Hyperoxia-induced bronchopulmonary dysplasia: better models for better therapies

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a chronic lung disease caused by exposure to high levels of oxygen (hyperoxia) and is the most common complication that affects preterm newborns. At present, there is no cure for BPD. Infants can recover from BPD; however, they will suffer from significant morbidity into adulthood in the form of neurodevelopmental impairment, asthma and emphysematous changes of the lung. The development of hyperoxia-induced lung injury models in small and large animals to test potential treatments for BPD has shown some success, yet a lack of standardization in approaches and methods makes clinical translation difficult. In vitro models have also been developed to investigate the molecular pathways altered during BPD and to address the pitfalls associated with animal models. Preclinical studies have investigated the efficacy of stem cell-based therapies to improve lung morphology after damage. However, variability regarding the type of animal model and duration of hyperoxia to elicit damage exists in the literature. These models should be further developed and standardized, to cover the degree and duration of hyperoxia, type of animal model, and lung injury endpoint, to improve their translational relevance. The purpose of this Review is to highlight concerns associated with current animal models of hyperoxia-induced BPD and to show the potential of in vitro models to complement in vivo studies in the significant improvement to our understanding of BPD pathogenesis and treatment. The status of current stem cell therapies for treatment of BPD is also discussed. We offer suggestions to optimize models and therapeutic modalities for treatment of hyperoxia-induced lung damage in order to advance the standardization of procedures for clinical translation.

KEY WORDS: Bronchopulmonary dysplasia, Stem cell therapy, Hyperoxia

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that is multifactorial in nature. It is the most common complication of preterm birth, occurring in 43% of infants born at or before 28 weeks gestation (Lignelli et al., 2019; Willis et al., 2018). BPD is characterized by arrested lung growth, alveolar simplification, impaired blood vessel development and abnormal pulmonary function (Banaldi and Filippone, 2007). It is associated with significant morbidity and mortality in premature infants, and survivors often exhibit persistent long-term effects into adulthood (Silva et al., 2015). Disease severity has a significant sex bias, whereby preterm males have a higher risk of developing more-severe symptoms compared to females of the same age (Collaco et al., 2017).

BPD incidence has increased owing to advances in neonatal care that improved the survival of lower gestational age infants. The condition has first been described by Northway et al. in 1967 (Northway et al., 1967). The ‘classic’ BPD, determined by intense inflammation, fibrosis and scarring of the lung, has now evolved to the ‘new’ BPD, determined by reduced alveolar development together with airway injury, inflammation and mild fibrosis (McEvoy and Aschner, 2015; Mosca et al., 2011). The severity of lung injury is determined by the duration of excess O₂ exposure and the degree of positive pressure during mechanical ventilation (Gibbs et al., 2020; Mann and Bär, 2011). Lung damage can range from early developmental arrest in patients diagnosed with new BPD to structural damage of the immature lung in neonates diagnosed with classic BPD (Mosca et al., 2011).

Exposure to high concentrations of O₂ is one of the most important factors in the development of BPD. It leads to the release of reactive oxygen species (ROS), which cause significant cellular and molecular damage to the lung (Wang and Dong, 2018) (see Fig. 1). Preterm infants are at high risk of developing BPD owing to their need for O₂ supplementation, since their immature lungs do not provide sufficient gas exchange (Chao et al., 2018). BPD pathogenesis involves a disruption of alveolar epithelial-mesenchymal signaling, causing alveolar fibroblasts to transdifferentiate into myofibroblasts. The latter lack the ability to maintain epithelial cell growth and differentiation, which results in reduced alveolarization (Hwang and Rehan, 2018). Premature infants are usually diagnosed with BPD 14–30 days after birth. Physical symptoms of disease include wheezing, rapid and labored breathing, repeated lung infections and difficulty in feeding (Principi et al., 2018). Currently, therapies, such as antenatal steroids, surfactant replacement and modified ventilation approaches, only treat the short-term effects of BPD. Preventative strategies include the use of lower inspired O₂ concentrations, non-invasive ventilation strategies such as continuous positive airflow pressure and improved resuscitation at birth. However, the incidence of BPD in premature infants born before 29 weeks gestational age remains at ~40% (Mandell et al., 2019). Currently, no treatments reverse or prevent the alveolar damage following hyperoxia.

The aim of this Review is to call attention to issues of modelling BPD induced by high levels of oxygen (hyperoxia) in animals, with a focus on outcome measurements. We discuss variations in the modelling of hyperoxia-induced BPD together with lung injury measurements, and highlight the use of in vitro models to evaluate the cellular and molecular pathways to show how these systems may complement current animal models. The status of potential therapies for treatment of BPD is also reviewed, followed by suggestions for the standardization of current methodologies to improve the therapeutic management of BPD in neonates.

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Lung development and BPD

In order to develop a disease model, it is important to understand both embryology and how the various animal models may relate to a human disease. There are five distinct stages to lung development that are conserved across species, and are categorized as embryonic, pseudoglandular, canalicular, saccular and alveolar (Schittny, 2017). Each stage entails defined anatomical and physical changes necessary for lung maturation, and the duration of each stage differs between species (Berger and Bhandari, 2014). Fig. 2 provides a timeline for the various stages of lung development in different species compared to those in humans. Premature infants susceptible to BPD are born during the canalicular-saccular stage of lung development, whereby branching morphogenesis and alveolar differentiation need yet to be completed (Thébaud et al., 2019).

Morphological changes

In response to high levels of O2 and to mechanical ventilation, the lung elicits a pro-inflammatory response that stimulates the release of free radicals. This disrupts alveolar architecture and results in simplified structures, fewer alveoli and a reduced surface area for gas exchange (Shahzad et al., 2016). Additionally, mild airway smooth muscle thickening develops together with alveolar septal fibrosis (Kalikkot Thekkeveedu et al., 2017). This significantly impairs lung function and, in severe cases, alters pulmonary vascular development. In ~25% of preterm infants diagnosed with BPD, pulmonary hypertension is a common finding (Shahzad et al., 2016). Lung biopsies from adolescents with a history of prematurity and BPD revealed peribronchial fibrosis and thickening of the submucosal layers, consistent with chronic obstructive lung disease and chronic abnormalities of the angiogenic process. These findings indicate that the morphological effects of BPD can persist into adulthood (Galderisi et al., 2019).

Molecular changes

A number of studies have indicated that expression levels of cytokines, chemokines, growth factors, apoptotic factors and proteases change profoundly during hyperoxia in the neonate and in relevant animal models of BPD (summarized in Table 1 and references therein). These changes are reflected in morphological and cellular perturbations seen in the lungs of patients with severe symptoms of BPD. Hyperoxia can lead to the initiation of an inflammatory cascade within immature lung tissue, thereby interfering with the normal course of septal and alveolar development, and often precedes clinical symptoms (Lignelli et al., 2019). Increased mRNA expression of pro-inflammatory cytokines has been identified in airway secretions and bronchoalveolar cells from human neonates with developing BPD (Köksal et al., 2012). One of the most important cytokines implicated in the pathogenesis of BPD is transforming growth factor β1 (TGFβ1), a multifunctional cytokine that activates fibroblast differentiation into myofibroblasts, regulates wound healing, and can control cell growth, proliferation, differentiation and apoptosis (Martin et al., 2016). Expression of TGFβ1 inhibits alveolar development and causes pulmonary fibrosis in lungs with advanced alveolarization. In human neonates with BPD, the TGFβ1 concentration in bronchoalveolar lavage fluid is increased and used as a predictor of BPD severity (Kwong et al., 2006). Moreover, transfer of the active Tgfb1 gene to the lungs of

Fig. 1. Molecular and cellular alterations associated with BDP. Hyperoxic conditions associated with the development of BPD lead to a release of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and superoxide (O2−), which trigger pro-inflammatory cytokine expression. This contributes to molecular and cellular damage within the lung, including damage to DNA. Additionally, pathways associated with vasculogenesis and alveolar development, as well as Notch signaling, are dysregulated, resulting in remodeling of the extracellular matrix, apoptosis and aberrant lung development. Morphological characteristics of disease include alveolar simplification and septal thickening.
neonatal rats shows areas of interstitial fibrosis and enlarged alveolar spaces (Gauldie et al., 2003). This suggests that overexpression of Tgfb1 during the crucial period of postnatal rat lung alveolarization gives rise to pathological, biochemical, and morphological changes consistent with BPD. Expression levels of Tgfb1 mRNA increase after hyperoxia in mice and rats, and significantly decrease after treatment with mesenchymal stem cells (MSCs) (Luan et al., 2015; Chang et al., 2011).

Another cytokine affected under hyperoxic conditions, and consistently cited in the literature, is interleukin-6 (IL-6). IL-6 is produced in response to inflammatory conditions, causes extensive damage to DNA, increases cell death regulator and angiogenic factor expression, and has both inflammatory and protective effects in hyperoxia (Ward et al., 2000). IL-6 is elevated in tracheal aspirates of human neonates, who subsequently developed BPD (von Bismarck et al., 2009). Elevated IL-6 concentrations are associated with BPD progression, which suggests that IL-6 expression may be used as a reliable biomarker of BPD (Li et al., 2019). When BPD is modeled in IL-6 knockout mice, the animals demonstrate a decreased severity of lung injury (Aziz et al., 2020). MSC treatment significantly decreases IL-6 secretion in neonatal rats exposed to high levels of O2 (Chang et al., 2013).

Several studies of BPD pathogenesis also note the importance of vascular endothelial growth factor (VEGF) in the regulation of pulmonary angiogenesis and alveolar development (Oak and Hilgendorff, 2017; Mariduena et al., 2020; Yang et al., 2015). VEGF is a widely expressed growth factor that is highly expressed in the lung. It is involved in the regulation of vascular growth and development, the stimulation of angiogenesis and the promotion of endothelial survival, all of which are particularly important for the proper functioning of the lungs. Aside from regulating lung vascularization, VEGF appears to be essential for the appropriate development of alveolar tissue (Bhandari, 2010). Hyperoxia triggers a biphasic VEGF response in both human neonates and

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**Fig. 2. Stages of lung development in humans and in animal species often used as disease models.** Although lung development is conserved across mammals, the individual developmental stages occur at different gestational age, which has implications for modeling pulmonary disorders, such as BPD. Stages of lung development are based on morphological criteria and do not have strict time lines; data were summarized from previous publications (Berger and Bhandari, 2014; Lau and Kavlock, 1994; Pinkerton and Joad, 2000; Schittny, 2017). PN, postnatal day; *, day when full-term gestation is reached.
Table 1. Proteins of interest in hyperoxia-induced BPD

| Protein                                      | Roles in hyperoxia/BPD                                                                 | Effects in hyperoxia/BPD                                                                 | References                                      |
|----------------------------------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------|
| Adrenomedullin (Adm)                         | Important role in angiogenesis, key factor in alveolar development.                     | Decreases in neonatal mice lungs exposed to hyperoxia.                                 | (Menon et al., 2017; Vadivel et al., 2010; Menon et al., 2017). |
| BCL2-associated X, apoptosis regulator (BAX) | Apoptosis is an important feature in hyperoxic lung injury.                             | BAX expression increases in neonatal rat lungs exposed to hyperoxia.                    | (Mantell and Lee, 2000).                        |
| BCL2 apoptosis regulator (BCL2)              | Apoptosis can be induced by elevated ROS levels, which occur in hyperoxia.             | Bone marrow mesenchymal stem cells protect against apoptosis in hyperoxia-exposed neonatal rat lung b upregulation of BCL2. | (Zhang et al., 2013).                          |
| Matrix metalloproteinase 9 (MMP-9)          | MMPs are proteolytic enzymes that degrade extracellular matrix components and fibrillar collagen. Extracellular matrix remodeling, driven in part by MMPs plays important roles during normal lung development; excess MMPs may alter lung remodeling and change its architecture. | Increases in human neonates during hyperoxia; associated with BPD.                     | (Bhandari, 2010; Sweet et al., 2004).           |
|                                              |                                                                                       | Increases in neonatal mice lungs during hyperoxia in association with impaired alveolar development. | (Chetty et al., 2008).                         |
|                                              |                                                                                       | MMP-9 knockout neonatal mice during hyperoxia have a larger gas exchange surface area and are protected from the effects of hyperoxia. | (Tambunting et al., 2005).                     |
|                                              |                                                                                       | Increases in premature baboon lungs during hyperoxia in association with changes in lung architecture. |                                               |

Growth factors and signaling pathways

| Protein                                      | Roles in hyperoxia/BPD                                                                 | Effects in hyperoxia/BPD                                                                 | References                                      |
|----------------------------------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------|
| Vascular endothelial growth factor A (VEGFA) | Widely expressed, with highest expression levels in the lung.                           | Biphasic response in hyperoxia: increased release may cause lung injury by altering vascular permeability, although a surge of VEGF also underlies lung healing and repair through angiogenesis and alveolarization. | (Bhandari, 2010; Luan et al., 2015).             |
|                                              | Involved in the regulation of pulmonary vascular growth and development, stimulation of angiogenesis, and promotion of endothelial survival. | VEGF antagonism in neonatal rats markedly impairs alveolarization, especially earlier in lung development. |                                               |
|                                              |                                                                                       | VEGF levels decrease in neonatal rat lungs during hyperoxia.                           | (Lassus et al., 2003).                         |
|                                              |                                                                                       | In experimental animals with acute lung injury, HGF expression is increased in the lung, suggesting an active role in the early events of lung repair. |                                               |
|                                              |                                                                                       | Decreased expression in preterm human neonates who developed BPD in the first two weeks of life. |                                               |
|                                              |                                                                                       |                                               |                                               |
| Connective tissue growth factor (CTGF, officially known as CCN2) | Involved in tissue repair and pulmonary fibrogenesis. Markedly elevated expression under fibrotic conditions. Stimulates deposition of extracellular matrix components, including collagen. | Increases in neonatal rat lungs exposed to hyperoxia. Fibrrosis in hyperoxia-exposed neonatal rat lungs is associated with CTGF increases, which precedes the increase in lung collagen levels. | (Chen et al., 2007). (Nauta et al., 2011).      |
|                                              | Important for both alveogenesis and angiogenesis of normally developing lung. Expression of different forms of PDGF peak at the canaliculare stage, suggesting a role in the development of gas-exchanging regions of the lung. |                                               |                                               |
| Platelet-derived growth factor B (PDGFB)    | Important for normal lung development.                                                 | Crical for normal lung development, but also associated with abnormal cellular hyperplasia and fibrosis in hyperoxia-exposed lungs. | (Zhang et al., 2005).                          |
|                                              | Important for differentiation, regeneration and repair of the airway epithelium.        | Hyperoxia suppresses total lung Pdgfb mRNA expression and protein levels in young growing piglet lungs. |                                               |
| Notch2                                       | Important for differentiation, regeneration and repair of the airway epithelium.        | Deficiencies in Notch2 expression in neonatal mice results in abnormal development of the alveolar and distal airway compartments. | (Tsao et al., 2016).                          |
| Fibroblast growth factor receptor (FGFR)     | Important for normal lung development.                                                 | In premature infants with BPD, expression of FGFR4 is upregulated. Fgfr4 knockout mice show no alveolarization. | (Rezvani et al., 2013).                       |
| Antioxidants                                 | Key component of the oxidative stress response pathway. Owing to the transition from conditions of intrauterine hypoxia to those of extrauterine hypoxia, preterm infants are particularly | Increases in neonatal mouse lung after hyperoxia recovery. | (Endesfelder et al., 2019).                    |

Continued
Table 1. Continued

| Protein | Roles in hyperoxia/BPD | Effects in hyperoxia/BPD | References |
|---------|------------------------|--------------------------|------------|
| Glutathione S-transferase mu 1 (GSTM1) | Key component of the oxidative stress response, of which preterm infants are at increased risk for. | Inactivating mutations in GSTM1 contribute to the development of BPD. In most tissues, activities of GSTM1 are not affected under hyperoxia but increase sharply during normoxic recovery. | (Wang et al., 2014) (Lushchak et al., 2005) |
| Interleukin 1 | Mixed inflammatory cytokine. | Increases in bronchoalveolar lavage fluid of human neonates with BPD and are a predictor of BPD severity. Increased levels in neonatal mice and rat lungs during hyperoxia. Mesenchymal stem cell treatment significantly decreases TGFβ1 levels in mice and rats. | (Gien and Kinsella, 2011) (Luan et al., 2015; Chang et al., 2011) |
| Monocyte chemoattractant protein 1 (CCL2) | Chemoattractant for macrophages and monocytes. | Increased levels in tracheal aspirates of neonatal humans who developed BPD. Increased in neonatal rat lungs exposed to hyperoxia. | (Baier et al., 2004) (Wagenaar et al., 2004) |
| Interleukin 6 (IL-6) | Produced in response to inflammatory conditions, causes extensive damage to DNA and increases expression of cell death regulator and angiogenic factor. | Elevated concentrations are associated with BPD progression, suggesting that IL-6 expression levels may reliably be used to predict progression of BPD. Il6-knockout mice exposed to high levels of O2 show decreased severity of lung injury in BPD. | (Li et al., 2019) (Aziz et al., 2020) (Ward et al., 2000) |
| Interleukin 1β (IL-1β) | Pro-inflammatory cytokines. | Increased concentration in amniotic fluid is associated with development of BPD. Increased in tracheal aspirates of human neonates who develop BPD. In neonate rabbits during hyperoxia, IL-1β was detected by 2-4 days of hyperoxia, peaking at 6-10 days and decreasing thereafter. Antagonism of IL-1 receptor reduces BPD in mice. IL-1α increased in the lungs of neonate mice with hyperoxia. | (Yoon et al., 1997) (Kotecha et al., 1996) (D’Angio et al., 1999) (Mulrooney et al., 2004) (Nold et al., 2013) (Warner et al., 1998) |
| Interleukin 10 (IL-10) | Anti-inflammatory cytokine. | Decreased in serum and tracheal aspirate of neonatal humans who developed BPD. Decreases in hyperoxic lungs of neonatal mice. No difference in expression in tracheal aspirates in a baboon model of BPD compared to those of healthy control. | (Thompson and Bhandari, 2008) (Lignelli et al., 2017) (Coalson et al., 1999) |
| Tumor necrosis factor α (TNFα) | Primary mediator of acute lung inflammation contributing to the pathophysiology of BPD. | Increases in neonatal mice and rat lungs during hyperoxia. Increased in tracheal aspirates of neonatal humans who developed BPD. | (Ben-Ari et al., 2000, Johnston et al., 1997) (Thompson and Bhandari, 2008) |

rodent models, where an initial increase in VEGF may cause lung injury, followed by a drop in VEGF. Subsequent increase of VEGF then promotes angiogenesis and alveolarization, necessary for lung healing and repair (Mura et al., 2004; Bhandari, 2010). Studies have shown that pups subcutaneously injected with the VEGF inhibitor VEGF-Trap show impaired alveolarization, especially, in early lung development (Thébaud et al., 2005). Also, rat pups exposed to hyperoxia have significantly lower levels of Vegf mRNA in their lungs at 14 days of age (Luan et al., 2015).

Inflammation and changes in angiogenic signaling are not the only consequences of hyperoxia. The release of pro-inflammatory cytokines and dysregulation of angiogenesis leads to significant cellular damage, which is evident in histology and transcription analyses of lung biopsies derived from BPD patients (Dishop, 2010). This damage occurs in the form of apoptosis, an important feature in hyperoxic lung injury, due to highly elevated levels of ROS (Mantell and Lee, 2000). The apoptotic signaling pathway involves a balance between BAX, i.e. the pro-apoptotic member of the BCL2 family, and the anti-apoptotic regulator BCL2, which is crucial to regulate apoptosis. The high levels of ROS upon hyperoxia can overwhelm the antioxidant systems in pulmonary cell types, which then become targets of ROS-induced injury and death. Studies show that hyperoxia-induced lung injury is associated with the death of alveolar epithelial cells, showing features of both apoptosis and necrosis (Das et al., 2004; Tong et al., 2021; Witkowski et al., 2016). Preterm human neonates who have received mechanical ventilation show higher levels of apoptosis, particularly, in epithelial cells (Lukkarinen et al., 2003). A correlation between increasing numbers of apoptotic cells and increasing mRNA levels of Bax during
hyperoxia was found in neonatal rat lung and, through upregulation of BCL2, administration of MSCs protects against apoptosis in this model (Zhang et al., 2013).

Extracellular matrix dynamics are another important factor in the response of lung tissue to hyperoxia, particularly regarding tissue regeneration and fibrosis. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix components and fibrillar collagen. They have important roles during normal lung development but, in excess, may alter lung remodeling and change lung architecture (Buckley and Warburton, 2002). MMP-9 is implicated in BPD, as studies in preterm human neonates support the argument that hyperoxia leads to increased MMP-9 levels in the lung (Bhandari, 2010; Sweet et al., 2004). Levels of MMP-9 also increase in lungs of neonatal mouse following hyperoxic injury and are associated with impaired alveolar development (Chetty et al., 2008). Compared with lungs from wild-type mice, lungs from MMP-9 knockout mice exposed to elevated O2 levels have an increased gas-exchange surface area and are protected from hyperoxia injury (Chetty et al., 2008). During hyperoxia, premature baboons also have increased MMP-9 levels and associated changes in lung architecture, compared with those of premature baboons under normoxic conditions (Tambunting et al., 2005). Upregulated MMP-9 expression during hyperoxia can lead to the activation of secreted glycoproteins, i.e. fibroblast growth factors (FGFs), that have an important role in normal pulmonary lung development and function, as well as in surfactant homeostasis (Rezvani et al., 2013). For instance, FGFR-HFc (HFc, heavy chain hinge and Fc domain of the mouse immunoglobulin) transgenic mice expressing a soluble FGF receptor are more susceptible to hyperoxic conditions following 95% O2 exposure compared to normal controls (Hokuto et al., 2004). Moreover, loss of FGFR signaling within type II alveolar epithelial cells leads to larger airspaces, enhanced collagen deposition and increased mortality of mice following lung injury (Dorry et al., 2020). Combined, these findings highlight how hyperoxia triggers a number of responses, which involve cell- and tissue-level signaling pathways that can result in BPD. Model systems, both in vivo and in vitro, have been crucial for these discoveries and remain instrumental in ongoing research. Additionally, this information is important because the identification of signaling pathways and molecular effects of BPD will aid in the identification of novel biomarkers and the development of therapeutics that reduce the negative effects of this disease.

Animal models
The majority of hyperoxia-induced BPD studies use mice and rats as their animal model. Both species are born during the saccular stage of lung development, which is characterized by the initiation of surfactant production and terminal airway dilatation and vascularization (Berger and Bhandari, 2014). In rodents, the saccular stage begins in utero and is completed by postnatal day 5 (PNS) (Berger and Bhandari, 2014). In humans, the saccular stage takes place exclusively in utero unless born prematurely. Humans born prematurely, when their lungs are in the saccular stage of development, are at high risk for complications due to pulmonary immaturity and surfactant deficiency (O’Reilly and Thébaud, 2014; Berger and Bhandari, 2014). This highlights a key difference between species. Rodents are born surfactant-sufficient, which limits their risk for respiratory distress during hyperoxic conditions (Berger and Bhandari, 2014). Despite this, rodents are the only animal models born during the saccular stage of lung development and, therefore, are well-suited to mimic the hallmark symptoms of BPD in neonates.

Fundamental differences between rodent species include total lung capacity, alveoli size and differential expression of genes that modulate lung processes (Berger and Bhandari, 2014). There are also strain- and sex-specific differences that need to be accounted for (Leary et al., 2019; Lingappan et al., 2015; Tiono et al., 2019; Dolma et al., 2020). Modeling of hyperoxic conditions in both outbred and inbred rodents, as well as in immunodeficient and immunocompetent ones, can lead to significant variability in results, posing a threat to the clinical applicability of these findings (Leary et al., 2019; Whitehead et al., 2006; Tiono et al., 2019; Will et al., 2019). Results may be irreproducible in outbred strains due to genotypic variations between different animals. In addition, different strains may exhibit distinct levels of gene expression. Whitehead and colleagues analyzed the genetic background of nine different mouse strains and discovered a profound effect on pathophysiologic responses to hyperoxic conditions (Whitehead et al., 2006). However, to study the heterogeneous responses of outbred animals to different drugs or interventions used to treat BPD may be beneficial because they more closely mimic the diverse responses seen in humans. In addition, on a molecular level, the response of rodents to hyperoxia may be protective in certain inbred strains compared to outbred strains, further skewing the translational relevancy of findings. There is also a potential for this to occur in immunocompetent vs deficient rodents, where immunodeficient mice may respond less well to hyperoxia.

Therefore, it is imperative to choose a strain showing a response that is most similar to that of a premature infant. For some studies, the use of rats may be beneficial as they are bigger, enabling improved handling during surgery and tissue harvesting, whereas using mice may have the advantage of widespread availability of biological reagents and their amenability to genetic manipulation (O’Reilly and Thébaud, 2014). Therefore, researchers need to consider a number of factors when choosing a rodent model for BPD research.

Unlike rodents, rabbits begin the alveolar stage of lung development in utero, similar to a full-term human infant (Richter et al., 2014; Pringle, 1986). Their larger lung capacity makes them better suited than rodents for the study of respiratory diseases affecting premature infants. However, since alveologenesis has already begun in the full-term rabbit, it may not be as well-suited to study this process. Compared to rodents, rabbits are costly and, in the United States, are regulated and protected by the US Department of Agriculture, which means that strict requirements and guidelines are in place to ensure they receive proper handling, care and treatment. However, when researchers need a larger model to study the effects of hyperoxia, rabbits do offer a suitable alternative when the use of larger non-human primates is impossible (D’Angio and Ryan, 2014).

Large animals, such as baboons and lambs are advantageous for studies of hyperoxia-induced lung damage, as they offer the opportunity to study disease progression over an extended time period, and to examine long-term effects of treatment. Pregnant animals can be induced, so that their babies are born prematurely during the saccular stage of lung development and can be placed on a mechanical ventilator at O2 levels similar to that delivered to human preterm infants (Albertine, 2015). Because of these attributes, large animals are best-suited for studies of hyperoxia-induced lung injury. Unfortunately, large animals are very expensive, require dedicated infrastructure and, with 150 days for lambs and 168 days for baboons compared to 21 days for rodents, have much longer gestation periods. On the basis of this information, researchers must carefully consider the stage of lung development, ability to manipulate the animal model, its size, cost and gestation time. Smaller animals (mice, rats, rabbits) are advantageous because of their short life cycle, low cost and the ability to manipulate their genomic and molecular pathways.
Differences in methodologies to model BPD in animals
To induce hyperoxic lung damage, animals are exposed to high levels of O$_2$ (60–95%) for a set period of time. As discussed previously, studies of animal models vary regarding species, O$_2$ concentration and duration of exposure, and lung injury measures (see Table 4). In rodents and rabbits, hyperoxia is achieved in a regulated chamber that mixes air and O$_2$. Some studies also employ mechanical ventilation to

Another benefit to the use of in vitro models for the study of BPD pathogenesis is that the stages of embryogenesis seen within the developing lung can be mimicked in culture. This allows for the identification of transcription factors affected by hyperoxia at each stage of development, which can further inform clinicians on the best course of action for treatment (Barkauskas et al., 2017). To mimic embryogenesis, bioengineered stem cells, such as induced pluripotent stem cells (iPSCs) are utilized as they can undergo a step-wise differentiation, using growth factors to promote pulmonary development. Our laboratory as well as others have successfully differentiated iPSCs to distal lung phenotypes that mimic type I and type II alveolar cells (Mitchell et al., 2017; Wang et al., 2020; Alyasdratos et al., 2020 preprint). Type I and type II alveolar cells are implicated in the functional regeneration of the distal lung after damage and are an excellent source for modeling hyperoxia-induced BPD (Jacob et al., 2017; Heo et al., 2019). In addition to studying cell development using cell culture, explant lung culture is another option to investigate specific pathways and novel interactions affected by hyperoxic conditions. Lung tissue explants can be studied before, after or during hyperoxia (Karekla et al., 2017; Asikainen et al., 2005b), and are used to study branching morphogenesis (Esquibies et al., 2008; Esquibies et al., 2014; Seedorf et al., 2016). Lung tissue explants can also be used to study the effects of transcription factors on lung repair pathways. In one study, researchers exposed fetal baboon explants to high (95%) levels of O$_2$, followed by treatment with prolyl hydroxylase domain-containing protein inhibitors with the aim to enhance angiogenic responses within the injured lung (Asikainen et al., 2005b). Their findings demonstrate increased expression of hypoxia-inducible factors 1α and 2α (HIF1A and HIF1B, respectively), as well as VEGF and platelet-endothelial cell adhesion molecule 1 (PECAM1), and are important because they provide a potential therapeutic option for the enhancement of lung growth during BPD. Overall, in vitro models are an attractive alternative to the use of in vivo models as they allow easy manipulation and direct visualization of lung development by using light or fluorescent microscopy (Wang et al., 2013).

In summary, in vitro models meet the need to reduce the use of animals in research, and provide an easily accessible platform in which to dissect the currently less understood molecular consequences of hyperoxia and to screen candidate therapeutics for treatment of BPD (Wu et al., 2018; Leeman et al., 2019; Knoll et al., 2013). Knowledge gained from these customizable models will allow investigators to manipulate developmental pathways within the lung, in order to improve clinical outcomes associated with BPD. However, these models should serve as a complement to in vivo studies, rather than a substitute, as they do not fully recapitulate the complexities of the in vivo microenvironment. Additionally, as evidenced from the studies cited in Table 2, considerable variability exists between different types of model, the source of tissue/cells and the duration and concentration of O$_2$ exposure used for hyperoxia experiments, i.e. caution should be taken when comparing these findings to in vivo conditions. Therefore, the continued use of animal models of hyperoxia-induced BPD remains essential for successful clinical progress.

In vitro models
To gain a deeper understanding of the molecular underpinnings of BPD pathogenesis, in vitro models of hyperoxia were developed that incorporate cells derived from human and rodent fetal lung tissues. Table 2 shows an overview of the different types of models as well as sources from which cells and tissue are commonly derived. These include 2D cultured cells or 3D-organotypic lung co-cultures as well as ex vivo anatomical scaffolds or models derived from live tissue or 3D-printed using biomimetic materials (Sucre et al., 2020; Montigaud et al., 2019; Möbius et al., 2018; Jiang et al., 2018; Leeman et al., 2019; Montemurro et al., 2006; Sucre et al., 2018; Knoll et al., 2013). Fetal primary epithelial cells isolated from human or rodent lung tissues can be cultured together with lung-derived fibroblasts or MSCs to study the effect of hyperoxia regarding the expression of transcription factors associated with pulmonary development. Sucre et al. have described a possible mechanistic link between hyperoxia and increased expression of Wnt5A – a protein involved in lung development – when human primary alveolar type II cells and fibroblasts were co-cultured under hyperoxic conditions (Sucre et al., 2020). Through this and other studies, it is now established that the Wnt5a signaling pathway is a mediator of chronic lung disease (Newman et al., 2016; Li et al., 2020; Lingappan and Savani, 2020). Because on molecular and transcriptional levels the above models show a response that is similar to that in animal models, they serve as a complementary resource to evaluate and manipulate the signaling pathways involved in the perturbation of lung development (Torday and Rehan, 2007; Asikainen et al., 2005a; Blackwell et al., 2011).

Response to certain inhibitors and treatments can first be tested in these in vitro models, followed by implementation in animal models. For example, Zhang and colleagues have demonstrated that isolated hyperoxic rat alveolar cells treated with the autophagy inducers rapamycin or LiCl show restored autophagy flux and increased survival of cells (Zhang et al., 2020). These types of study help to reduce the number of animals initially required to obtain the desired therapeutic response. In vitro model systems are also beneficial because they allow for the evaluation of parameters associated with epithelial-to-mesenchymal transition (Greer et al., 2014), cell migration (You et al., 2019) and angiogenesis (Zhang et al., 2018). The influence of sex on BPD severity has also been investigated by using a 2D in vitro model of hyperoxia (Zhang et al., 2017). Zhang and colleagues exposed human umbilical vein endothelial cells (HUVECs) derived from males and females to hyperoxic (95% O$_2$) and normoxic conditions (21% O$_2$) for up to 72 h. They discovered that mRNA and protein levels of the secreted cytokine growth and differentiation factor 15 (GDF15) – that plays a role in cell proliferation, angiogenesis and apoptosis – is upregulated in male-derived compared to female cells. Zhang et al. were able to reproduce these findings in vivo by exposing male and female C57BL/6 mice to the same conditions.
induce lung damage in small and large animals, with the aim to mimic the conditions experienced by at-risk neonates in the NICU. By itself, mechanical ventilation can greatly affect the inflammatory response within the lung (Helmerhorst et al., 2017). Therefore, the two methods for O2 delivery described above are not interchangeable. Treating them as such would pose a threat to the standardization of

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### Table 2. In vitro models of hyperoxia-induced BPD

| Type of model | Cells/material used | Concentration/duration of exposure to O2 | Primary findings | References |
|---------------|---------------------|-----------------------------------------|-----------------|------------|
| **3D co-cultures** | Primary human lung fibroblasts and epithelial cells | 70% for 96 h | Inhibition of Wnt5a signaling abrogated the BPD transcriptomic phenotype and improved alveolarization. | (Sucre et al., 2020) |
| | Fetal mouse lung mesenchyme and A549 epithelial cells | N/A | 3D peaks formed with similar cellular orientation as alveolar structures in vivo. LPS inhibited alveolar formation. | (Greer et al., 2014) |
| | MSCs and distal lung epithelial cells from mice | N/A | Co-culture with MSCs stimulated Epcam- and Sca1-positive derived distal lung epithelial organoid formation, increased alveolar differentiation and decreased self-renewal. Soluble factors from MSCs have demonstrable effects on alveolar organoid formation. | (Leeman et al., 2019) |
| | Rat bone marrow stem cells and primary fetal E21 rat type II cells, lung fibroblasts | N/A | Co-culture with BMSCs decreased type II cell and fibroblast proliferation and induced differentiation through paracrine release of soluble factors. | (Knoll et al., 2013) |
| | Human type II epithelial cells+fetal human PDGFRα+ lung stromal cells | N/A | Stromal cells enhanced growth and differentiation of type II alveolospheres. TEM analysis revealed presence of lamellar bodies. | (Barkauskas et al., 2013) |
| **3D-printed infant-like respiratory model** | Upper airways and larynx composed of SLA infant nasal replica obtained by reconstruction from CT scan of SAINT model, rabbit thorax | N/A | Used to assess aerosol regional deposition, breathing patterns obtained from model were similar to in vivo data of 3-month-old infants suffering from BPD. | (Montigaud et al., 2019) |
| **Ex vivo cultures** | Mouse lung endoderm explants | N/A | ERK1/2/Anxa4 signaling plays role in promoting migration of airway epithelial progenitor cells to distal airway tips. | (Jiang et al., 2018) |
| | Mouse lung explants | 95% O2 overnight | Fetal lung macrophage activation contributes to BPD by generating inflammatory response that disrupts developmental signals. | (Blackwell et al., 2011) |
| | Precision-cut lung slices from lungs of mouse pups (P4–P7) | 70% O2 for 96 h | Inhibition of Wnt5a signaling abrogated BPD transcriptomic phenotype and improved alveolarization. | (Sucre et al., 2020) |
| | Rat embryonic lung explants | 50% O2 for 4 days | Reduction in the number of terminal buds and branch length after hyperoxia. Significant increase in cellular apoptosis and decrease in proliferation. | (Leary et al., 2019) |
| | Mouse fetal lung explants and fetal alveolar and endothelial cells | 3% O2 for 72 h | Recombinant VEGF restores terminal lung bud formation in eNOS−/− mice. VEGF-HGF signaling between epithelial and endothelia is disrupted in infants with BPD. | (Seedorf et al., 2016) |
| | Mouse lung explants | N/A | Development of Sox2-positive progenitors in lung endoderm is regulated by HDAC1 and HDAC2. | (Wang et al., 2013) |
| **2D cultures** | MSCs isolated from human fetal lung tissue (16–18 weeks of gestation). | 5% or 60% O2 | Exposure to 60% O2 leads to excessive cellular proliferation, reduced colony-forming ability, decreased elastic deposition and impaired secretion of factors important for lung growth. | (Möbius et al., 2018) |
| | Calu-3 cells | 40%, 60% and 80% O2 for 72 h | Elevated secretion of IL-6 and IL-8 in all groups, as well as reduced cell viability and transepithelial resistance. | (Zhu et al., 2008) |
| | Human primary fetal lung fibroblasts | 21% or 40% O2 for 7 days. | Moderate hyperoxia enhances senescence and reduces autophagy in lung fibroblasts. Elevation of proinflammatory and profibrotic factors. | (You et al., 2019) |
| | Human lung microvascular endothelial cells and alveolar-epithelial-like cells | 21% O2 | Stimulation of HIFs and modulation of HIF-regulated angiogenic proteins through treatment with PHI-1, which enhances angiogenesis and lung growth, enhances angiogenesis and lung growth. | (Askainen et al., 2005a) |
| | HUVECs | 21% and 95% O2 for 72 h | Sex differences exist regarding expression and modulation of GDF15 by HIF1A in neonatal hypoxic injury. | (Zhang et al., 2014) |
| | Primary type II alveolar epithelial cells from premature rats | 21% and 95% O2 for 24 h | Hydrogen protects type II alveolar epithelial cells from hyperoxia-induced apoptosis by inhibiting apoptosis factors and promoting anti-apoptosis factors. | (Wu et al., 2018) |

*eNOS, endothelial nitric oxide synthase; PHI-1, prolyl 4-hydroxylase (HIF-specific inhibitor).*
methodologies, which is essential for a translationally relevant evaluation of hyperoxia-induced lung damage. This should be carefully considered when using a protocol that adopts a combination of the two types of O$_2$ exposure.

Silva et al. found that studies published between 2012 and 2015 described 41 different O$_2$ exposure protocols to induce lung damage in animal models (Silva et al., 2015). Silva’s review and Table 3 highlight that even these small subsets of studies substantially vary in the degree and duration of hyperoxia, and in the definition of lung injury associated with BPD (Silva et al., 2015), which greatly affects the translatability of findings. The expression of genes affected during hyperoxic conditions will vary significantly if the duration and concentration of O$_2$ exposure varies. For results to be reproducible, studies should focus on finding the optimal O$_2$ concentration and exposure time to mimic BPD symptoms without causing lethal injury. Based on our previously published findings, we have shown that exposing mice at PN1 to 75% O$_2$ for 14 days is sufficient to induce alveolar simplification and septal thickening similar to those seen in a premature infant suffering from BPD (Fig. 3), corroborating findings by others (Mitchell et al., 2020; Popova et al., 2014; Kindermann et al., 2019; Schmiedl et al., 2017). Nardiello et al. exposed C57/BL6 mouse pups to 85% O$_2$ for 14 days and used design-based stereology to analyze lung tissue samples (Nardiello et al., 2017b). They showed that – compared with pups under normoxic conditions – this level of hyperoxia sufficiently recapitulates the features of BPD, as it causes significant alveolar simplification, i.e. a 69% decrease in alveoli number and alveolar density, a 37% decrease in gas exchange area, and 25% thickening of the septal wall. Although exposure of mouse pups to 85% O$_2$ successfully induced a BPD phenotype, this dose might not be ideal when evaluating the effectiveness of therapeutic interventions. Nardiello et al., therefore recommend to evaluate the intervention by reducing the O$_2$ concentration to 60%, as this intervention is unable to reverse the damage caused by 85% O$_2$ (Nardiello et al., 2017b). This highlights the idea that severe disease phenotypes, like the murine BPD caused by exposure to 85% O$_2$, might not be the most appropriate vehicle to test the effectiveness of therapies, and that researchers must carefully select and evaluate their experimental parameters.

In addition to the lack of agreement regarding the optimal O$_2$ concentration to elicit appropriate lung damage, most current studies did not assess lung function after damage. BPD is no longer defined on the basis of morphological changes alone, but on the need for supplemental O$_2$ to live (Ibrahim and Bhandari, 2018). Accurate functional assessments in rodents are difficult as they are small and anatomically different compared to human neonates. However, accurate and clinically valid morphological and functional assessments are necessary for translationally relevant comparisons between species (Hoymann, 2012). Examples of tests used for functional assessments of the lung include whole-body plethysmography and forced oscillation. The whole-body plethysmography method involves an inner restraint chamber, in which the rodent is placed and positioned within a nose cone attached to a pneumotachograph, a flow-resistive device that quantitatively measures airflow. It contains plethysmography chambers with ports for bias flow, calibration and leak testing (Lofgren et al., 2006). It also allows to monitor changes in airway pressure and temperature at the nose of the rodent. Forced oscillation techniques are employed by using a flexiVent system made for small rodents. This method involves sedation and intubation of animals, followed by testing of lung function upon the application of forced oscillation signals over a set period. An example of this method was performed in neonatal rat pups exposed to three different concentrations of O$_2$ (fractions of inspired O$_2$: 0.6, 0.8, and 1.0) over a total of 18 days. Results showed altered tissue stiffness and reduced lung compliance following injury, as well as alveolar simplification, increased ACTA2 content in pulmonary vessels and fewer pulmonary arterioles (Greco et al., 2019). This study demonstrates that functional lung assessments are associated with structural changes within the lung and are, thus, a necessary parameter to consider when comparing results from animal models to premature infants.

**Lung injury measures in animal models**

A BPD diagnosis is based on the need for supplemental O$_2$ for at least 28 days after birth, whereas its severity is graded according to the respiratory support the infant requires at 36 postmenstrual weeks of age (Nardiello et al., 2017a). The clinical definition of BPD, thus, does not consider the structural damage hyperoxia causes to a developing lung. The goal of many BPD animal model studies is to explore interventions, with the aim of promoting lung growth and alveolarization. Hyperoxia-induced BPD models should show a combination of changes in lung morphology, and changes in the expression of relevant genes that mimic the multifactorial etiology of BPD. Currently, there is no standardization of lung injury measurement when exploring new interventions for BPD in animal models. Therefore, it is important to effectively quantify changes to the lung structure, especially the size and number of alveoli, the gas-exchange surface area, the vascularization of the lung, and the thickness of the septal walls (Nardiello et al., 2017a). Such quantitative information about lung architecture is necessary to assess pathological alterations as well as treatment effects, and to allow statistical comparisons among experimental groups (Ochs and Mühlfeld, 2013). A decrease in the available surface or an increase in the thickness of a barrier can result in functional impairment, leading to problems, such as emphysema and fibrosis (Mühlfeld et al., 2013). Additionally, transcriptomic and cellular alterations caused by BPD indicate disease severity and the standardization of markers to evaluate these changes in animal models is necessary to make the appropriate comparisons to neonates. Several of the current studies focus solely on morphological changes associated with hyperoxia-induced lung injury and neglect to highlight the molecular implications of BPD.

**Morphological assessments of the lung following hyperoxic damage**

In most studies, the mean linear intercept (MLI) or radial alveolar count (RAC) are techniques used for direct measurements of the distance between adjacent walls of an alveolus by means of a slide rule. MLI is used as an indirect method to measure air space enlargement, which includes both alveoli and alveolar ducts. RAC is a measure of the number of alveoli or degree of alveolarization within the lung. Unfortunately, there are downsides to using MLI and RAC in BPD animal models, such as inherent bias, especially if investigators are not double-blinded during their assessments. Moreover, dimensions within the distal air spaces are vastly different under physiological conditions compared to when the lungs are fixed for analysis (Knudsen et al., 2010). Distortion of the lung structure can occur during tissue processing, potentially introducing artifacts associated with non-uniform tissue dehydration and rehydration (Schneider and Ochs, 2014; Silva et al., 2015). Accurate morphological assessments in rodents is particularly difficult because of their small size and inability to mimic chronic respiratory conditions. Thus, the degree of lung inflation as well as length of tissue fixation need to be standardized to eliminate discrepancies in MLI and RAC findings.

Quantitative assessment of lung structure is essential to thoroughly understand the changes that occur in a diseased state (Mühlfeld et al., 2013). Microscopic study of the internal structure of the lung is an
### Table 3. Measurement of outcomes in a subset of hyperoxia animal models treated with cell therapy

| Species       | Strain | O₂ (in %) | Duration of hyperoxia | Alveolarization | Method used to measure lung injury/BPD | Genes expression | Other | References |
|---------------|--------|-----------|-----------------------|-----------------|--------------------------------------|-----------------|-------|------------|
| Mouse         | C57BL/6| 60        | 14 days (PN1–PN14)    | Radial alveolar count | IHC                    | TGFβ1, VEGF      |       | (Luan et al., 2015) |
|               | C57BL/6| 95        | 7 days (PN1–PN7)      | Mean cord length  |          | RT-PCR                          | Tgfβ1, Il1b, Il6 |       | (Fritzell et al., 2009) |
|               | C57BL/6| 85        | 14 days (PN1–PN14)    | Mean linear intercept | IHC            |                   | MPO, Mφ              |       | (Vosdoganes et al., 2013) |
|               | C57BL/6| 75        | 14 days (PN1–PN14)    | Septal volume     | qRT-PCR          | Tgfb1, Il1b, Il6, Pdgfa |       | (Mitchell et al., 2020) |
| SCID beige    | 90     | 7 days (PN3–PN10) | Mean cord length     | qRT-PCR          | IL6              | Tgfβ1, Nkb1       |       | Lung function testing (Liu et al., 2014a) |
| Rat           | Sprague Dawley | 95   | 7 days (PN3–PN10)    | Radial alveolar count | ELISA           | TGFβ1             |       | (Tian et al., 2007) |
|               |        | 95        | 14 days (PN1–PN14)    | Mean linear intercept | ELISA           |                   | TGFβ1              |       | (van Haafften et al., 2009) |
|               |        | 95        | 7 days (PN3–PN10)     | Radial alveolar count | ELISA           |                   | TGFβ1              |       | (Zhang et al., 2012) |
|               |        |           | 7 days (PN3–PN10)     | Mean linear intercept | ELISA           |                   | TGFβ1              |       | (Zhang et al., 2013) |
|               |        | 85        | 19 days (PN2–PN21)    | Radial alveolar count | ELISA           |                   | TGFβ1              |       | Pulmonary angiogenesis (Sammour et al., 2016) |
|               |        | 95        | 7 days (PN1–PN7)      | Radial alveolar count | ELISA           |                   | TGFβ1              |       | Pulmonary vascular remodeling (Tian et al., 2008) |
|               |        |           | 14 days (PN1–PN14)    | Mean linear intercept | ELISA           |                   | TGFβ1              |       | (Chang et al., 2011) |
|               |        |           | 14 days (PN1–PN14)    | Mean alveolar volume | ELISA           |                   | TGFβ1              |       | (Chang et al., 2013) |
| Rabbit        | White Dendermonde hybrid | 95 | 2 h  | Mean linear intercept | ELISA           |                   | TGFβ1              |       | Lung function testing (Jiménez et al., 2018) |
| Lamb          | 88–95 Vent |     | 2 h   | Mean wall thickness | ELISA           |                   | TGFβ1              |       | Lung function test Pulmonary artery micro-ultrasound Doppler (Melville et al., 2017) |

*EUSA, enzyme linked immunosorbent assay; IHC, immunohistochemistry; Mφ, macrophage; MPO, myeloperoxidase; PN, postnatal day; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Vent, ventilated; WB, western blot. ↔ denotes no change.*
indirect measurement of morphometry, whereby a small section of the lung is analyzed; this also can potentially introduce bias. Recommendations have shifted to the use of design-based stereology to assess changes to the lung morphometry. The American Thoracic Society and the European Respiratory Society have both defined the standards for performing quantitative assessments of lung structures by using this method (Hsia et al., 2010), which involves measuring 2D sections of tissue to characterize physical properties of irregular 3D objects. (Weibel et al., 2007). The use of computed tomography and magnetic resonance imaging may also help to identify and distinguish pulmonary abnormalities following hyperoxia-induced lung damage, which is not always representative of the changes that occur within the lung. Analysis of gene expression in the lung during hyperoxia will provide valuable information as to when molecular damage occurs – because this often precedes physical damage. This will provide a source of comparison to genes affected in the neonate, increasing the potential translational significance of the findings to better inform researchers and clinicians as to when therapeutic interventions will be most effective.

Molecular assessments of the lung following hyperoxic damage
Both morphologic and molecular assessments are necessary to ascertain the full effects of hyperoxia in animal models of BPD. Table 1 highlights the genes differentially expressed in neonatal humans and neonatal animal models, showing that BDP is multifactorial in nature. Therefore, it is imperative to develop an animal model that exhibits the same degree of lung injury as a preterm infant on the transcriptomic and molecular level. The majority of pre-clinical studies of hyperoxia focus on morphological parameters to assess lung damage, which is not always representative of the changes that occur within the lung. Analysis of gene expression in the lung during hyperoxia will provide valuable information as to when molecular damage occurs – because this often precedes physical damage. This will provide a source of comparison to genes affected in the neonate, increasing the potential translational significance of the findings to better inform researchers and clinicians as to when therapeutic interventions will be most effective.

Stem cells as a potential treatment for BPD
Although several different therapeutics have been tested for the treatment of BPD (Principi et al., 2018), the most promising – based on results from pre-clinical studies – are exogenous stem cells, which can be delivered via several different routes intravenously,
Table 4. Comparison of animal models of hyperoxia-induced BPD treated with cell therapy

| Source | Route of administration | Treatment day | Treatment results |
|--------|-------------------------|---------------|------------------|
| Mouse BM-MSC | 60 | 14 days (PN1–PN14) Mouse | 1×10^6 i.v. | Radial alveolar count (Luan et al., 2015) |
| UC-AMSC | 90 | 7 days (PN1–PN7) Mouse | 10×10^6 i.n. | Restoration of lung compliance, elastance, and tissue recoil associated with alveolar septal widening (Liu et al., 2014a) |
| Human UC-MSC | 90 | 14 days (PN1–PN14) Human | 0.1, 0.5, 1×10^6 i.p. | Restoration of lung compliance, elastance, and tissue recoil associated with alveolar septal widening (Vosdoganes et al., 2013) |
| Human iPSC or diPSC | 90 | 14 days (PN1–PN14) Human | 1.5×10^6 | Differentiation of MSC into vascular endothelial cells (Mitchell et al., 2020a) |
| Rat BM-MSC | 85 | 7 days (PN1–PN7) Rat | 5×10^4 i.v. | Radial alveolar count (Tian et al., 2007) |
| Rat UC-MSC | 95 | 7 days (PN1–PN7) Rat | 1×10^5 | Radial alveolar count (Zhang et al., 2012) |
| Human UC-MSC, A-MSC, or UC-PCs | 90 | 14 days (PN1–PN14) Human | 60% PH | Improved lung compliance (Pierro et al., 2013) |
| Human AEC | 85 | 2 h Human | 60% | Improved lung compliance (Melville et al., 2017) |
| Human AEC | 88 | 2 h Human | 60% | Improved lung compliance (Melville et al., 2017) |

References:
- Luan et al., 2015
- Vosdoganes et al., 2013
- Mitchell et al., 2020a
- Tian et al., 2007
- Zhang et al., 2012
- Pierro et al., 2013
- Melville et al., 2017
intraperitoneally, intramuscularly, intraorally or intranasally. Stem cells are an attractive therapeutic option owing to their excellent anti-inflammatory properties and regenerative abilities mediated by their paracrine effects on neighboring cells (Namba, 2019). Progenitor-like stem cells are depleted in infants suffering from BPD, making exogenous administration of allogenic or autologous stem cells beneficial (Borghesi et al., 2013). The types of stem cell utilized include amnion epithelial cells (AECs), MSCs and induced pluripotent stem cells (iPSCs) (Möbius and Thébaud, 2015). Pre-clinical studies have shown that delivery of stem cells can protect the lungs and restore damage in different animal models of BPD (Table 3).

**Amnion epithelial cells**

Amnion epithelial cells (AECs) are pluripotent and derived from the placenta. In both mouse intraperitoneal and lamb intravenous AEC administration models, the AECs attenuated damage caused by hyperoxia-induced inflammation, and improved lung function and structure (Melville et al., 2017; Vosdoganes et al., 2013). Clinical trials are currently in progress to assess their efficacy in the treatment of BPD in neonates (Baker et al., 2019; Lim et al., 2018).

**Mesenchymal stromal/stem cells**

MSCs are multipotent stem cells that can be isolated from many tissue types (Ee and Thébaud, 2018); MSC therapy is the most extensively studied cell therapy for BPD in hyperoxia models. This cell therapy focus has been placed on MSCs owing to their pleiotropic effects of immune modulation, angiogenesis and anti-oxidant activity – key hallmarks of BPD (Ee and Thébaud, 2018; Davis et al., 2012; Kuchroo et al., 2015; Dey et al., 2012). A concern with the use of certain stem cells is their ability to form non-cancerous tumors called teratomas upon *in vivo* administration (Hentze et al., 2009; Gutierrez-Aranda et al., 2010). However, MSCs are safe and non-teratogenic, making them an attractive therapeutic option.

Results from studies in which MSCs were administered to animal models of hyperoxia-induced lung injury have been promising. MSCs administered either intravenously, intraperitoneally or intratracheally have mitigated neonatal lung injury – as shown by decreased lung inflammation (O'Reilly and Thébaud, 2014), prevention of vascular damage and of alveolar growth arrest (Aslam et al., 2009), inhibition of lung fibrosis (Tropea et al., 2012), and improvement of exercise tolerance (Hansmann et al., 2012; Zhang et al., 2012).

**Induced pluripotent stem cells**

Another type of stem cell investigated as a potential therapeutic are induced pluripotent stem cells (iPSCs). These cells can differentiate into all three embryonic germ layers and have immunogenic properties similar to those of MSCs, but show more potent immunomodulatory effects *in vitro* (Schnabel et al., 2014). iPSCs are capable of unlimited *in vitro* proliferation and can be differentiated into virtually any cell type of the body.
iPSCs have been found to attenuate acute lung injury caused by mechanical ventilation, by suppressing oxidative stress, acute inflammation and apoptosis (Liu et al., 2014b). Data from our laboratory has shown that iPSCs, when administered intratraurally after exposing mice to hyperoxic conditions (75% O₂ for 14 days), attenuate alveolar simplification (Fig. 2A) and restore lung morphology to that seen in normoxic control animals (Fig. 2B) (Mitchell et al., 2020).

A limitation of transplanting undifferentiated iPSCs is their potential to form teratomas in vivo, whereas differentiated iPSCs do not, as previously published by our laboratory (Mitchell et al., 2019). Studies from our group and others have evaluated the effect of transplanting iPSCs that had differentiated – i.e. diPSCs – into an alveolar-like phenotype in attenuating hyperoxia-induced lung injury (Mitchell et al., 2019, 2017; Shafa et al., 2018; Huang et al., 2014). Our lab showed that, although alveolar-like diPSCs can prevent hyperoxia-induced impairment of lung function and alveolar growth in mice, they are not as efficacious as undifferentiated iPSCs (Fig. 2B) (Mitchell et al., 2020; Shafa et al., 2018).

Stem cell-based therapy for the treatment of hyperoxia-induced lung disease is an exciting and evolving field. Studies have shown that the anti-inflammatory effect of stem cells benefit lung repair; however, there are still inherent risks associated with their use (Chang et al., 2014; Mitchell et al., 2020; Shafa et al., 2018; Liu et al., 2014b). Certain cell types can transform into inappropriate phenotypes that may lead to cancer, and may have pro-arrhythmic side effects (Toh et al., 2018). Unfortunately, as with animal models, their variability in terms of cell type, source species and route of administration as well as the age of the recipient at treatment affect outcomes. MSCs isolated from bone marrow, umbilical cord, Wharton’s jelly and adipose tissue have been tested in hyperoxia-induced BPD animal studies together with AECs, iPSCs and differentiated iPSCs. These studies show great promise and some groups are testing MSCs in clinical trials for safety and possible efficacy (Ahn et al., 2017; Powell and Silvestri, 2019). However, it is imperative that the field standardize these studies to determine which combination of parameters best mimics the BPD seen in human neonates, and to test possible treatment options.

Conclusions
Considerable differences in animal models and methodologies used to analyze the effects of hyperoxia-induced lung damage exist, hampering the potential to develop reliable and effective therapies for the treatment of BPD in neonates. In vitro models have the potential to address many of the shortcomings associated with current animal models, by revealing the cellular and molecular pathways involved in the progression of BPD. 3D-organotypic models are able to replicate embryogenesis of the lung, and provide excellent platforms for the safe and efficient testing of different therapeutics.

Based on a thorough analysis of the current hyperoxia literature, rodent models of BPD seem best-suited for the initial study of hyperoxia-induced lung damage and potential therapeutic treatments (Box 1). Not only are they suitable in terms of cost and gestation length, but they more closely mimic the stage of lung development seen in a preterm infant. Based on our previously published study, standardized models should be developed – implementing at least 75–85% O₂ for 14 days – to achieve the morphological alterations associated with BPD, such as alveolar simplification, fibrosis and septal wall thickening (Mitchell et al., 2020). Even though stem cell therapy shows promise in treatment of BPD, there is still a long way to go in terms of safety and efficacy in clinical trials. However, MSCs and iPSCs are consistently cited in the literature as having beneficial effects. The route of administration for cell-based therapies should be one that successfully delivers cells directly to the lung for the greatest tissue regeneration benefits. Our own work showed that the intratracheal or intraoral routes are most effective in the amelioration of lung damage within animal models (Mitchell et al., 2020). Analysis of changes to gene expression during hyperoxia-induced lung damage in rodent models should be comprehensive and mirror the genes affected in the neonate to make direct comparisons. Techniques standardized and validated in rodent models should be implemented in large animals, such as sheep, to evaluate reproducibility and potential for clinical translation. Standardized functional assessments of lungs following damage should also be included in preclinical studies, as BPD is no longer solely defined on the basis of morphological changes. In vitro systems can complement animal models to study the molecular mechanisms and pathways related to BPD progression, and for development and validation of potential therapeutics. Once a rigorous set of guidelines and methodologies are established and efficiently reproduced, the findings in both preclinical animal and in vitro models can be translated into potential therapeutic options for neonates suffering from BPD.

In conclusion, considerable work still needs to be accomplished to improve the current models of BPD. However, substantial progress has been made in the identification of molecular and cellular pathways, as well as transcription factors and cytokines implicated in BPD pathogenesis in both in vivo and in vitro models. This information has contributed to a deeper understanding of disease progression and lung development overall. Implementation of a multidisciplinary approach, involving researchers, developmental biologists, clinicians and pathologists, will help to advance the discovery of viable therapeutic options and appropriate interventions for BPD and, potentially, other chronic respiratory diseases that currently lack sufficient treatment options.

Competing interests
The authors declare no competing or financial interests.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Box 1. Recommendations for the standardization of current models of hyperoxia-induced tissue damage

- Develop a set of guidelines to improve animal reporting that will encourage the development of rigorous experimental design standards, similar to the ARRIVE essential 10 checklist, a minimum set of guidelines that need to be reported to improve research involving animals. This will maximize the reproducibility, quality and reliability of published research (see Table 5).
- Determine appropriate model species based on translational relevancy and cost (small rodent vs large animal).
- Establish the appropriate O₂ concentration and duration of exposure to elicit morphological and genotypic changes in in vitro and in vivo models of bronchopulmonary dysplasia.
- Establish appropriate methods of O₂ administration, e.g. mechanical ventilation vs regulated chamber, or both.
- Standardize techniques for morphological analysis of the damaged lung, e.g. mean linear intercept vs radial alveolar count vs design-based stereology.
- Include functional assessments of the lung post damage.
- Analyze changes in gene expression by investigating markers affected in neonates suffering from bronchopulmonary dysplasia.
- Standardize the source of cells for in vitro models and therapeutic studies, e.g. stem cells vs primary cells from the lung.
- Standardize the route and frequency of stem cell administration in in vivo treatments.
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Lung injury and reparative events can be modeled in vitro and in vivo. In vitro, the use of organotypic cultures, human lung tissue, cell lines, primary cells, or induced pluripotent stem cells (iPSCs) has demonstrated the utility of these models for studying lung disease. These models are advantageous because they allow for the control of environmental conditions and the manipulation of genetic factors, which can provide insights into the mechanisms underlying lung injury and repair.

In vivo models, on the other hand, offer more physiological relevance as they mimic the human condition. Rodent models, such as the hyperoxia model in neonatal rats, provide a platform to study the effects of environmental factors, such as hyperoxia, on lung development and injury. Additionally, models of human bronchopulmonary dysplasia (BPD) in preterm infants have been developed to understand the pathophysiology and potential therapies for this disease.

The selection of the appropriate model depends on the specific research question and the experimental goals. Both in vitro and in vivo models have contributed significantly to our understanding of lung injury and repair, and they continue to be refined and expanded to better simulate the complexities of human lung disease.
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