National Heart, Lung and Blood Institute and Building Respiratory Epithelium and Tissue for Health (BREATH) Consortium Workshop Report: Moving Forward in Lung Regeneration

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Abstract

The National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health, along with the Longfonds BREATH consortium convened a working group to review the field of lung regeneration and suggest avenues for future research. The meeting took place on May 22, 2019 at the American Thoracic Society 2019 conference in Dallas, Texas, USA, and brought together investigators studying lung development, adult stem cell biology, induced pluripotent stem cells, biomaterials and respiratory disease. The purpose of the working group was 1) to examine the present status of basic science approaches to tackling lung disease and promoting lung regeneration in patients and 2) to determine priorities for future research in the field.

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Introduction

During healthy life, the lung is a quiescent but highly complex organ with capacity for regeneration following injury. However, the mechanisms of lung regeneration and the factors that determine it's outcome, i.e. productive restoration of homeostasis or destructive remodeling and scarring in disease, need to be better understood to realise the ambition of pro-regenerative therapies. Stem cell populations have been described in the submucosal glands, the conducting airways and the alveolar epithelium, with basal cells (proximal airways), club cells (distal airways), and alveolar type II cells (AT2; alveolus) capable of self-renewal and differentiation towards region-specific cell types (1). These stem cells equip the lung to regenerate functional tissue from resident progenitors during homeostasis. However, with age and after severe or repetitive injury these mechanisms can be overwhelmed and the response becomes characterized by remodeling, an adaptive but often irreversible process that prevents severe respiratory failure at the expense of long-term tissue function (2). As an example, mouse lungs exposed to severe infection with a mouse-adapted H1N1 strain of influenza A demonstrate abnormal appearance of KRT5+ cells in the distal airways and alveoli (3). Whilst these KRT5 'pods' – which bear striking similarity to peribronchiolar basaloid pods described in human cases (4) – might help to restore barrier function immediately post-infection, they can persist and likely preclude optimal gas exchange (5).

Chronic lung diseases in patients are characterized by aberrant regeneration and failure to restore tissue homeostasis; thus, therapy development hinges on understanding how disease pathogenesis causes regenerative failure, at the same time as understanding how the lung is created and matures throughout pre- and post-

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natal lung development. To advance this cause, the NHLBI and the Lung Foundation Netherlands (Longfonds) assembled a multidisciplinary working group for advancing lung regeneration work, involving basic, translational, and clinical investigators in May 2019. The working group brainstormed opportunities for future lung regeneration research with a focus on identifying knowledge gaps where resolution would help to bring research progress closer to the clinic for patients with chronic lung diseases. The meeting comprised four sessions, which addressed the subjects of technology development in lung research, cell therapy, bioengineering and the challenges of understanding the human lung, followed by a group discussion on emerging themes and priorities. This report summarizes the topics highlighted, suggests areas of future investigation and provides recommendations for moving the field forward.

Developing tools to study lung regeneration

In order to treat respiratory disease, it will be necessary to understand the mechanisms of development, adult homeostasis and pathological deviations from these. Thanks to rapid advances in 'omic' technologies and decreasing associated costs, it is becoming possible to study human patients in new detail. Practical and affordable whole-genome and whole-exome sequencing allow us to understand genotype and somatic alteration, while single cell and single nucleus RNA sequencing technologies reveal the cellular actors of disease in new depths of resolution. In the lung, these technologies have already described epithelial cell diversity and processes relevant to regeneration, for example by mapping the differentiation trajectories of epithelial progenitors during homeostasis (6, 7) and regeneration (8), defining rare cell populations such as the ionocyte (9, 10), a population of CFTR-rich airway epithelial cells, and allowing deep phenotyping of lung tissues in smokers (11, 12), asthmatics

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(13), COPD patients (14-16) and pulmonary fibrosis patients (16-21). Consortia efforts are now underway to characterize the processes of post-natal lung development (LungMAP (22)), homeostasis and disease (Human Lung Cell Atlas (23)) and it will also be informative to analyse human embryonic and foetal lung development *in utero* (24). Since a majority of patients recover fully from serious insults during bacterial or viral pneumonia and ARDS, insights might also be gained by characterizing successful regeneration in the context of disease resolution.

Over the past decade technology development has also substantially broadened the range of *in vitro* model systems available for the study of primary lung epithelial cells. New or improved human cell culture systems allow expansion of fetal lung progenitor cells (25, 26), adult regional epithelial stem cells (27-29) and induced pluripotent stem cell-derived airway (30-32) and alveolar (33) epithelial cells. Notably, human alveolar epithelial cells change to resemble AT1-like cells in 2D cultures and largely fail to establish the specialized cell types of the alveolus in 3D organoids. However, methods for culturing murine and human AT2 cells as long-term proliferating organoid cultures have been a significant advance (34-37). The air-liquid interface culture system (38), in which freshly isolated or cultured basal cells are differentiated recapitulate the pseudostratified epithelium, affords the airway field a robust primary cell culture system for studying differentiation and for exposing multiciliated epithelium to apical exposures. However, the throughput and cellular complexity of such cultures is low. Recent advances mean that it is now possible to culture lung epithelium in a variety of other 3D formats: organoids are predominantly reliant upon culture in Matrigel basement membrane extract but increasingly can be cultured in biological (39) or synthetic (40) alternatives, are feasible in high-throughput assays and can be derived from both human (41, 42) and mouse (43) lungs. Lung-on-a-chip models have been

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developed which, whilst low-throughput, allow recombination of the epithelium with mesenchymal and immune populations, the generation of directional flow and the application of force to mimic the physical influence of breathing (44). At larger scale, bioreactor culture systems have the potential to allow tissue- and organ-scale investigations. An additional approach has been to maintain human tissue *ex vivo* for sufficient periods to perform experiments. At the whole organ level this is possible using *ex vivo* lung perfusion (45), a technique used to recondition and preserve lungs in the context of transplantation or, following surgical resection of lung tissue, precision-cut lung slices can be maintained for periods of weeks to study complex cellular interactions within the native tissue organization (46). These approaches are potentially useful when tissue from diseased human lungs are available (47), but primary tissue is a scarce resource in many centers. Further, there is a lack of standardization of many of these assays between laboratories at present making it difficult to compare data.

Induced pluripotent stem cells (iPSCs) have added to the toolset available to model human lung disease, in theory having the capacity to provide an unlimited source of autologous cells for both *in vitro* modeling of disease and with the potential for cellular therapy. iPSC can offer a number of advantages over primary cells or cell lines: they reflect the complete genotype of an individual, they can be propagated long-term *in vitro*, and they can be readily gene-edited and clonally expanded. Purification of specific cell types throughout the differentiation process has been challenging due to a lack of cell specific cell surface markers; protocols have been aided by the use of gene editing approaches to knock in reporters specifically for primordial lung progenitor marker NKX2.1 and pro-SFTPC (Surfactant Protein C) (33, 48). While significant progress has been made in defining protocols to differentiate iPSC into

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mature lung cells, more needs to be done to establish conditions representing all mature adult human lung cells (25, 30, 32, 48-51) and the field is only just starting to appreciate the extent of epithelial cell heterogeneity in the human airway and alveolus. How close the iPS-derived lung cells are to the cells of the adult human lung is currently under investigation. While distal airway protocols have advanced to the point where a self-renewing population of ATII cells can be generated through passage in spheroid format (33), it remains to be determined if they represent the full differentiation and functional potential of adult human AT2 cells.

In vivo animal models of lung injury have been a mainstay of regeneration research and much has been gleaned from mouse models. However, given the limitations imposed upon the preclinical development pathway by differences between murine and human lung biology (discussed below), the gap between basic lung biology and clinical translation is considerable with just 3% of respiratory drugs reaching patients (52). Rabbits, ferrets, pigs, sheep and rhesus monkeys all have respiratory systems that more closely resemble humans in some aspect, but large animal models are expensive and impractical for the majority of centers to develop and use (53). These less frequently studied species also have downsides relating to genomic annotation, reagent availability and protocol development. For example, iPSCs from pigs are still difficult to maintain in their undifferentiated state and iPSCs from ferrets have yet to be established, limiting the tools available to study lung biology in these species. Regardless of species, histopathology has been the traditional means to determine the extent of regeneration in animals but technology advances now allow more nuanced read outs, for example using genetic barcoding, advanced imaging (54) or scRNAseq (55). These techniques will allow investigators to unravel the heterogeneity that is intrinsic to models and also present in patients. Regeneration occurring in

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severely damaged areas is likely to be governed by different rules to that occurring in less damaged regions and there might be cross-talk between these that we do not yet fully appreciate – could a future therapy promote replenishment of damaged tissue by progenitors from less affected regions? Such technologies will also be powerful tools in combination with assays that assess functional regeneration versus remodeling responses, such as pulmonary function tests including assessments of airflow and gas exchange.

Future cell therapies for lung disease

Multiple approaches have been taken to deliver cells to lungs with the ultimate aim of functional restoration of lung tissue. Mesenchymal stromal cells (MSCs) have been widely used in regenerative medicine approaches as a result of the ease of their isolation and culture, their immunomodulatory effects and the opportunity to use allogeneic cells as a result of immune privilege (56). MSCs have been widely trialed and shown to be safe in early phase trials, including in chronic lung diseases, where they might be of particular relevance given that they rapidly localize to the lungs following infusion. While pre-clinical studies suggest safety in settings such as acute respiratory distress syndrome (ARDS), allergic asthma, and emphysema, clinical trials have thus far failed to demonstrate therapeutic value. An overall lack of mechanistic insight into the therapeutic effects of MSCs in pre-clinical studies means that a large number of possible protocol modifications are now being explored to improve outcomes, including MSC source (bone-marrow, adipose, umbilical cord etc.), isolation/culture methods, pre-conditioning (using hypoxia, serum starvation, oxidative stress etc.), genetic modification approaches and using MSC derivatives (exosomes, conditioned medium etc.).

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A major limitation of MSC therapy in the context of regeneration might be their short engraftment period. Live MSCs have been detected in the lungs two weeks post-infusion (and two months post-infusion in skin) but the long-term engraftment of MSCs is limited (57). Although circulating bone marrow-derived MSCs were previously suggested to engraft and transdifferentiate within the lung epithelium, these cells were subsequently shown to definitively lack epithelial lineage potential (58). Thus, while MSCs might be used as a tool to cyclically dampen inflammation and promote repair, they have not yet been validated as a truly corrective therapy. In pursuit of long-term engraftment in the lung and encouraged by improved cell culture methodologies, investigators are now considering alternative cell sources, including embryonic stem (ES) cell-derived AT2 cells, adult AT2 cells, lineage-negative epithelial progenitor cells and fetal lung tissue. It is important to note that – despite the nascency of this field – epithelial cell transplantation has been performed in patients, with culture expanded SOX9+ human basal cells delivered to patients with bronchiectasis (59). Given these developments, it will be important for the field to acknowledge the potential to generate enthusiasm among patients for unlicensed stem cell therapies (60) and attempt to direct resources and attention towards studied, validated therapies and further research and development of techniques (61).

With current techniques it should be possible to assess the safety and potential of human progenitor cells in murine transplantation models and lung disease models, while also comparing alternative delivery methods and immunosuppressed mouse strains. Efforts to compare cell types and culture protocols directly would be of benefit, particularly using methods that unambiguously and comprehensively locate and quantify cells after xenotransplantation. In moving from small animal models into large animals and patients, cell delivery is a key challenge because delivery methods used

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in rodents are poorly applicable to large animal models and humans. To date, little cell engraftment is observed in the absence of recipient conditioning, generally achieved by causing extensive lung injury that would be unacceptable in clinical studies. Therefore, translational studies that investigate plausible cell delivery methods, perhaps drawing from the successful translation of bone marrow, epidermal and corneal stem cell-based therapies or building on existing bronchoscopic or surgical techniques, will be required to advance the field. Gene editing technology might also be used to determine donor cell characteristics that promote engraftment and therapeutic effect.

Scale is also challenging for manufacture and care must be taken that favorable characteristics of culture-expanded cells are not lost during scale-up. Using a similar rationale to gene therapy approaches, total replacement of a cell population may not be necessary if introducing a subset can suffice. Indeed, restoring CFTR in 25% of epithelial cells can restore function *in vitro* (62) and recent evidence shows that correcting around 20% of lung epithelial cells (including AT2 stem cells) using *in utero*, intra-amniotic delivery of adenoviral CRISPR-Cas9 gene editing vectors can rescue the perinatal lethal congenital lung disease phenotype of pro-Sftpc mutations in mice (63).

While most efforts focus on epithelium, it may become interesting to consider other cell types as more becomes understood about lung mesenchymal heterogeneity (64). Future therapies might enable the normalization of the diseased matrix, a particular problem in the context of cell therapy as delivering cells to a disease niche may limit the efficiency of engraftment and the efficacy of therapies. There is some evidence for this concept from *in vitro* studies of recellularized lung scaffolds, where donor age and

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disease status affects the efficiency of re-seeding (65) and from MSC infusions in patients, where lungs with milder disease retain MSCs for longer (66).

Whilst cell therapy clearly needs substantial pre-clinical work before it becomes a feasible in routine clinical practice, an alternative avenue for more immediate exploitation is the mobilization of endogenous progenitor cells for repair. Deciphering pathways that enable progenitor cell expansion in the context of diseased lungs will require not only an improved understanding of the pathways that control lung progenitor self-renewal and differentiation but also work to determine how these cellular processes become dysfunctional during healthy ageing and in disease microenvironments. Perhaps separable from future use in cell therapy, it is imperative for the field to have robust methods to test the potential of lung cell populations *in vivo* as another strategy to delineate key pathways and molecules that may be altered in lung disease.

Bio-engineering the lung

The long-term objective of the field of lung bioengineering is to combine cell and materials approaches to produce functional, engineered lung tissue that could alleviate donor shortages and the requirement for immunosuppression in lung transplantation, which is indicated in end-stage chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and cystic fibrosis, among other lung diseases. This clinical aspiration remains distant, in large part due to the cellular complexity of the lung (> 50 cell types), but valuable insights into the lung extracellular matrix (ECM), the nature of cell-ECM interfaces and the effects of mechanical force in lung regeneration are being generated. Technology development has led to novel approaches to expand cells at different scales in lung-on-a-chip devices or

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bioreactors, to maintain lung tissue in *ex vivo* lung slices or in *ex-vivo* lung perfusion (EVLP) models and to isolate high-quality lung ECM for basic and translational research.

Lung decellularization techniques have improved markedly, with species-, tissue- and disease-specific protocols developed. Healthy and diseased ECM can be compared using this approach and since intra-individual differences are retained in patient decellularized lung scaffolds, these represent an opportunity to study the diseased ECM in detail. Efforts to recellularize scaffolds are currently relatively simplistic with much focus on the ability of scaffolds to support cells in a broad sense, often using immortalized cell lines. Increasingly these studies have turned to primary cells to improve human relevance, seeding epithelial or endothelial cells. Although maintenance of both has been shown, the targeting of cells to particular tissue locations is challenging and the functionality of regeneration in these studies is difficult to ascertain. Restoration of functional interactions between the epithelium and the endothelial, mesenchymal and immune cell compartments are likely to be critically important given their roles as niche cells *in vivo* (67-69) and the crucial role of ECM remodeling in lung disease pathogenesis (70), so efforts to generate increasingly complex recellularized structures will reveal new biology.

Additional insight might be gained by applying the bioengineering approach more broadly within regeneration research; investigating encapsulation, homing and targeting of cells could overcome challenges in cell therapy, methods to focally decellularize and recellularized lung tissue would be beneficial and engineered organoid techniques to assess hydrogel substrates, determine the influences of physical force and acquire scalable platforms would expand our repertoire. 3D printing

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potentially also offers opportunities for progress either by generating custom-made laboratory/surgical tools or even by patterning cells and scaffolds.

Beyond model organisms

Much of our understanding of lung regeneration has been gleaned from studies in mice, whose genetic traceability has allowed precise delineation of stem cell populations and interrogation of the roles of candidate genes, but there are major differences between mouse and human lung biology and anatomy (summarized in Table 1). Although there is broad conservation of stem/progenitor populations, with basal cells serving as upper airway progenitors and AT2 cells maintaining the alveolus, the relevance of club cells to human epithelial turnover remains unclear. While club cells are generated from basal cells in human small airways (71), the composition and maintenance of the respiratory bronchiole, a simple epithelium within the smallest airways that is unique to humans, are very poorly understood and might be maintained by club cell progenitors as is seen in the intrapulmonary airways of mice.

	Human	Mouse
Size	Trachea 1.5-2cm, >20 airway generations	Trachea 1.5mm, 13-17 airway generations
Mechanical forces	Mostly upright	Mostly prone
Cartilage	Trachea and intrapulmonary for several bronchial generations	Trachea and only extrapulmonary bronchi
Submucosal glands	Throughout cartilaginous airways	First 3 cartilage rings of trachea
Epithelial composition	Goblet cells, low number of club cells restricted to small airways	Few goblet cells (unless injured), club cells line all conducting airways
Basal cells	Trachea, extrapulmonary and intrapulmonary bronchi,	Trachea, extrapulmonary bronchi only

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	extending to terminal bronchioles	
Neuroendocrine cells	Cells found throughout airway epithelium, clusters found only within intrapulmonary airways	Mostly clustered
Respiratory bronchiole	Present	Absent
Proximal-distal patterning	SOX2/SOX9 co-expression in tip progenitors in pseudoglandular stage	Sox2 expression absent in Sox9+ tip progenitors in pseudoglandular and canalicular stages
Developmental timing	Alveologenesis initiates pre-birth	Alveologenesis initiates post-birth

Table 1 – Summary of major differences between human and mouse lungs.

Perhaps owing to these differences in underlying biology, modelling some human lung disease in mice has proved challenging. In monogenic lung disease, there is variability in different genes for the similarity observed in mouse models and related human patients. When CFTR knockout mice were created, some tissues recapitulated the phenotype well but the critical airway ion transport defect that is the source of destructive lung disease in patients was not present due to differences in the acidity of human and mouse airway surface liquid (72). Conversely, recent data has indicated that a mouse model with a knock-in of the familial fibrosis associated mutation $SFTPC^{I73T}$ can recapitulate several pulmonary and biomarker aspects of human fibrotic disease (73). Additionally, injury models of clear utility in studying mice have variable relation to human disease. The bleomycin-induced lung injury model has been used as a surrogate for pulmonary fibrosis research but the model has many drawbacks, including the fact that it is an acute injury that resolves in younger mice, making it poorly representative of the progressive disease course seen in patients. Conversely, the H1N1 influenza model has been widely adopted in recent years due to the similarity of injury seen in mice and humans. The regenerative capacity of

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mouse models has been seen as a drawback, but in fact this resolution phase may provide an opportunity to understand new aspects that have heretofore been poorly characterized (74), potentially offering insight into how to achieve successful regeneration. Increasingly, chronic injury models have been sought, including using repeated, lower dose exposure to bleomycin, but the acute nature of mouse injury models is a widespread limitation. Of note, the fibrotic phenotype of older mice is markedly different than the typically young mice studied in most applications, and few studies have focused on age-related decline in regeneration capacity due to the cost of working with aged animals. Such data would likely be relevant as most lung disease occurs on an aged lung background.

Conclusions

Despite significant recent advances, it is clear that extensive further work on lung regeneration is required to provide an effective and realistic treatment option for the various pathologies causing lung tissue destruction, many of which are a direct consequence of failed regeneration. Considering the complexity of the lung, progress can only be made through a multidisciplinary approach, combining expertise in studying lung development, adult stem cell biology, induced pluripotent stem cells, biomaterials and respiratory disease (Figure 1). We have set the following specific scientific priorities and recommendations to bring us closer to effective lung regeneration.

Scientific Priorities to Advance Lung Regeneration

- Deeply phenotype well-annotated patient samples using the suite of genomic, transcriptomic epigenomic and proteomic tools available, following up discoveries using *in vitro* and *in vivo* model systems.

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- Recognise and further investigate possible differences in lung biology between patients of different ages, sexes and ethnic backgrounds.

- Develop small and large animal models that recapitulate key aspects of human lung disease.

- Take a multi-center approach to developing and implementing large animal models of lung disease.

- Develop standards for *in vitro* model systems and *in vivo* transplantation methods, promoting working with well-characterized and transferable techniques

- Develop more complex model systems based on patients, considering ageing, co-morbidities and exacerbations.

- Refine human airway and alveolar *in vitro* model systems.

- Develop new models and assays for understanding the cellular constituents and their cell-cell interactions that regulate the ***human*** gas exchange alveolar compartment/niche and compare this to both chronic high-incidence as well as rare lung diseases with related phenotypes such as COPD and alpha-1 anti-trypsin disorder.

- Optimize emerging *ex vivo* assays for studying ***human*** lung cell regeneration including organoids, tissue explants, and artificial matrices to include additional cell types and architecture.

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- Develop new technologies for tracking cellular identity and responses in *ex vivo* *human* assay systems including cell barcoding, emerging single cell assays, and bioinformatic assessment of cell identity and relationships.

- Leverage high throughput screening technologies to identify transcriptional and signaling pathways that will awaken the facultative regenerative response in the lung for development of future therapies.

- Better understand the role of the lung scaffold in cellular regeneration and the requirements needed for lung repair.

- Delineate the factors governing organized functional repair and dysfunctional remodeling.

Recommendations to Advance these Aims

- Actively promote the cause of evidence-based policy and education on the subject of stem cell-based therapies.

- Develop and validate biobank platforms to enable more widespread access to viable human cells, viable and fixed tissue and extracellular matrix, including from patients with lung disease.

- Encourage multidisciplinary team building that will be required to unravel new complexity; in particular, support integrated studies linking cell and developmental biologists with (bio)engineers and (bio)informaticians to tackle complexities of lung disease and regeneration.

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Figure Legend

Figure 1 : An integrated approach to the development of lung regenerative therapies. Abbreviations : EVLP = ex vivo lung perfusion ; iPSCs = induced pluripotent stem cells ; HTS = high-throughput screening.

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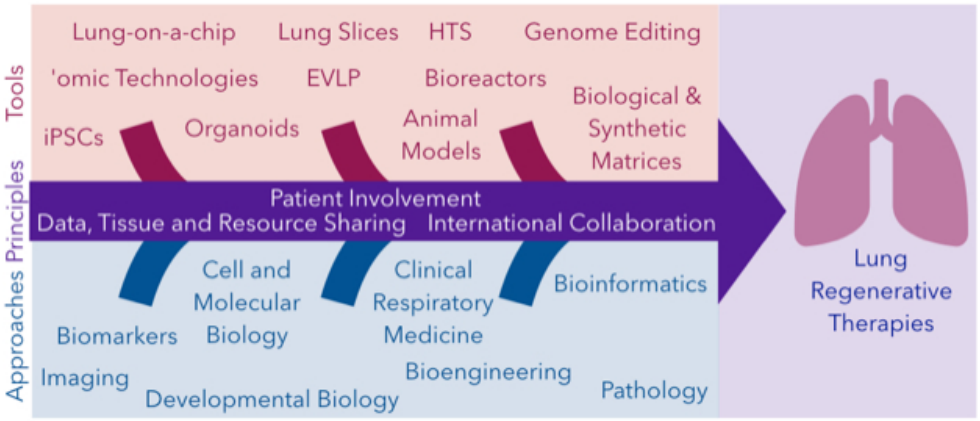

Figure 1

225x99mm (72 x 72 DPI)

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