by the Perinatal Pathology staff at Women and Infants Hospital according to standard methods. The gender of the fetuses was recorded. The gestational age was confirmed by fetal foot-length measurements. After thorough *in situ* examination and preparation of lung and blood culture specimens, the lungs were dissected and weighed. Samples for transplantation were taken from the peripheral parenchyma of the right lung. Tracheal and cartilaginous bronchial tissue was excluded. The lung tissue was cut into 1–3 mm³ pieces under sterile conditions. The lung samples were rinsed in Hanks' Balanced Salt Solution (HBSS) and transported to the Xenotransplant Core Facility at Brown University (Providence, Rhode Island) in ice cold Leibovitz's L-15 Medium, supplemented with gentamicin (50 µg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml), all from Gibco/Invitrogen, Carlsbad, CA). In addition, samples from the right lung were treated with RNA*later* (Ambion Inc., Austin, TX) and stored at –80°C for molecular analyses, as described previously¹³.

Preimplantation lung tissues: Processing and histologic analysis

Part of the right lung and the entire left lung were immersed in formalin. After overnight fixation, the preimplantation tissues were embedded in paraffin, sectioned at a thickness of 4 µm, and stained with hematoxylin and eosin. The developmental stage was determined based on well-established morphologic criteria¹⁴. The degree of autolysis was estimated based on the detachment of the bronchial epithelium from the basement membrane¹⁵ and scored as absent, focal (< 10% of high-power fields), multifocal (10–75%) or diffuse (>75%). The presence or absence of neutrophils in the airspaces, indicative of intrauterine infection with amniotic infection, was noted.

Fetal lung xenografts: Transplantation and processing

Transplantation was performed at the Xenotransplant Core Facility at Brown University within 1–2 hours after harvesting. Recipients of the renal subcapsular xenografts were four-to-eight week-old male immunodeficient rats (CrI:NIH-Foxn1^{nu}, Charles River, Wilmington, MA). Animals were anesthetized with isoflurane anesthesia (Baxter Healthcare Corporation, Deerfield, IL). Three to four lung samples measuring 1–2 mm were placed under the capsule of both kidneys. All animal research was conducted in accordance with Brown University institutional guidelines for the care and use of laboratory animals in compliance with National Institute of Health guidelines.

The xenografts were analyzed two weeks after implantation. The rats were euthanized by overdose of isoflurane. The grafts of one kidney were dissected free from the surrounding kidney and placed in RNA^{later} for molecular studies. The other kidney, with implants, was formalin-fixed and paraffin-embedded *in toto* for histologic and immunohistochemical studies.

Analysis of proliferative activity

Cell proliferation in preimplantation lung tissues and xenografts was studied by immunohistochemistry using anti-Ki-67 antibody (DakoCytomation, Glostrup, Denmark) in an avidin-biotin-peroxidase system as described before¹⁶. Controls for specificity consisted of omission of the primary antibody, which abolished all staining. The fraction of Ki-67-positive epithelial cells lining the airspaces and airways was manually counted in at least 25 randomly selected high power fields, collected from all available graft sites per recipient. In addition, the proliferative rate of epithelial cells and alveolar epithelial type II cells in preimplantation tissues and grafts was assessed by double immunofluorescence staining for Ki-67 combined with staining for cytokeratin (as epithelial marker) or surfactant protein-C (SPC) (as type II cell marker), respectively, as described before¹⁶.

Analysis of SP-C mRNA expression

Surfactant protein-C mRNA levels were quantified by real-time PCR analysis. Total cellular RNA was extracted from lung homogenates using TRIzol reagent (Invitrogen). Total RNA (2 μ g) was DNase-treated (Turbo DNA-free kit, Ambion, Austin, TX) and reverse-transcribed using the reverse transcriptase² first strand kit (SuperArray Bioscience/Qiagen) according to the manufacturer's protocols. The cDNA templates were amplified with GAPDH (SuperArray catalog no. PPH00150E) and SP-C (PPH07047A) primer pairs in independent sets of PCR using reverse transcriptase² real-time SYBR Green PCR master mix (SuperArray) on an Eppendorf Mastercycler ep realplex (Westbury, NY) according to the manufacturer's protocols. Each sample was run in triplicate and mRNA levels were analyzed relative to the GAPDH housekeeping gene. Relative gene expression ratios were calculated according to the SuperArray-recommended $\Delta\Delta C_t$ protocol¹⁷.

Data analysis

Values are expressed as mean \pm standard deviation (SD) or median (range). The significance of differences between groups was determined by unpaired Student *t*-test where applicable. The significance level was set at $P < 0.05$.

RESULTS

Patients

The gestational age of the stillborn fetuses at the time of delivery ranged between 13 and 22 weeks (median age: 17 weeks) (Table 1). Seven fetuses were male; four female. Complete

postmortem and placental examinations were performed in 7/11 cases; in the remaining 4/11 cases only the placenta was examined. In all cases, fetal demise had occurred prior to delivery. In two cases (cases 5 and 9), fetal demise occurred following admission to the hospital and was known to have happened within one hour prior to delivery, as documented by loss of cardiac activity and ultrasonographic confirmation of fetal demise during monitoring. In all other cases, fetal demise had occurred prior to admission to the hospital. While the precise timing was unknown, fetal death in these remaining cases had occurred at least several hours before delivery. Spontaneous miscarriage was attributable to intrauterine infection and/or acute chorioamnionitis in 7/11 cases. In two cases (cases 4 and 9), there was a known clinical suspicion of chorioamnionitis. The cause of fetal death in the four apparently non-infectious cases was preterm rupture of membranes (1 case), preterm rupture of membranes with abruption (1 case) or undetermined (2 cases). Postmortem cultures were negative in all seven cases subjected to full postmortem examination.

The post-delivery interval ranged from 10 to 41 hours (median interval: 14 hours). With respect to post-delivery interval, two distinct groups could be identified. In six cases, further described as cases with 'short post-delivery interval', the post-delivery interval was less than 14 hours (median gestational age for this group: 19.5 weeks). In the remaining five cases ('long post-delivery interval'), the post-delivery interval was greater than 30 hours (median gestational age: 17 weeks). All fetuses included in the study were non-macerated by external examination.

Histology of preimplantation lung tissue

The preimplantation lung tissue displayed the architectural and cellular characteristics of pseudoglandular, early canalicular or late canalicular stages of development, corresponding to the respective gestational ages at the time of fetal demise (Table 1, Fig. 1). Autolysis, histopathologically defined by the presence of bronchial epithelial detachment from the basement membrane, was noted in 4/6 cases with short post-delivery interval and in 4/5 cases with long post-delivery interval. Autolysis was focal in all cases, except for case 4, which displayed marked autolysis with diffuse bronchial epithelial detachment, cell lysis and diffuse interstitial hemorrhages even though the post-delivery interval was short (12 hours) (Fig. 2). The prominent autolysis of this case is suggestive of prolonged fetal retention prior to delivery, even though overt external signs of intrauterine retention (maceration) were absent.

The susceptibility to undergo autolysis appeared to correlate with gestational age. Autolysis was noted in only 4/7 (57%) of cases between 13 and 17 weeks' gestation but in all 4/4 cases between 19 and 22 weeks' gestation, although the post-delivery interval was short for all cases of the latter gestational age group. Polymorphonuclear leukocytes were identified in the pulmonary airspaces in 3/7 cases with evidence of intrauterine infection, supporting the diagnosis of amniotic fluid infection syndrome.

Histology of xenografts

The renal subcapsular grafts were studied two weeks after transplantation. All xenografts showed significant architectural maturation compared with their respective preimplantation tissues, characterized by thinning of the alveolar septa, flattening of the epithelium and widening of the airspaces (Fig. 1). Most grafts showed abundant capillaries in the septa, indicative of prominent angiogenesis. The appearance of the grafts at post-transplantation week 2 was similar in all cases, regardless of post-delivery interval or gestational age. While epithelial flattening and occasional secondary crest formation suggested evidence of maturation in the xenografts, the airspaces were abnormally enlarged and rounded and appeared dysplastic (Fig. 1). In one case (case # 2), the graft sites revealed only a single

layer of flattened airspaces, associated with some scarring and foreign body giant cell reaction. Graft failure in this exceptional case was likely due to implantation of excessively small lung fragments.

Analysis of proliferative activity of epithelial cells and alveolar type II cells

The proliferative activity of epithelial cells in pre-implantation fetal lungs and renal subcapsular grafts was determined by manual counting of the fraction of Ki-67-positive nuclei in the epithelial lining of airspaces and airways. In further descriptions, the term 'proliferative rate' will indicate the specific proliferative rate of epithelial cells.

Proliferation analysis of preimplantation and graft tissues was performed in 10/11 cases (in case 2, insufficient graft tissue was available for proliferation studies). Overall, the proliferative rate of the grafts was significantly higher than that of the preimplantation tissues (average Ki-67-labeling index $26.7 \pm 7.7\%$ in grafts versus $14.7 \pm 10.5\%$ in preimplantation tissues, $P < 0.01$) (Fig. 1). The Ki-67 labeling index in the preimplantation tissues was highly variable, ranging between 1.2% and 36.5%. The Ki-67 labeling index in the xenografts was more uniform and ranged between 14.6% and 38.0%. In all individual cases, the proliferative activity of the grafts was higher than that of the corresponding preimplantation tissue; the increase in proliferative activity in grafts versus preimplantation tissues ranged from 4.1% to 2083% (average: $309 \pm 634\%$). Case 4, previously shown to display severe autolysis, had low proliferative activity in the preimplantation tissue (Ki-67 labeling index: 1.2%) despite a short post-delivery interval, which supports prolonged *in utero* retention of the stillborn prior to delivery. After two weeks of transplantation, the proliferative rate of this originally very autolyzed tissue had increased to a striking 26.2% (Fig. 2). While not formally studied, the proliferative activity of interstitial (non-epithelial) cells also appeared consistently higher in xenografts than in preimplantation tissues.

In the previous proliferation studies, epithelial proliferation was identified by Ki-67-labeling of cells lining the airways and airspaces. To confirm the unequivocal occurrence of proliferation in epithelial cells and, in particular, in alveolar epithelial type II cells, we performed double immunofluorescence studies combining anti-Ki-67 and anti-cytokeratin (AE1/3) or anti-SP-C antibodies. Robust proliferative activity was present in epithelial cells, identified by anti-cytokeratin staining, as well as in alveolar epithelial type II cells, identified by anti-SP-C staining in grafts at post-transplantation week 2 (Fig. 2E-F and Fig. 3).

Correlation of proliferative activity with post-delivery interval, gestational age and intrauterine infection/inflammation

We then determined the association between duration of post-delivery interval and proliferative activity in preimplantation tissues and grafts (Fig. 4, left). Due to logistical and organizational factors, lung tissues fell into two distinct categories with respect to duration of post-delivery interval: those with a post-delivery interval ≤ 14 hours (designated "short PDI"), and those with a post-delivery interval > 30 hours (designated "long PDI"). The Ki-67-labeling index of preimplantation tissues was similar in cases with short and long post-delivery interval (Fig. 4, left; $n = 5$ in both groups). For tissues obtained after a short post-delivery interval, the proliferative rate was equally high in the graft and preimplantation tissue. For tissues harvested after a long post-delivery interval, the proliferative rate of the grafts was significantly more than two-fold higher than that of the original lung tissue ($P < 0.01$). The proliferative rates of grafts obtained after long versus short post-delivery interval were strikingly similar ($27.5 \pm 8.8\%$ for short post-delivery interval versus $26.0 \pm 7.4\%$ for long post-delivery interval). These findings suggest that the duration of the post-delivery interval does not affect the proliferative activity of the fetal lung graft two weeks post-

transplantation, at least within the range of post-delivery intervals considered in this study (up to 41 hours).

We then determined the association between gestational age and proliferation in preimplantation tissues and grafts. The cases were divided into two groups of roughly similar size, designated “early gestational age” (13–17 weeks’ gestational age, pseudoglandular and early canalicular stages of development, $n = 6$) and “late gestational age” (19–22 weeks’ gestational age, late canalicular stage of development, $n = 4$) (Fig. 4, right). As expected, the proliferative activity of the preimplantation lungs tended to be higher at earlier gestational age: the Ki-67 labeling index of lungs between 13 and 17 weeks’ gestation was $19.3 \pm 10.4\%$, compared with $7.8 \pm 6.6\%$ for lungs between 19 and 22 weeks’ gestation ($P = 0.08$). In both age groups, the proliferative activity was significantly higher in the grafts than in the preimplantation tissue ($P < 0.05$ and $P < 0.02$ for early and late gestational ages, respectively). The proliferative rate of the grafts was significantly lower for tissues harvested at later gestational age than for tissues harvested at early gestational age ($P < 0.05$). These findings suggest that second trimester fetal lung tissues demonstrate a significant capacity for recovery of proliferative activity following xenograft culture, regardless of the gestational age at time of tissue harvesting. However, the maximal proliferative capacity following transplantation appears to be greater for tissues harvested at earlier gestational age (13–17 weeks, corresponding to pseudoglandular and early canalicular stages) than for tissues harvested at later gestational age (20–22 weeks, late canalicular stage).

Finally, we determined the association between intrauterine infection/chorioamnionitis and the proliferative activity of preimplantation and transplanted lung tissues. Preimplantation and graft tissues were available from 7 cases with evidence of intrauterine infection (gestational age: 16.9 ± 2.4 weeks) and from 3 cases without evidence of infection (gestational age: 19.0 ± 4.2 weeks). The proliferation rates of preimplantation tissues were comparable for cases with or without intrauterine infection ($12.1 \pm 8.7\%$ versus $20.9 \pm 13.8\%$ for cases with and without infection, respectively; difference not statistically significant). Similarly, the proliferative activity of the grafts was equivalent in both groups ($27.0 \pm 6.6\%$ versus $26.1 \pm 11.7\%$ for cases with and without infection, respectively). These findings suggest that the capacity of fetal lung tissues to undergo epithelial cell replication following xenografting is not influenced by the presence or absence of intrauterine infection or chorioamnionitis.

Analysis of surfactant protein-C mRNA expression

Our studies so far demonstrated that fetal lung tissues display robust proliferative activity of epithelial cells, including alveolar epithelial type II cells, after short-term xenograft culture. To assess the capacity of alveolar type II cells to regain *function* after culture, we studied the SP-C mRNA expression of preimplantation and graft lung lysates by real time PCR analysis. Preimplantation lungs and grafts were available for expression studies in 7/11 cases. The gestational age at time of harvest for these cases ranged between 16 and 22 weeks; the post-delivery interval between 11 and 38 hours.

There was no correlation between post-delivery interval and SP-C mRNA levels in preimplantation tissue or grafts (Fig. 5, left). The SP-C mRNA levels of preimplantation lungs at 19–22 weeks’ gestation (late canalicular stages) tended to be higher than those of preimplantation lungs at 13–17 weeks’ gestation (pseudoglandular and canalicular stages) (Fig. 5, right), which is consistent with alveolar epithelial cytodifferentiation and maturation during transition towards the late canalicular and saccular stages of development. For lungs obtained at earlier gestational ages, the SP-C expression in the grafts was significantly higher than that of the corresponding original preimplantation tissue, suggestive of ongoing

epithelial maturation of the grafted lung tissue following transplantation. For lungs harvested at later gestational ages, the SP-C expression levels in the grafts were equivalent to those of the original lung tissues (Fig. 5, right). Taken together, our findings suggest that short-term xenograft culture of fetal lungs obtained from mid-pregnancy stillbirths can achieve proliferation, as well as functionality (surfactant production) and cytodifferentiation of alveolar epithelial cells.

DISCUSSION

We investigated the regenerative capacity of human fetal lung tissues obtained after spontaneous second trimester stillbirths with the ultimate goal to utilize these tissues for pulmonary cell therapy or tissue engineering approaches. Using a combination of molecular and immunohistochemical techniques, we determined that fetal lung tissues from spontaneous miscarriages can recuperate the normally high proliferative activity and surfactant protein production of fetal lungs *in situ* following short-term xenograft culture. These results suggest that fetal lung tissues derived from mid-pregnancy stillbirths may represent a formidable source of alveolar epithelial cells for pulmonary regenerative medicine.

To begin to determine the potential usefulness of human fetal lungs for cell therapy and tissue engineering applications, we studied the effects of three important clinical variables on the regenerative capacity of fetal lungs: duration of post-delivery interval, gestational age, and presence of intrauterine inflammation/chorioamnionitis. First, we determined the correlation between post-delivery interval and lung recovery after xenograft culture. As we aimed to respect the parents' grieving process as much as possible, the post-delivery interval of our cases was relatively long, ranging from 10 hours to a maximum of 41 hours (not including the antenatal postmortem time). Parenthetically, a significant part of these post-delivery intervals occurred at room temperature, in theory further enhancing autolysis and diminishing the regenerative capacity of the lungs.

Interestingly, we found no correlation between the duration of the post-delivery interval and the proliferative activity and surfactant expression of grafts two weeks post-transplantation. All grafts showed a striking capacity for increased epithelial cell proliferation and architectural maturation and surfactant expression, regardless of the duration of the post-delivery interval. In fact, robust alveolar epithelial cell proliferation could be restored in fetal lungs that were harvested more than 36 hours after delivery, and even in lungs that were severely autolyzed at the time of delivery. The remarkable resilience of fetal lungs, even when obtained relatively long after delivery (and even longer after actual fetal demise), implies that there will be no need to unreasonably hasten the tissue harvesting process, should the use of fetal lung tissues for regenerative purposes become a clinical reality. Consequently, adequate time may be available for the grieving parents to process the stillbirth and to consider the possibility of organ or tissue donation without the pressure of time constraints characteristic of cadaveric organ donations.

We also determined the effect of gestational age at time of delivery on the regenerative ability of the fetal lung tissues. Fetal lungs were harvested from fetuses between 13 and 22 weeks' gestation, spanning the pseudoglandular and canalicular stages of lung development. As expected, the proliferative activity of preimplantation lungs of earlier gestational ages (13–17 weeks, pseudoglandular and early canalicular stages of development) tended to be higher than that of fetuses at later gestational ages (19–22 weeks, late canalicular stage). While at all ages the proliferative activity in the grafts exceeded that seen in the preimplantation tissue, the eventual proliferative activity at two weeks post-transplantation remained greater for tissues harvested at younger gestational age. Importantly, alveolar

epithelial type II cells at all ages demonstrated capacity for surfactant expression and, at younger ages, displayed evidence of significant cytodifferentiation and maturation, as suggested by increased surfactant expression in grafts compared with preimplantation tissue.

Finally, we determined the effect of intrauterine infection/inflammation or chorioamnionitis on the regenerative capacity of the fetal lung tissues. In concordance with the known high prevalence of infection in second trimester stillborns^{18,19}, the majority of fetal lung tissues in our study were derived from pregnancies complicated by intrauterine infection. We found no correlation between intrauterine infection/chorioamnionitis and epithelial cell proliferation in preimplantation lung tissues or xenografts. It needs to be emphasized that this negative statistical analysis has limited power, given the relatively low number of subjects. Additional studies may be needed to determine whether these observations hold true with larger case numbers. Interestingly, the presumably infected lung tissues were well tolerated by the immune suppressed recipient rats, which may be attributable, in part, to exposure to antibacterial reagents during preimplantation tissue processing.

The use of fetal lung tissue from second – and possibly third - trimester stillbirths for alveolar cell therapy may be within reach from clinical, practical and ethical perspectives. First, fetal tissues may significantly narrow the gap between need and availability of lung and other tissues for regenerative purposes. Pregnancy loss is one of the most common adverse pregnancy outcomes. In the United States, 1 in 160 pregnancies is complicated by stillbirth, defined as fetal death at or after 20 weeks' gestation, and approximately 25,000 stillbirths at 20 weeks' gestation or greater are reported annually^{20,21}. The incidence of early fetal death is less clear, as the reporting requirement for fetal deaths under 20 weeks' gestation are less stringent in the United States. A Norwegian population-based study reported a fetal death rate of 3.4 fetal deaths per 1,000 pregnancies at early gestation (16–22 weeks) in 2006²². Second, in addition to their higher proliferative capacity, fetal tissues are characterized by lower immunogenicity than adult tissues. Third, in contrast with fetal tissues procured after induced or medical abortions, the use of fetal tissues obtained after spontaneous, non-induced pregnancy losses or stillbirths may be able to gain wider acceptance based on religious, sociocultural and ethical grounds.

For this proof-of-principle study, we took advantage of the near-physiological incubator conditions provided by the renal subcapsular milieu of immune suppressed rodents^{11,12} to investigate the full growth potential of postmortem fetal lungs. Our study has demonstrated that under the ideal culture conditions provided by this xenograft model, fetal alveolar epithelial cells can be recovered from stillborn lungs and can regain – and even exceed – the proliferative activity and function seen in the original preimplantation tissues. Future research endeavors need to be aimed at dissecting the various physical, biochemical and biological factors that contribute to the excellent culture conditions provided by the renal subcapsular xenograft milieu to allow replication of similar growth and maturation-promoting conditions for large scale *in vitro* culture of human fetal lungs.

In conclusion, fetal lungs from second trimester stillbirths display a robust capacity for alveolar epithelial regeneration, even after a prolonged postmortem interval. The potential implications of our observations for regenerative pulmonary medicine may be significant. Wider expansion of cell based therapies and tissue engineering approaches, especially those targeting reconstitution of the alveolar or bronchial epithelium, will benefit not only end stage adult lung diseases, but any acute or chronic pulmonary disease characterized by injured, defective or deficient parenchyma, including pediatric diseases such as bronchopulmonary dysplasia, pulmonary hypoplasia and alveolar proteinoses caused by genetic surfactant deficiencies.

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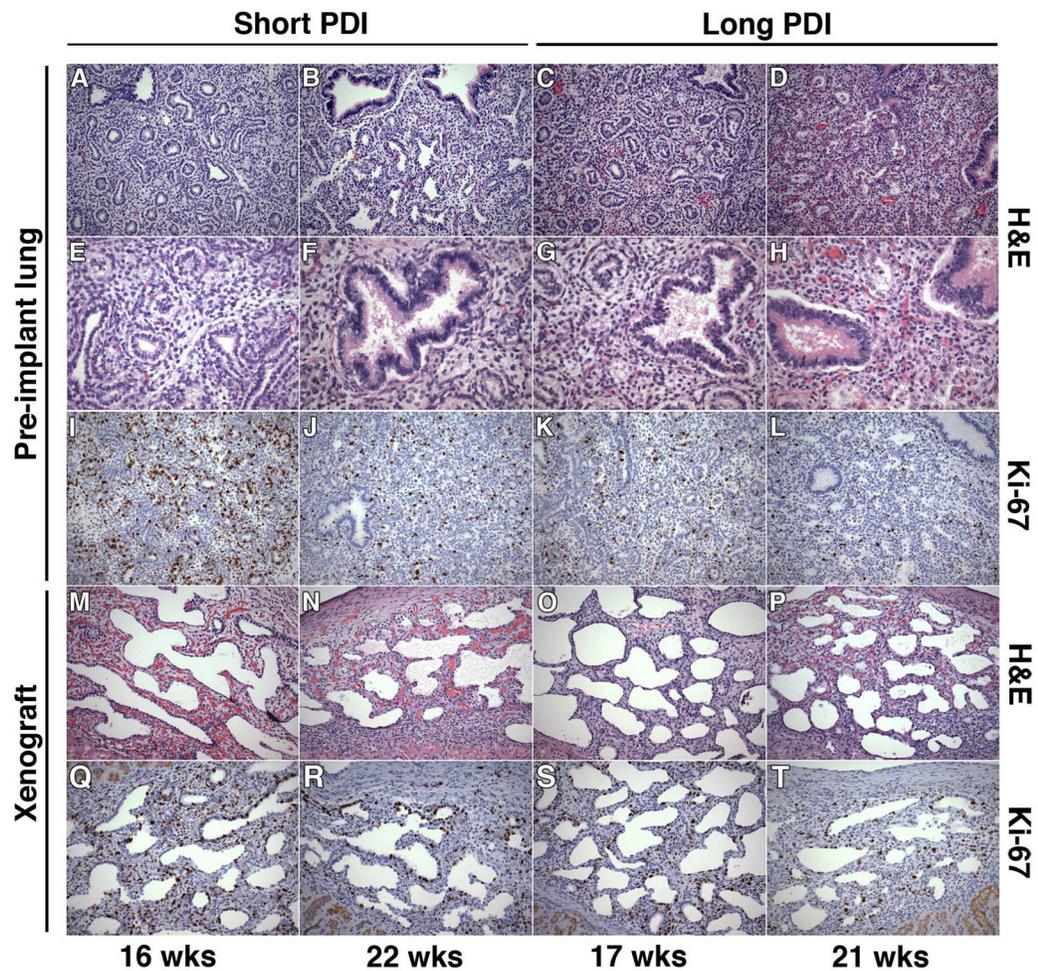


Figure 1. Morphology and proliferative activity of representative preimplantation lung tissues and corresponding renal subcapsular xenografts at post-transplant week 2

Column 1: Case 3: 16 weeks' gestation; PDI: 12 h. (A) and (E) Preimplantation tissue showing well preserved lungs in late pseudoglandular stage of development. (I) Preimplantation tissue showing brisk proliferative activity in epithelial cells lining the airspaces and in stromal cells. (M) Xenograft at post-transplant week 2 showing large, dilated airspaces and thin septa, imparting a mature, pseudosaccular morphology to the lung tissue. The airspace septa appear well vascularized. (O) Ki-67 labeling of xenografts showing abundant proliferating epithelial and interstitial cells.

Column 2: Case 5: 22 weeks' gestation; PDI: 12 h. (B) and (F) Lungs in late canalicular stage of development showing more advanced autolysis, characterized by focal detachment of bronchial epithelium (B, left upper corner, and F), tissue shearing and erythrocyte extravasation. (J) Corresponding Ki-67 labeling showing residual proliferative activity in epithelial and stromal cells. (N) and (R) Xenograft of case 5 showing architectural maturation with occasional secondary crest formation. Robust epithelial cell proliferation is noted.

Column 3: Case 11: 17 weeks' gestation, PDI: 41 h. (C) and (G) Pseudoglandular stage with focal evidence of autolysis, characterized by bronchial epithelial detachment. (K) Moderate proliferative activity is noted in epithelial and interstitial cells. (O) and (S) Xenograft of case 11 showing marked architectural maturation and prominent epithelial proliferation.

Column 4. Case 9: 21 weeks' gestation, PDI: 36 h. (D) and (H) Lungs in late canalicular stage of development showing evidence of autolysis (bronchial epithelial detachment). Neutrophils in the airspaces are consistent with intrauterine infection. (L) Corresponding Ki-67 labeling showing relatively modest epithelial cell proliferation. (P) and (T) Xenograft of case 9 with a mature, pseudosaccular appearance. Proliferative activity of the epithelial cells is increased compared with preimplantation tissue.

A–H and M–P: Hematoxylin-eosin staining; I–L and O–T: Anti-Ki67 immunohistochemistry, ABC-peroxidase technique with DAB detection and hematoxylin counterstain. Original magnification: X200, except E–H: X400.

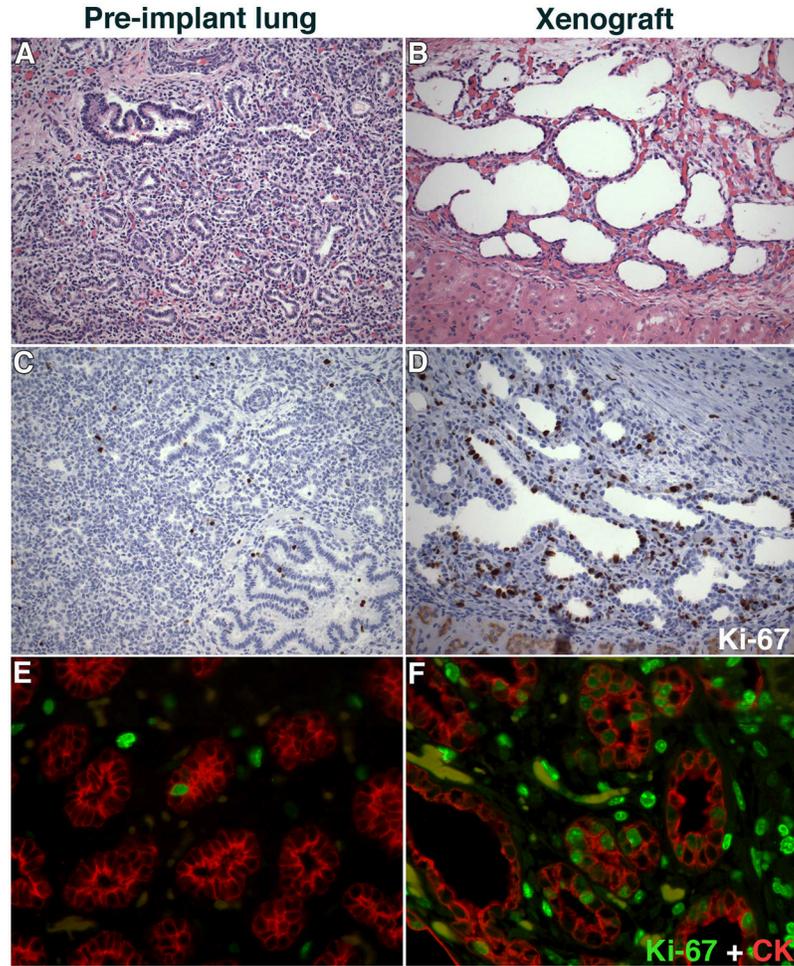


Figure 2. Case 4 (20 weeks' gestation; post-delivery interval: 12 hours). Morphology and proliferative activity of preimplantation lung tissue and corresponding renal subcapsular xenograft at post-transplant week 2

(A) Preimplantation lung tissue at canalicular stage of development showing marked autolysis with widespread detachment and intraluminal infolding of the bronchial epithelium. (C) and (E) Ki-67 and combined Ki-67/cytokeratin stains of the preimplantation tissue showing very limited proliferative activity in epithelial cells. (B) Xenograft at post-transplant week 2 showing striking architectural remodeling characterized by large-sized airspaces separated by thin and well-vascularized septa. (D) and (F) Ki-67 and combined Ki-67/cytokeratin stains of the xenografts showing markedly increased epithelial cell proliferation compared with the original preimplantation tissue.

A–B: Hematoxylin-eosin staining; C–D: Anti-Ki67 immunohistochemistry, ABC-peroxidase technique with DAB detection and hematoxylin counterstain; E–F: cytokeratin (AE1/3 Cy3, red) and Ki-67 (Alexa Fluor 488, green) double immunofluorescence.

A–D: original magnification: X200; E–F: original magnification: X400.

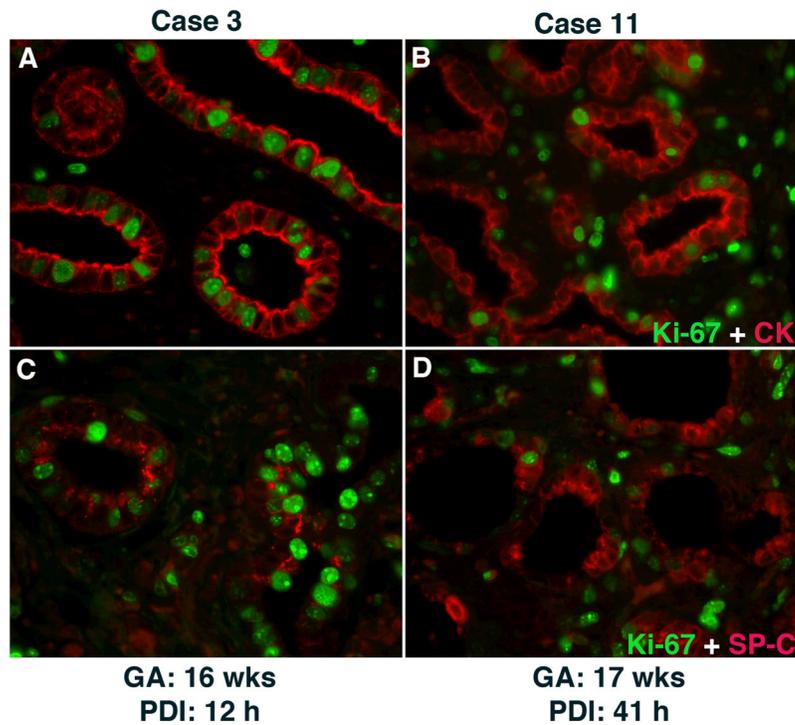


Figure 3. Proliferative activity of epithelial cells and alveolar epithelial type II cells in renal subcapsular xenografts at post-transplant week 2
 Representative cases with short and long post-delivery interval showing evidence of proliferation in epithelial (A–B) and, in particular, alveolar epithelial type II cells (C–D). GA: gestational age; PDI: post-delivery interval.
 A–B: cytokeratin (AE1/3; Cy3, red) and Ki-67 (Alexa Fluor 488, green) double immunofluorescence; C–D: surfactant protein-C (Cy3, red) and Ki-67 (Alexa Fluor 488, green) double immunofluorescence. Original magnification: X400.

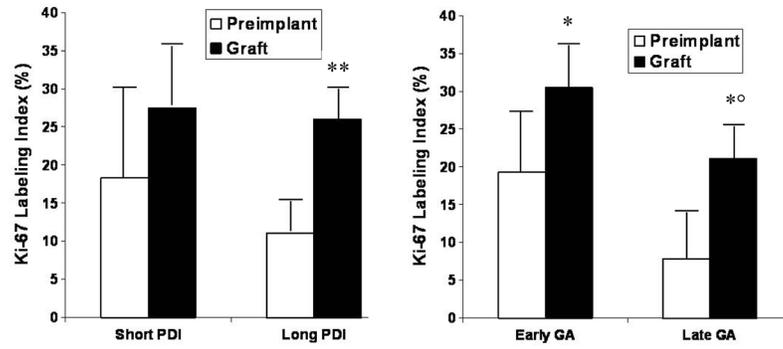


Figure 4. Correlation between proliferative activity and post-delivery interval (PDI) (left) or gestational age (right) in preimplantation lung tissue and renal subcapsular xenografts at post-transplant week 2

Proliferation was assessed by anti-Ki67 immunohistochemical analysis and expressed as fraction (percentage) of Ki67-positive epithelial cells. Values represent mean \pm SD of 4–6 cases per group.

PDI: post-delivery interval (short PDI: 14 hours; long PDI: > 30 hours); GA: gestational age (early GA: 13–17 weeks' gestation; late GA: 19–22 weeks' gestation).

*: $P < 0.05$; **: $P < 0.005$ versus corresponding preimplantation tissue

°: $P < 0.05$ versus grafts at early gestational age

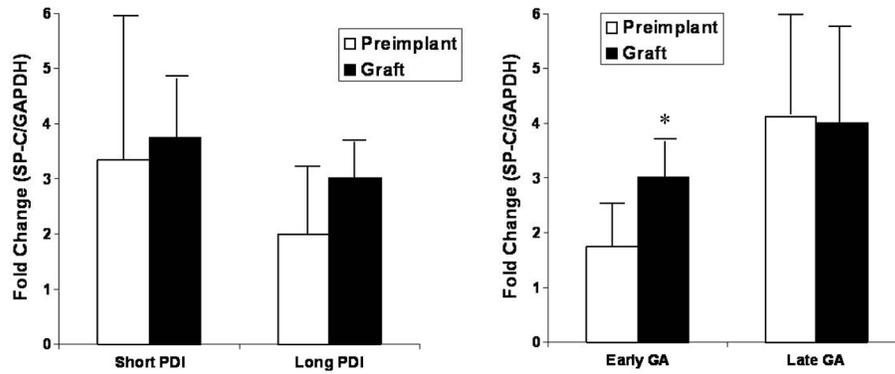


Figure 5. Correlation between SP-C mRNA expression and post-delivery interval (PDI) (left) or gestational age (right) in preimplantation lung tissue and renal subcapsular xenografts at post-transplant week 2

SP-C mRNA levels were assessed by real-time PCR analysis.

Data shown are from grafts retrieved from one to three recipient rats per case. The number of recipients is shown above the bars. Levels were normalized to those of one of the 16-week preimplant tissues. Values represent mean \pm SD of 3–4 cases per group.

PDI: post-delivery interval (short PDI: 14 hours; long PDI: > 30 hours); GA: gestational age (early GA: 13–17 weeks' gestation; late GA: 19–22 weeks' gestation).

*: $P < 0.05$ versus corresponding preimplantation tissue

Table 1

Clinical data and histologic analysis of preimplantation lung tissue.

Case #	PDI (h)	Gest. Age (wks)	Gender	COD	PM exam	Developmental stage	Bronchial epithelial detachment	Neutrophils in airspaces
1	10	13	M	ACA	Y	Pseudoglandular	0	N
2	11	22	M	PPROM, no evidence of infection/inflammation	Y	Late canalicular	Focal	N
3	12	16	F	COD undetermined, no evidence of infection/inflammation	N	Early canalicular	0	N
4	12	20	F	ACA, abruption	Y	Canalicular	Diffuse	Y
5	12	22	M	PPROM, abruption, no evidence of infection/inflammation	N	Late canalicular	Focal	N
6	14	19	M	No evidence of infection/inflammation, lung hypoplasia	Y	Canalicular	Focal	N
7	30	16	F	ACA	Y	Early canalicular	0	Y
8	36	17	M	ACA	N	Early canalicular	Focal	N
9	36	21	F	ACA, abruption	Y	Late canalicular	Focal	Y
10	38	17	M	ACA	N	Early canalicular	Focal	N
11	41	17	M	ACA	Y	Early canalicular	Focal	N

Abbreviations: M: male; F: female; PDI: post-delivery interval; COD: cause of death or preterm delivery; PPRM: preterm premature rupture of membranes; ACA: acute chorioamnionitis; PM exam: postmortem exam. Gestational age reflects reflect postmenstrual age.