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Culture nTAD: A New Avenue for Research in Late-stage Human Lung Development and Perinatal Lung Disease

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Airway Fluids as a Resource for the Acquisition of Patients-Specific and Disease-Specific Airway Epithelium

Primary cell culture is a laboratory process that has been practiced for more than a century by which the cells of interest are isolated from tissues and grown under the appropriate conditions to achieve a certain cell number. In conducting airway, basal cells function as stem cells that self-renew and differentiate to maintain tissue homeostasis and to regenerate after injuries [1]. Patient-specific and disease-specific airway basal cells provide valuable resources for human basic and translational research. Culture methods of primary human airway basal stem cells have reached remarkable achievements over the past several years [2-7]. Human airway tissues (rejected lungs for transplantation, surgical and bronchoscopy biopsies, postmortem tissues, etc.) are available for cell dissociation and subsequent culture. The existing method limits the easy establishment of a physiological cellular platform to tackle questions related to developmental and perinatal lung diseases. Infant tissues are difficult to access since invasive biopsy is unlikely and postmortem tissues are extremely rare. We reported previously that noninvasively obtained clinic fluids including induced sputum and bronchoalveolar lavage (BAL) were used to derive patient airway basal cells for a cystic fibrosis study [5]. BAL can be obtained from pathologists as discarded samples after diagnosis. Induced sputa can be easily collected when patients visit their physicians' offices. The article by the Lerou group and associates recently published in *Pediatric Research* [8] demonstrated that tracheal aspirate fluid from mechanically ventilated infants in the Neonatal Intensive Care Unit (NICU) can serve as a valuable alternative to tissues when generating neonatal airway basal cells. These cells are referred as neonatal tracheal aspirate-derived (nTAD) epithelial basal cell-like cells. The aspirates are collected from neonates intubated due to a variety of complications who are regularly suctioned to clear the tube. By using single-cell RNA-sequencing, we confirmed the presence of epithelial cells in tracheal aspirates based on epithelial gene signatures including epithelial cell adhesion molecule (EpCAM) and cytokeratin [8]. Despite for presence of epithelial cell markers, we identified that the majority of cells in each sample are differentiated cells, mixed with a few basal stem cells. Since aspirates from intubated neonates are produced on a daily basis, a fast and effective way to grow out those basal stem cells is necessary. We did so by directly plating the filtered aspirates within hours after suctioning (any additional procedures including cell sorting would cause cell death and stress, resulting in decreased success with cell derivation). Based on our experience, the terminally differentiated airway epithelial cells and hematopoietic cells cannot attach to the plates and therefore are removed by medium change next day. Those transient amplifying cells can be eradicated from cell population after a couple of passages. The stem cells will then dominate the cell pool and continue propagating to produce a large quantity of cells [8]. We demonstrated that by using this approach we have successfully established a repository of nTAD basal cell lines from infants ranging in gestational age from 24 weeks to full term and with a variety of clinical conditions [8]. With this successful example, we expect that the similar approach can be deployed to derive primary airway

cell lines from other clinical fluids such as nasopharyngeal fluids that are sucked from neonates immediately from birth in delivery rooms, as long as the fluids contain viable stem cells, thus providing an opportunity to generate airway basal cell lines from even healthy neonates.

There are two caveats for using fluids and the direct culture method to derive airway basal cells. One is that the regional information is lost, and all stem cells are expanded as a pool. Single cell analysis done directly on aspirates can be a parallel way to answer questions related to anatomic and regional cell heterogeneity in the airways of neonates. Another caveat is that a portion of basal cells might come from secretory cells. It has been reported in mice that a subset of club cells can undergo dedifferentiation to basal cells *in vivo* [9] and *in vitro* culture [5]. However, once the differentiated club cells acquire basal cell signatures they are functionally and morphologically equivalent to original basal cells [5,9]. The dedifferentiation of human club cells to basal cells has not yet been reported in literature. We have detected club cell signatures in aspirates based on single cell sequencing data; therefore, we cannot exclude the possibility of dedifferentiation of club cells in our culture. However, based on previous results in mice we have reasons to believe that the "club cell originated basal cells" should not cause too much concerns.

Long-Term Expansion of nTAD Cells: Achieved with Rapamycin

Since only a small population of stem cells can be identified in aspirates, the application of nTAD cells in basic and translational research is dependent on the ability to expand them extensively. We have reported that ROCK inhibition and SMAD signaling inhibition maintains cell stemness to achieve serial expansion of adult epithelial basal cells of multiple organs including lung [5]. These conditions also supported the initial outgrowth of basal cells from the aspirates. However, we identified that nTAD cells are easily deteriorated after a few passages unlike their adult counterparts. The mechanisms underlying the difference between adult and neonatal basal cell behaviors in culture are unclear. One of possibilities we can postulate is that the immaturity of neonatal cells makes them easy to differentiate or undertake morphological alteration (i.e. epithelial-mesenchymal transition or EMT). Likely one or more additional factors are needed to sustain long-term nTAD expansion. After searching for several possible candidates, we identified that rapamycin, an inhibitor for the mammalian target of rapamycin (mTOR) pathway, fits our purpose. The mTOR pathway has been well studied and is a critical pathway for tissue homeostasis, injury repair and regenerative medicine [10-14]. Inhibition of the mTOR pathway using rapamycin prevents epithelial stem cell senescence [15,16] and modulates the epidermal and tracheal basal cell pool *in vivo* [17, 18]. Rapamycin has been used in culture to prolong the growth and proliferation of multiple epithelial stem cells and progenitors [19]. In Lu et al., 2020, we provided evidence that rapamycin effectively sustains the cell growth of neonatal airway basal cells. In the presence of rapamycin, nTAD cells could be maintained in culture for at least 15 passages (or ~50 population doublings at 1:8-1:10 splitting ratio) [8]. In addition, we used transcriptomic and protein analysis to show that rapamycin was responsible for preventing cellular differentiation pathways, in particular by upregulating RSK3. Interestingly, transcriptomic analysis also indicated that the suppression of epithelial-mesenchymal transition is not implicated as a beneficial effect in cell cultures. Despite this rapamycin plays an essential role in EMT in numerous studies (Review in [20]).

Rapamycin must be included in the medium for continuous culture, withdrawal of this compound quickly causes the serial cell expansion to deteriorate. One may question that constitutive treatment with rapamycin could alter airway basal cell functions since mTOR and related pathways participate in pleiotropic cellular processes. Our work suggested that basal cells cultured in rapamycin retain characteristic morphology and stem cell biomarkers over passages [8]. Air-liquid interface (ALI) culture further verified that basal cells cultured in rapamycin can effectively differentiate into a pseudostratified epithelium with appropriate cellular composition and epithelial barrier functions [8]. These data in aggregate demonstrated that nTAD cells cultured in rapamycin genuinely recapitulate expected basal cell functions and are suitable for the study of lung development, stem cell function and diseases. Whether such cells can be also utilized for reparative cell therapy in infants with lung complications requires additional securitization to ensure their safety.

Prospective Application of nTAD Culture: Study Late Stage of Human Lung Development, Perinatal Diseases, and Beyond

The study of human lung development is important for understanding mechanisms underlying both congenital and acquired lung diseases that affect newborns and children [21]. Animal genetic models have merit by providing insights for detailed cellular and molecular events across temporal progression of lung development [22,23]. However, mouse and human lungs differ drastically in anatomic architecture and timeframe of organogenesis [24,25]. This suggests that a human cellular model is critical and should be used as a gold standard to validate the mechanisms derived from mouse work and to develop the therapeutic drugs and strategies to treat infant diseases. Previously, the knowledge of

human lung development and abnormalities mainly relied on histological and biomarker examination on embryonic and perinatal lung tissues. In recent years, human pluripotent stem cell (hPSCs) differentiation and the subsequently derived cells have provided valuable models for further understanding of human lung development and diseases (Review in [24,26,27]). Our ability to derive a large repository of perinatal airway basal cells provides us a unique opportunity to study late-stage human lung development. Lerou and colleagues have reported that the mesenchymal stromal cells (MSCs) derived from intubated aspirates maintained their developmental memory despite extensive expansion [28], setting the stage to use nTAD cells to study cellular phenotypes during lung development.

Another foreseeable application is that we can use nTAD cells to interrogate the similarities and differences of adult and infant airway epithelium responses to environmental stimuli and genetic mutations. It is well recognized that pediatric and adult asthmatic diseases exhibit different clinical manifestations and may be under regulation by distinct mechanisms [29-31]. In addition, respiratory viral infections often elicit different clinical outcomes in infants and adults. For example, human respiratory syncytial virus (RSV), the most important viral agent causing serious bronchiolitis in infants younger than 2, only causes mild symptoms in adults. Conversely, the current coronavirus disease 2019 (COVID-19) rarely induces lung symptoms in infants and young kids and the severity of disease increases with patients' age (based on Data & Statistics | CDC). This age-dependent and virus-specific disease severity in infants/children and adults is intriguing. One of the mechanisms could lie in epithelial cells themselves including cellular architecture and composition, barrier function, transcriptome, susceptibility to infections, innate immunity. In the past, such comparative studies were not realizable due to the difficulty in procuring infant and pediatric airway basal cells. Now this type of study can be engaged by our groups with the new culture method. Meanwhile, we will continue our nTAD biobanking with the hope of utilizing them for many other valuable applications.

Competing Financial Interests

The author declaims no conflict of interest.

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