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A 3-Dimensional Human Model of Bronchopulmonary Dysplasia

Identifies Notch-Mediated Pathophysiology

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Running title: A human 3D model of BPD

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a leading complication of premature birth and occurs primarily in infants delivered during the saccular stage of lung development. Histopathology shows decreased alveolarization and a pattern of fibroblast proliferation and differentiation to the myofibroblast phenotype. Little is known about the molecular pathways and cellular mechanisms that define BPD pathophysiology and progression. We have developed a novel 3-dimensional human model of BPD and using this model we have identified the Notch pathway as a key driver of disease pathogenesis. Fetal lung fibroblasts were cultured on sodium alginate beads to generate lung organoids. After exposure to alternating hypoxia and hyperoxia, the organoids developed a phenotypic response characterized by increased α-SMA expression and other genes known to be upregulated in BPD and also demonstrated increased expression of downstream effectors of the Notch pathway. Inhibition of Notch with a gamma-secretase inhibitor prevented the development of the pattern of cellular proliferation and α-SMA expression in our model. Analysis of human autopsy tissue from the lungs of infants who expired with BPD demonstrated evidence of Notch activation within fibrotic areas of the alveolar septae, suggesting that Notch may be a key driver of BPD pathophysiology.
INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a form of chronic lung disease that is both a leading cause of morbidity in infants born prematurely and the most common chronic respiratory disease of infancy (3, 40). Between 23 and 32 weeks gestation, fetal lungs are in the canalicular and saccular stages of development. At this developmental stage, a combination of environmental insults to the lungs of premature infants, including hyperoxia, mechanical ventilation, inflammation, and infection, causes a pattern of lung injury and scarring that is characterized by arrest of alveolarization, vascular hypoplasia, increased proliferation of fibroblasts, and development of fibrosis (34). The cellular mechanisms that promote the development of BPD are not well understood nor are the factors that promote rapid progression of the disease (17). BPD is a complex disorder with clinical heterogeneity: not every extremely premature infant develops BPD, and the pathophysiology likely arises from a process involving gene-environment interaction, in addition to known environmental triggers.

While there are multiple animal models that approximate BPD by exposing developing newborn animals to hyperoxia, infection, or mechanical ventilation, there is currently no human model of the disease (15, 18). Although much has been learned from the animal models, human lung development has a different trajectory than the mouse, the species used most commonly to model BPD (15). Term mouse pups are born in the saccular stage of lung development but unlike humans at this stage, they are surfactant sufficient and capable of normal gas exchange (34). This and other interspecies developmental differences underscore the need for a human model of BPD, both for improved
understanding of the disease pathophysiology and for the development of targeted
disease-modifying therapeutics. The pathogenesis of BPD likely includes multiple cell-
cell signaling events and cross-talk between neighboring cells (14). In contrast to 2D
traditional cell culture methods, a 3D tissue structure more closely approximates the *in
vivo* architecture of the lung parenchyma and provides cells with the ability to grow in a
geometry that mimics their *in vivo* environment. Our 3D disease model provides the
opportunity to observe the development and pathogenesis of myofibroblast differentiation
and gene expression changes in a way that more closely models the *in vivo* cell-cell
signaling events that are thought to be involved in disease pathogenesis (33).

The Notch pathway plays an essential role in normal lung development and has been
previously described to be upregulated in response to airway injury and in response to
oxidative stress in the proximal airways (32, 38). Here we describe a unique human 3D
culture system that we have developed to model BPD and which we have used to identify
the involvement of the Notch developmental pathway in the disease pathophysiology.
METHODS

Human fibroblast isolation and cell culture

Human fetal lung fibroblasts were isolated from 18-20 week old fetal lungs (Advanced Bioscience Resources, Inc.). Neonatal skin fibroblasts were isolated from foreskins discarded after the circumcision of former premature infants. Tissues were dissociated and then minced using 1 mg/ml Collagenase/Dispase (Roche) and 0.1 mg/ml DNase with rotation for 45 minutes at 37°C. After washing using media containing 1% fetal bovine serum, a single cell suspension was generated using 100 and 40 micron cell strainers. To remove red blood cells, the suspension was incubated in lysis buffer (BD Pharmingen) for 15 minutes at room temperature. Cells were then plated in 6-well tissue culture plates and cultured in DMEM/F12 containing 10% fetal calf serum.

3D Culture on Alginate Beads

Alginate beads were generated using an electrostatic droplet generator with 3% alginate (Sigma) in the presence of an electric field of 9000V. Alginate beads were functionalized by incubating with high-concentration rat tail collagen 1 (9.37 mg/ml, Corning) for 6 days and then coated with Tris-buffered dopamine hydrochloride (2mg/mL, Sigma) at pH 8.5. (please see Wilkinson et al for additional details) to promote cellular adhesion. 0.5ml of sedimented alginate beads were loaded into a 2ml rotating bioreactor (Synthecon) along with 2 x 10^6 fibroblasts. Organoid formation occurred for 96hrs, after which time the organoid was removed and divided into two pieces, one of which was grown in normoxic conditions and the other was placed in the hypoxic incubator. We exposed the organoids to alternating hypoxia (10% O_2) and hyperoxia (70% O_2). We oscillated the
organoids between the two conditions every 24 hours for a total of 4 days. For the HT 96-well organoids the same protocol was followed except 150 μl volume of beads and 1 x 10^5 number of cells were seeded in each well of the 96-well plate.

**Immunofluorescence (IF)**

IF was performed as described previously (32). Organoid cultures were fixed in 4% paraformaldehyde, washed and either stained in the well or embedded in Histogel and subsequently paraffin embedded and sectioned (5 μm thickness). IF was done after Tris-EDTA-Tween/Citrate buffer-mediated antigen retrieval followed by permeabilization with 0.3% Triton X-100 in protein blocking buffer (Dako, North America Inc., USA) for at least 30 minutes at RT. Sections were incubated with primary antibodies diluted in blocking solution, overnight at 4°C. After several washes in TBST, sections were incubated with secondary antibodies for 1–2 hr at RT or overnight at 4°C, washed, counterstained with DAPI (Vector labs - Burlingame, CA) placed under a coverslip and the edges sealed with nail polish. Slides were then analyzed by fluorescent microscopy with a LSM 780 Zeiss confocal microscope (Carl Zeiss, Jena, Germany). The following primary antibodies were used: mouse anti-α-SMA (Sigma, A2547), rabbit anti-vimentin (Bioss, bs-0756r), rabbit anti-NICD (Abcam, ab8925).

**Real time quantitative PCR (qPCR)**

Total RNA was extracted from the cells around the beads using the RNeasy micro kit (Qiagen) according to the manufacturer’s instructions. DNAse treatment was performed with RQ1 RNase-Free DNase (Promega). Reverse transcription was performed using
SuperScript II First Strand Kit (Invitrogen). qPCR was performed with the Taqman PCR Master Mix (Applied Biosystems) or Sybr Green Supermix (Biorad) on an Applied Biosystems StepOne-plus Real-Time PCR System. Each RNA sample was reverse-transcribed in triplicate, and appropriate negative controls were included in each run. Gene-specific primer pairs and probes were obtained from Applied Biosystems (See table in supplemental appendix for probe catalog numbers and primer sequences). For analysis, the ΔCT method was applied with 18S and B2M as endogenous controls. Relative gene expression, presented as a ratio of a target gene to reference control, was used for analysis.

Statistics

Triplicate samples were used in each experiment. Experiments were repeated a minimum of three times. All values are reported as mean with error bars representing +/- SEM. Statistical analysis was performed using Microsoft Excel, with two-tailed Student’s t-test being used for two-group comparisons. For all measurements, p-values less than 0.05 were considered statistically significant.

Study Approval

Approval for this research was obtained by the UCLA Institutional Review Board.
RESULTS

The 3D lung organoid model created an alveolar template that was scalable for high-throughput applications. To recapitulate the architecture of distal lung tissue, alginate beads were functionalized with collagen I and poly-dopamine (Figure 1A) to create alveolar templates for primary human fetal lung cells attachment and growth (Wilkinson et al., submitted). Fetal lung fibroblasts (FLFs) were added to the beads in a rotating 2mL bioreactor at 37 degrees, and 24 hours later the beads were uniformly coated with the FLFs (Figure 1B). The beads and cells were spun together in the bioreactor for a total of four days at 37 degrees, generating a lobular organoid structure (Figure 1C) held together by the mesenchymal cells proliferating on and around the beads. Cross sections of the lung organoid viewed with H&E staining showed structural homology between the lung organoid and the distal human lung (Figures 1D and E). Variable coating and proliferation of fibroblasts around the beads was seen. Some beads were separated by a single cell, and other areas showed proliferation of fibroblasts with multiple cell layers in the interstitium between the beads. The geometry and 3D arrangement of the fibroblasts in our model are important for allowing the cell coated beads to aggregate together and contract to form the lung organoid (Figure 1F). We also generated lung organoids in a 96-well plate that were identical in structure to the organoids made in the 2mL bioreactor in order to utilize this model for high throughput (HT) applications for BPD drug discovery (Figure 1G).

Exposure of 3D lung organoids to alternating hypoxia and hyperoxia recapitulates the phenotype of fibroblast activation seen in the fibrotic component of human BPD.
In response to hypoxia-hyperoxia, we saw a phenotype of increased fibroblast activation as demonstrated by markedly increased expression of alpha-smooth muscle actin (α-SMA) by immunofluorescence (IF) when compared with organoids exposed to normoxia (Figure 2A). This pattern of stress-fiber α-SMA expression strongly resembled that seen in the lungs of human infants who died with BPD (Figure 2C). Whereas fetal lung (Figure 2C) and healthy newborn lungs have very little α-SMA expression in the alveolar spaces, the lungs of human infants with BPD have been previously described to have bands of α-SMA expressing fibroblasts at the alveolar septae (4). This band-like pattern of α-SMA expression in response to hypoxia-hyperoxia is recapitulated in our model in both the large organoids and in the HT organoids made in the 96-well plates (Figure 2B).

We then used real-time quantitative PCR (qPCR) to examine the expression of ten genes from multiple gene families previously described to be upregulated in either human infants with BPD or in animal models of BPD (7, 11, 19, 21, 22, 28, 31). We found all ten genes to have significantly increased transcription in our model when compared with the normoxia control (Figure 3). As a negative control, three genes known to be expressed by fibroblasts but without known increased expression in BPD or in response to oxidative stress; vimentin, fibropectin, and N-cadherin, were analyzed and these genes all showed no increase in expression in response to hypoxia-hyperoxia (Figure 3). In order to ensure that the changes in ambient oxygen in our incubator created changes in oxidative stress in the organoid, we measured expression of NQO1, a phase II enzyme that has been previously shown to be activated by Nrf2 in response to oxidative stress (9). NQO1 showed significantly increased expression in the organoid exposed to hypoxia-hyperoxia.
when compared with normoxic controls, suggesting that the fluctuation in oxygen levels in the incubator was experienced by the cells in the submerged organoid cultures.

As a control experiment, FLFs were cultured in 2D using standard tissue culture techniques and were exposed to the same pattern of alternating hypoxia and hyperoxia. When comparing the hypoxia-hyperoxia exposed 2D cells to normoxic controls, we did observe changes in fibroblast morphology including elongation of the cells, however we saw no changes in α-SMA expression by IF or at the level of gene expression for multiple targets known to be upregulated in BPD (Figure 4A). We did observe significantly increased expression of NQO1, indicating a cellular response to oxidative stress, and that the 2D cells were exposed to the increased oxygen levels (Figure 4A). In order to ensure that the phenotype we saw was both lung-specific and not an artifact of the 3D culture method itself, we isolated skin fibroblasts from neonatal foreskin and cultured these cells on the functionalized alginate beads, generating an organoid structure populated by skin fibroblasts. When exposed to alternating hypoxia-hyperoxia, we saw no changes in α-SMA expression by IF or in expression of the panel of BPD related genes by qPCR (Figure 4B). Lung organoids comprised of primary adult lung fibroblasts did not demonstrate the pattern of fibroblast activation and proliferation in response to alternating hypoxia and hyperoxia that was observed in the fetal lung fibroblasts (Figure 4C). In fact, the adult lung fibroblast organoids exposed to variable oxygen exposure had significantly decreased expression of extracellular matrix genes such as Elastin, Col1A1, and Col3A1, with no change in α-SMA expression by qPCR or immunofluorescence.
Therefore, the fibroblast proliferation and activation seen in the 3D BPD model was specific to fetal lung fibroblasts.

Activation of the Notch pathway is a key driver of the fibrotic phenotype in our model and is also seen in human infants with BPD. The Notch pathway has been shown by prior studies to play a critical role in lung development and septation, repair after injury to the airway, and oxidative stress response, and therefore, we hypothesized that the Notch pathway may be involved in the development of fibroblast activation and differentiation in our model (16, 24, 32, 37, 38).

We found that in response to hypoxia-hyperoxia exposure, the areas of increased α-SMA expression in our model as observed by IF also demonstrated intranuclear staining for the activated form of Notch, Notch intracellular domain (NICD) (Figure 5A). When we pre-treated organoids with dibenzazepine (DBZ), a gamma-secretase specific inhibitor of Notch activation and signaling, the organoids did not develop increased areas of α-SMA expression following exposure to hypoxia-hyperoxia, as was seen in the organoids that were not treated with DBZ (Figure 5A). Additionally, the organoids pre-treated with DBZ had no NICD demonstrated in the nuclei (Figure 5A).

We also found evidence of Notch pathway upregulation in our model at the level of gene expression. Analyzing RNA from our model showed significantly increased transcription of NOTCH1 and JAG1, and downstream mediators HES1, HEY1 (Figure 5B). HES1, one of the key downstream effectors of Notch signaling, is known to be expressed in fetal lung (20). Treating our lung organoid BPD model with DBZ decreased the expression of
HES1 and HEY1 in response to hypoxia-hyperoxia as would be expected with inhibition of gamma-secretase. NOTCH and JAG1, which are upstream of this enzyme, did not change their expression in response to DBZ following exposure to hypoxia-hyperoxia (Figure 5B), which provides evidence that the inhibition by DBZ of the phenotype of fibroblast activation and α-SMA expression was occurring via the Notch pathway and not through off-target effects. Indeed, inhibiting Notch appeared to prevent the development of the hypoxia-hyperoxia phenotype completely, both at the level of α-SMA expression by immunostaining and at the level of gene expression for the panel of genes known to be upregulated in BPD (Figure 5C). Thus inhibiting Notch signaling altered the FLF myofibroblast differentiation and gene-expression pattern that define phenotype in our model.

To determine if the Notch pathway identified in our model was of clinical relevance, we examined NICD expression in fetal lung tissue and in lung tissue taken at autopsy from 4 infants who died with BPD. In the autopsy tissue from infants with BPD, we observed Notch activation, as evidenced by nuclear NICD expression by IF in the fibrotic alveolar septae in cells that were also expressing α-SMA (Figure 6A). Examination of fetal lung tissue by IF showed very little baseline expression of α-SMA and no intranuclear NICD (Figure 6B), which suggests that the FLF used in our model do not have increased expression of α-SMA or Notch signaling prior to exposure to hypoxia-hyperoxia. As a histologic comparison, we examined the lungs from term infants who were stillborn and found lower levels of α-SMA expression when compared with the BPD patients and no
evidence of Notch activation as there was no intranuclear expression of NICD observed (Figure 6C).

Another important aspect of the BPD phenotype in human infants is increased fibroblast proliferation (7). By PCNA staining, organoids exposed to hypoxia-hyperoxia demonstrated increased cellular proliferation compared to normoxic controls (Figure 7). DBZ inhibition of Notch signaling altered the cellular proliferation of FLFs exposed to hypoxia-hyperoxia as evidenced by PCNA staining of the BPD lung organoids cultured with and without DBZ (Figure 7). In response to hypoxia-hyperoxia exposure, lung organoids showed an increase in mitotic activity that decreased even below normoxia controls when the organoids were treated with DBZ (Figure 7).
DISCUSSION

Much of our understanding of BPD pathophysiology comes from several highly developed animal models, including sheep, rat, mouse, rabbit, pig, and baboon, that are exposed to various environmental stimuli thought to be implicated in BPD, including hyperoxia, mechanical ventilation, and maternal infection (1, 5, 8, 12, 18, 30, 39). While these animal models have taught us a great deal about BPD, there are key differences between other mammalian species and humans; for example, mice are born at term with lungs fully functional for gas exchange while still in the saccular stage of lung development, unlike their human counterparts (18). Of additional note, several targeted therapies have shown promise in animal models but have not shown benefit in humans, likely in part due to these developmental differences (18), which underscores the need for a human, in vitro model for the disease.

In order to create a human model of BPD, we used a new 3D primary cell lung organoid model developed by our lab to create a cellular scaffold that replicates the geometry of the interstitial region between alveoli. In this model, alternating hypoxia and hyperoxia resulted in fibroblast proliferation, myofibroblast differentiation, and gene expression changes that replicate many of the changes seen in human infants with BPD. Alternating hypoxia and hyperoxia has been used in prior animal models (5) and appears consistent with the clinical course of premature infants, who are exposed to widely variable oxygen saturations, especially in the initial period after birth. The observed changes in α-SMA gene expression and protein expression by IF in response to hypoxia-hyperoxia are found in human infants with BPD and in animal models of BPD, thus validating the phenotypic
response seen in our model in response to hypoxia-hyperoxia as a model of the mesenchymal component of BPD (4, 11, 19, 21, 28).

Prior work has demonstrated that pulmonary mesenchyme is a critical driver of normal lung development (25). Specifically, mesenchymal cells in the developing lung have been shown to direct the processes of branching morphogenesis, epithelial differentiation, pulmonary vascular development, and secondary septation (4, 25-27). During the saccular stage of lung development, the alveolar septae are thickened and enriched in fibroblasts, and signaling events from these fibroblasts drives the process of alveolarization (6). Pulmonary fibroblasts are important cells in the pathogenesis of BPD, as the fibroblasts are thought to respond to environmental stress through paracrine signaling which drives their proliferation, activation to myofibroblasts, migration into the alveolar septae, and deposition of extracellular matrix (ECM) (4, 23). In developing our human model of BPD, we looked to the fetal lung fibroblast as the cell type that could best generate an observable myofibroblast phenotype in response to variable oxygen concentrations.

One limitation of our model is that it includes a single mesenchymal cell type with the notable absence of the contribution from alveolar epithelial and endothelial cells. However, by focusing on the contribution of one isolated cell type, our 3D lung model allowed us to specifically assess the mesenchymal component of the disease as well as the role of pulmonary mesenchyme in expressing various genes that contribute to BPD pathophysiology, such as PDGFRα, α-SMA, and TGFβ. In the future, we plan to exploit
the modularity of this model and incorporate additional lung cell types to study their interactions, e.g. mesenchymal cells, endothelial cells and epithelial cells, to gain insight into how communication between different cell types can drive the pathophysiology of this disease.

Our lung organoid recreates the 3D niche microenvironment of fetal lung fibroblasts growing in close proximity in the alveolar interstitium. One possible reason for differences between the results in the cells cultured in 3D when compared to 2D is that the 3D culture system likely creates a microenvironment that allows the FLFs to engage in cell signaling by direct contact between adjacent cells, and through activation via paracrine signaling(33), which we speculate drives the pathophysiology both in the model of the disease and in vivo(33, 35). In order for Notch signaling to occur, the cell expressing Notch ligand must be in direct contact with a neighboring cell expressing the Notch receptor. Our 3D model affords cells the opportunity to grow in close contact in the anatomically correct locations that allow this specific cell-cell contact and therefore these signaling events to take place.

While the creation of this 3D microenvironment in our organoid was necessary to develop the mesenchymal phenotype in response to hypoxia-hyperoxia, it was not sufficient. The fetal lung fibroblast cell type grown in the organoid was critical for differentiation to myofibroblasts and expression of genes known to be upregulated in BPD as we did not observe this phenotype with neonatal skin fibroblasts or adult pulmonary fibroblasts. This is consistent with the development of BPD occurring only
during a specific window in development in preterm infants and is congruous with the
observation that adults exposed to hyperoxia and mechanical ventilation do not develop
the same pathology as premature infants with the same exposures (10, 13). It is also
consistent with the pathogenesis of BPD being dependent on developmental pathways. In
addition, the recreation of the 3D fibroblast microenvironment in our model is scalable to
a 96-well format, which provides a critical advance for developing potential future
applications, such as a high throughput drug screen to prevent differentiation to
myofibroblasts. These HT organoids also can provide a platform for the future study of
multiple molecular pathways.

While major advances in lung cellular biology and development have been made over the
past fifty years, the precise cellular pathways and properties that regulate the
pathophysiology of BPD remain elusive (14). Since BPD is defined by a developmental
arrest in alveolar septation, we were interested in examining our model to see if there
were developmental programs involved in the mechanism of the development of the
fibrotic phenotype as well. It has been previously shown that in response to oxidative
stress, Nrf2 activates the Notch pathway in other cell types in the lung (32) and liver (36,
37) by binding to antioxidant response elements in the Notch promoter and that the Notch
pathway is known to be activated in response to airway injury (38); overexpression of
Notch is also known to inhibit alveolar septation (38), a process that is disrupted in
BPD (3). Moreover, the Notch pathway has also been implicated in myofibroblast
differentiation and the development of fibrosis in other pulmonary diseases (24). We
found that the Notch pathway drove the cellular proliferation and differentiation to

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SMA positive myofibroblasts in our model and also demonstrated evidence for increased activated Notch expression by myofibroblasts in the lungs of human infants with BPD, and we did not see the presence of activated Notch in the lungs of term infants. The identification of the Notch pathway in BPD is significant; while Notch pathway expression in response to airway injury in the proximal airways has been well-described, Notch has not previously been identified in BPD pathophysiology and presents a new opportunity to look for druggable targets that interact with this pathway and its downstream effectors.

In summary, we have developed an *in vitro* 3D human model of BPD in a novel culture system that replicates the alveolar architecture found in human distal lung. The phenotypic changes in our model have been validated by comparison with human BPD lung autopsy tissue and qPCR for specifically upregulated genes in BPD. Using our model, we have been able to augment our understanding of BPD pathophysiology, identifying the Notch pathway as being a key driver of the development of the phenotype of myofibroblast differentiation and gene expression in our model. The model has allowed us to identify that the Notch pathway is activated in the distal lung in BPD and may be a driver of the pathophysiology seen in BPD. As we are now able to generate HT organoids, in the future we will increase the scale of our investigation to identify additional molecular pathways and targets that may be perturbed in the clinical setting of changes in oxygen tension. By creating this HT human *in vitro* model of the disease we plan to create a 3D HT drug screen that will foster the discovery of novel therapies to improve the survival and outcomes for premature infants.
### APPENDIX

Primer and probe details from qPCR experiments:

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DISCLOSURES

The authors report no conflicts of interest, financial or otherwise.
AUTHOR CONTRIBUTIONS

JMSS designed the research study, conducted experiments, acquired data, performed data analysis, and wrote the manuscript. DW developed experiments and experimental protocols, conducted experiments, and contributed to manuscript writing and review. PV contributed to experimental design, performed experiments, acquired data, and edited manuscript. MP contributed to experimental design, performed experiments, acquired data, and edited manuscript. BD contributed to experimental design. JA performed experiments, acquired data, contributed to manuscript writing and review. BG designed the study, analyzed the data, and wrote the manuscript.
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REFERENCES


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FIGURE CAPTIONS:

Figure 1: Development of the 3D lung organoid: A) Brightfield micrograph of alginate beads, generated by cross-linking with barium in the presence of an electric field. Scale bar = 200μm. B) Calcein intravital stain of beads after 24 hours in the bioreactor showing FLFs coating the beads. Scale bar = 150μm. C) Photograph of lung organoid generated after 4 days in rotating bioreactor. Scale bar = 1cm. D) Hematoxylin and eosin stain of a cross section of a lung organoid showing FLFs growing around alginate beads. Scale bar = 200μm. E) Hematoxylin and eosin stain of a cross section of normal human lung. Scale bar = 200μm. F) Brightfield microscopy of lung organoid. Scale bar = 100μm. G) Brightfield microscopy of HT lung organoid growing in a well of a 96-well plate. Scale bar = 1.6mm.

Figure 2: α-SMA IF of the 3D BPD model. A) α-SMA IF of 3D lung organoids exposed to hypoxia-hyperoxia (bottom panel) vs. normoxia controls (top panel). B) α-SMA IF of high throughput 3D lung organoids exposed to hypoxia-hyperoxia (bottom panel) vs. normoxia controls (top panel). C) α-SMA IF of fetal lung tissue (top panel) vs lung tissue from an infant with severe BPD (bottom panel). Scale bar = 50μm

Figure 3: Validation of the 3D BPD model at the level of gene expression. Comparison of gene expression differences of molecular targets known to upregulated in BPD by qPCR in 3D lung organoids grown in normoxia vs exposed to hypoxia-hyperoxia. Triplicate samples were used in each experiment. Experiments were repeated a minimum of three times. *p<0.001.
Figure 4: Changes in α-SMA expression and other genes associated with BPD were specific to fetal lung fibroblasts cultured in 3D. A) IF for α-SMA and vimentin expression and BPD target gene expression pattern by qPCR in 2D FLF cultured in normoxia and hypoxia-hyperoxia conditions. Scale bar = 50μm. B) IF for α-SMA and vimentin expression and BPD target gene expression pattern by qPCR in 3D skin fibroblasts cultured in normoxia and hypoxia-hyperoxia conditions. Scale bar = 50μm. C) IF for α-SMA and vimentin expression and BPD target gene expression pattern by qPCR in 3D adult primary fibroblasts cultured in normoxia and hypoxia-hyperoxia conditions. Scale bar = 50μm. Triplicate samples were used in each experiment. Experiments were repeated a minimum of three times. *p<0.001.

Figure 5: Notch pathway involvement in BPD pathophysiology in the 3D lung model: A) IF for α-SMA and NICD expression in 3D FLF lung organoids comparing normoxia to hypoxia-hyperoxia conditions, with and without pretreatment with Notch inhibitor DBZ. Examination of α-SMA positive fibroblasts (inset) showed evidence of intranuclear presence of NICD. Scale bar = 50μm B) Expression of downstream effectors of the Notch pathway, HES1 and HEY1, upstream targets JAG1 and NOTCH1 and oxidative stress enzyme NQO1 as measured by qPCR in response to hypoxia-hyperoxia and treatment with DBZ, *p<0.01. C) Comparison by qPCR of the gene expression pattern of molecular targets known to be upregulated in BPD between 3D organoids grown in normoxia and organoids exposed to hypoxia-hyperoxia, with and without pretreatment.
with Notch inhibitor DBZ, *p<0.01. Triplicate samples were used in each experiment. Experiments were repeated a minimum of three times.

Figure 6: Notch pathway involvement in patient samples: A) IF for α-SMA and NICD expression in autopsy tissue from the lungs of 4 human infants who died with BPD. Scale bar = 25µm. B) IF for baseline α-SMA and NICD expression in fetal lung. Scale bar = 20µm. C) IF for α-SMA and NICD expression in autopsy tissue from the lung of 3 stillborn term human infants. Scale bar = 25µm.

Figure 7: Inhibition of Notch decreased myofibroblast differentiation as well as cellular proliferation as measured by intranuclear PCNA expression. PCNA and α-SMA IF of organoids cultured in normoxia and hypoxia-hyperoxia exposure, with and without pretreatment with Notch inhibitor DBZ. Scale bar = 20 µm. 200 cells were counted in triplicate for each condition and reported as average % positive for each group, *p<0.01. Triplicate samples were used in each experiment. Experiments were repeated a minimum of three times.