ANIMAL MODELS OF BRONCHOPULMONARY DYSPLASIA.

I: THE TERM MOUSE MODELS

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ABSTRACT

The etiology of BPD is multifactorial with genetics, ante- and post-natal sepsis, invasive mechanical ventilation and exposure to hyperoxia being well-described as contributing factors. Much of what is known about the pathogenesis of BPD is derived from animal models being exposed to the environmental factors noted above. This review will briefly cover the various mouse models of BPD, focusing mainly on the hyperoxia-induced lung injury models. We will also include hypoxia, hypoxia/hyperoxia, inflammation-induced and transgenic models in room air. Attention to the stage of lung development at the timing of the initiation of the environmental insult and the duration of lung injury is critical to attempt to mimic the human disease pulmonary phenotype -- both in the short-term and outcomes extending into childhood, adolescence and adult. The various indices of alveolar and vascular development as well as pulmonary function including pulmonary hypertension will be highlighted. The advantages (and limitations) of using such approaches will be discussed in the context of understanding the pathogenesis of, and targeting therapeutic interventions to ameliorate human BPD.
Bronchopulmonary Dysplasia (BPD)

BPD is a clinically significant morbidity associated with preterm birth. The current clinical definition, derived from conclusions by the 2001 NIH Consensus Group, defines BPD based on the number of postnatal (PN) days of supplemental oxygen received, as well as oxygen and positive pressure ventilation use at 36 weeks’ post-menstrual age (PMA) (43). Though oxygen requirement, and thus diagnosis of BPD, may be clinically subjective, one validated method to characterize supplemental oxygen requirement is the “physiologic definition of BPD” described by Walsh et al. This method aims to evaluate and quantify the infant’s true oxygen needs as compared to their clinically-determined settings through an oxygen reduction trial culminating in a room air challenge at 36 weeks’ PMA (86).

BPD is characterized by decreased alveolar septation and dysregulated development of the pulmonary microvasculature (13, 43). This histologic description is often labeled “new” BPD and differs from “old” BPD in several ways. Under the microscope, lungs with “new” BPD have larger, simplified, cystic alveoli and irregular pulmonary vessels. Less prominent are the widespread fibrosis and smooth muscle hypertrophy that were hallmarks of “old” BPD. This change is thought to reflect differences in the timing and mechanism of lung insult and injury compared to decades past (11).

BPD is common among preterm neonates, with rates in this population ranging from 22-68% (27, 77). Infants afflicted with BPD often suffer from respiratory (12, 20, 32, 59) and neurocognitive sequelae (74, 75) that may persist in the short-term or for years after initial diagnosis.

The etiology of BPD is multifactorial. Genetic susceptibility (17, 49, 67) and environmental factors have all been implicated in the etiology of BPD (3, 12). Inflammation and trauma at
critical stages of lung development lead to dysmorphic lung tissue with impaired functionality
(12, 33, 47). Many believe that prenatal infections such as chorioamnionitis drive pro-
inflammatory changes including increased cytokine concentrations that may predispose infants to
prematurity as well as impaired lung maturation (3, 42), though this is controversial. Recent
evidence suggests that confounders and bias may have overestimated the link between
chorioamnionitis and BPD (48). Postnatally, invasive ventilation and supplemental oxygen are
mainstays in the early management of preterm neonates. Paradoxically, prolonged exposure to
these life-saving therapies is a known cause of BPD. Invasive ventilation damages lung tissue by
several mechanisms, including barotrauma and presence of a foreign body in the airway.
Prolonged exposure to supraphysiologic concentrations of oxygen may damage lung tissue
through free radical formation (12, 33, 47).
To date, much of what is known about the etiology of BPD originates in clinical studies of
infants or animal models of BPD using rodents or non-human primates. The use of animal
models, particularly rodent models, to study pulmonary disease in neonates dates back several
decades. As early as the 1930s, Smith et al exposed rats of varied ages to high oxygen tension
greater than 80% to examine the effect on lung morphology as well as the period of recovery.
The mice used were divided into “young” and “old” using a cutoff of 100 days of life, though
none of the mice were neonates. The investigators identified pulmonary hyperplasia and alveolar
hypercellularity, particularly in the older mice, and also observed hyaline cartilage deposition in
the alveolar walls (76). Early pioneering studies such as this paved the way for further study of
pulmonary disease in the neonate using younger and younger animals to better model the human
condition.
By the mid-1950s, experimental studies using rabbits were describing the condition known as hyaline membrane disease by exposing neonatal animals to high concentrations of oxygen (10).

Nearly a decade later, in 1965, Helström and Nergårdh sought to study the toxic effects of high environmental oxygen concentrations and to investigate the potential protective effects of hypothermia. The investigators used newborn mice within 24 hours of life and exposed some to continuous oxygen at 90% concentration. Others were exposed to high oxygen concentrations for five days followed by 10 days of normoxia. A subset of each group was also treated with hypothermia at different time points in the experimental period. Upon sacrifice of the animals, the lungs were sectioned and examined. Animals exposed to prolonged oxygen had poorly developed, emphysematous lungs with fewer alveolar vessels and scattered hemorrhage, as compared to control animals whose lungs were morphologically normal. Those exposed to a brief period of oxygen followed by room air had normal-appearing lungs, suggesting signs of recovery. In all study groups, hypothermia had little impact on the damage done by continuous oxygen exposure (36).

In addition to studying morphology, investigators in the 1970s began to investigate the biochemical effects of high oxygen concentrations on the lungs, a precursor to the studies of today that investigate mRNA and protein expression as markers of disease. In 1970, Rosan et al demonstrated increased synthesis of ribosomes with abnormal protein structures in newborn guinea pigs exposed to 80-100% oxygen. By microscopy, the investigators observed that the rapid synthesis of abnormal ribosomes preceded lung metaplasia and emphysema (73). Northway et al also described a decrease in DNA synthesis and cell replication in mice exposed to 96-100% O₂, as represented by decreased incorporation of radio-labeled thymidine into lung DNA. In light of these findings, Northway et al postulated that lower concentrations of O₂ might have similar
This process can be reversed in animals who survive the initial insult (63). In the late 1970s, the biochemistry of BPD was investigated further as specific enzyme activity was quantified in the setting of exposure to hyperoxia. Studies quantifying the activity of superoxide dismutase, catalase, and glutathione peroxidase in multiple rodent species and rabbits propagated the free radical theory of lung injury in response to hyperoxia (29).

These early studies and those that followed manipulated known or suspected elements in the development of BPD to gain a better understanding of the complex interplay of factors that contribute to this multi-faceted and challenging disease.

This review aims to add to the existing body of literature summarizing and analyzing current murine models of bronchopulmonary dysplasia. This review will describe several of the various methods of producing BPD-like disease in mice, including hyperoxia, hypoxia, invasive ventilation, inflammation, and genetic manipulation. Considering the limitations of methodology and technology, this review will assess the advantages and disadvantages of each technique and will discuss the cutting edge ways in which investigators are working to make these model systems more faithful to human disease.

Rodent Models of Pulmonary Disease

Rodents, for practical reasons, make excellent experimental animals. In addition to small size, other factors, such as short life cycle, ease of animal husbandry, and ready availability of rodent antibodies make mice easier to work with than larger animals. Low cost of purchase and upkeep allows for larger sample sizes for study (26).
It should be noted that, though they share many similarities, rats and mice are not interchangeable as animal models. There is a paucity of published work comparing mice to their rodent relatives, but basic examinations of lung anatomy suggest that the lung structure of the mouse is not identical to that of the rat. The mouse has a smaller total lung capacity than the rat (1mL compared to 10mL). The lung parenchyma of the mouse represents only 18% of the total lung volume, compared to 24% in the rat, whereas airway space occupies nearly twice as much of the total lung volume in the mouse (11%) than the rat (5.7%). Mice have also been shown to have smaller alveoli than rats (40).

Few studies have compared mice and rats as experimental models of BPD, but one study by Hoshikawa et al showed different patterns of gene expression and lung tissue morphology in mice and rats exposed to similar hypoxic conditions. By exposing young, but not neonatal, Sprague-Dawley rats and C57BL/6 mice to chronic hypoxia, investigators showed that rats developed significant arterial wall thickening, whereas mice did not. Additionally, the expression of genes modulating a wide variety of processes, including vascular proliferation, apoptosis, vascular tone, and antioxidation differed between species (39). These data suggest that different experimental animal species may not respond equivalently to the same interventions, a fact that should be considered when comparing studies. Indeed, even different strains of mice may respond differently to experimental exposures (83). Additional work in this area, however, demonstrated similar patterns of both mRNA expression and direct changes to cellular number and morphometry in different strains of mice (C57B1/6J, 129/J, and C3H/HeJ) exposed to >95% O₂ despite minor changes in the rate of change (44).

Mouse Lung Development
Previous studies in the area of vertebrate embryogenesis have identified five distinct stages of lung development that tend to be conserved across species. These stages temporally group the anatomical and physiologic changes necessary for lung maturation, including vascularization, alveolar septation, and surfactant synthesis. Neonatal mice and humans undergo similar stages of lung development, but differ with respect to the duration of each stage and its temporal relationship to gestational age (Table 1) (37, 46, 52-54, 80). Of note, there is considerable overlap in reported ranges for each phase of pulmonary development.

The penultimate or saccular stage of lung development is crucial in preparing the fetus for extra-uterine life. In this stage, surfactant production is initiated and the terminal airways dilate and vascularize (46, 47, 52). For neonatal mice and humans, the embryonic, canalicular, and pseudoglandular stages take place within the uterus. For mice, however, the saccular stage begins in the uterus and continues through PN day five (52). This is a critical difference between the two species. Whereas mouse pups delivered during the saccular stage do not have respiratory distress and are fully capable of extra-uterine survival, human neonates delivered during the saccular stage are preterm infants and are at high risk for development of respiratory distress syndrome due to pulmonary immaturity (47). It is important to mention here that while the lungs of the mouse born at term are in the saccular stage and surfactant-sufficient, this can be considered somewhat akin to a human preterm neonate in the same stage of lung development that has been exposed to a full complement of antenatal steroids (which is known to enhance surfactant production).

The distinction between the pulmonary developmental stages of mice and humans is meaningful to the design of experimental mouse models that aim to mimic BPD. As such, the timing of
exposures or interventions must be considered when evaluating the validity of any model system of BPD.

Mouse Models of BPD

This review will discuss numerous types of experimental mouse models that exist in the current literature that aim to reproduce the clinical and histologic stages of BPD. Some of the models discussed include pure hyperoxia, invasive ventilation, mixed hypoxia/hyperoxia, pure hypoxia, inflammation/chorioamnionitis, and transgenic mice in room air. As the number of studies in the literature is tremendous, we aimed to be as inclusive as possible while highlighting key examples of each model and the significant findings from the studies. We created a table to broaden the number of studies described beyond those that are discussed within the text itself (Table 2).

Hyperoxia

The current body of literature describes several mouse models designed on the concept that hyperoxia, or exposure to high fractions of inspired oxygen (FiO₂), is associated with the development of BPD. On a cellular level, hyperoxia causes direct injury to cells via reactive oxygen species, recruits inflammatory cells to the lung and sets in motion pro-apoptotic pathways (15).

Many studies of the effects of hyperoxia use continuous exposure to high concentrations of oxygen in order to induce lung injury. These studies vary based on the duration of exposure and
the concentration of oxygen, both of which influence the degree of congruence of the BPD mouse model.

In an early study, Warner et al showed significant changes to the morphology of lung tissue in mouse pups exposed to 85% oxygen from PN day 0-28, including decreased septation of the alveoli, enlarged and simplified terminal air spaces, and a greater degree of pulmonary fibrosis. These anatomical changes were present as early as PN7 and worsened over time. Additionally, this study demonstrated decreased pulmonary cell proliferation in hyperoxia-exposed subjects at PN14 as compared to room air controls, though cell counts normalized between PN14 and PN28. RT-PCR analysis showed increased inflammation in hyperoxic mice, including higher levels of interleukin (IL)-1α and macrophage inflammatory protein-1α, as compared to controls (88).

Administration of an IL-1α receptor antagonist in another model of BPD induced with prenatal lipopolysaccharide (LPS) and PN hyperoxia showed decreases in many inflammatory mediators, including but not limited to IL-1 (61). These data suggest that prolonged exposure to hyperoxia produces a physiologic and histologic picture similar to BPD, which may be mediated in part by inflammatory cytokines. Other groups have used similar models based on 28 days of hyperoxia exposure (81, 90). However, the success of the 28-day hyperoxia model is limited in its ability to mimic “new” BPD, which is not characterized by as significant an increase in pulmonary fibrosis as the “old” definition of BPD (11).

As described above, 28 days of hyperoxia produces a model that is closer histologically to pulmonary fibrosis and “old” BPD than to the currently accepted “new” BPD. This remains true for 14 or 21 days of hyperoxia, which also extend beyond the saccular phase of development. Zhang et al produced a hyperoxia model analyzing the microRNA profile of mice exposed to hyperoxia for 21 days compared to room air controls. Subsequently, this group used the same
model to assess the efficacy of mesenchymal stem cell therapy on the BPD phenotype (92, 93).

Velten et al devised a 14-day hyperoxia followed by a 14-day normoxia model to assess the effects of a shorter, but still prolonged period of hyperoxia followed by recovery. Velten et al also studied the effect of prenatal inflammation on lung tissue by injecting pregnant dams with LPS or saline controls. At birth, pups were then exposed to either room air or 0.85 FiO₂ followed by a recovery period. The study concluded that the combination of prenatal inflammation and PN hyperoxia produced a greater degree of lung injury, with significantly enlarged alveoli, pulmonary fibrosis, and macrophage infiltrate, than either a prenatal or PN insult alone (84).

Fourteen day models have also been used to study the effects of mesenchymal stem cells (34). In terms of mimicking the human condition, the most successful hyperoxia models are those that limit exposure to hyperoxia to the saccular stage of pulmonary development. Yee et al produced a mouse model of BPD by exposing mice to hyperoxia of varying concentrations (40%, 60%, 80%, 100%) between PN1-4 and allowing the mice to recover at room air until postnatal week eight. At week eight, mouse lung tissue had enlarged alveoli and increased lung compliance.

Protein levels and surfactant phospholipid composition of pulmonary secretions were measured by bronchoalveolar lavage and were not significantly affected by hyperoxia. Data also showed a dose-dependent effect of hyperoxia on lung tissue, with lower concentrations corresponding to less severe disease than higher concentrations (91). The model by Yee et al simulated the clinical course of many neonates in an intensive care unit who are exposed to a brief, intense period of supplemental oxygen therapy and are then weaned off supplemental oxygen as they recover. In limiting hyperoxia to the saccular stage of development and studying the lung tissue after several weeks of recovery, Yee et al were able to show the persistent, deleterious effects of hyperoxia early in PN life. Additional work by this group showed that mice exposed to hyperoxia between
PN1-4 developed increased levels of inflammatory markers and mortality in response to infection with the Influenza A virus as compared to room air controls (64). This result, which was also dose-dependent (23), mimics the increased susceptibility to respiratory tract infections that has been well-studied in formerly preterm human infants with BPD (12). The same BPD model has also been reported by other investigators (25, 50).

Other studies have also looked at the dose-dependent effects of hyperoxia over various durations of time. The degree of hyperoxia plays a significant role in the level of impairment seen. Studies show dose-dependent derangements of pulmonary structure and function after exposure to varying concentrations of oxygen. Neonatal mice exposed to more than 0.9 FiO\textsubscript{2} showed decreased alveolarization and impaired weight gain by PN14 as compared to mice exposed to 80% O\textsubscript{2}. The higher oxygen group also had a greater degree of neutrophilic infiltration. By PN7, the higher oxygen group had higher levels of prostaglandins, though there were no significant differences between groups in other downstream products of arachidonic acid breakdown, suggesting a specific effect produced by hyperoxia on this branch of the pathway\cite{72}. As described above, Yee et al demonstrated significant differences in outcomes at lower concentrations of oxygen, as well \cite{91}.

In addition to the studies mentioned above, several recent studies have used varying days of hyperoxia exposure to identify changes in cellular biochemistry, including abnormal cross-linking of essential enzymes in mice with a BPD phenotype \cite{89} and changes in the cellular regulation of smooth muscle actin expression, which indicates deranged myofibroblast differentiation \cite{68}.

There are several advantages to using a hyperoxia-induced model of BPD. Hyperoxia is a known factor in the development of BPD. Hyperoxic conditions are easily reproduced and levels of FiO\textsubscript{2}
are easily manipulated to study dose dependence. Hyperoxia can also be combined with other interventions to generate more nuanced models of BPD that better mimic clinical conditions. There are limitations to hyperoxia models. Based on the correlation between mouse and human lung development, timing of such experiments may preferably be restricted to the first 4-5 days of PN life, which limits hyperoxia exposure to the saccular stage and the transition to the alveolar stage and reduces fibrosis, which is not a prominent histologic characteristic of “new” BPD (11). Experimental data suggest that variable concentrations of hyperoxia exposure could mimic the differential severity of BPD (23, 50, 91). As such, subsequent studies should continue to use a wide range of oxygen concentrations to better understand the effects of commonly used doses in clinical settings.

**Invasive Ventilation Model**

As previously discussed, invasive ventilation is often needed in preterm infants and may contribute to the development of BPD. Mokres et al were successful in creating a mouse model to mimic some of the characteristics of BPD using brief periods of invasive ventilation with air starting at PN5-6. This study showed that mechanical ventilation led to an increase in alveolar area and a decrease in number of alveoli and septae. On a cellular level, the study identified increased apoptotic activity, higher concentrations of elastase in the alveolar walls, and impaired angiogenesis, as evident by decreased vascular endothelial growth factor (VEGF) receptor 2 expression (56). These data are similar to previous work by this group using a combination of invasive ventilation and mild hyperoxia (0.4 FiO₂) (18, 19). The novelty of this work, in addition to developing a protocol for invasively ventilating rodent subjects (18, 19), is the ability to isolate the effects of ventilation from the effects of hyperoxia.
There are numerous advantages to an invasive ventilation model in mice, including the ability to manipulate the degree of hyperoxia delivered through an invasive ventilator. This allows scientists to more closely approximate interventions used by clinicians in the intensive care setting. There are a few significant limitations of such a model at the present time, most of which are hindered by technological capabilities. At present, invasive ventilation of mice during the saccular stage prior to PN5 is extremely challenging. As a result, this type of model may induce a brief volutrauma with and without hyperoxia but cannot truly mimic the conditions that produce BPD in human infants. Additionally, the limited duration of exposure to mechanical ventilation (24 hours) would not correlate with the entire saccular stage of lung development even if initiated prior to PN5. Though the investigators are correct in appreciating that even gentle mechanical ventilation produces microscopic and gross changes in pulmonary function (56), the model would be a better correlate for human BPD with the capability to ventilate mice from PN1 through PN4-PN5.

**Hyperoxia/Hypoxia Models**

As previously described, hyperoxia is a well-studied model of BPD. Ratner et al studied the effect of intermittent hypoxia on mice in a hyperoxia-induced BPD model. Starting on PN3, mice were exposed either to continuous hyperoxia with 0.65 FiO₂ for four weeks postnatally or hyperoxia with intermittent hypoxemic episodes of 10 minutes each at 0.08 FiO₂. The hypoxemic episodes occurred daily for the first PN week of life and alternated days for the second PN week of life in order to maximize exposure to hypoxia during the late saccular and early alveolar stages. Overall, mice exposed to intermittent hypoxia had fewer alveoli and increased granulocytes in the lung tissue. Mice exposed to hypoxia also had greater oxidative
damage represented by total/oxidized glutathione ratio. In the clinical setting, preterm infants in a relatively hyperoxic environment (compared to the in utero environment) often experience periodic bouts of apnea in which they may become hypoxemic. By creating a mouse model that introduces intermittent hypoxemia into a hyperoxic model, the authors mimic a real clinical scenario and suggest that episodes of hypoxemia actually potentiate the deleterious effects of hyperoxia (71).

In order to more closely approximate the full spectrum of clinical events that precipitate BPD, some research groups have developed a “two-hit” model, which combines the insults of prenatal hypoxia and PN hyperoxia. Mice in the “two-hit” model are exposed to 0.1 FiO₂ for four days prenatally followed by 0.7 FiO₂ for two weeks after birth. In addition to producing a histological picture of BPD with decreased alveolar septation and simplified terminal airway structures, this model also leads to significant growth restriction by PN14, as represented by decreased total body length and weight and decreased lung and brain weight. Compared to mice exposed to prenatal normoxia, the prenatal hypoxia mice were born at decreased weight, which mimics intrauterine growth restriction seen in many preterm infants (31). Mice in the “double-hit” model subsequently treated with umbilical cord mononuclear cells at PN7 showed decreased inflammatory markers such as IL-1β compared to untreated “double-hit” mice and normalization of alveolar septal thickness when compared to normoxic controls (57).

Hypoxia Model

Compared to models that combine both hyperoxia and hypoxia, prenatal hypoxia models are often less successful. One such model exposed fetal mice to hypoxia at 0.1 FiO₂ from embryonic day 14 to 17.5, at which time the fetuses were delivered preterm and sacrificed. Compared to normoxic controls, the hypoxic mice did show significant intrauterine growth restriction but did
not demonstrate significant differences in lung tissue architecture. Results also showed reduction in mRNA levels of three out of four surfactant protein genes associated with surfactant production compared to normoxic controls (30). There are several limitations to this type of model, however, including its termination at embryonic day 17.5, which precludes the study of the PN lung or potential implications of decreased surfactant. Additionally, intrauterine growth restriction and BPD may develop independently of one another, thus decreasing the potential link between hypoxia in the model and subsequent BPD.

In comparison to prenatal hypoxia models, PN hypoxia models have successfully induced changes in lung architecture similar to that of BPD. Ambalavanan et al exposed C57BL/6 wild type and inducible transforming growth factor (TGF)-β dominant negative mutants (with ZnSO4) to hypoxia at 12% O2 versus normoxia (21% O2) during postnatal days 0-14. Investigators measured mean linear intercepts (MLI) and radial alveolar counts (RAC) from randomly chosen tissues sections to assess alveolar development and showed increased MLI and decreased RAC, changes consistent with impaired alveolar development, in the hypoxia-exposed wild type mice as compared to normoxia-exposed mice. Inhibiting TGF-β in the transgenic model with hypoxia exposure resulted in slightly increased RACs compared to wild type. Investigators also measured pulmonary arterial thickness and right ventricular thickness in sectioned hearts and vessels to show increased vessel thickness and right ventricular hypertrophy in wild type mice exposed to hypoxia (5). The success of this model is reflected in the fact that it produces impaired alveolarization and dysregulated pulmonary vascularization in the mouse lung, both features of BPD. Additionally, the presence of pulmonary hypertension as represented by increased pulmonary vascular muscularization and right ventricular hypertrophy appropriately mimics human BPD. This model hits the mice with hypoxia during the saccular and alveolar phases,
which would correspond to in utero and PN hypoxia in the human neonate. The experimental
interventions used by these investigators produce a picture that closely resembles human BPD.
This model was used for further study as to whether altered expression of particular factors, i.e.
Thy-1 (60) or Endothelin-1 (66) attenuate the effect of chronic postnatal hypoxia. The main
limitation of a purely postnatal hypoxia model is that it does not account for exposure to
treatment with supraphysiologic levels of oxygen in the clinical setting postnatally.

**Inflammation/Chorioamnionitis Model**

Though the controversy related to the link between prenatal inflammation and BPD in neonates
has been described above, investigators have successfully created models of BPD by creating
prenatal inflammation. Benjamin et al showed that LPS exposure resulted in decreased
expression of fibroblast growth factor-10 (FGF-10), a critical protein in bronchial and
bronchiolar development. Mice exposed to anti-FGF-10 antibodies showed a similar effect.
However, C.C3H.Tlr4<sup>ldsd</sup> mice with a loss-of-function mutation in the Tlr4 gene did not have
altered FGF-10 expression. Using laser scanning confocal microscopy, investigators showed that
the saccular airways in mice exposed to LPS with decreased FGF-10 expression were shorter
than controls. By staining for α-SMA, investigators also showed that positioning of
myofibroblasts in the airways of treated animals is altered, which may affect terminal airway
branching and future alveolarization (9). By exposing fetal mice at embryonic day 15 to E. coli
LPS, investigators showed increased levels of pro-angiogenic chemokines macrophage
inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) in
explanted fetal lung tissue, which corresponded to increased levels in tracheal aspirates of infants
exposed to in-utero chorioamnionitis (55). Velten et al’s model, as described above, works
towards the goal of better mimicking the confluence of factors that contribute to BPD by combining prenatal inflammation with PN hyperoxia. By measuring alveolar width and septal thickness, investigators showed poor alveolarization. Lung compliance was also decreased, using snapshot perturbation and pressure-volume loops. Additionally, there was widespread fibrosis shown by measuring alpha-hydroxyproline content in the fetal mouse lung (84). Prince et al, however, suggest that exposure to LPS and in-utero chorioamnionitis may have the effect of increasing the number of type-II pneumocytes in alveoli, thus contributing to surfactant production and lung maturation in the setting of NF-κB and TLR-4 activation (69).

Transgenic Models

Previous work in hyperoxia-induced injury models in mice showed up-regulation of key cytokines leading to activation of pro-inflammatory pathways or inactivation of inflammatory mediators in lung tissue. Elevated levels of particular cytokines, including interleukin (IL)-6 and TGF-β, have also been identified in tracheal aspirates of preterm infants displaying a BPD phenotype (15). Hyperoxia studies in mice have enabled the extrapolation of regulatable targets to study pulmonary phenotypes in room air using transgenic animal technology. One advantage of using transgenic models to confirm the effects of genetic manipulation is that it excludes the ancillary effects of hyperoxia. Additionally, transgenic models may be exposed to hyperoxia or other mediators to explore the effects of gene overexpression in multiple physiologic or pathologic conditions.

Bry et al were the first to demonstrate an association between increased levels of the cytokine IL-1 and the development of a BPD-like phenotype. Using a doxycycline-inducible system controlled by the Clara cell secretory protein promoter, Bry et al developed a model to conditionally express IL-1β perinatally in mouse pup lungs. The conditional expression of IL-1β
produced a cascade of effects, both on a clinical, histological, and molecular level. As compared to control pups, pups over-expressing IL-1β developed symptoms of respiratory distress, including chest retractions. These pups also exhibited poor postnatal growth despite being born at comparable weight, suggesting that the negative effect of IL-1β on growth may be limited prenatally. The IL-1β-expressing mice also had a significantly increased rate of mortality by PN7. Bry et al also found decreased septation and abnormal vascularization of alveolar tissue, as well as goblet cell metaplasia and airway smooth muscle hyperplasia, consistent with the histology of BPD. Finally, expression of chemokines specific to neutrophils and macrophages was increased in the lung tissue of mice expressing IL-1β (22). Inhibition of one of these chemokines, CXCR2, in an IL-1β transgenic mouse produced drastically different effects on alveolar septation and survival depending on the timing of exposure (38).

Further studies by this group used a similar model to elucidate the effect of pulmonary IL-1β expression on expression of cellular retinoic acid binding proteins (CRABP) and nuclear retinoic acid receptors (RAR), both of which play a role in alveolar septation. This study demonstrated decreased levels of CRABP-1 and RAR-γ2 mRNA in mouse pups expressing IL-1β perinatally. Using immunohistochemistry, the study also showed a decrease at the protein level (21). This study suggests that an increased quantity of inflammatory markers in the injured lung may result in down-regulation of proteins critical for the subdivision of the developing lung into complex alveolar structures. However, it has not been fully elucidated whether inflammation is causative or correlated with the changes seen histologically in BPD.

Timing of expression was found to be of particular significance. High maternal levels of inflammation produced by induced expression of IL-1β at embryonic day zero actually decreased the deleterious effects of IL-1β expression in pups postnatally (8). This study highlights the
importance of timing induction in a developmentally appropriate way. When compared side-by-side, mouse pups overexpressing IL-1β in the mid-saccular stage demonstrated the most significant alterations to pulmonary architecture and had the greatest mortality. Overexpression in earlier phases of development such as the late canalicular/early saccular stage had little impact on lung morphology or survival. Delayed overexpression starting in the late saccular and early alveolar stages also showed less tissue damage than the mid-saccular stage (7).

Other targets of transgenic overexpression have also been shown to produce a BPD-like phenotype. Elevated levels of interferon (IFN)-γ have been seen in tracheal aspirates of human subjects with BPD, prompting investigation in transgenic mouse models (2, 35). Overexpression of IFN-γ was induced in the lungs of newborn mice in the saccular stage of lung development, which resulted in increased markers of apoptosis, including caspases 3, 8, and 9. Increased mRNA levels of angiopoietin 2 were also seen, which reflect previous studies in human subjects (1, 16). Additionally, increased levels of matrix metalloproteinases (MMPs) were seen in lung tissue, which may also contribute to the structural simplicity of alveoli seen in BPD. This study took further steps to characterize the impact of MMPs on lung tissue in the setting of overexpressed IFN-γ by comparing mice with a partial deficiency in MMP-9 to wild type (WT) mice. Ultimately, mouse pups overexpressing IFN-γ with a partial deficiency in MMP-9 demonstrated relatively normal lung architecture and reduced levels of pro-apoptotic and angiogenic factors. When exposed to 48 hours of hyperoxia during PN0-2, a partial deficiency in MMP-9 provided a significant survival benefit in the setting of overexpressed IFN-γ (35). Additional work in IFN-γ transgenic mice exposed to hyperoxia showed a rescue effect with celecoxib, an inhibitor of cyclooxygenase-2 (Cox2). Inhibition of Cox2 had a profound effect on reversing the BPD phenotype perhaps because it is playing a role in activating the endoplasmic
reticulum (ER) stress pathway associated with BPD. As such, a similar rescue effect was produced with use of C/EBP homologous protein (which is a critical signaling molecule in the ER stress pathway) siRNA. These data further suggest that targeted inhibition of downstream pathways of IFN-γ may ameliorate lung damage in hyperoxia (25).

TGF-β1 is another regulatable target believed to contribute to BPD. Vicencio et al created a triple transgenic mouse designed to express TGF-β1 in lung tissue between PN7 and PN14, which corresponds to the alveolar stage of development. PN pulmonary expression of TGF-β1 produced similar respiratory symptoms and disruptions to the pulmonary architecture as previously described studies, including impaired septation and capillary structure (85). A study by Li et al also explored the effects of pulmonary TGF-β1 expression in PN mouse pups. Based on previous data showing that TGF-β1 expression mediates activation of the c-JunNH2-terminal kinase (JNK) pathway in hyperoxic conditions, Li et al created transgenic TGF-β1 expressing mice with either intact or inhibited JNK pathways. Transgenic and WT mice with inhibited JNK pathways showed significantly reduced mortality in the setting of hyperoxia as compared to mice with intact JNK, as well as partial improvement in lung architecture (50).

Recent studies have also suggested that macrophage migration inhibitory factor (MIF) might play a protective role in BPD. Tracheal aspirates of neonates with respiratory distress syndrome (RDS) showed an association between high levels of MIF and a reduced incidence of BPD (45). MIF knockout mice demonstrated less mature lungs than WT mice based on histology and levels of corticosterone and vascular endothelial growth factor (VEGF), both of which promote maturity. MIF knockout mice also had increased mortality compared to WT mice (45). Using a hyperoxia-induced injury model, this group also studied MIF knockout and MIF transgenic mice to better understand the role MIF plays in lung development. When exposed to seven days of
hyperoxia, MIF knockout mice had the highest mortality rate, followed by the WT and then the transgenic mouse. Of note, mortality correlated with increased angiopoietin-2 levels. In room air, both the knockout and transgenic mice had decreased levels of angiopoietin-1 and Tie-2 proteins, which corresponded with increased chord length. Taken together, these data suggest that MIF plays a regulatory role in the developing lung that impacts other key proteins, including angiopoietins 1 & 2 and Tie 2 (79). WT mice exposed to hyperoxia showed some recovery of normal pulmonary architecture after treatment with a MIF agonist, whereas MIF transgenic mice had improved pulmonary phenotype after being administered a MIF antagonist. These data reflect the delicate balance of regulatory MIF protein levels in producing a healthy neonatal lung (78).

The use of transgenic mouse models has provided invaluable information about the effect of single gene modification on the BPD phenotype. In addition to the ability to isolate the model from the global effects of hyperoxia, transgenic models are valuable in that they can be temporal- and tissue-specific. Using inducible models allows for the induction of gene expression at various time points during or after gestation, thus enabling specific targeting of the saccular stage of lung development. Although prematurity and intrauterine inflammation causes global effects in the neonate, tissue specificity in transgenic models is also an advantage, as induced systemic overexpression of pro-inflammatory cytokines may be undesirable and unpredictable in effect. There are drawbacks and limitations to the use of transgenic mouse models for BPD. Though tracheal aspirates of newborns with BPD confirm elevated levels of multiple cytokines (15), the relative levels of cytokines in the lung tissue of transgenic mice likely exceed levels in preterm human lungs, which may overestimate the effect of the cytokine. However, this can be potentially overcome as the cytokine levels can be manipulated to mimic human lung
concentrations in the inducible transgenic mice models by controlling the dose and duration of
doxycycline (or other agent modulating expression) exposure. As BPD is a truly multifactorial
disease, overexpression of single genes in isolation cannot truly mimic BPD. This limitation is
mitigated by the ability of scientists to further modify their transgenic mouse models through
exposure to hyperoxia, inhibition of downstream pathways, or perhaps even invasive ventilation.

Quantifying the Rodent Lung: Pulmonary Measurements and the Challenges of the Mouse

The success of experimental models of BPD hinges on the accuracy and precision of the
pulmonary and vascular measurements used to identify phenotypic changes in the lungs and
vessels. Many challenges exist in systematically selecting tissue samples and acquiring
reproducible measurements. Ochs and Muhlfeld do an admirable job of reviewing the challenges
and solutions present in the currently available technology to quantify various aspects of lung
structure. The challenge of circumventing sampling bias and measurement bias is addressed well,
with discussion of the fractionator and proportionator as methods to generate unbiased
measurements (65). Tissue shrinkage during preservation is one particular problem which can
seriously alter and bias reference measurements for comparison with tissue exposed to
experimental interventions (65). In particular, Ochs and Muhlfeld provide a useful discussion of
some of the challenges of evaluating common pulmonary diseases, including emphysema,
pulmonary artery hypertension, and asthma (58). Pulmonary arterial hypertension is of particular
interest when discussing animal models of BPD, as many of the studies addressed in this review
comment on vascular remodeling and increased pulmonary arterial muscularity as a consequence
of hypoxia or hyperoxia. Ochs and Muhlfeld discuss common misperceptions and traps where
inaccuracies may occur. Though many investigators use the percentage of medial thickness to represent vascular wall hypertrophy, many factors can affect this variable including luminal diameter and overall wall thickness, thus preventing consistent measurements. As such, the authors suggest alternative ways of reporting arterial wall thickening, namely relating wall volume to endothelial basal lamina surface area (58).

One recent study published by Madurga et al utilized a novel method for preserving lung tissue, which eliminates concerns for tissue artifact present in many previous studies. Many studies in the past have embedded tissue samples in paraffin and observed changes in the MLI and septal thickness as their main metrics for assessing changes in lung structure. Madurga et al describe the disadvantages of this system, which may predispose tissue to shrinkage and artifact. Using a novel method for tissue preservation and systemic uniform random sampling, investigators were able to count the total number of alveoli and use this reference number to identify a significant decrease in the number of alveoli after hyperoxia exposure (51). The establishment of a reference for alveolar number using methodology that avoids artifact and bias is an obvious boon to the field and will no doubt further the study of lung morphometry. The use of computed tomography angiography with 3D reconstruction is also an excellent way to visualize gross morphologic changes in the pulmonary vasculature, as shown by Hansmann et al (34).

Many methods exist to quantify changes in the lungs and hearts of living mice prior to sacrifice. In addition to measuring the muscularity and remodeling of fixed and sectioned vessels by staining for α-SMA with select antibodies, Chen et al also compared the weight of the right ventricle to the left ventricle and septum, also known as Fulton’s index (28), as a means of identifying pulmonary hypertension (24). Using a pressure transducer with Gould polygraph, Chen et al were also able to measure right ventricular systolic pressures (RVSP) of live sedated
mice. This methodology enabled Chen et al to document a significant increase in the density of
α-SMA in vessels of mice overexpressing connective tissue growth factor (CTGF). Mice
overexpressing CTGF also had significant increases in RVSP as compared to wild type mice
(24). There is a distinct benefit to being able to document elevated RVSP in pulmonary
hypertension, as this provides clear evidence of the downstream sequelae of abnormal vascular
development in BPD.

In the past several years, investigators have started using techniques also used in cardiology and
pulmonology, namely echocardiography, to evaluate for clinical evidence in impaired cardiac
function as a result of the BPD phenotype, particularly pulmonary artery acceleration time
(PAAT) and pulmonary artery ejection time (PAET). Both Hansmann et al and Alphonse et al
were able to show, using trans-thoracic echocardiography, decreases in PAAT, a strong indicator
of pulmonary hypertension in animals exposed to hyperoxia (4, 34).

Use of pulmonary function testing is one way of quantifying changes in lung function as a result
of experimental interventions and is made possible by using the Flexivent. Several recent studies
have used this technology to cannulate the neonatal mouse trachea in order to measure
pulmonary resistance and compliance (4, 34, 70, 87). In addition to permitting measurement of
baseline lung function, this technology enables investigators to measure response to inhaled
therapies such as β2 agonists (70). However, technology is currently limited by the size of the
mice, and the majority of studies in the present body of literature focus on mice aged PN14-21
and no younger.

Summary/Conclusions
In the past decades, we have been able to derive a wealth of knowledge about BPD from experimental animal models using mice. In light of what is known about mouse lung development and its correlation with human lung development, developmentally appropriate models should study responses to noxious stimuli during the saccular stage when subjects are most prone to developing BPD. Models using prolonged hyperoxia may not be optimal, as this exposes animals to noxious stimuli well into the alveolar stage when lung tissue is less susceptible to injury and may already be showing signs of recovery.

One of the most important advances in mouse models has been the ability to create transgenic animals that conditionally over- or under-express specific genes in a temporal- and tissue-specific fashion. As mentioned, these models utilize room air, allowing for the isolated study of specific proteins or pathways independent of hyperoxia or mechanical stretch injury. To truly mirror the multifactorial clinical and genetic conditions contributing to human BPD, an ideal model system would aim to deftly combine multiple factors. This remains a significant obstacle, which can only partially be overcome by optimizing study methods and more closely matching them to clinical scenarios. As technology improves, we believe that the ability to invasively ventilate animal models through the end of the saccular phase in combination with other techniques like genetic manipulation and exposure to hyperoxia will yield a wealth of information.

One of the greatest challenges of using experimental animal models will remain; those in this field must continue to think critically about the clinical significance of the findings in order to use the data to develop focused interventions to reduce the human burden of this highly morbid disease (14).
Table 1: Stages of Mouse and Human Lung Development (37, 46, 52-54, 80)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Mouse Gestational Age (days)</th>
<th>Human Gestational Age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>E9-11.5</td>
<td>3-7</td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td>E11.5-16.5</td>
<td>5-17</td>
</tr>
<tr>
<td>Canalicular</td>
<td>E16.5-17.5</td>
<td>16-26</td>
</tr>
<tr>
<td>Saccular</td>
<td>E17.5-PN5</td>
<td>24-38</td>
</tr>
<tr>
<td>Alveolar</td>
<td>PN5-28</td>
<td>32 weeks- 8 years PN*</td>
</tr>
</tbody>
</table>

E, embryonic; PN, postnatal. * Most of the alveolar multiplication ceases by 2-3 years of PN age.
### Table 2: Mouse Models of Bronchopulmonary Dysplasia

<table>
<thead>
<tr>
<th>Strain</th>
<th>Experimental Model</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyperoxia Alone</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Warner 1998 (88) FVB/N | Hyperoxia for 28 days | - Bronchopulmonary dysplasia (BPD) pulmonary phenotype  
- Decreased cell proliferation over 14 days, which normalized by day 28  
- Increased inflammatory markers (IL-1α and MIP-1α) |
| Nold 2013 (61) C57BL/6J | Postnatal hyperoxia at different concentrations for 3, 14, or 21 days  
 +/- prenatal LPS | - BPD pulmonary phenotype |
| Tibboel (81) C57BL/6 | Hyperoxia for 28 days followed by room air for 7, 14, or 28 days | - BPD pulmonary phenotype |
| Woyda (90) C57BL/6N | Hyperoxia for 28 days | - BPD pulmonary phenotype  
- Upregulation of phosphodiesterases (PDE) 1A and 4A; downregulation of phosphodiesterase 5A |
| Zhang (92, 93) KunMing | Hyperoxia for 15 days | - BPD pulmonary phenotype  
- Differential expression of mRNA |
| Velten (84) C3H/HeN | Hyperoxia for 14 days followed by room air for 14 days  
 +/- prenatal LPS | - Hyperoxia alone produced BPD pulmonary phenotype  
- Combination of hyperoxia and prenatal LPS did not result in a more severe phenotype but the effects were more prolonged than either exposure alone  
- Combined exposure produced more inflammation, as measured by pulmonary macrophages |
| Hansmann (34) FVB | Hyperoxia for 14 days | - BPD pulmonary phenotype |
| Yee, O'Reilly, Buczynski (23, 64, 91) C57BL/6J | Hyperoxia for 4 days | - Pulmonary fibrosis  
- Increased inflammation, measured by lymphocyte, neutrophil and macrophage counts  
- Increased levels of monocyte chemotactic protein-1 (MCP-1)  
- Increased mortality by DOL 14  
- Increased sensitivity to influenza A as adults |
| Rogers (72) C3H/HeN | 14 days hyperoxia at varying concentration | - Increased severity of BPD pulmonary phenotype with higher concentration O₂  
- Higher O₂ concentration associated with increased neutrophil counts  
- Stunted growth, worse with higher concentrations O₂ |
| **Hyperoxia/Hypoxia** |                                            |                                                                                                                                                           |
| Ratner (71) C57BL/6J | Continuous hyperoxia for 4 weeks or hyperoxia with 10 minutes daily hypoxia | - Intermittent hypoxia worsens BPD pulmonary phenotype compared to hyperoxia alone |
| Gortner 2013, Monz (31, 57) C57BL/6N | 4 days prenatal hypoxia followed by 14 days postnatal hyperoxia | - Growth restriction  
- BPD pulmonary phenotype without significant inflammation based on cytokine levels |
| **Hypoxia** |                                            |                                                                                                                                                           |
| Gortner 2005 (30) C57BL/6 | 4 days prenatal hypoxia | - Significant growth restriction  
- No difference in lung histomorphometry |
<table>
<thead>
<tr>
<th>Study</th>
<th>Genotype</th>
<th>Treatment/Manipulation</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambalavana (5)</td>
<td>C57BL/6</td>
<td>Transforming growth factor (TGF)-β transgenic mice Postnatal hypoxia</td>
<td>Mice under-expressing TGF-β showed attenuation of BPD phenotype</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Benjamin, Prince (9, 69)</td>
<td>BALB/cJ, C3H.7Br-4 (Lpsd)</td>
<td>LPS exposure in utero to wild type and toll-like receptor (TLR) 4 deficient mice</td>
</tr>
<tr>
<td></td>
<td>Miller (55)</td>
<td>Tie2-LacZ, Tie-2-green fluorescent protein (GFP), BALB/cJ, ROSA26 enhanced yellow fluorescent protein (EYFP)</td>
<td>Prenatal exposure to LPS</td>
</tr>
<tr>
<td>Invasive Ventilation</td>
<td>Mokres, Bland (18, 19, 56)</td>
<td>BALB/c</td>
<td>24 hours invasive mechanical ventilation</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Bry, Backstrom, Hogmalm (7, 8, 21, 22, 38)</td>
<td>FVB/N</td>
<td>Interleukin (IL)-1β transgenic mice Additional gene knockouts</td>
</tr>
<tr>
<td></td>
<td>Kevill (45), Sun (78, 79)</td>
<td>C57BL/6</td>
<td>Hyperoxia + macrophage migration inhibitory factor (MIF) knockout or transgene</td>
</tr>
<tr>
<td></td>
<td>Choo-Wing (25, 50), Harijith (35)</td>
<td>C57BL/6J</td>
<td>Transgenic interferon (IFN)-γ over-expressing mice</td>
</tr>
<tr>
<td></td>
<td>Li (50), Vicencio (85)</td>
<td>C57BL/6J</td>
<td>Transforming growth factor (TGF)-β transgenic mice</td>
</tr>
<tr>
<td></td>
<td>James (41)</td>
<td>C57BL/6</td>
<td>7-14 days hyperoxia +/- exposure to vitamin A and retinoic acid (VARA)</td>
</tr>
<tr>
<td></td>
<td>Fernandez-Gonzalez (28)</td>
<td>FVB/N</td>
<td>Heme oxygenase (HO)-1 transgenic mice 14 days hyperoxia</td>
</tr>
<tr>
<td></td>
<td>Tropea (82)</td>
<td>FVB</td>
<td>10 days hyperoxia Treatment with mesenchymal stromal cells (MSC) or MSC-conditioned media (MSC-CM)</td>
</tr>
<tr>
<td></td>
<td>Bachiller (6)</td>
<td>C57BL/6</td>
<td>Soluble guanylyl cyclase (sGC)-α1 knockout mice Exposure to hyperoxia</td>
</tr>
<tr>
<td>Study</td>
<td>Model</td>
<td>Treatment</td>
<td>Phenotype</td>
</tr>
<tr>
<td>------------------</td>
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<td>---------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Witsch (89), Popova (68)</td>
<td>C57BL/6</td>
<td>Varying days of hyperoxia</td>
<td>BPD pulmonary phenotype</td>
</tr>
</tbody>
</table>

BPD pulmonary phenotype: characterized by indices of decreased alveolarization and dysregulated vascularization ("new" BPD), with variable fibrosis ("old" BPD); see text/referenced articles for additional details of the individual studies.


