UCLA
UCLA Previously Published Works

Title

A three-dimensional human model of the fibroblast activation that accompanies bronchopulmonary dysplasia identifies Notch-mediated pathophysiology

Permalink
https://escholarship.org/uc/item/7997m4xh

Journal
American Journal of Physiology - Lung Cellular and Molecular Physiology, 310(10)

ISSN
1040-0605

Authors
Sucre, Jennifer MS
Wilkinson, Dan
Vijayaraj, Preethi
et al.

Publication Date
2016-05-15

DOI
10.1152/ajplung.00446.2015

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

A 3-Dimensional Human Model of Bronchopulmonary Dysplasia Identifies Notch-Mediated Pathophysiology

Jennifer M. S. Sucre[1], Dan Wilkinson[2], Preethi Vijayaraj[1,4], Manash Paul[1], Bruce Dunn[2], Jackelyn A. Alva-Ornelas[1], Brigitte N. Gomperts[1,3,4,5]

[1] UCLA Children's Discovery and Innovation Institute, Mattel Children's Hospital UCLA, Department of Pediatrics, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA, 90095

[2] UCLA Department of Materials Science and Engineering, UCLA, Los Angeles, CA, USA, 90095

[3] Pulmonary Medicine, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA, 90095

[4] Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, CA, USA, 90095

[5] Eli and Edythe Broad Stem Cell Research Center, UCLA, Los Angeles, CA, USA, 90095

Running title: A human 3D model of BPD

Corresponding author: bgomperts@mednet.ucla.edu

Phone number: (310) 206-0772; Fax number: (310) 206-8089

24**ABSTRACT**

25Bronchopulmonary dysplasia (BPD) is a leading complication of premature birth and 26occurs primarily in infants delivered during the saccular stage of lung development. 27Histopathology shows decreased alveolarization and a pattern of fibroblast proliferation 28and differentiation to the myofibroblast phenotype. Little is known about the molecular 29pathways and cellular mechanisms that define BPD pathophysiology and progression. 30We have developed a novel 3-dimensional human model of BPD and using this model we 31have identified the Notch pathway as a key driver of disease pathogenesis. Fetal lung 32fibroblasts were cultured on sodium alginate beads to generate lung organoids. After 33exposure to alternating hypoxia and hyperoxia, the organoids developed a phenotypic 34response characterized by increased α-SMA expression and other genes known to be 35upregulated in BPD and also demonstrated increased expression of downstream effctors 36of the Notch pathway. Inhibition of Notch with a gamma-secretase inhibitor prevented 37the development of the pattern of cellular proliferation and α-SMA expression in our 38model. Analysis of human autopsy tissue from the lungs of infants who expired with BPD 39demonstrated evidence of Notch activation within fibrotic areas of the alveolar septae, 40suggesting that Notch may be a key driver of BPD pathophysiology.

41**INTRODUCTION**
42
43Bronchopulmonary dysplasia (BPD) is a form of chronic lung disease that is both a
44leading cause of morbidity in infants born prematurely and the most common chronic
45respiratory disease of infancy (3, 40). Between 23 and 32 weeks gestation, fetal lungs are
46in the canalicular and saccular stages of development. At this developmental stage, a
47combination of environmental insults to the lungs of premature infants, including
48hyperoxia, mechanical ventilation, inflammation, and infection, causes a pattern of lung
49injury and scarring that is characterized by arrest of alveolarization, vascular hypoplasia,
50increased proliferation of fibroblasts, and development of fibrosis (34). The cellular
51mechanisms that promote the development of BPD are not well understood nor are the
52factors that promote rapid progression of the disease (17). BPD is a complex disorder
53with clinical heterogeneity: not every extremely premature infant develops BPD, and the
54pathophysiology likely arises from a process involving gene-enviroment interaction, in
55addition to known environmental triggers.

56

57While there are multiple animal models that approximate BPD by exposing developing
58newborn animals to hyperoxia, infection, or mechanical ventilation, there is currently no
59human model of the disease (15, 18). Although much has been learned from the animal
60models, human lung development has a different trajectory than the mouse, the species
61used most commonly to model BPD (15). Term mouse pups are born in the saccular
62stage of lung development but unlike humans at this stage, they are surfactant sufficient
63and capable of normal gas exchange(34). This and other interspecies developmental
64differences underscore the need for a human model of BPD, both for improved

65understanding of the disease pathophysiology and for the development of targeted
66disease-modifying therapeutics. The pathogenesis of BPD likely includes multiple cell-
67cell signaling events and cross-talk between neighboring cells (14). In contrast to 2D
68traditional cell culture methods, a 3D tissue structure more closely approximates the *in*
69*vivo* architecture of the lung parenchyma and provides cells with the ability to grow in a
70geometry that mimics their *in vivo* environment. Our 3D disease model provides the
71opportunity to observe the development and pathogenesis of myofibroblast differentiation
72and gene expression changes in a way that more closely models the *in vivo* cell-cell
73signaling events that are thought to be involved in disease pathogenesis (33).

74

75The Notch pathway plays an essential role in normal lung development and has been
76previously described to be upregulated in response to airway injury and in response to
77oxidative stress in the proximal airways (32, 38). Here we describe a unique human 3D
78culture system that we have developed to model BPD and which we have used to identify
79the involvement of the Notch developmental pathway in the disease pathophysiology.

80 **METHODS**

81 *Human fibroblast isolation and cell culture*

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

91

92 *3D Culture on Alginate Beads*

93 Alginate beads were generated using an electrostatic droplet generator with 3% alginate
94 (Sigma) in the presence of an electric field of 9000V. Alginate beads were functionalized
95 by incubating with high-concentration rat tail collagen 1 (9.37 mg/ml, Corning) for 6
96 days and then coated with Tris-buffered dopamine hydrochloride (2mg/mL, Sigma) at pH
97 8.5. (please see Wilkinson *et al* for additional details) to promote cellular adhesion. 0.5ml
98 of sedimented alginate beads were loaded into a 2ml rotating bioreactor (Synthecon)
99 along with 2 x 10^6 fibroblasts. Organoid formation occurred for 96hrs, after which time
100 the organoid was removed and divided into two pieces, one of which was grown in
101 normoxic conditions and the other was placed in the hypoxic incubator. We exposed the
102 organoids to alternating hypoxia (10% O_2) and hyperoxia (70% O_2). We oscillated the

103organoids between the two conditions every 24 hours for a total of 4 days. For the HT 96-
104well organoids the same protocol was followed except 150 µl volume of beads and 1 x
105$10^5$ number of cells were seeded in each well of the 96-well plate.
106
107*Immunofluorescence (IF)*
108IF was performed as described previously (32). Organoid cultures were fixed in 4%
109paraformaldehyde, washed and either stained in the well or embedded in Histogel and
110subsequently paraffin embedded and sectioned (5 µm thickness). IF was done after Tris-
111EDTA-Tween/Citrate buffer-mediated antigen retrieval followed by permeabilization
112with 0.3% Triton X-100 in protein blocking buffer (Dako, North America Inc., USA) for
113at least 30 minutes at RT. Sections were incubated with primary antibodies diluted in
114blocking solution, overnight at 4°C. After several washes in TBST, sections were
115incubated with secondary antibodies for 1–2 hr at RT or overnight at 4°C, washed,
116counterstained with DAPI (Vector labs - Burlingame, CA) placed under a coverslip and
117the edges sealed with nail polish. Slides were then analyzed by fluorescent microscopy
118with a LSM 780 Zeiss confocal microscope (Carl Zeiss, Jena, Germany). The following
119primary antibodies were used: mouse anti-α-SMA (Sigma, A2547), rabbit anti-vimentin
120(Bioss, bs-0756r), rabbit anti-NICD (Abcam, ab8925).
121
122*Real time quantitative PCR (qPCR)*
123Total RNA was extracted from the cells around the beads using the RNeasy micro kit
124(Qiagen) according to the manufacturer's instructions. DNAse treatment was performed
125with RQ1 RNase-Free DNase (Promega). Reverse transcription was performed using

126SuperScript II First Strand Kit (Invitrogen). qPCR was performed with the Taqman PCR
127Master Mix (Applied Biosystems) or Sybr Green Supermix (Biorad) on an Applied
128Biosystems StepOne-plus Real-Time PCR System. Each RNA sample was reverse-
129transcribed in triplicate, and appropriate negative controls were included in each run.
130Gene-specific primer pairs and probes were obtained from Applied Biosystems (See table
131in supplemental appendix for probe catalog numbers and primer sequences). For analysis,
132the ΔCT method was applied with 18S and B2M as endogenous controls. Relative gene
133expression, presented as a ratio of a target gene to reference control, was used for analysis
134(32).

135

136*Statistics*

137Triplicate samples were used in each experiment. Experiments were repeated a minimum
138of three times. All values are reported as mean with error bars representing +/- SEM.
139Statistical analysis was performed using Microsoft Excel, with two-tailed Student's t-test
140being used for two-group comparisons. For all measurements, p-values less than 0.05
141were considered statistically significant.

142

143*Study Approval*

144Approval for this research was obtained by the UCLA Institutional Review Board.

145 **RESULTS**

146 *The 3D lung organoid model created an alveolar template that was scalable for high*
147 *throughput applications*

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

165

166 *Exposure of 3D lung organoids to alternating hypoxia and hyperoxia recapitulates the*
167 *phenotype of fibroblast activation seen in the fibrotic component of human BPD*

168In response to hypoxia-hyperoxia, we saw a phenotype of increased fibroblast activation
169as demonstrated by markedly increased expression of alpha-smooth muscle actin (α-
170SMA) by immunofluorescence (IF) when compared with organoids exposed to normoxia
171(Figure 2A). This pattern of stress-fiber α-SMA expression strongly resembled that seen
172in the lungs of human infants who died with BPD (Figure 2C). Whereas fetal lung
173(Figure 2C) and healthy newborn lungs have very little α-SMA expression in the alveolar
174spaces, the lungs of human infants with BPD have been previously described to have
175bands of α-SMA expressing fibroblasts at the alveolar septae(4). This band-like pattern
176of α-SMA expression in response to hypoxia-hyperoxia is recapitulated in our model in
177both the large organoids and in the HT organoids made in the 96-well plates (Figure 2B).
178
179We then used real-time quantitative PCR (qPCR) to examine the expression of ten genes
180from multiple gene families previously described to be upregulated in either human
181infants with BPD or in animal models of BPD(7, 11, 19, 21, 22, 28, 31). We found all ten
182genes to have significantly increased transcription in our model when compared with the
183normoxia control (Figure 3). As a negative control, three genes known to be expressed
184by fibroblasts but without known increased expression in BPD or in response to oxidative
185stress; vimentin, fibrobectin, and N-cadherin, were analyzed and these genes all showed
186no increase in expression in response to hypoxia-hyperoxia (Figure 3). In order to ensure
187that the changes in ambient oxygen in our incubator created changes in oxidative stress in
188the organoid, we measured expression of NQO1, a phase II enzyme that has been
189previously shown to be activated by Nrf2 in response to oxidative stress(9). NQO1
190showed significantly increased expression in the organoid exposed to hypoxia-hyperoxia

191when compared with normoxic controls, suggesting that the fluctuation in oxygen levels
192in the incubator was experienced by the cells in the submerged organoid cultures.
193
194As a control experiment, FLFs were cultured in 2D using standard tissue culture
195techniques and were exposed to the same pattern of alternating hypoxia and hyperoxia.
196When comparing the hypoxia-hyperoxia exposed 2D cells to normoxic controls, we did
197observe changes in fibroblast morphology including elongation of the cells, however we
198saw no changes in α-SMA expression by IF or at the level of gene expression for multiple
199targets known to be upregulated in BPD (Figure 4A). We did observe significantly
200increased expression of NQO1, indicating a cellular response to oxidative stress, and that
201the 2D cells were exposed to the increased oxygen levels (Figure 4A). In order to ensure
202that the phenotype we saw was both lung-specific and not an artifact of the 3D culture
203method itself, we isolated skin fibroblasts from neonatal foreskin and cultured these cells
204on the functionalized alginate beads, generating an organoid structure populated by skin
205fibroblasts. When exposed to alternating hypoxia-hyperoxia, we saw no changes in α-
206SMA expression by IF or in expression of the panel of BPD related genes by qPCR
207(Figure 4B). Lung organoids comprised of primary adult lung fibroblasts did not
208demonstrate the pattern of fibroblast activation and proliferation in response to
209alternating hypoxia and hyperoxia that was observed in the fetal lung fibroblasts (Figure
2104C). In fact, the adult lung fibroblast organoids exposed to variable oxygen exposure had
211significantly decreased expression of extracellular matrix genes such as Elastin, Col1A1,
212and Col3A1, with no change in a-SMA expression by qPCR or immunofluorescence.

213Therefore, the fibroblast proliferation and activation seen in the 3D BPD model was
214specific to fetal lung fibroblasts.
215
216*Activation of the Notch pathway is a key driver of the fibrotic phenotype in our model*
217*and is also seen in human infants with BPD*
218The Notch pathway has been shown by prior studies to play a critical role in lung
219development and septation, repair after injury to the airway, and oxidative stress
220response, and therefore, we hypothesized that the Notch pathway may be involved in the
221development of fibroblast activation and differentiation in our model(16, 24, 32, 37, 38).
222We found that in response to hypoxia-hyperoxia exposure, the areas of increased α-SMA
223expression in our model as observed by IF also demonstrated intranuclear staining for the
224activated form of Notch, Notch intracellular domain (NICD) (Figure 5A). When we pre-
225treated organoids with dibenzazepine (DBZ), a gamma-secretase specific inhibitor of
226Notch activation and signaling, the organoids did not develop increased areas of α-SMA
227expression following exposure to hypoxia-hyperoxia, as was seen in the organoids that
228were not treated with DBZ (Figure 5A). Additionally, the organoids pre-treated with DBZ
229had no NICD demonstrated in the nuclei (Figure 5A).
230
231We also found evidence of Notch pathway upregulation in our model at the level of gene
232expression. Analyzing RNA from our model showed significantly increased transcription
233of *NOTCH1* and *JAG1*, and downstream mediators *HES1, HEY1* (Figure 5B). *HES1*, one
234of the key downstream effectors of Notch signaling, is known to be expressed in fetal
235lung(20). Treating our lung organoid BPD model with DBZ decreased the expression of

236*HES1* and *HEY1* in response to hypoxia-hyperoxia as would be expected with inhibition
237of gamma-secretase. *NOTCH* and *JAG1*, which are upstream of this enzyme, did not
238change their expression in response to DBZ following exposure to hypoxia-hyperoxia
239(Figure 5B), which provides evidence that the inhibition by DBZ of the phenotype of
240fibroblast activation and a-SMA expression was occurring via the Notch pathway and not
241through off-target effects. Indeed, inhibiting Notch appeared to prevent the development
242of the hypoxia-hyperoixa phenotype completely, both at the level of α-SMA expression
243by immunostaining and at the level of gene expression for the panel of genes known to be
244upregulated in BPD (Figure 5C). Thus inhibiting Notch signaling altered the FLF
245myofibroblast differentiation and gene-expression pattern that define phenotype in our
246model.

247

248To determine if the Notch pathway identified in our model was of clinical relevance, we
249examined NICD expression in fetal lung tissue and in lung tissue taken at autopsy from 4
250infants who died with BPD. In the autopsy tissue from infants with BPD, we observed
251Notch activation, as evidenced by nuclear NICD expression by IF in the fibrotic alveolar
252septae in cells that were also expressing α-SMA (Figure 6A). Examination of fetal lung
253tissue by IF showed very little baseline expression of α-SMA and no intranuclear NICD
254(Figure 6B), which suggests that the FLF used in our model do not have increased
255expression of a-SMA or Notch signaling prior to exposure to hypoxia-hyperoxia. As a
256histologic comparison, we examined the lungs from term infants who were stillborn and
257found lower levels of a-SMA expression when compared with the BPD patients and no

258evidence of Notch activation as there was no intranuclear expression of NICD observed
259(Figure 6C).
260
261Another important aspect of the BPD phenotype in human infants is increased fibroblast
262proliferation (7). By PCNA staining, organoids exposed to hypoxia-hyperoxia
263demonstrated increased cellular proliferation compared to normoxic controls (Figure 7).
264DBZ inhibition of Notch signaling altered the cellular proliferation of FLFs exposed to
265hypoxia-hyperoxia as evidenced by PCNA staining of the BPD lung organoids cultured
266with and without DBZ (Figure 7). In response to hypoxia-hyperoxia exposure, lung
267organoids showed an increase in mitotic activity that decreased even below normoxia
268controls when the organoids were treated with DBZ (Figure 7).

269**DISCUSSION**

270Much of our understanding of BPD pathophysiology comes from several highly
271developed animal models, including sheep, rat, mouse, rabbit, pig, and baboon, that are
272exposed to various environmental stimuli thought to be implicated in BPD, including
273hyperoxia, mechanical ventilation, and maternal infection(1, 5, 8, 12, 18, 30, 39). While
274these animal models have taught us a great deal about BPD, there are key differences
275between other mammalian species and humans; for example, mice are born at term with
276lungs fully functional for gas exchange while still in the saccular stage of lung
277development, unlike their human counterparts(18). Of additional note, several targeted
278therapies have shown promise in animal models but have not shown benefit in humans,
279likely in part due to these developmental differences(18), which underscores the need for
280a human, *in vitro* model for the disease.

281

282In order to create a human model of BPD, we used a new 3D primary cell lung organoid
283model developed by our lab to create a cellular scaffold that replicates the geometry of
284the interstitial region between alveoli. In this model, alternating hypoxia and hyperoxia
285resulted in fibroblast proliferation, myofibroblast differentiation, and gene expression
286changes that replicate many of the changes seen in human infants with BPD. Alternating
287hypoxia and hyperoxia has been used in prior animal models(5) and appears consistent
288with the clinical course of premature infants, who are exposed to widely variable oxygen
289saturations, especially in the initial period after birth. The observed changes in α-SMA
290gene expression and protein expression by IF in response to hypoxia-hyperoxia are found
291in human infants with BPD and in animal models of BPD, thus validating the phenotypic

292response seen in our model in response to hypoxia-hyperoxia as a model of the
293mesenchymal component of BPD (4, 11, 19, 21, 28).
294
295Prior work has demonstrated that pulmonary mesenchyme is a critical driver of normal
296lung development (25). Specifically, mesenchymal cells in the developing lung have been
297shown to direct the processes of branching morphogenesis, epithelial differentiation,
298pulmonary vascular development, and secondary septation (4, 25-27). During the
299saccular stage of lung development, the alveolar septae are thickened and enriched in
300fibroblasts, and signaling events from these fibroblasts drives the process of
301alveolarization (6). Pulmonary fibroblasts are important cells in the pathogenesis of BPD,
302as the fibroblasts are thought to respond to environmental stress through paracrine
303signaling which drives their proliferation, activation to myofibroblasts, migration into the
304alveolar septae, and deposition of extracellular matrix (ECM) (4, 23). In developing our
305human model of BPD, we looked to the fetal lung fibroblast as the cell type that could
306best generate an observable myofibroblast phenotype in response to variable oxygen
307concentrations.
308
309One limitation of our model is that it includes a single mesenchymal cell type with the
310notable absence of the contribution from alveolar epithelial and endothelial cells.
311However, by focusing on the contribution of one isolated cell type, our 3D lung model
312allowed us to specifically assess the mesenchymal component of the disease as well as
313the role of pulmonary mesenchyme in expressing various genes that contribute to BPD
314phathophysiology, such as PDGFR□, α-SMA, and TGFβ. In the future, we plan to exploit

315the modularity of this model and incorporate additional lung cell types to study their
316interactions, e.g. mesenchymal cells, endothelial cells and epithelial cells, to gain insight
317into how communication between different cell types can drive the pathophysiology of
318this disease.

319

320Our lung organoid recreates the 3D niche microenvironment of fetal lung fibroblasts
321growing in close proximity in the alveolar interstitium. One possible reason for
322differences between the results in the cells cultured in 3D when compared to 2D is that
323the 3D culture system likely creates a microenviroment that allows the FLFs to engage in
324cell signaling by direct contact between adjacent cells, and through activation via
325paracrine signaling(33), which we speculate drives the pathophysiology both in the model
326of the disease and *in vivo(33, 35)*. In order for Notch signaling to occur, the cell
327expressing Notch ligand must be in direct contact with a neighboring cell expressing the
328Notch receptor. Our 3D model affords cells the opportunity to grow in close contact in
329the anatomically correct locations that allow this specific cell-cell contact and therefore
330these signaling events to take place.

331

332While the creation of this 3D microenvironment in our organoid was necessary to
333develop the mesenchymal phenotype in response to hypoxia-hyperoxia, it was not
334sufficient. The fetal lung fibroblast cell type grown in the organoid was critical for
335differentiation to myofibroblasts and expression of genes known to be upregulated in
336BPD as we did not observe this phenotype with neonatal skin fibroblasts or adult
337pulmonary fibroblasts. This is consistent with the development of BPD occurring only

338during a specific window in development in preterm infants and is congruous with the
339observation that adults exposed to hyperoxia and mechanical ventilation do not develop
340the same pathology as premature infants with the same exposures (10, 13). It is also
341consistent with the pathogenesis of BPD being dependent on developmental pathways. In
342addition, the recreation of the 3D fibroblast microenvironment in our model is scalable to
343a 96-well format, which provides a critical advance for developing potential future
344applications, such as a high throughput drug screen to prevent differentiation to
345myofibroblasts. These HT organoids also can provide a platform for the future study of
346multiple molecular pathways.

347

348While major advances in lung cellular biology and development have been made over the
349past fifty years, the precise cellular pathways and properties that regulate the
350pathophysiology of BPD remain elusive(14). Since BPD is defined by a developmental
351arrest in alveolar septation, we were interested in examining our model to see if there
352were developmental programs involved in the mechanism of the development of the
353fibrotic phenotype as well. It has been previously shown that in response to oxidative
354stress, Nrf2 activates the Notch pathway in other cell types in the lung(32) and liver(36,
35537) by binding to antioxidant response elements in the Notch promoter and that the Notch
356pathway is known to be activated in response to airway injury(38); overexpression of
357Notch is also known to inhibit alveolar septation(38), a process that is disrupted in
358BPD(3). Moreover, the Notch pathway has also been implicated in myofibroblast
359differentiation and the development of fibrosis in other pulmonary diseases(24). We
360found that the Notch pathway drove the cellular proliferation and differentiation to □-

361SMA positive myofibroblasts in our model and also demonstrated evidence for increased
362activated Notch expression by myofibroblasts in the lungs of human infants with BPD,
363and we did not see the presence of activated Notch in the lungs of term infants. The
364identification of the Notch pathway in BPD is significant; while Notch pathway
365expression in response to airway injury in the proximal airways has been well-
366described(29), Notch has not previously been identified in BPD pathophysiology and
367presents a new opportunity to look for druggable targets that interact with this pathway
368and its downstream effectors.

369

370In summary, we have developed an *in vitro* 3D human model of BPD in a novel culture
371system that replicates the alveolar architecture found in human distal lung. The
372phenotypic changes in our model have been validated by comparison with human BPD
373lung autopsy tissue and qPCR for specifically upregulated genes in BPD. Using our
374model, we have been able to augment our understanding of BPD pathophysiology,
375identifying the Notch pathway as being a key driver of the development of the phenotype
376of myofibroblast differentiation and gene expression in our model. The model has
377allowed us to identify that the Notch pathway is activated in the distal lung in BPD and
378may be a driver of the pathophysiology seen in BPD. As we are now able to generate HT
379organoids, in the future we will increase the scale of our investigation to identify
380additional molecular pathways and targets that may be perturbed in the clinical setting of
381changes in oxygen tension. By creating this HT human *in vitro* model of the disease we
382plan to create a 3D HT drug screen that will foster the discovery of novel therapies to
383improve the survival and outcomes for premature infants.

384**APPENDIX**

385Primer and probe details from qPCR experiments:

Gene	Catalog Number
18S	4318839
JAG1	hs01070032
HES1	hs00172878
HEY1	hs01114113
NQO1	Hs02512143

386

Gene	Primer Sequence
α-SMA	AAAAGACAGCTACGTGGGTG GCCATGTTCTATCGGGTACTTC
B2M	CGTGTGAACCATGTGACTTTG G CATCTTCAAACCTCCATG
TGFB1	CAATTCCTGGCGATACCTCAG GCACAACTCCG TGACATCAA
PDGFRa	TGGCAGTACCCCATGTCTGAA CCAAGACCGTCACAAA AGGC
PDE5a	GCAGAGTCCTCGTGCAGATAA GTCTAAGAGGCCGGTCAAATTC
LOX	CGGCGGAGGAAAACTGTCT TCGGCTGGGTAAGAAATCTGA
LOXL2	GGGTGGAGGTGTACTATGATGG CTTGCCGTAGGAGGAGCTG
COL1	GAGCGGTAACAAGGGTGAGC

	CTTCCCCATTAGGGCCTCTC
ET1	AGAGTGTGTCTACTTCTGCCA CTTCCAAGTCCATACGGAACAA
COL3	GGAGCTGGCTACTTCTCGC GGGAACATCCTCCTTCAACAG
TNC	TCCCAGTGTTCGGTGGATCT TTGATGCGATGTGTGAAGACA
ELN	GCAGGAGTTAAGCCCAAGG TGTAGGGCAGTCCATAGCCA

387

388**GRANTS**
389
390This work was supported by a Clinical Fellowship Training Grant from the Eli and

391Edythe Broad Center of Regerative Medicine and Stem Cell Research and the California

392Institute of Regenerative Medicine and through the Scholars in Translational Medicine

393Program at the Broad Stem Cell Research Center (JMSS). The work was also supported

394by R01 GM114259-01 and California Institute for Regenerative Medicine (CIRM) 12-02

395(BNG) and NSF DGE-1144087 (DW).

396**DISCLOSURES**

397The authors report no conflicts of interest, financial or otherwise.

398

AUTHOR CONTRIBUTIONS

399 JMSS designed the research study, conducted experiments, acquired data, performed data
400 analysis, and wrote the manuscript. DW developed experiments and experimental
401 protocols, conducted experiments, and contributed to manuscript writing and review. PV
402 contributed to experimental design, performed experiments, acquired data, and edited
403 manuscript. MP contributed to experimental design, performed experiments, acquired
404 data, and edited manuscript. BD contributed to experimental design. JA performed
405 experiments, acquired data, contributed to manuscript writing and review. BG designed
406 the study, analyzed the data, and wrote the manuscript.,

407**ACKNOWLEDGEMENTS**

408We are grateful to Ved Londhe, April Pyle, and Sherin Devaskar for helpful discussions
409about our model and experiments and to Brian Hackett for careful and thoughtful review
410of the manuscript.

411 **REFERENCES**

412 1. **Albertine KH**. Utility of large-animal models of BPD: chronically ventilated
413 preterm lambs. *Am J Physiol Lung Cell Mol Physiol* 308: L983-L1001, 2015.
414 2. **Ascolani G, and Lio P**. Modeling TGF-beta in early stages of cancer tissue
415 dynamics. *PLoS One* 9: e88533, 2014.
416 3. **Baraldi E, and Filippone M**. Chronic lung disease after premature birth. *N Engl*
417 *J Med* 357: 1946-1955, 2007.
418 4. **Benjamin JT, Smith RJ, Halloran BA, Day TJ, Kelly DR, and Prince LS**.
419 FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor
420 activation. *Am J Physiol Lung Cell Mol Physiol* 292: L550-558, 2007.
421 5. **Berger J, and Bhandari V**. Animal models of bronchopulmonary dysplasia. The
422 term mouse models. *Am J Physiol Lung Cell Mol Physiol* 307: L936-947, 2014.
423 6. **Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna**
424 **M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J,**
425 **Heath JK, and Betsholtz C**. PDGF-A signaling is a critical event in lung alveolar
426 myofibroblast development and alveogenesis. *Cell* 85: 863-873, 1996.
427 7. **Bourbon J, Boucherat O, Chailley-Heu B, and Delacourt C**. Control
428 mechanisms of lung alveolar development and their disorders in bronchopulmonary
429 dysplasia. *Pediatr Res* 57: 38R-46R, 2005.
430 8. **Caminita F, van der Merwe M, Hance B, Krishnan R, Miller S, Buddington**
431 **K, and Buddington RK**. A preterm pig model of lung immaturity and spontaneous infant
432 respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 308: L118-129, 2015.
433 9. **Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, and**
434 **Kleeberger SR**. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J*
435 *Respir Cell Mol Biol* 26: 175-182, 2002.
436 10. **Cho HY, Reddy SP, Debiase A, Yamamoto M, and Kleeberger SR**. Gene
437 expression profiling of NRF2-mediated protection against oxidative injury. *Free Radic*
438 *Biol Med* 38: 325-343, 2005.
439 11. **Cuna A, Halloran B, Faye-Petersen O, Kelly D, Crossman DK, Cui X, Pandit**
440 **K, Kaminski N, Bhattacharya S, Ahmad A, Mariani TJ, and Ambalavanan N**.
441 Alterations in gene expression and DNA methylation during murine and human lung
442 alveolar septation. *Am J Respir Cell Mol Biol* 53: 60-73, 2015.
443 12. **D'Angio CT, and Ryan RM**. Animal models of bronchopulmonary dysplasia.
444 The preterm and term rabbit models. *Am J Physiol Lung Cell Mol Physiol* 307: L959-969,
445 2014.
446 13. **Datta A, Kim GA, Taylor JM, Gugino SF, Farrow KN, Schumacker PT, and**
447 **Berkelhamer SK**. Mouse lung development and NOX1 induction during hyperoxia are
448 developmentally regulated and mitochondrial ROS dependent. *Am J Physiol Lung Cell*
449 *Mol Physiol* 309: L369-377, 2015.
450 14. **Hadchouel A, Franco-Montoya ML, and Delacourt C**. Altered lung
451 development in bronchopulmonary dysplasia. *Birth Defects Res A Clin Mol Teratol* 100:
452 158-167, 2014.
453 15. **Hilgendorff A, Reiss I, Ehrhardt H, Eickelberg O, and Alvira CM**. Chronic
454 lung disease in the preterm infant. Lessons learned from animal models. *Am J Respir Cell*
455 *Mol Biol* 50: 233-245, 2014.

45616. **Hu B, Wu Z, Bai D, Liu T, Ullenbruch MR, and Phan SH**. Mesenchymal
457deficiency of Notch1 attenuates bleomycin-induced pulmonary fibrosis. *Am J Pathol* 185:
4583066-3075, 2015.
45917. **Jensen EA, and Schmidt B**. Epidemiology of bronchopulmonary dysplasia.
460*Birth Defects Res A Clin Mol Teratol* 100: 145-157, 2014.
46118. **Jobe AH**. Animal Models, Learning Lessons to Prevent and Treat Neonatal
462Chronic Lung Disease. *Front Med (Lausanne)* 2: 49, 2015.
46319. **Kaarteenaho-Wiik R, Kinnula VL, Herva R, Soini Y, Pollanen R, and
464Paakko P**. Tenascin-C is highly expressed in respiratory distress syndrome and
465bronchopulmonary dysplasia. *J Histochem Cytochem* 50: 423-431, 2002.
46620. **Katoh M, and Katoh M**. Integrative genomic analyses on HES/HEY family:
467Notch-independent HES1, HES3 transcription in undifferentiated ES cells, and Notch-
468dependent HES1, HES5, HEY1, HEY2, HEYL transcription in fetal tissues, adult tissues,
469or cancer. *Int J Oncol* 31: 461-466, 2007.
47021. **Kumarasamy A, Schmitt I, Nave AH, Reiss I, van der Horst I, Dony E,
471Roberts JD, Jr., de Krijger RR, Tibboel D, Seeger W, Schermuly RT, Eickelberg O,
472and Morty RE**. Lysyl oxidase activity is dysregulated during impaired alveolarization of
473mouse and human lungs. *Am J Respir Crit Care Med* 180: 1239-1252, 2009.
47422. **Lee KJ, Berkelhamer SK, Kim GA, Taylor JM, O'Shea KM, Steinhorn RH,
475and Farrow KN**. Disrupted pulmonary artery cyclic guanosine monophosphate signaling
476in mice with hyperoxia-induced pulmonary hypertension. *Am J Respir Cell Mol Biol* 50:
477369-378, 2014.
47823. **Li J, Li Y, He H, Liu C, Li W, Xie L, and Zhang Y**. Csk / Src / EGFR Signaling
479regulates migration of myofibroblasts and alveolarization. *Am J Physiol Lung Cell Mol
480Physiol* ajplung 00162 02015, 2016.
48124. **Liu T, Hu B, Choi YY, Chung M, Ullenbruch M, Yu H, Lowe JB, and Phan
482SH**. Notch1 signaling in FIZZ1 induction of myofibroblast differentiation. *Am J Pathol*
483174: 1745-1755, 2009.
48425. **McCulley D, Wienhold M, and Sun X**. The pulmonary mesenchyme directs lung
485development. *Curr Opin Genet Dev* 32: 98-105, 2015.
48626. **McGowan SE**. Paracrine cellular and extracellular matrix interactions with
487mesenchymal progenitors during pulmonary alveolar septation. *Birth Defects Res A Clin
488Mol Teratol* 100: 227-239, 2014.
48927. **McGowan SE, and McCoy DM**. Fibroblast growth factor signaling in
490myofibroblasts differs from lipofibroblasts during alveolar septation in mice. *Am J
491Physiol Lung Cell Mol Physiol* 309: L463-474, 2015.
49228. **McGowan SE, and McCoy DM**. Fibroblasts expressing PDGF-receptor-alpha
493diminish during alveolar septal thinning in mice. *Pediatr Res* 70: 44-49, 2011.
49429. **Mori M, Mahoney JE, Stupnikov MR, Paez-Cortez JR, Szymaniak AD,
495Varelas X, Herrick DB, Schwob J, Zhang H, and Cardoso WV**. Notch3-Jagged
496signaling controls the pool of undifferentiated airway progenitors. *Development* 142: 258-
497267, 2015.
49830. **O'Reilly M, and Thebaud B**. Animal models of bronchopulmonary dysplasia.
499The term rat models. *Am J Physiol Lung Cell Mol Physiol* 307: L948-958, 2014.
50031. **Olave N, Lal CV, Halloran B, Pandit K, Cuna AC, Faye-Petersen OM, Kelly
501DR, Nicola T, Benos P, Kaminski N, and Ambalavanan N**. Regulation of Alveolar

502Septation by MicroRNA-489. *Am J Physiol Lung Cell Mol Physiol* ajplung 00145 02015, 5032015.
50432. **Paul MK, Bisht B, Darmawan DO, Chiou R, Ha VL, Wallace WD, Chon AT, 505Hegab AE, Grogan T, Elashoff DA, Alva-Ornelas JA, and Gomperts BN**. Dynamic 506changes in intracellular ROS levels regulate airway basal stem cell homeostasis through 507Nrf2-dependent Notch signaling. *Cell Stem Cell* 15: 199-214, 2014.
50833. **Ravi M, Paramesh V, Kaviya SR, Anuradha E, and Solomon FD**. 3D cell 509culture systems: advantages and applications. *J Cell Physiol* 230: 16-26, 2015.
51034. **Silva DM, Nardiello C, Pozarska A, and Morty RE**. Recent advances in the 511mechanisms of lung alveolarization and the pathogenesis of bronchopulmonary dysplasia. 512*Am J Physiol Lung Cell Mol Physiol* 309: L1239-1272, 2015.
51335. **Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas 514C, Mendiola M, Hardisson D, and Eccles SA**. Advances in establishment and analysis 515of three-dimensional tumor spheroid-based functional assays for target validation and 516drug evaluation. *BMC Biol* 10: 29, 2012.
51736. **Wakabayashi N, Chartoumpekis DV, and Kensler TW**. Crosstalk between 518Nrf2 and Notch signaling. *Free Radic Biol Med* 88: 158-167, 2015.
51937. **Wakabayashi N, Skoko JJ, Chartoumpekis DV, Kimura S, Slocum SL, Noda 520K, Palliyaguru DL, Fujimuro M, Boley PA, Tanaka Y, Shigemura N, Biswal S, 521Yamamoto M, and Kensler TW**. Notch-Nrf2 axis: regulation of Nrf2 gene expression 522and cytoprotection by notch signaling. *Mol Cell Biol* 34: 653-663, 2014.
52338. **Xu K, Moghal N, and Egan SE**. Notch signaling in lung development and 524disease. *Adv Exp Med Biol* 727: 89-98, 2012.
52539. **Yoder BA, and Coalson JJ**. Animal models of bronchopulmonary dysplasia. The 526preterm baboon models. *Am J Physiol Lung Cell Mol Physiol* 307: L970-977, 2014.
52740. **Zysman-Colman Z, Tremblay GM, Bandeali S, and Landry JS**. 528Bronchopulmonary dysplasia - trends over three decades. *Paediatr Child Health* 18: 86-52990, 2013.
530

531**FIGURE CAPTIONS:**

532Figure 1: Development of the 3D lung organoid: A) Brightfield micrograph of alginate 533beads, generated by cross-linking with barium in the presence of an electric field. Scale 534bar = 200µm. B) Calcein intravital stain of beads after 24 hours in the bioreactor showing 535FLFs coating the beads. Scale bar = 150µm. C) Photograph of lung organoid generated 536after 4 days in rotating bioreactor. Scale bar = 1cm. D) Hematoxylin and eosin stain of a 537cross section of a lung organoid showing FLFs growing around alginate beads. Scale bar 538= 200µm. E) Hematoxylin and eosin stain of a cross section of normal human lung. Scale 539bar = 200µm. F) Brightfield microscopy of lung organoid. Scale bar = 100µm. G) 540Brightfield microscopy of HT lung organoid growing in a well of a 96-well plate. Scale 541bar = 1.6mm.

542

543Figure 2: α-SMA IF of the 3D BPD model. A) α-SMA IF of 3D lung organoids exposed 544to hypoxia-hyperoxia (bottom panel) vs. normoxia controls (top panel). B) α-SMA IF of 545high throughput 3D lung organoids exposed to hypoxia-hyperoxia (bottom panel) vs. 546normoxia controls (top panel) . C) α-SMA IF of fetal lung tissue (top panel) vs lung tissue 547from an infant with severe BPD (bottom panel). Scale bar = 50µm

548

549Figure 3: Validation of the 3D BPD model at the level of gene expression. Comparison 550of gene expression differences of molecular targets known to upregulated in BPD by 551qPCR in 3D lung organoids grown in normoxia vs exposed to hypoxia-hyperoxia. 552Triplicate samples were used in each experiment. Experiments were repeated a minimum 553of three times. *p<0.001.

554

555Figure 4: Changes in α-SMA expression and other genes associated with BPD were
556specific to fetal lung fibroblasts cultured in 3D. A) IF for α-SMA and vimentin expression
557and BPD target gene expression pattern by qPCR in 2D FLF cultured in normoxia and
558hypoxia-hyperoxia conditions. Scale bar = 50μm. B) IF for α-SMA and vimentin
559expression and BPD target gene expression pattern by qPCR in 3D skin fibroblasts
560cultured in normoxia and hypoxia-hyperoxia conditions. Scale bar = 50μm. C) IF for α-
561SMA and vimentin expression and BPD target gene expression pattern by qPCR in 3D
562adult primary fibroblasts cultured in normoxia and hypoxia-hyperoxia conditions. Scale
563bar = 50μm. Triplicate samples were used in each experiment. Experiments were repeated
564a minimum of three times. *p<0.001.

565

566

567Figure 5: Notch pathway involvement in BPD pathophysiology in the 3D lung model*:* A)
568IF for α-SMA and NICD expression in 3D FLF lung organoids comparing normoxia to
569hypoxia-hyperoxia conditions, with and without pretreatment with Notch inhibitor DBZ.
570Examination of α-SMA positive fibroblasts (inset) showed evidence of intranuclear
571presence of NICD. Scale bar = 50μm B) Expression of downstream effectors of the
572Notch pathway, HES1 and HEY1, upstream targets JAG1 and NOTCH1 and oxidative
573stress enzyme NQO1 as measured by qPCR in response to hypoxia-hyperoxia and
574treatment with DBZ, *p<0.01. C) Comparison by qPCR of the gene expression pattern
575of molecular targets known to be upregulated in BPD between 3D organoids grown in
576normoxia and organoids exposed to hypoxia-hyperoxia, with and without pretreatment

577with Notch inhibitor DBZ, *p<0.01. Triplicate samples were used in each experiment.
578Experiments were repeated a minimum of three times.

579

580Figure 6: Notch pathway involvement in patient samples*:* A) IF for α-SMA and NICD
581expression in autopsy tissue from the lungs of 4 human infants who died with BPD. Scale
582bar = 25μm. B) IF for baseline α-SMA and NICD expression in fetal lung. Scale bar =
58320μm. C) IF for α-SMA and NICD expression in autopsy tissue from the lung of 3
584stillborn term human infants. Scale bar = 25μm.

585

586Figure 7: Inhibition of Notch decreased myofibroblast differentiation as well as cellular
587proliferation as measured by intranuclear PCNA expression. PCNA and α-SMA IF of
588organoids cultured in normoxia and hypoxia-hyperoxia exposure, with and without
589pretreatment with Notch inhibitor DBZ. Scale bar = 20 μm. 200 cells were counted in
590triplicate for each condition and reported as average % positive for each group, *p<0.01.
591Triplicate samples were used in each experiment. Experiments were repeated a minimum
592of three times.