Recent advances in the mechanisms of lung alveolarization and the pathogenesis of bronchopulmonary dysplasia

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Submitted 5 August 2015; accepted in final form 9 September 2015

Silva DM, Nardiello C, Pozarska A, Morty RE. Recent advances in the mechanisms of lung alveolarization and the pathogenesis of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 309: L1239–L1272, 2015. First published September 11, 2015; doi:10.1152/ajplung.00268.2015.—Alveolarization is the process by which the alveoli, the principal gas exchange units of the lung, are formed. Along with the maturation of the pulmonary vasculature, alveolarization is the objective of late lung development. The terminal airspaces that were formed during early lung development are divided by the process of secondary septation, progressively generating an increasing number of alveoli that are of smaller size, which substantially increases the surface area over which gas exchange can take place. Disturbances to alveolarization occur in bronchopulmonary dysplasia (BPD), which can be complicated by perturbations to the pulmonary vasculature that are associated with the development of pulmonary hypertension. Disturbances to lung development may also occur in persistent pulmonary hypertension of the newborn in term newborn infants, as well as in patients with congenital diaphragmatic hernia. These disturbances can lead to the formation of lungs with fewer and larger alveoli and a dysmorphic pulmonary vasculature. Consequently, affected lungs exhibit a reduced capacity for gas exchange, with important implications for morbidity and mortality in the immediate postnatal period and respiratory health consequences that may persist into adulthood. It is the objective of this Perspectives article to update the reader about recent developments in our understanding of the molecular mechanisms of alveolarization and the pathogenesis of BPD.

bronchopulmonary dysplasia; persistent pulmonary hypertension of the newborn; pulmonary hypertension; lung development; alveolarization; hyperoxia

THE TRANSPORT OF OXYGEN from the atmosphere into the bloodstream, and conversely carbon dioxide from the bloodstream into the atmosphere, is the primary function of the lung. To optimize gas exchange, the barrier across which gas exchange takes place, the alveolo-capillary barrier, must have a surface area as large as possible and must be as thin as possible. Thus the objective of lung development is to generate this optimized gas-exchange structure (131, 358). The development of the mammalian lung is broadly divided into two phases, namely, early lung development (357), when the core lung structure containing the branching conducting airways with their attendant vasculature develops. This is followed by the structural refinement of the gas-exchange surface, through the generation of alveoli, the principal gas-exchange units of the lung. The alveoli, which were first described by Marcello Malpighi in 1661 (210, 211), are generated by a process of secondary septation that occurs during late lung development and are uniquely designed to facilitate gas exchange (364). A recent report has indicated that these two phases of lung development are intimately coordinated (61).

Perturbations to lung development result in lungs that are defective for gas exchange. This is exemplified in the clinical setting by bronchopulmonary dysplasia (BPD) (169, 218), a common complication of preterm birth originally described by Northway and coworkers in 1967 (258). Preterm infants with compromised respiratory function attributable to respiratory distress syndrome (RDS) require oxygen supplementation, often by mechanical ventilation. In these infants, infection, inflammation, oxygen toxicity, and volu- and baro-trauma from ventilation, together with other factors, stunt the postnatal maturation of the lung (154). This maturational stunting includes blunted alveolarization and the generation of a dysmorphic pulmonary vasculature, accompanied by aberrant pulmonary vascular wall remodeling, causing pulmonary hypertension (125). BPD is associated with significant morbidity and mortality in the neonatal intensive care unit, and survivors exhibit long-term consequences that persist into adulthood. Similar disturbances to alveolar structure and the pulmonary vasculature are noted in infants with congenital diaphragmatic hernia (CDH) (317, 344). The improved medical management of preterm birth has interestingly led to an increase in the incidence of BPD (158, 159). This highlights the need to better understand normal and aberrant late lung development. In particular, the molecular mechanisms underlying the associated blunted alveolarization and disturbed vascular development and homeostasis associated with BPD remain largely unclear.

To this end, there is much activity within the lung development and pediatric communities to better understand the molecular mechanisms of normal lung development (242) and the factors that influence the postnatal maturation of the mammalian lung (366). It is the objective of this Perspectives article to update the reader about advances in our understanding of lung alveolarization reported in the literature since the beginning of 2013. It is intended that this update will pick up where the last update, published in 2013 (206), ended. This Perspectives article will first address recent developments in methodologies, including new state-of-the-art approaches to quantify the lung architecture, as well as an overview and critique of the current animal models employed to study alveolarization. These elements of this Perspectives article will be followed by a report on novel players in normal and aberrant late lung development, either identified by transgenic mouse approaches or pharma-
cological interventions in animal models of arrested lung development. Recent advances in our understanding of the long-term sequelae of arrested alveolarization and the process of neoalveolarization in developed lungs, as well as recent advances in cell therapy for arrested alveolarization, will also be addressed.

Quantitative Assessment of Lung Structure

Being able to robustly quantify structural changes to the lung architecture is central to the study of normal and pathological lung development (361). Historically, this has been performed by many investigators using paraffin-embedded lung tissue, which is then assessed by light microscopy, usually with the determination of the mean linear intercept (MLI, or the related mean chord length, $L_m$) or radial alveolar count (RAC) as a surrogate for alveolar size and, hence, alveolar number. Additionally, the septal thickness has been directly determined, usually at the “thinnest” point of the septal wall. Providing a wealth of meaningful and valuable data, this approach is facing increasing valid criticism. Among the critiques are the lack of randomization in the sampling, the introduction of artifacts associated with nonuniform tissue dehydration and rehydration (“shrinkage”) during classical fixations protocols, and subsequent paraffin embedding. Another concern is the range in hydrostatic pressure used for pressure fixation via the trachea, with some investigators preferring 20 cmH$_2$O, whereas others employ 25 cmH$_2$O. Presently, there is much enthusiasm in the field for the implementation of improved fixation and embedding techniques and stereological approaches to address these problems and to provide robust quantitative data.

A very important study comparing three methods of lung tissue preparation was published by Schneider and Ochs (309), in which lung tissue was embedded in paraffin, in glycol methacrylate alone (“simple”), or in glycol methacrylate with osmium tetroxide and uranyl acetate. The authors documented a 40% shrinkage of cut face areas in the paraffin-embedded tissue, a 30% shrinkage with the simple glycol methacrylate, and <3% shrinkage with glycol methacrylate with osmium tetroxide and uranyl acetate. The authors also documented the superiority of glutaraldehyde-based fixative protocols. This study clearly demonstrated the need to move from a paraffin-based to a glycol methacrylate with osmium tetroxide and uranyl acetate-based tissue-embedding system, with glutaraldehyde-based fixation. With this in mind, two review articles have been published in the pages of the American Journal of Physiology Lung Cellular and Molecular Physiology recently, highlighting the challenges presently faced by investigators interested in the quantitative determination of lung structure and solutions to these challenges. These articles take a problem-based approach to illustrate basic principles of stereology (267) and to highlight a stereological approach to the study of lung structure (248). The stereological and morphological analyses of lung tissue, including recent developments, have also recently been reviewed (246, 247, 361).

Using a stereological approach, Herring and coworkers (134) studied postnatal alveolar growth in humans aged 2 mo to 15 yr and demonstrated that alveolar growth continues well into adolescence. Furthermore, by comparing these data with historical data derived from rats and Rhesus macaques, the investigators confirmed that Rhesus macaques represent a better model for postnatal lung growth in humans than do rats. Furthermore, the studies revealed that the kinetics of postnatal lung growth and alveolarization in rats were more comparable to prenatal lung growth and alveolarization in humans. This idea that alveolarization persists into adolescence has been supported by limited radiological evidence that alveolarization in school-age survivors of extreme prematurity and term-born children is similar, suggesting that “catch-up” alveolarization took place in the preterm group (252), although the suitability of $^3$He diffusion MRI to reach this conclusion has been questioned (271). As such, the idea of catch-up alveolarization remains controversial. Together, these observations reinforce the utility of combining state-of-the-art lung structural analyses with alternative (radiological or physiological) supportive approaches.

Presently, the use of stereological approaches to quantify structures in the developing lung is used by relatively few groups. This methodology has been pioneered by Tschanz and coworkers (341) to identify a biphasic formation of new alveoli during postnatal rat lung development, in which a bulk formation of new alveoli was observed between postnatal day 4 (P4) and P21. This was followed by a second phase of continued alveolarization between P21 and P60. The stereology approach has also been applied to assess total alveoli number and mean septal wall thickness after pharmacological interventions in developing mouse pup lungs in a BPD model that relies on exposure to hyperoxia (described in detail in the next section) (207, 238) and to assess lung vessel wall thickness during normal lung development in transgenic mice (205). Presently, no stereological approach is available for the quantification of capillaries in the lung. The development of such a methodology is highly desirable, given the drawbacks of the currently used surrogates. These surrogates include number of platelet/endothelial cell adhesion molecule (PECAM), or von Willebrand factor (vWF), stained vessels per microscopic high-power field. In those approaches, differences in lung inflation, artifacts of fixation and embedding, and biased selection of fields may impact the robustness of the data. The stereological analysis of lung structure is both technically complicated (in terms of lung sample preparation) and time consuming, which has limited the enthusiasm of the lung community to embrace the methodology despite the clear advantages to acquiring robust, unbiased data. Looking to the future, the time constraints may be circumvented by automation of the tissue section analysis. Efforts in this direction are already underway by the Tremblay group (307); however, there is most likely a long and difficult road ahead before this goal is attained to the satisfaction of the stereology community.

Animal Models of Arrested Alveolarization

The study of lung development is entirely reliant on the use of animal models, and a broad range of animal models has been employed in the past (139). All the presently available BPD animal models have been recently reviewed in the American Journal of Physiology Lung Cellular and Molecular Physiology, including the use of mice (33), rats (264), rabbits (80), lambs (15), and baboons (377). Although it is generally acknowledged that the large animal models, notably preterm lambs and preterm baboons, probably represent the best mod-
els of human disease, there is considerable cost and ethical debate associated with the use of these models. For this reason, the mouse and rat models have found the most widespread use. The key advantages of the mouse and rat models are the relatively low costs, as well as access to transgenic strains, particularly for mice. Additionally, the small size of newborn rodent pups translates to lower costs (because of the lower quantity required) of pharmacological agents delivered. However, critical evaluation of the rodent models also reveals key concerns that are becoming more apparent. The mouse and rat BPD models are often promoted because mouse and rat pups are born in a stage of lung development comparable to the stages in which premature humans are born that go on to develop BPD. As such, these are seen as “relevant” models. However, it is also true that these term mouse and rat pups are born with lungs that are already fully competent for gas exchange, which is not the case for premature human infants. Furthermore, another significant drawback of the term mouse and rat models is the difficulty with prolonged mechanical ventilation, which can be undertaken for short periods (up to about 24 h) (33, 38) but not beyond. In this sense, the lamb and baboon models are far superior although these models are also complicated by high costs and ethical considerations. The recent demonstration that infant baboons infected with respiratory syncytial virus (RSV) develop clinical and pathological changes that parallel those of human infants (269) highlights the potential utility of the baboon model, given the relationship between RSV and BPD in a clinical setting, in which infants with BPD are particularly susceptible to RSV infection (57).

Another key concern, largely with the rodent models, is a lack of standardization. This is particularly evident with the hyperoxia models. In the many studies reported in this Perspectives article, 1) investigators employed a wide variety of mouse strains, including BALB/c, C57BL/6, FVB/N, C3H/HeN, as well as mixed backgrounds. However, there is notorious variability in the susceptibility of mouse strains to hyperoxia-induced lung injury (147, 365), making comparisons of studies performed with different strains very difficult. 2) This is exacerbated by the use of a broad range of oxygen concentrations in the hyperoxia-based models, in which term mouse pups are exposed to a range of hyperoxia levels, between 40% O2 and 100% O2 in the inspired air. 3) A further confounding factor impacting standardization is the duration and window of hyperoxia exposure, in which term mouse pups may be exposed to hyperoxia from the day of birth for up to 28 days. Alternatively, term mouse pups are exposed for a specific window (for example, P4 to P8) of hyperoxia, followed by variable durations of normoxia. This variability in the hyperoxia models extracted from the studies reported in this Perspectives article, published between 2013 and 2015, is made clear in Fig. 1. There is a pressing need for a systematic comparison of the effects of different oxygen levels (assessed over the same time range) on lung development. Additionally, it remains important to assess the critical time window in which lung development is exquisitely susceptible to oxygen injury, should such a window exist. It may well be that oxygen exposure over the first few hours of postnatal life (in mice) is sufficient to irreversibly perturb lung development, but this remains to be experimentally demonstrated. This idea is supported by the observations of Firsova and coworkers (102) that exposure of newborn mouse pups to 90% O2 over the first 4 days of life led to a persistent disturbance to alveolarization (assessed by MLI) that was evident 56 days after birth. It is clearly important to better understand how much oxygen is required, and when to provoke a particular structural abnormality in the lung and what impact this has on the magnitude of that disturbance (346). To this end, Wang and coworkers (355) have examined the impact of exposure to 40% O2 and 70% O2 over the first 7 days of life, followed by recovery in room air for 14 days, in which no differences were observed in alveolar structure or number (assessed by RAC) comparing the 40% O2 and 70% O2 groups. However, the alveolar structure was not assessed at the conclusion of hyperoxia exposure (P7).

Another issue of standardization relates to nomenclature, in which some investigators consider P1 as the day of birth, whereas others report P1 as the day after birth. Clearly, there is much room for improvement of the standardization and reporting of experimental protocols and nomenclature in rodent models of BPD.

Efforts are underway to further characterize and optimize the hyperoxia-exposure protocols in animal models of BPD. Rieger-Fackeldy and coworkers (300) demonstrated the impact of abrupt vs. gradual weaning of FVB/N mouse pups from hyperoxia (85% O2) to normoxia (21% O2). Interestingly, a gradual weaning (10% reduction in O2 levels per day, over 6 days) resulted in double the number of alveoli compared with age-matched controls subjected to abrupt return to room air. These data indicate that, not only the oxygen levels and the duration of exposure, but also the steepness of the oxygen gradient posthyperoxia exposure can influence alveolarization. These ideas have also received attention in the pioneering work of Michael O’Reilly’s group (52), which continues to explore the role of different oxygen levels on lung structural development, particularly in relation to influenza A virus infection.

There is also a need to further develop alternative animal models of BPD, not based (exclusively) on hyperoxia. The impact of cell stretch and lung distention is likely to be a key cause of damage to the developing lung (151), highlighting the need for increased use of ventilation-based models to study the impact of mechanical ventilation on the developing lung. Along these lines, Ratner and coworkers (294) demonstrated that an 8-h period of aggressive mechanical ventilation (tidal volume 15 μl/g) of 5-day-old mouse pups with room air caused pronounced mitochondrial dysfunction accompanied by blunted alveolarization (measured by RAC) that was evident 10 days later, which are important findings given the increasing appreciation for mitochondria being more than just a “powerhouse” (311). However, using the same protocol employing gentle mechanical ventilation (tidal volume 8 μl/g), no effect on alveolarization or mitochondrial dysfunction was noted. Similarly, Young and coworkers (379) demonstrated that lung elastase activity can be locally regulated by stretch during alveolar septation, which, together with the other studies reported here, highlights the usefulness of mechanical ventilation models of BPD.

The rabbit is often seen as a compromise between the advantages of small rodents (rats and mice) and larger vertebrates (sheep and nonhuman primates). The rabbit affords the possibility of preterm delivery of rabbit kittens, followed by hyperoxia exposure or other interventions. Additionally, the rabbit lung is anatomically closer to human lungs than are rat or mouse lungs. The development of a preterm [at embryonic...
day (E)28 vs. the E31 term date) rabbit model, followed by hyperoxia exposure (95% O2 for 7 days), has been carefully described by Richter and coworkers (299), highlighting the potential utility of this model for studies on arrested alveolarization. Further refinement of this model has also been undertaken (215), demonstrating that caesarean delivery of preterm rabbit kittens at E29 vs. E28 was accompanied by reduced mortality although the E29 group maintained a reduction in alveoli number and thus remained suitable for modelling structural changes to the lung associated with preterm birth. These efforts to further develop the rabbit model are also being facilitated by transcriptional studies in rabbit lungs from preterm rabbit kittens exposed to hyperoxia (306).

So long as rodents cannot be ventilated for prolonged periods, the preterm lamb model remains the method of choice. This model has recently been used to demonstrate the usefulness of high-frequency nasal ventilation to maintain adequate gas exchange and to promote alveolarization in preterm lambs (260), which would not have been possible with the rodent ventilation approach. To this end, the ventilated preterm lamb model continues to be developed (49). Collins and coworkers (73) have clarified the need for the optimization of this model, given the different transforming growth factor (TGF)-β responses of Merino ewes to intrauterine LPS vs. Ureaplasma parvum exposure. Ureaplasma is commonly isolated from pregnant mothers with chorioamnionitis and thus may pose an increased risk for preterm birth and the development of BPD in the neonate (114, 161). The preterm lamb model has also been used to study ventilation mechanics and most recently the utility of recruitment maneuvers before mechanical ventilation of preterm lambs to limit ventilator-induced lung injury (140). In that study, Hillman and coworkers (140) found that, irrespective of the functional residual capacity recruited, the recruitment maneuvers did not alter the acute phase and proinflammatory responses to mechanical ventilation at birth; however, alveolar development was not quantified in that study.

Antenatal inflammation can lead to pulmonary inflammation, with important consequences for pre- and postnatal lung development.
development (179), although chorioamnionitis as a risk factor for BPD remains controversial (180, 333). With antenatal inflammation in mind, several animal models of BPD have been developed to include an intratracheal inflammation component, which is translatable to the clinic, as described above for intratracheal Ureaplasma infection in sheep (73). Related intratracheal inflammation models have been reported in rats to study the utility of antenatal glucocorticoids to influence lung development in the background of antenatal inflammation (378). Studies have also examined NF-κB function in the endothelium (329). Further work in preterm lambs examined inflammatory signaling and glucocorticoid-growth factor signaling in fetal sheep lungs (73, 74). These are important and clinically relevant models that demand more widespread use.

To date, BPD has not been modeled in other large vertebrates apart from nonhuman primates and lambs; however, work has been initiated in piglets, with a recent report by Bartlett and coworkers (31) on the proteomic analysis of bronchoalveolar lavage fluid and epithelial lining fluid in newborn piglets. These authors suggest that the relative similarity of human and porcine lungs supports the further development of animal models in the porcine system. This idea has been reinforced by Caminita and coworkers (55), who have elegantly demonstrated that, between gestational days E98 and E102, preterm piglets exhibited ventilation inadequacies as well as risks for the development of RDS that mimic those of preterm infants born during the saccular phase of lung development. The investigators concluded that these piglets were compatible with modern neonatal intensive care management approaches and thus represented an alternative to nonhuman primates and lambs.

Although use of the primate models is steadily declining, largely attributable to cost and ethical issues, macaques have recently been used to evaluate the impact of ozone on postnatal lung maturation in nonhuman primates by Murphy and coworkers (250), who noted that susceptibility to ozone damage is age dependent and that prior ozone exposure modulates the response to subsequent ozone exposure. These data have clearly translatable information to how children living in polluted metropolitan areas during key stages of lung development may respond to ozone exposure in later life.

Returning to rodents, intrauterine growth restriction (IUGR) also impacts alveolarization, and thus IUGR is also occasionally employed as a BPD model. Zana-Taieb and coworkers (383) compared two IUGR models, namely protein deprivation and nitric oxide synthase (NOS) inhibition. The investigators concluded that only the protein-deprivation approach yielded a sustained impairment of alveolarization (assessed by MLI and RAC) in rat pups, whereas NOS inhibition blunted early lung development before alveolarization. The same investigators followed up with a transcriptional profiling study in the protein-deprivation IUGR model (384) and identified the peroxisome proliferator-activated receptor (PPAR) pathway as a likely key mediator of impaired alveolarization. Prenatal growth restriction also forms the basis of a new double-hit mouse model of BPD, where pregnant mothers are exposed between E14 and E18 to hypoxia (10% O2), after which offspring were exposed to 75% O2 for the first 14 days of life. Although not yet in widespread use, this model was used to demonstrate the therapeutic potential of umbilical cord mononuclear cells to promote proper lung alveolarization (assessed by stereology, using airspace volume density and surface density of septa as surrogates for alveolarization) (239), and, on the basis the expression of surfactant protein and other genes, this model is considered to closely mimic the clinical situation (120). This concept of fetal priming has also been observed in the rodent hyperoxia model using maternal diabetes as a first hit, in which streptozotocin-induced diabetes in rats before pregnancy appeared to modulate the effects of hyperoxia on septal wall thickness in pup lungs, although the precise implications of this are not clear (173). In terms of other new models, the observation that maternal deprivation (that is, separation of mother and pups) in rats also led to a pronounced blunting of lung development (assessed by MLI), ostensibly mediated by dipeptidyl peptidase IV, suggests that maternal deprivation may represent a new (stress-induced) rat model of BPD (150). This idea possibly intersects with the recent observation that maternal stress during pregnancy alters the predisposition of pups to allergy susceptibility, in which a role for glucocorticoids was implicated (193). This idea has yet to be tested with regard to lung parenchymal and vascular development. Additionally, Ayala de la Peña and coworkers (26) have suggested that the expression of a KrasG12D variant in the urothelium may represent a new BPD model, an idea based on lung histopathology assessed at P1. The utility of this transgenic mouse strain as a BPD model awaits further evaluation, and further genetic models will be discussed later in this Perspectives article.

As an alternative to animal models, several recent reports have detailed the use of ex vivo lung culture (277), epithelial-mesenchymal cocultures (122), in vitro-generated human pluripotent stem cell-derived lung organoids (90), 3D multicell microtissue culture models of airway smooth muscle (363), and a biomaterial-templated alveoli culture system (189) for the study of alveolarization and alveolar biology. Although not yet in widespread use, these systems potentially provide useful alternatives to in vivo studies, in which high-throughput screening of pharmacological or genetic interventions could be conducted to identify pathways relevant to alveolarization and to limit the use of experimental animals. Among these approaches is the coculture of fetal (but not adult) mesenchymal cells with A549 cells (a human lung epithelial cell line), in which three-dimensional peaks formed that had a similar cellular orientation to alveolar structures in vivo (122). Additionally, there is evidence that secondary septation continues in ex vivo lung slices maintained in culture (277). This provides an alternative model for the in vitro analysis of signaling pathways and pharmacological or genetic interventions to study secondary septation. Clearly, this model lacks input from physiological blood flow and cyclic stretch from breathing motions, suggesting areas where this potentially interesting in vitro approach could be further refined. Along these lines, the reproducible uniform equibiaxial stretch of precision-cut lung slices has recently been demonstrated (82).

Last, concerning the analysis of gene expression in tissues harvested from animal models, several investigators have systematically addressed the selection of reference genes in real-time PCR analysis. Mehta and coworkers (234) assessed the utility of Tubal, Actb, Gapdh, Rn18S, and Hist4h4 and identified Tubal as the least variable in terms of expression levels over the course of mouse lung development (E11.5 to P28). Unfortunately, other popularly used references genes,
including \textit{Hmbs} and \textit{Poir2a}, were not evaluated. In a related study, Bouhaddioui and coworkers (43) screened reference genes for the normalization of microRNA gene expression and concluded from the panel of \textit{Sno135}, \textit{Sno142}, \textit{Sno202}, \textit{Sno234}, and \textit{Sno251} that the expression of \textit{Sno234} was the most stable over the course of mouse lung development studied (E15.5 to P30) although other popularly used reference genes, such as the U6 small nuclear 1 RNA (\textit{Rnu6-1}), were not assessed.

\textbf{Imaging Lung Structure and Function}

The imaging of the lung in real time is an emerging area of interest and technological development, leading to a special call by the \textit{American Journal of Physiology Lung Cellular and Molecular Physiology} for papers, “Real-time visualization of lung function: from micro to macro” (244). In response to this call, a review article that broadly described the imaging of the lung using a spectrum of tomographic and X-ray approaches was recently published (116). Recent advances in lung imaging include the development of noninvasive MRI approaches to quantify lung volume changes in mice and the application of this approach to monitor interventional studies, in this case, the impact of the somatostatin analog pasireotide (SOM230) on the progress of bleomycin-induced lung injury (91). This methodological approach has not yet been extended to the study of postnatal lung development in mice. Very recently, a study has emerged (354) that demonstrated that pulmonary MRI can reveal quantifiable and significant differences between patients with BPD, premature patients without BPD, and full-term controls. These new data suggest that pulmonary MRI might be implemented to individually phenotype disease, which would be most useful for clinical care and to predict outcome.

Other types of imaging are also emerging for the study of lung development and BPD. For example, optical cryoimaging has been employed to address the mitochondrial redox state of mouse lungs during lung development in Bcl-2-deficient mice, in which the authors determined that Bcl-2 deficiency was associated with oxidative stress that correlated with perturbed alveolarization (221). Thus these authors have added fluorescent imaging to our lung development methodology toolbox. Additional recent reports reveal the exciting potential of contrast-enhanced micro-CT in rodents (77) and automated intravital microscopy to study vascular function (129); however, these approaches have not yet been applied to developing lungs. Other tried and tested imaging approaches continue to support studies on lung vascular development, for example, barium angiography, which allows the extraordinary visualization of vascular pruning in the rat hyperoxia model of BPD (342).

Although not imaging per se, the three-dimensional reconstruction of serial sections of lungs from patients with BPD has been reported by Galambos and coworkers (111) and facilitated the identification of intrapulmonary anastomoses in affected lungs. These investigators employed the same methodology to describe curious pathological perturbations to lung vascular structure in patients with alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) (112, 113) and CDH (2). This approach has not yet been employed in animal models of arrested lung development.

\textbf{New Mediators and Modulators of Impaired Alveolarization}

The past 2.5 yr have seen the addition of several new mediators of alveolarization that have been identified in animal models of BPD. Among these is soluble guanylate cyclase (sGC), in which lung development was particularly impaired after hyperoxic injury in mouse pups carrying a targeted deletion of the gene encoding the \(\alpha_1\) subunit of sGC (27). These data support the idea of activating sGC as a management strategy in hyperoxia-induced lung injury. Along similar lines, McKenna and coworkers (231) demonstrated that sustained NF-\(\kappa B\) activation, through overexpression of the NF-\(\kappa B\) inhibitor-\(\beta\) (Ik-B\(\beta\)), protected mice against the deleterious effects of hyperoxia (95% \(O_2\)) on alveolarization (assessed by ML and RAC), suggesting that the potentiation of NF-\(\kappa B\) activity may also represent a management strategy in hyperoxia-induced lung injury. Although not yet studied in a BPD model, overexpression of the receptor for glycation end products (RAGE) blunted alveolarization (assessed by ML) in mouse pups, adding RAGE to the catalog of negative regulators of alveolarization (101). Although the receptor for RAGE is expressed in alveolar type I cells (304), other studies have indicated that RAGE expression in the lung is suppressed by exposure of rat pups to hyperoxia (198); as such, it remains unclear whether RAGE plays a functional role in limiting alveolarization in experimental BPD. Further studies on RAGE in lung development are clearly indicated, particularly in light of the recent report that RAGE also acts as a mediator of endothelial activation (214).

Isoforms of hypoxia-inducible factor (HIF) continue to receive attention as mediators of lung development, including HIF-1\(\alpha\) (335), HIF-2\(\alpha\) (270), and HIF-3\(\alpha\) (146). The expression of HIF-1 is known to be downregulated in patients with BPD, leading Tibboel and coworkers (335) to overexpress a constitutively active HIF-1\(\alpha\) subunit (HIF-1\(\alpha\Delta ODD)) in the distal lung epithelium. This increased lung vessel density upregulated the expression of proangiogenic factors and drove increased alveolarization in mice maintained in room air. However, contrary to expectations, when these HIF-1\(\alpha\Delta ODD\)-expressing C57BL/6 mice were exposed to hyperoxia (80% \(O_2\) for 28 days), lung function worsened, and the surfactant and lung architecture abnormalities (assessed by ML and RAC) seen in hyperoxia-exposed wild-type mice persisted. Opposite results were obtained by Vadivel and coworkers (342), who delivered wild-type HIF-1\(\alpha\) via adenovirus in the rat hyperoxia model (95% \(O_2\) for 14 days) and, in doing so, conferred striking protection against the deleterious impact of hyperoxia on alveolarization (assessed by ML), which was comparable to that of room air-exposed rat pups. The reasons for the discordant observations reported by Tibboel and coworkers (335) vs. Vadivel and coworkers (342) might be due to the epithelial-restricted overexpression of constitutively active HIF-1\(\alpha\) in the Tibboel study. This idea is supported by the dramatically improved vascularization seen in the Vadivel study, which might suggest that the HIF-1\(\alpha\) must be delivered to the endothelium to confer protection and promote lung maturation in the background of hyperoxia.

MicroRNA are also emerging as candidate mediators of fibroblast (41, 148) and pulmonary artery smooth muscle cell behavior (157), alveolarization, and BPD (165); however, to date, this idea is supported only by the observed changes in...
expression of some microRNA species that correlate with arrested alveolarization. These candidate microRNA species have recently been reviewed (165, 249, 375), and integration of microRNA pathways into disease processes by modeling (310) and systems biology approaches (36, 127, 235) continues to be developed; however, the field awaits the first demonstration of a causal role for any microRNA species in arrested late lung development. Narasaraju and coworkers (251) have suggested a role for miR-150 in blunted alveolarization associated with hyperoxia, using a miR-150−/− mouse strain, which, after hyperoxia exposure (95% O2 from P1 to P10), exhibited transient rescue of alveolarization (assessed by MLI) seen at P6, but this rescue did not persist to P10. Capillary density was apparently improved in this model; however, the strain background of the miR-150−/− mouse strain was not declared, and, as such, the appropriateness of the C57BL/6J background as a control in these studies cannot be assessed. Some studies in clinical BPD material have indicated that the expression of miR-206 is downregulated in the lungs of patients with BPD (388); however, miR-206 has yet to be causally implicated in arrested alveolarization. A role in alveolarization for long noncoding RNA, which are involved in early lung development (132), is yet to be addressed.

The mechanisms leading to cell death in the lungs in patients with BPD and experimental (hyperoxia-based) animal models of BPD have also been addressed. One notable recent finding is that exposure to hyperoxia leads to elevated interferon-γ levels, which in turn drive endoplasmic reticulum stress-dependent cell death in a manner that relies on cyclooxygenase-2 (COX-2) (72). This represents a novel cell-death pathway that is relevant to clinical and experimental BPD. These findings support the exploration of COX-2- and DNA-damage-inducible transcript 3 (Ddit3; also called Chop)-based approaches in the management of BPD, and, indeed, efforts along these lines are already underway, with the report of Masood and coworkers (220), who employed a highly selective COX-2 inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone in the rat hyperoxia model (60% O2 from P1 to P14). In that study, COX-2 inhibition blunted inflammatory infiltration provoked by hyperoxia and improved lung vascularity and alveolarization (assessed by MLI). Conflicting data have been obtained in an alternative study by Britt and coworkers (50), who could also demonstrate the ability of both aspirin and the COX-2-specific inhibitor celecoxib to limit pulmonary inflammation provoked by hyperoxia (85% O2 from P1 to P14) in C3H/HeN mice; however, pharmacological inhibition of COX-2 did not improve lung structure (assessed by number of alveoli per high-power field) in the background of hyperoxia. These data were supported using Cox2−/− and Cox2+/− C57BL/6 mice, which were also protected against pulmonary inflammation provoked by hyperoxia; however, genetic ablation of COX-2 expression did not improve lung structure in the background of hyperoxia. The reasons for these divergent observations are not clear; however, it is apparent that no impact of COX-2 inhibition (or gene ablation) was seen at the higher oxygen level, highlighting a possible influence of the model (different O2 levels) on the outcome. Additionally, one study was performed in rats, whereas the other was performed in mice. As such, although the data clearly implicate COX-2 as a candidate druggable target to limit lung inflammation in the background of hyperoxia, it remains unclear whether an impact on alveolarization accompanies this effect.

Sex differences are emerging as possible confounders in studies on clinical and experimental BPD (121), as recently discussed in the context of pulmonary hypertension (182), and it is important that the sex of experimental animals is considered in the design of experimental studies and interpretation of data. There is a growing body of evidence that both androgens and steroidogenic enzymes may influence postnatal lung development (339). The importance of considering sex bias in studies in animal models of BPD is underscored by the observations that adult (C57BL/6J) mice exhibit sex-specific dimorphic effects on inflammation and antioxidant enzyme expression in response to hyperoxia (196), with male animals exhibiting greater lung injury, neutrophilic inflammation, and apoptosis, all of which are also characteristic of clinical BPD. In support of this idea, Martin and coworkers (219) reported a pronounced impact of hyperoxia on estradiol metabolism in postnatal airway smooth muscle cells. Additionally, the effects of glucocorticoids on alveolar type II cell function in vitro were sex dependent (167). To date, no study has addressed sex differences in animal models of BPD, and this represents a priority area for research for future work in the field, particularly given the differences in the kinetics of structural changes in the ageing lung for male vs. female Rhesus macaques (133).

Gasotransmitters including NO, carbon monoxide (CO), and hydrogen sulfide (H2S) are also emerging as mediators of late lung development and, possibly, arrested lung development associated with BPD. NO has a long history of use in premature infants with or at risk for BPD (168, 340). One recent report has indicated that treatment of newborn rats with high-dose (20 ppm) inhaled NO improved lung growth, most likely through increased expression of hemeoxygenase-1 and VEGF/VEGF receptor 2 (VEGFR2), as well as matrix metalloproteinases (MMPs). Interestingly, increased gene expression was seen over the course of NO administration but was lost after discontinuation of NO (89). The immediate implication of this study is not clear because NO administration was made in room air to healthy, properly developing rat lungs. Interestingly, 20 ppm NO inhalation yielded an ~20% increase in RAC, suggesting that healthy lungs are indeed capable of hyperalveolarization. In a different line, continued work on the inhibition of phosphodiesterase (PDE)5 by Park and coworkers (270) demonstrated that sildenafil modulation of the NO/cGMP pathway promoted alveolarization (assessed by Lm) in the background of hyperoxia. These data highlight PDE5 as a candidate druggable target to drive alveolarization, and this has stimulated additional studies on the interaction between different cGMP- or cAMP-dependent agonists in the pulmonary vasculature in newborn rats (96). Interestingly, working in adult mice, Czövek and coworkers (79) have demonstrated the utility of VIP to stimulate the NO pathway and preserve lung function and structure in the background of hyperoxia in adult rats. The modulation of NO signaling withVIP in neonates awaits investigation. Future work on inhaled NO approaches in hyperoxia-based animal models will have to take into account the impact of hyperoxia on S-nitrosothiol accumulation and L-type amino acid transporter 1 expression and function, given the recent important report from Richard Auten’s group that the oxidative stress generated by hyperoxia blocks a major route of inhaled NO action (45, 46).

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00268.2015 • www.ajplung.org
The alternative gasotransmitter CO has also received attention by Anyanwu and coworkers (23), who reported the protective effects of CO administration concomitant with hyperoxia (75% O₂, from P3, for up to 21 days) exposure in C57BL/6j mice on alveolar development. These studies were supported with hemeoxygenase-1 knockout mice, which were demonstrably more susceptible to the injurious effects of hyperoxia. This phenomenon has also been reported by Yang and coworkers (374), who suggested that the β-catenin/heterogeneous nuclear ribonucleoprotein K axis mediated the protective effects of hemeoxygenase-1 during hyperoxia exposure. The protective effects of CO were attributed to the modulation of a broad spectrum of inflammatory cytokines, in which the expression of proinflammatory cytokines was suppressed, and the expression of anti-inflammatory cytokines (IL-10) was increased by CO treatment. These studies highlight the CO/hemeoxygenase-1 axis as a potentially exciting lung-protective axis and also, together with work by Luo and coworkers (201), emphasize a role for regulators of the Wnt/β-catenin system in regulating lung development. Indeed, given the recent reports of the β-catenin system regulating epithelial repair (185, 385) and smooth muscle responses to hypoxia (381), β-catenin is emerging as an exciting mediator of the postnatal lung response to injury, which might impact lung maturation.

The third gasotransmitter H₂S has also received attention. In pioneering work by Vadivel and coworkers (343), exogenous administration of H₂S to rats in the hyperoxia model (95% O₂ from P1 to P14) improved lung alveolarization (assessed by MLI), limited hyperoxia-provoked increases in pulmonary arteriole medial wall thickness, restored the pulmonary arterial acceleration time/right ventricular ejection time ratio, and partially restored the Fulton index. These data support a protective role for H₂S administration, primarily related to restoration of vascular remodeling and pulmonary hemodynamics in the hyperoxia model. In a subsequent study by Madurga and coworkers (207), daily administration of the H₂S donor GYY4137 in the mouse hyperoxia model (85% O₂ from P1 to P14) protected C57BL/6 mice against the damaging impact of hyperoxia (85% O₂ from P1 to P10) on alveolarization (assessed by stereology). This appeared to be correlated with blunted inflammatory responses provoked by hyperoxia, perhaps mediated by increased IL-10 levels. Taken together, these reports underscore H₂S as a mediator of lung development that warrants further study. This idea was recently reinforced by another report by Madurga and coworkers (205), who demonstrated that two of the three endogenous H₂S-generating enzyme systems, cystathionine β-synthase (Cbs) and cystathionine γ-lyase (Cth), are mediators of normal lung alveolarization and vascular development. These studies are not conclusive, however, because, although the necessity of Cbs and Cth for alveolarization was documented using knockout mice, it remains unclear whether the defective alveolarization and vascular development seen in knockout mouse pups was due to a lack of H₂S production or a toxic buildup of enzyme substrates. Furthermore, the contribution of the third H₂S-generating enzyme, mercaptopyruvate sulfurtransferase, awaits study.

Singh and coworkers (316) have recently added cigarette smoke to the list of potential inducers of arrested alveolarization. Interestingly, gestational (maternal) but not postnatal exposure to cigarette smoke blunted alveolar development in both BALB/c and C57BL/6 mice. This could be attenuated by administration of mecamylamine, a nicotinic acetylcholine receptor antagonist, to pregnant mothers during gestation, which concomitantly with cigarette smoke exposure attenuated the impact of cigarette smoke on alveolarization (assessed by MLI) in mouse pups. Some components of cigarette smoke have also been studied in isolation; Thankur and coworkers (331) administered benzo[a]pyrene to pregnant rats at E17, E18, and E19. After delivery, newborn pups were exposed to hyperoxia (85% O₂ from P1 to P14). Maternal benzo[a]pyrene exposure appeared to impact alveolarization; however, lung structure was not quantified in that study. Additionally, the translatability of the very high benzo[a]pyrene dose (25 mg/kg per day, for 3 days) is unclear, as the comparability of this dose to benzo[a]pyrene levels in smoking mothers is not reported. Still much remains to be investigated about how exactly cigarette smoke impacts lung development; however, some lead studies, such as the observation that cigarette smoke drives hyperplasia and extracellular matrix (ECM) production by fetal airway smooth muscle cells (347), are laying important groundwork in this area. Studies have not been confined to tobacco-containing cigarettes, with a recent report (229) that exposure of newborn C57BL/6j mouse pups to E-cigarette vapor (containing nicotine in propylene glycol vehicle) once per day on days 2 and 3 of life, followed by twice daily on days 4–10 of life, led to an attenuation of alveolarization (assessed by MLI), compared with mice exposed to propylene glycol vehicle alone. The alveolarization defect was accompanied by detectable systemic levels of cotinine. These data add to the argument that exposure to both tobacco and E-cigarettes is an important consideration in postnatal lung maturation and assume further importance in light of recent reports that perinatal nicotine can induce transgenerational asthma (297) and that nicotine drives epithelial-mesenchymal transition through Wnt/β-catenin signaling in human airway epithelial cells (390). Apart from cigarette smoke, nanoparticles are another environmental factor that can impact lung development. Nanoparticles are present in cosmetics, paint, sunscreens, and food (287). Using titanium oxide nanoparticles (average diameter 8–10 nm), Ambalavanan and coworkers (22) reported that exposure of mouse pups at P4 to single or multiple doses of nanoparticles provoked an increase in the expression of proinflammatory mediators and blunted lung development (assessed at P14; by MLI and RAC). In a related study, Chan and coworkers (60) described the muted expression of Cyp1A1, a cytochrome P-450 superfamily member, in neonatal, but not adult rats, after exposure to ultrafine particular matter, which is a key component of vehicle exhaust. These data underscore the need for further investigation into the public health impact of exposure of children to nanoparticles and particulate matter during periods of postnatal lung growth.

A genome-wide transcriptional analysis approach has been used by Bhattacharya and coworkers (37) in the mouse hyperoxia model to identify the aryl hydrocarbon receptor (Ahr) as a candidate key regulator of responses to hyperoxia in C57BL/6 mouse pups. This observation is interesting because Shang and coworkers (387) have reported that Ahr is necessary to protect fetal human pulmonary microvascular endothelial cells against hyperoxic injury. Genome-wide association studies (GWAS) have been used by Nichols and coworkers (254) to evaluate the role played by genetic background in the
response of mouse pups from 36 inbred mouse strains to hyperoxia (95% O\textsubscript{2} from P1 to P3). These investigators identified the muscarinic acetylcholine receptor M2 gene (Chrm2) as a susceptibility gene, and mouse strains carrying a Chrm2 single-nucleotide polymorphism (SNP; causing a P265L substitution in Chrm2) exhibited reduced hyperoxia-induced inflammation. Importantly, that study employed hyperoxia-induced lung inflammation as an endpoint readout, and similar GWAS studies using perturbations to lung architecture have not yet been performed. GWAS have been less successful in patients with BPD, in whom, in one recent report, no new SNPs were identified that could be associated with BPD; furthermore, this study could not validate any previously identified SNPs (356). In another GWAS involving material from patients with BPD, no new SNPs were identified, but Ambalavanan and coworkers (20) highlighted the miR-219 pathway [which includes platelet-derived growth factor (PDGF)-α] and the IGF family pathway as areas that warrant further study in BPD. Studies on DNA methylation patterns are also emerging (78), which have implicated epigenetic regulation of growth factor, ECM, and antioxidant enzyme expression during normal lung development and epigenetic (dys)regulation of detoxifying enzymes and growth factors in clinical BPD (126).

Interestingly, whole-genome expression studies in plasma samples in patients with BPD revealed alteration of the expression of nearly 10% of the genome in affected patients (279).

Recent studies have also shed new light on classical mediators and pathways that remain controversial players in lung development and candidate druggable targets and pathways in BPD. Lykkedegn and coworkers (203) thoughtfully appraised the impact of vitamin D on lung maturation in a systematic review and highlighted the discord between observations made in animal models, in which vitamin D dramatically stimulated lung maturation, whereas clinical data currently do not support a role for vitamin D in the prevention or treatment of infant RDS or BPD. This report highlights a need for further studies on vitamin D deficiency or insufficiency as a risk factor for RDS and BPD and supported further investigations into the optimal dosing (in terms of dose and timeframe) of vitamin D and its analogs for the management of infant RDS and BPD.

To this end, Mandell and coworkers (213) reported that antenatal vitamin D administration improved survival and lung structure (assessed by RAC) after intra-amniotic endotoxin exposure in rats [which is also known to modulate airway reactivity in mice following hyperoxia exposure (71)], suggesting a potential role for vitamin D in the prevention of arrested alveolarization in this rat model. Along these lines, Yurt and coworkers (382) demonstrated a biphasic response to cholecalciferol nutritional supplementation, in which both alveolar development (assessed by RAC and MLI) as well as PPAR-γ and CCAAT/enhancer binding protein-α expression in the lung peaked at a supplementation of 500 U/kg cholecalciferol, which were all reduced at both 250 U/kg and 1,000 U/kg. These data highlight the concerns about dose in vitamin D supplementation studies. In the context of the impact of nutrition on lung development, Mayor and coworkers (222) identified maternal high-fat diet as a risk factor for alveolar simplification (assessed by MLI), possibly related to placental inflammation. These studies provide a firm basis for further work on the role of maternal nutrition on postnatal lung maturation.

In addition to these newly identified mediators of alveolarization, several recent studies have identified changes in the expression of further candidate mediators that accompany disturbances to lung alveolarization, which have yet to be validated in interventional or transgenic mouse studies. These candidate mediators include neuronal Per Arnt Sim domain protein 3 (274) and SOD-3 (283). SOD-3 is an antioxidant that is curiously targeted to the ECM and epithelial lining fluid of the lung and is not regulated by nuclear factor, erythroid-derived 2-like 2 (Nfe2LI2, also called Nrf2) (283) and adds SOD-3 and thioredoxin-1 (66) to the list of antioxidants that might play a role in perturbed lung alveolarization in response to hyperoxia (103, 337). This idea is all the more interesting given the recent report by Gupta and coworkers (124) that loss of SOD-2, studied in Sod2\textsuperscript{-/-} C57BL/6 mice, did not impact arrested alveolarization (assessed by changes in alveolar area), changes in Fulton index, or pulmonary arteriole medial wall thickness that were caused by hyperoxia (75% O\textsubscript{2}, from P1 to P14) exposure, leading these authors to conclude that other antioxidant systems may compensate the loss of SOD-2 or that a single sod allele generated sufficient SOD to elicit a wild-type response.

**Epithelial Cells and Epithelial Cell Transdifferentiation**

Epithelial growth and differentiation and epithelial interactions with the ECM are key events in alveolarization. Using a lung epithelium-specific [surfactant-associated protein C (Sfpce)-driven] deletion of integrin-β1, Plosa and coworkers (281) demonstrated a pivotal role for integrin-β1 in branching morphogenesis and alveolarization. The alveolarization defect was accompanied by abnormal epithelial differentiation, as well as blunted secondary septation (assessed by MLI), excessive ECM production, hypercellularity of the mesenchyme, and persistent alveolar inflammation. Using a clonodronate approach, the investigators further demonstrated that disturbed alveolarization after loss of integrin-β1 in the epithelium was secondary to persistent inflammation. These investigators proposed that loss of integrin-β1 in the epithelium increased epithelial production of chemokine (C-C motif) ligand 2 (Ccl2), chemokine (C-X-C motif) ligand 1 (Cxc1), and reactive oxygen species (ROS), which drove increased recruitment of macrophages, blunting alveolar development. These observations add to a growing body of data, discussed in detail below, implicating inflammation as a key mediator of both normal and aberrant lung alveolarization. Lafemina and coworkers (181) addressed a role for a related cell-communication and adhesion molecule, claudin 18, in postnatal lung growth. Claudin 18 is reported to be a lung-specific tight junction protein that is abundant on alveolar type I cells. Genetic ablation of claudin 18 expression had no impact on newborn mouse lung structure but did stunt postnatal lung maturation (assessed by MLI). The precise role played by claudin 18 in postnatal lung growth is not yet clear; however, loss of Cldn18 did upregulate the expression of amphiregulin, sonic hedgehog (Shh), and elastin and downregulated the expression of fibroblast growth factor (FGF) receptor (FGFR4), adrenomedullin, and VEGF-A, hinting at a possible mechanism. Along these lines, p66\textsuperscript{SHC} (188), IL-1β (142), and homeobox proteins Hox3a and Hox5a (42) have also recently been added to the list of new mediators of lung epithelial cell differentiation that impact the structural
development of the lung although the underlying mechanisms remain to be fully elucidated. Additionally, lung epithelial TNF-α-converting enzyme (TACE) was implicated by Xu and coworkers (373) as a regulator of lung saccul formation, in which genetic ablation of epithelial TACE retarded fetal lung development but not postnatal alveolarization. As yet, the molecular mechanisms at play in this process have not been defined.

The dynamics of type II to type I cell differentiation are currently a hot topic in lung developmental biology, and Hou and coworkers (143) have provided data to suggest that hyperoxia drives type II cell transdifferentiation in neonatal rat pups although the relevance to disease and the molecular mechanisms at play have not been clarified. Zhao and coworkers (389) made the striking observation that the balance between TGF-β and bone morphogenetic protein signaling directs the transdifferentiation of type II cells into type I cells, perhaps hinting at a mechanism to explain the observations of Hou and coworkers (143). Further to this idea, Ghosh and coworkers (117) identified antagonism between Wnt3a and Wnt5a as a mediator of alveolar epithelial cell phenotype and proposed that IGF-1 stimulated type II to type I cell transdifferentiation through activation of Wnt5a. This process also drove cell wound repair in vitro. These ideas are integrated into a scheme in Fig. 3, are emphasized by the demonstration by Keith Tanswell’s group that the IGF/IGF receptor (IGFR)1 system is a key regulator of alveolarization (192), and tie into earlier work by Tom Mariani’s group (318), in which epithelial/mesenchymal cross talk and IGF-1 contributed to FGFR-dependent alveolar elastogenesis and proper airspace formation. The ability to drive repair is likely to be relevant to the lung response to ventilation because ventilation and cyclic stretch have recently been described by Kroon and coworkers (176) to drive alveolar type II cell (but not fibroblast) death by the extrinsic apoptotic pathway. These and other data reinforce the idea that promoting epithelial repair is a candidate strategy to manage aberrant alveolarization following hyperoxia and ventilator injury to the lung. Consistent with this idea, Kim and coworkers (166) identified tripartite motif-containing 72 (Trim72) as a mediator of alveolar repair in ventilated adult mouse lungs, in which overexpression of Trim72 protected against, and genetic ablation of Trim72 increased, susceptibility to ventilator-induced lung injury. The molecular mechanisms of the protection conferred by Trim72 are not known; however, these observations made in adult mice highlight Trim72 as a molecule that warrants investigation in neonatal models of hyperoxic and ventilator-induced lung injury.

Several investigators have recently built on the key finding of Barkauskas and coworkers (30), who demonstrated that type II cells are stem cells in the adult lung. Among key developments in this fast-moving field are observations recently made by Ochieng and coworkers (162), who documented that induction of sex determining region Y-box 2 (Sox2) in type II cells in vivo could “deprogram” type II cells, which reverted to a less differentiated cell type that expressed the stem cell markers lymphocyte antigen 6 complex, locus A (also called Sca1), and fucosyltransferase 4 (also called Ssea1), and coexpressed SftpC and secretoglobin, family 1A, member 1 (Scgb1a1; also called club (formerly Clara) cell protein 10), which are characteristics of bronchoalveolar stem cells. The ability of Sox2 to regulate transformation-related protein 6 and GATA-binding protein 6 accounted, at least in part, for the effects of Sox2 on stem-cell phenotype (266). This finding most likely has relevance for the regeneration lung tissue, perhaps by modulating Sox2 levels. Along the lines of this thinking, Jain and coworkers (155) demonstrated a surprising plasticity of type I cells, which up until now have been considered terminally differentiated, as well as a bidirectional relationship between differentiated distal alveolar epithelial cells in a process regulated by TGF-β. With these ideas in mind, Desai and colleagues (66) have challenged the view that type I cells arise from type II cells, providing data to suggest that type I and type II cells arise directly from a bipotent progenitor in the prenatal lung but can arise from type II cells in the postnatal lung, a stem cell function that is activated by type I cell injury. The next few years will no doubt see much progress in our understanding of the interrelated plasticity of type I and type II cells, and this work has received further clinical impetus by the recent report that alveolar stem cell failure may be related to telomere dysfunction (17).

Work in primary type I and type II cells continues to be hampered by the lack of 1) availability of type I cells for in vitro studies and 2) efficient delivery of genes to alveolar type II cells, other than by viral-mediated transduction. Some progress has been made on both fronts over the past two years. Kim and coworkers (166) have reported the enrichment of alveolar type I cell preparations for in vitro studies by panning with commercially available anti-rat podoplanin (T10) IgG. Grzesik and coworkers (123) have also recently reported the ability to deliver genes on plasmids to primary alveolar epithelial cells by nucleofection technology, and Ramachandran and coworkers (291) have described the efficient delivery of RNA interference oligonucleotides to polarized airway epithelia in vitro. Although none of these methodologies has yet found widespread use, these important advances will no doubt facilitate further studies with alveolar type I and alveolar type II cells in the future. Work is also currently ongoing to develop unbiased protein screening approaches to detect quantitative changes in protein expression in type II cells in response to hyperoxia using isobaric labeling (isobaric tags for relative and absolute quantitation) with tandem mass spectrometry (35). To date, this approach remains at the proof-of-principle stage.

Fibroblasts and Fibroblast Differentiation

McGowan and McCoy (227) have examined the molecular pathways that direct fibroblast migration during alveologenesis. These investigators reported data that suggest that Shh, which is also known to play a role in lung fibrosis (209), directs the uniform orientation of lung fibroblasts and organizes the migration of lung fibroblasts toward the distal secondary septum. These investigators also made the observation that a larger proportion of PDGF receptor (PDGFR)α-expressing lung fibroblasts bore more primary cilia than other cells present in the alveolar did, and this may impart increased migratory capacity to these cells and all these cells to respond to PDGF-A chemotactic gradients. Lu and coworkers (200) further explored upstream mediators of Shh signaling and, using $\text{Eya1}^{-/-}$ and $\text{Six1}^{-/-}$ knockout mice and $\text{Eya1}^{-/-}/\text{Six1}^{-/-}$ double-knockout mice, identified that the transcription factors Eya1 and Six1 act together to regulate saccular development by modulating Shh levels.
The characterization of mesenchymal progenitors in the embryonic lung as well as the postnatal lung continues to be an area of intensive efforts. Recent advances include the demonstration by El Agha and coworkers (92, 93) that Fgf10-expressing cells represent a pool of mesenchymal progenitors in both the embryonic and the postnatal lung, possibly suggesting that Fgf10-positive cells might be useful for the development of stem cell-based therapies for parenchymal lung diseases (65). Furthermore, using tracing based on the expression of the Wilm’s tumor suppressor gene, a reliable marker for cells derived from the mesothelium, Cano and coworkers (56) described the extraordinary heterogeneity of mesenchymal-derived cells during lung morphogenesis. These studies continue to set the stage for our understanding of the original of “the cellular players” in late lung development.

Fibroblast differentiation and the existence and function of fibroblast subsets are an exciting area of intensive work in the lung development field. Lipofibroblasts in particular have received much attention. This fibroblast subset is characterized by the presence of lipid bodies although the presence of lipofibroblasts in the human lung remains controversial. Using light and electron microscopy, Tahedl and colleagues (327) reported that, although lipofibroblasts could be detected in adult rodent lungs, lipofibroblasts were not noted in any other mammalian lungs examined, including human, dog, goat, and horse lungs, among others. Furthermore, lipofibroblasts were not seen in postnatal human lungs although this cell type was abundant during postnatal mouse lung development. Ahlbrecht and McGowan (7) subsequently pointed out that these data conflicted with reports of lipofibroblasts in human lungs (298) and suggested that alternative methodologies to identify lipofibroblasts in autopsy tissue may play a role in whether lipofibroblasts are detected or not. Clearly, additional work remains to be done to clarify the existence of lipofibroblasts at particular time points in the development of the lung across several species and also to clarify a functional role for lipofibroblasts in alveolarization.

The presence or role of the lipofibroblast and a role for FGF and PDGF signaling in arrested lung development associated with BPD (228) and CDH (104–108) have also received recent attention, with one recent report highlighting the temporal commitment of the PDGFR-α+ cell lineage to lipofibroblasts and myofibroblasts during late lung development (259). Continuing in this line, McGowan and McCoy (228) also suggested that a transition from lipofibroblasts to myofibroblasts in BPD drives thickened alveolar septa attributable to collagen deposition. These investigators further suggested that PDGFR-α-driven Sox9 expression maintained the lipofibroblast state, and that Sox9 expression was lost with the acquisition of the myofibroblast state. As such, targeting PDGFR-α downstream signaling pathways (involving Sox9) may represent a strategy to drive fibroblasts from the myofibroblast back to the fibroblast phenotype, which would limit profibrotic activity and possibly drive lung (re)generation. These ideas are presented schematically in Fig. 2. These data have a clinical implication as well, given the report of Popova and colleagues (284), who documented that mesenchymal stem cells from patients with BPD hold stable alterations to PDGFR-α gene expression that drive hypovaleolarization.

Several new mediators of fibroblast motility, proliferation, and ECM production have recently been identified, including the tumor-associated calcium signal transducer 2 (224, 225) and cysteine-rich secretory protein LCCL domain containing 2 (also Lgll1) (386). How these mediators function to impact lung development awaits clarification. Other elements of fibroblast behavior have also received attention; for example, MMP-9 was identified as a mediator of fibroblast contractility in a TGF-β-dependent mechanism (171). The lung mesenchyme is regarded as a regulator of lung development (223), and coming work in this area will no doubt address roles for fibroblast subsets in directing alveolarization, not only through the production of the lung ECM “skeleton”, but also through cross talk between the mesenchyme, in particular, as a regulator of epithelial cell behavior (152, 348). Looking ahead, Li and coworkers (190) have recently reported that progenitors of secondary crest myofibroblasts are developmentally committed in the early lung mesoderm and highlighted the utility of the Gli1-CreERT2 line for the study of formation of the alveoli.

**ECM Production and Remodeling**

Alveolarization occurs through a complex process of cellular and ECM interactions (226) assisted by mesenchymal and other progenitors and physical forces such as breathing motions. Recent work addressing roles for the ECM in lung development has examined both the production of ECM components (Fig. 3), as well as subsequent assembly and remodeling of the ECM, building on the pioneering work of Richard Bland and Kurt Albertine and colleagues (16, 38–40). One recent study by Hilgendorff and coworkers (138) reported that elastin haploinsufficient C57BL/6 mice exhibited perturbed lung capillary growth and attenuated elastin deposition in the developing parenchyma although no alveolarization defect was noted (assessed by Lm). This study provides further impetus for the study of collagen dynamics as well as elastin and collagen cross linking in alveolarization.

In this line, there is currently much interest in the balance between increased ECM production and impaired ECM degradation, in the context of fibrotic lung disease (97). This topic has been reviewed in detail recently, while asking whether idiopathic pulmonary fibrosis is a disease of impaired ECM degradation, rather than the classical view of increased ECM production (232). This idea may be extended to lung development. Several recent studies have addressed the posttranslational processing of collagen and elastin fibers in experimental and clinical BPD. Studies have included the examination of enzyme systems that direct the cross linking of collagen and elastin fibers, which include the lysyl oxidase, lysyl hydroxylase, and transglutaminase enzyme families. The expression of lysyl oxidases (namely Lox and LoxL1) (178), lysyl hydroxylases (namely lysyl hydroxylase 2, also called Plod2) (369), and transglutaminase 2 (368) has been associated with BPD in clinical subjects and with arrested alveolarization in the hyperoxia-based (85% O2 for 14 days) C57BL6 mouse model of BPD. Studies employing pharmacological interventions or knockout mouse strains are required to causally implicate these enzyme families in aberrant lung development. To this end, Mižíková and coworkers (238) set out to test a pathogenic role for lysyl oxidases in arrested alveolarization associated with hyperoxia exposure. Inhibition of lysyl oxidases with β-amino-propionitrile (BAPN) in developing C57BL6 mouse pups in the hyperoxia BPD model was without effect on alveolariza-
tion (assessed by stereology) at P10 and worsened lung development at P20, perhaps indicating a need for a careful balance of lysyl oxidase activity during organogenesis (238). Indeed, BAPN proved to be very toxic in mouse pups, limiting the suitability of BAPN as an interventional tool in developing animals (238), although BAPN is well tolerated in adult mice when organogenesis is complete (253). Contrasting data were obtained by Mammoto and coworkers (212), who reported that lysyl oxidase inhibition with BAPN improved alveolarization (assessed by MLI) in the mouse hyperoxia model. Both groups

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**Fig. 2.** Integration of selected, recently identified signaling pathways that can be induced by either hyperoxia or mechanical ventilation, which might impact fibroblast function during alveolarization. The scheme only details new developments that were published between 1 January 2013 and 30 June 2015. Crispld2, cysteine-rich secretory protein LCCL domain containing 2; MMP-2/9, matrix metalloproteinase 2 and 9; TGF-β, transforming growth factor-β; Sox9, sex determining region Y-box 9; sCG, soluble guanylate cyclase; Trop2, trophoblast antigen 2; HIF-2α, hypoxia-inducible factor 2α; ECM, extracellular matrix; miR, microRNA; BAPN, β-aminopropionitrile; MNT, MAX network transcriptional repressor.
employed C57BL/6 mice and a 10-day hyperoxia-exposure period; however, Mižíková (238) employed 85% O2 and a stereological analysis, whereas Mammoto (212) employed 75% O2 and a morphometric (MLI) analysis. Whether these differences explain the discordant observations is unclear. A weakness of the Mammoto study (212) is that the investigators supplemented maternal nutrition with BAPN and assumed maternal transmission to pups through the breastmilk; however, no data on BAPN levels or lysyl oxidase activity levels in the lungs of pups were provided. As such, it is not known whether lysyl oxidase activity levels were impacted at all in hyperoxia-exposed neonates. The investigators claimed to have repeated the intervention with intraperitoneal application of BAPN every other day to mouse pups and apparently obtained comparable effects on protecting the developing lung from hyperoxia-induced arrest of alveolarization. However, these data are neither provided in the manuscript proper nor in the online supplement. These studies highlight the need for adequate supporting data, in this case, to demonstrate the delivery of pharmacological agents to the target organs or at least to demonstrate that pharmacological agents that were applied did have the desired impact in the target organs; and 2) to provide supporting data, in this case, the intraperitoneal application of BAPN in the Mammoto study (212), which could have been compared with the analogous study by Mižíková (238).

Other ECM components such as peristin, a ligand for αvβ3 and αvβ5 integrins to support the adhesion and migration of epithelial cells, have also received attention (9, 44). Both studies localized peristin to myofibroblasts and clearly indicated that peristin (Postn) expression is driven by hyperoxia. Ahlfield and coworkers (9) demonstrated that hyperoxia-induced arrest of alveolarization has not been impacted (assessed by MLI) by global loss of peristin in C57BL/6J Postn−/− mice, when mice were exposed to 60% O2 or 85% O2 between P1 and P7 and assessed at P7. Opposing data were obtained by Boyzk and coworkers (44), who reported that exposure of Postn−/− mice to 75% O2 from P2 or P3 for 14 days fully protected against hyperoxia-induced arrest of alveolarization (assessed by Lm). These investigators also described a circle of peristin driving TGF-β and TGF-β driving peristin expression (highlighted in Fig. 2). Given the opposing trends noted in the two studies, it remains unclear whether peristin participates in aberrant alveolarization. There are several possible explanations for the opposing trends observed by the two groups of investigators. It is clear that different oxygen levels and different exposure protocols were employed. Additionally, two different mouse strains and backgrounds have been employed, in which Ahlfield and coworkers (9) employed a mouse strain (301) in which exon 1 of the peristin gene was insertionally inactivated with a LacZ cassette. Additionally, this strain was backcrossed over eight generations onto a C57BL/6 background. In contrast, Boyzk and coworkers (44) employed the B6;129-Postn tm1Jmol/J strain, in which the exons 4–10 of the peristin gene were replaced with a neomycin resistance cassette (268), and the strain was maintained on a mixed C57BL/6 and 129 background. As a further potential confounding variable, periodontal disease was present in Postn−/− mice, wild-type mice, and knockout animals fed with powdered chow by Boyzk and coworkers (44). These studies exemplify intermodel comparisons that arrive at opposite conclusions, possibly attributable to the use of different oxygen injury protocols or different background strains, as highlighted in Animal Models of Arrested Alveolarization, or attributable to different approaches to targeting genes in model animals.

Changes in ECM abundance in the airways have recently been correlated with lung function in equine heaves (314), and the influence of mechanical ventilation on ECM production in neonatal lungs has also recently been addressed. Kroon and coworkers (177) demonstrated that mechanical ventilation of 8-day-old mouse pups with low (3.5 ml/kg), moderate (8.5 ml/kg), or high (25 ml/kg) tidal volumes impacted ECM gene expression. Increased expression of the tropoelastin, fibulin 5, lysyl oxidase-like 1, and tenasin C genes was noted after an 8-h high tidal volume ventilation with room air. This was correlated with an altered appearance of parenchymal elastin fibers and reduced vessel density; however, lung structure was not quantified. These data demonstrate the power of mechanical ventilation to drive ECM gene expression, but the impact of altered gene expression reported in this study on alveolarization remains unclear.

The ability to therapeutically modulate ECM production by fibroblasts continues to be an area of interest in terms of

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**Fig. 3.** Some recently described pathways by which hyperoxia might modulate type II cell-type I cell transdifferentiation. The proposed roles for bone morphogenetic protein (BMP) signaling, IGF, IL-1β, TGF-β signaling, tripar-tite motif-containing 72 (Trim72), Wnt5a and Wnt3a, are indicated. The scheme only details new developments that were published between 1 January 2013 and 30 June 2015.
influencing aberrant late lung development in premature neonates. Among the key reports that have emerged in the past 2.5 yr are the exciting finding that glucocorticoids such as fluticasone can have divergent effects on ECM production by fibroblasts, in which fluticasone drove decorin production in airway fibroblasts but inhibited biglycan and procollagen production by parenchymal fibroblasts (47). These data also highlight the idea of compartmentalized responses to therapy in different cell types. More generally, it has also recently emerged that administration of dexamethasone to newborn mice over the first 4 days of postnatal life impaired alveolarization, ostensibly by the suppression of tenascin C and elastin expression (303). Glucocorticoids have also recently been demonstrated to modulate TGF-β signaling by redirecting TGF-β activity from the TGF-β receptor 1 (Tgbr1)/smooth muscle actin/mothers against decapentaplegic (Smad)3/4 axis to the activin A receptor type II-like 1 (Acvrl1)/Smad1 axis in lung fibroblasts. This may represent a relevant drug-pathway interaction in lung disease and development (312); however, ECM component expression was not addressed in that study. Preliminary evidence has also been provided for some new players in the regulation of ECM production and reorganization during lung development, including the serine peptidase chymotrypsin-like elastase 1 (197) and cathepsin K (170) and the possible interaction between integrins and the ECM (128); however, the relevance of these candidates in alveolarization remains to be demonstrated.

Growth Factors

Along with physical forces (stretch and breathing motions), the ECM, and coordinated transcription factor expression, growth factors are credited with regulatory roles in alveolarization (243). Belcastro and coworkers (32) have added to a growing wealth of evidence implicating disturbances to growth factor dynamics in arrested alveolarization. Using a hyperoxia-based mouse model of BPD (60% O₂ for 14 days), the investigators confirmed the increased parenchymal expression of TGF-β in hyperoxia-exposed mouse lungs and further demonstrated that in vivo inhibition of TGF-β signaling with SB431542 concomitant with hyperoxia exposure limited proximal TGF-β signaling (as assessed by Smad3 phosphorylation), and this was accompanied by an increase in secondary crest formation. These investigators further documented that increased peroxynitrite, thought to be generated by macrophages, drove increased TGF-β production. Furthermore, the peroxynitrite caused nitration of IGF1R, thereby preventing binding of IGF-1 to its cognate receptor and hence limiting downstream IGF-1 signaling, with deleterious consequences for alveolarization. In support of a role for IGF-1 in alveolarization, Galvis and coworkers (115) described that the repression of IGF-1 expression by the histone methyl transferase Ezh2 prevented basal cell differentiation in the developing lung. However, this idea has not yet been assessed in postnatal lung maturation. This study, along with the work of Brechbuhl and coworkers (48), adds the IGF system and the EGF system to the cocktail of modulators of basal cell differentiation and proliferation.

The TGF-β system, which is activated by exposure to a hyperoxic environment (18), continues to receive attention as a key mediator of alveolarization (359) and has recently been identified as the mediator of epithelial-to-mesenchymal transition in the lungs of rat pups exposed to hyperoxia (85% O₂ from P1 to P7) (350). Using transgenic mice that overexpress TGF-β or mice with genetic ablation of the type II TGF-β receptor (Tgbr2) in the lung epithelium, Sureshbabu and coworkers (323) described a pivotal role for the TGF-β/Tgbr2 axis in mediating arrested alveolarization in the background of hyperoxia. To this end, the ability of several pharmacological agents to limit hyperoxia-induced arrest of alveolarization has been attributed to the ability of these agents to blunt hyperoxia-induced TGF-β signaling, as was the case with curcumin (305) and nutritional supplementation with docosahexaenoic acid (345) and an activin A receptor type IIB-Fc antagonist (194).

Other studies have addressed drug-cell interactions in the context of growth factors; for example, Schwartz and coworkers (312) demonstrated that glucocorticoids modulated TGF-β signaling in lung fibroblasts and could shift TGF-β signaling from the Tgbr1/Tgbr2/Tgfbr2/Smad3 axis to the Acvrl1/Smad1 axis and, in doing so, promote lung myofibroblast differentiation. Given the widespread use of steroids in the management of patients with BPD, these studies reveal a drug-cell interaction of potential clinical significance. The interaction between TGF-β signaling and glucocorticoids has also been studied by Collins and coworkers (74) in the preterm lamb model in the context of intrauterine inflammation, in which betamethasone was documented to blunt TGF-β signaling in fetal lungs. The potential importance of these observations is underscored by the report of Alanis and coworkers (12), who described that the boundary separating the two distinct lung compartments, the proximal conducting airways and the peripheral gas exchange regions, the bronchoalveolar duct junction, may undergo a proximal or distal positional shift after premature activation or loss of glucocorticoid signaling. Other modulators of TGF-β signaling in developing lungs have been identified, including all-trans-retinoic acid, which dampened aberrantly high TGF-β signaling in a caloric restriction model of BPD in rats (1, 199). Some recent observations made in early lung development, such as the control of epithelial differentiation by the hippo pathway, in which yes-associated protein 1 regulates the ability of epithelial progenitor cells to respond to TGF-β-induced cues (208), await exploration in a postnatal context.

Inflammation

Inflammation is a common complication of BPD, both in a clinical setting and in experimental animal models that use inflammation as an injurious stimulus to mimic choorioamnionitis (34). The role played by inflammation in limiting lung development in other BPD models, notably those based on hyperoxia and mechanical ventilation, remains relatively unexplored. The direct (parenteral) attenuation of inflammation has been undertaken with daily, subcutaneous administration of an IL-1 receptor antagonist (IL-1Ra) in two slightly different BPD models (257). Interestingly, in a perinatal inflammation plus hyperoxia model, in which pregnant dams were first treated with LPS and then newborn pups were exposed to 85% O₂ for 28 days, no impact of IL-1Ra administration on lung development (assessed by calculation of the surface area-to-volume ratio using ImageJ software) was noted. However, in a “gentler” alternative model, in which, instead of 85% O₂, newborn mouse pups were exposed to 65% O₂, a dramatic
improvement in lung structure was noted. Similarly, administration of resolvin D1 and lipoxin A4 every third day to C57BL/6 pups exposed to 95% O2 from P1 to P10 improved alveolarization (assessed by MLI and RAC), which was associated with reduced proinflammatory cytokine gene expression (217). These data indicate 1) the potential benefit of attenuating inflammation on alveolarization in BPD models and 2) that the selection of oxygen levels may dramatically influence the outcome of interventional studies. It is clear that broadly blunting inflammation in BPD models improves alveolarization. Future work must address how inflammation modulates alveolarization.

Jagarapu and coworkers (153) evaluated leukadherin-1 (LA1; applied by daily intraperitoneal injection), a novel agonist of leucocyte surface integrins, and CD11b/CD18, which blunts transendothelial leukocyte migration to sites of injury in the hyperoxia-based (85% O2 from P1 to P14) rat model of BPD. LA1 administration decreased neutrophil and macrophage infiltration into the lungs, provoked hyperoxia, improved alveolarization (assessed by MLI and RAC) and angiogenesis, and limited aberrant pulmonary vascular remodeling. These data clearly demonstrate that inflammation plays a key role in aberrant alveolarization in response to hyperoxia. Identical observations were made by Drummond and coworkers (88), who applied AMD3100, an antagonist of Cxcr4 in a similar rat hyperoxia model (90% O2 from P1 to P16, AMD3100 applied between P5 to P15, in a therapeutic regimen). Cxcr4 antagonism also decreased neutrophil and macrophage infiltration into the lungs, provoked hyperoxia, and improved alveolarization (assessed by MLI) and angiogenesis, and limited aberrant pulmonary vascular remodeling. A role for epithelial integrins in macrophage recruitment has already been described earlier in this Perspectives article, relating the work of Plosa and coworkers (281) using an epithelial-specific (Sftpc-driven) deletion of integrin-β1, which promoted macrophage recruitment and blunted alveolarization (assessed by MLI) in mice, ostensibly through increased proinflammatory cytokine (Ccl2 and Cxcl1) and ROS production by lung epithelial cells. These data highlight the regulatory cross talk between the epithelium and inflammatory cells during alveolarization and add to a growing body of data highlighting the connection between inflammatory signaling and ROS production (130). Further studies by Carver and coworkers (58) have linked innate immune signaling and regulatory developmental programs in the lung, in which a link between NF-κB signaling and the expression of FGF-10, a regulator of lung development, was demonstrated. The authors suggested that NF-κB-driven inflammatory pathways may suppress the expression of growth factors and other mediators of alveolarization, which would negatively impact lung structure. These data take on increased importance in light of the documented regulation of lung epithelial stem cell growth by TGF-β and FGF-10 (93, 233). Interestingly, in contrast to the ideas of Carver and coworkers (58), Hou and coworkers (144), using BAY 11-7082, which is an inhibitor of NF-κB signaling, have indicated that it may be desirable to promote some elements of NF-κB inflammatory signaling to suppress damaging chemokine (C-X-C motif) ligand 2 (Cxcl2) [also called macrophage inflammatory protein (MIP)-2] production. Clearly much remains to be learned about the contribution of NF-κB signaling to postnatal lung maturation.

Efforts have been made to further delineate the specific inflammatory players that impact lung maturation. Among recent studies, Stouch and coworkers (320) identified IκB as a mediator of fetal macrophage maturation and suggested that the maturation of fetal lung macrophages into mature alveolar macrophages might include proinflammatory features, hitherto unrecognized aspects of macrophage activation. Syed and Bhandari (326) have revealed that macrophage polarization was skewed in favor of an M1 phenotype after hyperoxia exposure (100% O2 for 7 days). These studies support a role for macrophage polarization in promoting aberrant alveolarization (assessed by Lm) in response to hyperoxia and add to a growing body of data defining very diverse roles for macrophages in lung disease (6). This idea was recently elaborated upon by Eldredge and coworkers (94), who report existence of an anti-inflammatory population of CD11b+ mononuclear cells that are protective against hyperoxia-induced injury to neonatal lungs; however, only survival and inflammation were assessed in that study, without consideration of changes to the lung architecture. A follow-up study is eagerly awaited, particularly considering the emerging roles of inflammatory cell types and subpopulations in lung development and disease (6, 367), to identify causal roles for specific inflammatory cell subsets in lung development. By way of examples, Podolin and coworkers (282) recently demonstrated that T-cell depletion protects against alveolar destruction attributable to chronic cigarette smoke exposure in mice, but the deletion of T cells has yet to be evaluated in the context of lung development.

Efforts to modulate regulators of inflammation in experimental BPD have also received attention, with McGrath-Morrow and coworkers (230) focusing on Nfe2l2 (also called Nrf2), which was formerly demonstrated to be protective in hyperoxia-induced arrest of alveolarization (assessed by Lm). Interestingly, using sulforaphane, an inducer of Nfe2l2, the investigators demonstrated that, although inflammation could be attenuated by Nfe2l2 induction, alveolar growth was not improved, possibly attributable to proliferation-suppressive effects of Nfe2l2. This study makes the important point that caution should be exercised when anti-inflammatory strategies may have the side effect of growth inhibition during a critical phase of rapid postnatal growth and highlights the need for additional studies both on Nfe2l2 and other molecules that protect against oxidative stress, such as Sirt1 (376). An interesting angle on the Nfe2l2 line has been developed in adult mice by Kawamura and coworkers (163), who reported that the anti-inflammatory effects of hydrogen gas [2% (vol/vol)] in the background of hyperoxia (98% for 60 h) were Nfe2l2 dependent. The use of hydrogen gas to limit hyperoxia-induced damage in neonates has not yet been reported. Another regulator of inflammation that has received recent attention is macrophage migration inhibitory factor (MIF) (321, 322), in which it was demonstrated that both too much (overexpression in transgenic mice) and too little (MIF knockout mice) MIF arrested alveolarization (assessed by Lm) in both room air-exposed and hyperoxia-exposed mice. Thus the correct balance of MIF levels in the developing lung was essential for proper lung development. Administration of a small molecular MIF agonist to wild-type mice in the hyperoxia BPD model partially restored normal lung alveolarization, further supporting the idea that modulating inflammation represents a viable approach for the restoration of lung development in BPD.
In addition to a role for inflammation in modulating alveolarization, innate immunity has also been addressed in the context of BPD, in which where Raffay and coworkers (288) demonstrated that hyperoxia exposure (85% O2 for 14 days, with a posthyperoxia recovery phase) influenced club (formerly Clara) cell function, evident by decreased secretoglobin, family 3A, member 1 (Sgb3a1) and Sgb1a1 protein expression, which would have implications for the innate immune response. These studies may indicate a mechanism by which infants with BPD are more susceptible to lung infections. Along the lines of this study, Cai and coworkers (54) demonstrated that administration of a recombinant-related secretoglobin, secretoglobin, family 3A, member 2 (Sgb3a2), to pregnant mice promoted the development of terminal air sacs in preterm (E17.5) pups (as assessed by RAC). Together, these data might suggest that the decreased expression of secretoglobin in the hyperoxia model observed by Raffay and colleagues may have also directly negatively impacted terminal air-sac development or alveolarization, in addition to limiting innate immune responses.

Pulmonary Vascular Development and PPHN

Pulmonary vascular development and vascular endothelial and smooth muscle cell function are perturbed in a spectrum of lung developmental abnormalities, including BPD (21, 245, 319), CDH (3), ACD (172, 302), and persistent pulmonary hypertension of the newborn (PPHN) (84, 183, 237). Understanding the molecular regulation of pulmonary vascular development remains a major challenge (273). The past 2 yr have seen the identification of several new mediators of pulmonary vascular development, including Wntless (75) and Sox17 (184), which play a role in embryonic vessel development, but a function for either mediator in normal or aberrant late lung development is not yet clear. In contrast, the semaphorin/neuropilin 1 axis was demonstrated to play a role in fetal, but not postnatal, pulmonary vessel development (160), and pigment epithelium-derived factor appeared to be involved in perturbed vascular development associated with hyperoxia (69). Additionally, antagonism of PDE5 with sildenafil promoted pulmonary vascular development and alveolarization, ostensibly via activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt/mammalian target of rapamycin pathway and enhanced HIF-1α and HIF-2α activity (270). Other candidate regulators of lung vessel cell behavior have also been identified in vitro, including heat-shock protein 70 (5), the oxygen-sensitive voltage-gated potassium channel Kv1.5 (202), and AMPK (330); however, a role for these molecules in pulmonary vascular development awaits clarification.

Along with reports that the transcription factor forkhead box (Fox)M1 regulates pulmonary inflammatory responses to hyperoxia (371), FoxF1 has also been recently implicated as a candidate regulator of aberrant pulmonary vascular development in a spectrum of congenital diseases. FoxF1 haploinsufficiency is characteristic of ACDMPV. Therefore, a transcriptional profiling in a spectrum of congenital diseases. FoxF1 haploinsufficiency is peroxia (371), FoxF1 has also been recently implicated as a (Fox)M1 regulates pulmonary inflammatory responses to hyperoxia (202), and AMPK (330); however, a role for these molecules in pulmonary vascular resistance in an experimental sheep model of PPHN via the 5-HT2A receptor. The angiotsin system has also received attention in the context of postnatal lung development, in which Castro and coworkers (59) describe the downregulation of angiotensin-converting enzyme (ACE) expression in the lungs of patients with BPD, suggesting a pathophysiological contribution of diminished ACE levels to the development of clinical BPD. This idea is supported by Wagenaar and coworkers (352), who reported that stimulation of the MAS oncogene with cyclic (Ang)1–7 or stimulation of the type II angiotensin receptor with [d-lysine]cyclic(Ang)1–7 in the rat hyperoxia model (100% O2 for 10 days, from P1) partially protected against hyperoxia-induced damage to alveolarization, with an increased number of alveolar crests and decreased septal wall thickness observed. Additionally, the arteriolar medial wall thickness and Fulton index were partially normalized. Collectively, these observations support a role for dysregulation of the angiotsin system in clinical and experimental BPD. In another study, Nijmeh and coworkers (256) revealed the existence of high proliferative potential colony-forming cells (HPP-CFC) in the adventitial vaso vasorum of the pulmonary arteries in a hyperoxia-induced PPHN model in calves. These authors proposed that, given the high proliferative potential and vasculogenic capacity of HPP-CFC, these cells may be pathogenic mediators and perhaps targets for the management of pulmonary vascular remodeling associated with hyperoxia exposure in neonates.

Several lines of evidence point to PPAR-γ as a key contributor to perturbed vascular development and homeostasis in PPHN. Wolf and colleagues (370) demonstrated that increased levels of endothelin (ET)-1, which are indeed observed in clinical PPHN, decreased PPAR-γ signaling and blocked pulmonary artery endothelial cell tube formation. PPAR-γ agonists promoted endothelial tube formation in pulmonary artery endothelial cells in an NO-dependent manner. These observations suggest that targeting ET-1 production may restore angiogenesis in PPHN. In another study, Wagenaar and coworkers (351) also addressed the ET-1 system using ambrisentan (an endothelin receptor type A antagonist) in the rat hyperoxia model (100% O2 from P1 to P10), which improved survival, promoted alveolar development by normalizing septal wall thickness (without impacting the number of alveolar cresps per field), limited excessive ECM deposition, and dramatically improved aberrant remodeling of vessel walls and the heart that were provoked by hyperoxia exposure. Interestingly, these positive effects were not seen in a late treatment protocol (pups were exposed to 100% O2 for 9 days, followed by 9 days of recovery in room air, with daily ambrisentan application initiated on day 6). A potential connection between the HIF-1α and ET-1, as demonstrated by Larissa Shimoda’s group in smooth muscle cells in adult rats (280), has not yet been explored in the context of PPHN.

Returning to PPAR-γ, Gien and coworkers (118) revealed an interaction between PPAR-γ and Rho kinase (ROCK) that increased pulmonary artery smooth muscle cell proliferation
and proposed that this interaction was relevant to the aberrant vascular remodeling seen in PPHN. These data are very interesting considering the observations of Lee and coworkers (186), who reported that inhibition of ROCK with Y-27632, not only attenuated the deleterious impact of bleomycin in alveolarization in rat pups, but also normalized pulmonary vascular resistance, decreased right-ventricular hypertrophy, and attenuated vascular remodeling. Thus PPAR-γ and ROCK are exciting candidate targets in the management of pulmonary hypertension associated with BPD, as well as PPHN; however, the demonstration that some elements of pulmonary artery contraction are likely Rho independent (98) suggests that other mediators are also involved. It is worth noting at this junction that stimulation of PPAR-γ signaling can rescue alveolarization and vessel development in the hyperoxia model, in which Lee and coworkers (187) applied Escherichia coli LPS to the amniotic sac of pregnant mice. After spontaneous delivery, pups were exposed to hyperoxia (80% O₂ from P1 to P7) followed by recovery in room air (P8 to P14), which blunted both alveolarization (assessed by Lm) and vessel density (assessed by visual inspection and PECAM immunohistochemistry). Administration of the PPAR-γ agonist rosiglitazone rescued both vascularization and vascular growth. Taking this idea further, Morales and coworkers (240) nebulized the PPAR-γ agonists rosiglitazone and pioglitazone into the lungs of 1-day-old rat pups, which were subsequently exposed to hyperoxia (95% O₂ for 3 days, intervention once daily). Nebulized PPAR-γ agonists protected against hyperoxia-induced retardation of alveolarization (assessed by MLI and RAC) and blunted hyperoxia-provoked lung inflammation. Although not assessed in pulmonary vascular development, in the context of sickle cell disease-associated pulmonary hypertension, Li and coworkers (191) described the downregulation of ET-1 by fenofibrate-stimulated PPAR-γ, which upregulated miR-199a2, which in turn targeted ET-1 expression. This pathway may also be relevant to the ET-1/PPAR-γ axis in the context of vascular development as well. Thus the PPAR-γ system represents an exciting druggable target to drive lung alveolar and vascular development. These ideas are integrated into a scheme in Fig. 4. Apart from PPAR-γ/ET-1, AMPK has also recently been introduced as a candidate target to drive angiogenesis in utero (330).

In an effort to delineate the impact of congenital heart defects on lung development, Razavi and coworkers (296)

Fig. 4. Recently identified roles for peroxisome proliferator-activated receptor-γ (PPAR-γ) in pulmonary vascular development and homeostasis associated with persistent pulmonary hypertension of the newborn (PPHN). Modulation of PPAR-γ is potentially relevant to pulmonary vascular development and homeostasis in bronchopulmonary dysplasia (BPD) and PPHN. PPAR-γ can be modulated by activators (green) or by the suppression of inhibitory pathways (red) by attenuating the effects of endothelin (ET)-1 mediated by the endothelin 1A (ET₁A) and 1B (ET₁B) receptors. The effects of PPAR-γ agonism may impact smooth muscle cells (SMC) and endothelial cells (EC) by modulating endothelial nitric oxide synthase (eNOS) or Rho kinase (ROCK) activity. The scheme only details new developments that were published between 1 January 2013 and 30 June 2015.
employed a model of pulmonary artery stenosis in rats. After the left pulmonary artery was banded at 21 days of life, lung structure was assessed at 160 days. Left pulmonary artery banding resulted in an apparent decrease in alveolar size compared with sham-operated animals, suggesting that disturbed blood flow to the lung can limit alveolarization. It must be kept in mind that arterial banding study was performed after the phase of bulk alveolarization but before the continued alveolarization described by Tschanz and coworkers (341) that occurs between P21 and P60. As such, it remains unclear whether the apparently reduced alveolar size results from a failure of late alveolar development or from alveolar destruction in response to blood flow limitation.

The role of perturbed ECM deposition and remodeling, which result in vessel stiffness, is an exciting and new area of research in the pulmonary vasculature. Recent work by Nave and coworkers (253) described aberrant collagen and elastin cross linking in pulmonary vessels in pulmonary arterial hypertension in adult mice. Additionally, vascular stiffening is presently being debated as a cause or effect of pulmonary hypertension in human adults (328). Along these lines, Dodson and coworkers (87) reported that the main pulmonary arteries in lambs with PPHN exhibited increased stiffness and excessive ECM remodeling. These exciting data point to hitherto unrecognized roles for perturbations to vessel stiffness in proximal (as opposed to distal) pulmonary vessels as a pathogenic factor in PPHN and highlight the examination of ECM deposition and the activity of ECM cross-linking enzymes as a worthwhile avenue to pursue in understanding the mechanistic basis of PPHN.

A Role for the Small Airways?

The development of the alveoli and the pulmonary vascular tree has received much attention in the context of late lung development. However, despite the pioneering reports in the early 1990s by Marc Hershenson and Julian Solway demonstrating that exposure to hyperoxia in the immediate postnatal period causes airway hyperresponsiveness and aberrant airway remodeling (135, 136) and studies by Shenberger and colleagues (315) that demonstrated pulmonary neuroendocrine cell hyperplasia in developing rats in response to hyperoxia, the small airway compartment has been very neglected. Given the intimate relationship that must exist between the developing airways and the associated parenchyma and vessels (53), together with many recent advances in airway smooth muscle cell biology (286), further investigations into airway biology during normal and aberrant late lung development are warranted. This idea is supported by the predisposition of preterm neonates to clinical problems related to the airways, such as wheeze and asthma (50), and the recent exciting observation from Wolfgang Kübler’s group that upper airway distension triggers proinflammatory responses in mechanically ventilated adult C57BL/6 mice (255).

Some recent reports have addressed the role of postnatal hyperoxia exposure on bronchiolar remodeling that is evident in adult experimental animals (261–263, 293); however, a comprehensive assessment of small airway architecture in neonates has not been undertaken. This appears all the more important given the recent summary by Hye-Youn Cho and Steven Kleeberger highlighting the perspective of Nfe2l2 (also called Nrf2) in the airways as a mediator of oxidant damage responses in the airways (70). The recently described role of the transcription factors Spdef and FoxA3 in goblet cell metaplasia and Th2-mediated inflammatory responses in the postnatal lung (290) provides impetus for such investigations. A role for the pulmonary neuroendocrine cells in aberrant lung alveolarization also awaits attention.

Some studies have started to address the impact of hyperoxia on small airway structure. Wang and coworkers (355) described the differential impact of elevated oxygen levels on smooth muscle mass and collagen deposition in the developing airways in mice exposed to 40% O2 or 70% O2 from P1 to P7, followed by recovery in room air between P8 and P21. Other reports, for example those documenting the impact of cigarette smoke on mitochondrial function (24, 25) and the proliferation and ECM production (347) in airway smooth muscle cells, as well as the impact of hyperoxia on estradiol metabolism in postnatal airway smooth muscle (219), highlight the potential relevance of perturbations to airway development to lung maturation. Furthermore, Chen and colleagues (67) provide evidence that endogenous retinoic acid, a key player in lung development, restricts airway smooth muscle cell differentiation during formation of the airways. Similarly, Xie and coworkers (372) connected Wnt/β-catenin and Sox2, two other key players in lung development already discussed in this Perspectives article, in airway submucosal gland morphogenesis. Clearly, much interesting work in this direction lies ahead.

Long-Term Sequelae of Hyperoxia Exposure

There is currently much interest in the long-term follow-up of patients with BPD to assess the implications of having had BPD on morbidity in later life (137, 263). One recent report suggested that early classification of BPD severity in preterm infants did not provide useful information about future lung function, when patient lung function was assessed at 6 and 18 mo after preterm birth (334); however, additional studies are clearly indicated. Similar studies are also now being conducted in animal models of BPD, in which the time course to adulthood is relatively shorter. Some notable studies have revealed that perturbed late lung development, for example after hyperoxia exposure in the postnatal period, does indeed have consequences for the lung architecture in adult animals. O’Reilly and coworkers (262) demonstrated that exposure of mouse pups to 65% O2 over the first 7 days of life resulted in remodeling of the bronchiolar walls and loss of bronchiolar-alveolar attachments that was evident at 10 mo of age, leading the investigators to propose that immediate postnatal hyperoxia exposure could lead to impaired lung function and airway hyperreactivity in adulthood. In a related study, these investigators demonstrated that the exposure to hyperoxia caused persistent alteration to the bronchiolar wall that contributed to perturbed lung function that had a stronger impact on the male sex (261). These data thus also contribute to current thinking that sex plays a key role in lung responses to hyperoxic injury. These observations are supported by a report from Ramani and coworkers (293), in which exposure of C57BL/6 mouse pups to either hyperoxia (85% O2) or normoxia (12% O2) over the period P2-P14, followed by a 10-wk recovery period under normoxic (21% O2) conditions, caused stunted alveolar development (assessed by MLI and RAC), loss of bronchiolar-
alveolar attachments, increased lung compliance, and, in the case of the hyperoxia group, increased arteriolar medial wall thickening that persisted into adulthood. An important structure-function correlate has also been reported by Ahlfeldt and coworkers (8), in which mouse pups were exposed to hyperoxia (90% O₂ from P1 to P7) followed by recovery in room air from P8 to P56. At P56, an alveolarization (assessed by ML1) and vessel density defect persisted. Furthermore, the alveolarization defect corresponded with a reduced functional gas exchange (measured by the diffusing factor for carbon monoxide, DfCO). This report also highlights the usefulness of combining structural analyses with physiological measures of lung function, which together provide complementary views on the same idea, contributing to the robustness of the argument. To this end, the measurement of pressure-volume curves in mouse lungs has recently been considered in detail (195).

Experimental studies on susceptibility to viral infection in later life, after exposure to hyperoxia in the neonatal period have also been undertaken by Maduekwe and coworkers (204), who demonstrated that exposure to a particular threshold of hyperoxia in the neonatal period altered host susceptibility to influenza virus infection in the long term. Taking this idea further, Bucynski and coworkers (51) explored a protective role for SOD during hyperoxia exposure of mouse pups in the immediate postnatal period (100% O₂ between P1 and P4) on alveolar development quantified in late life (8–10 wk). Over-expression of SOD in alveolar type II cells during hyperoxia exposure conferred protection against hyperoxia-induced damage to lung structure, with a partial normalization of alveolar development reported, although this was assessed by visual inspection of lung sections, in which lung structure was not quantified. Exposure of these mice, as adults, to influenza A virus indicated that wild-type mice with postnatal hyperoxia exposure were more sensitive to influenza A virus infection, exhibiting increased mortality and increased lung collagen production, which was prevented in transgenic mice overexpressing SOD in alveolar type II cells. In sum, these observations support the idea that immediate postnatal exposure predisposes adults to respiratory viral infection.

Neoalveolarization

Postpneumonectomy lung growth is an increasingly used model to study lung development in which mice regenerate lung tissue (neoalveolarization) after surgical resection (pneumonectomy) (332). There is currently controversy about the molecular pathways that drive neoalveolarization. To date, lung regeneration after pneumonectomy has been attributed to reinitiation or acceleration of cellular growth, with the proposal that, analogous to limb regeneration, neoalveolarization would result from reactivation of lung organogenesis genes (145, 360). In a transcriptional profiling study, Kho and colleagues (164) make the observation that, rather than the engagement of early lung organogenesis pathways, postpneumonectomy lung growth was characterized by the engagement of pathways driving later stages of lung development (alveolarization) rather than pathways that direct branching and primary airway enlargement. In addition to transcriptional control of postpneumonectomy lung growth, Ysasi and coworkers (380), using a unilateral diaphragmatic paralysis approach, have also demonstrated the critical importance of cyclic stretch associated with diaphragmatic contraction as a controlling factor in compensatory lung growth. On the vascular side, Ackermann and coworkers (4) have used remarkable imaging approaches to demonstrate sprouting and intussusceptive angiogenesis as important mechanisms of lung alveolarization during post-pneumonectomy lung growth. A key mechanistic finding was provided by Rafii and coworkers (289), who documented that platelets drive postpneumonectomy lung regeneration by supplying stromal cell-derived factor (SDF)-1, which activates the SDF-1 receptors Cxcr4 and Cxcr7 on capillary endothelial cells, which in turn stimulate alveolar epithelial cell expansion, via the angiocrine membrane-type metalloproteinase. This idea also hints at worthwhile avenues to pursue for the stimulation of lung maturation in neonates.

Pharmacological Interventions to Identify New Pathogenic Pathways

Several interventional pharmacological studies have identified candidate pathways or molecules that might be targeted in a translational sense to promote better lung growth or to identify new pathways relevant to normal or aberrant late lung development. These targets are summarized in Table 1 and have identified possible lead compounds for further development, with the aim to drive lung maturation by pharmacological interventions. Additionally, these studies have highlighted new pathways relevant to normal and aberrant alveolarization, which warrant further study.

One area of current intensive investigation is the impact of caffeine and other xanthine derivatives on lung maturation (218), highlighted in Fig. 5. Weichert and colleagues (362) administered caffeine [10 mg/kg per day, ip; from P6, concomitant with the onset of hyperoxia (80% O₂ for up to 48 h) exposure] to rat pups and observed an apparent improvement in alveolarization by visual inspection of sections (lung structure was not quantified). Additionally, blunted neutrophil and macrophage infiltration and a normalization of Cxcl1, Cxcl2 (also called MIP-2), Ccl2, TNF-α, and IL-6 levels were observed in the caffeine-treated group in the background of hyperoxia. In another study, Dayanim and colleagues (83) administered caffeine (20 mg/kg on P1, followed by 10 mg/kg per day thereafter) to FVB/N mouse pups in a dosing regimen directly translated from clinical studies (272, 308). In the latter study, caffeine administration has only deleterious effects, with increased pulmonary inflammation and increased epithelial apoptosis, as well as worsening of the lung architecture (as assessed by RAC) in the background of hyperoxia. Clearly, these two studies yield conflicting results, which may be attributable to a lack of dose range finding, as acknowledged by Dayanim and colleagues in their report (83). Interestingly, both studies reported that caffeine applied under room-air conditions dysregulated Cxcl1 expression, perhaps suggesting a proinflammatory activity of caffeine in the lungs under room air conditions. Comparing these studies is difficult because of the different animal models and oxygen exposure protocols, and the impact of caffeine on alveolar architecture in the Weichert study (362) remains to be validated because these investigators did not quantify changes in lung structure, which were observed by visual inspection only. Clearly, the potential utility of caffeine to manage lung inflammation and promote
### L1258 ADVANCES IN LUNG ALVEOLARIZATION AND BPD

Table 1. **Pharmacological interventions in animal models of bronchopulmonary dysplasia and associated persistent pulmonary hypertension of the newborn undertaken since 1 January 2013**

<table>
<thead>
<tr>
<th>Drug (dose and frequency)</th>
<th>Species and Strain</th>
<th>Injury Stimulus</th>
<th>Molecular/Pathway Targets</th>
<th>Lung Pathological Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActRIIB-Fc (5 mg/kg tiw, ip)</td>
<td>Mouse C57BL/6</td>
<td>85% O₂; P1-P14</td>
<td>Activin A receptor antagonist</td>
<td>Improved alveolarization (194)</td>
<td></td>
</tr>
<tr>
<td>Ambrisentan (1-20 mg/kg daily, sc)</td>
<td>Rat S/N</td>
<td>100% O₂; P1-P10</td>
<td>Blunted TGF-β signaling</td>
<td>Improved septal thickness (351)</td>
<td></td>
</tr>
<tr>
<td>AMD3100 (240 μg/kg daily, sc)</td>
<td>Rat S-D</td>
<td>90% O₂; P2-P16</td>
<td>ET receptor type A antagonist</td>
<td>Improved vascular structure (88)</td>
<td></td>
</tr>
<tr>
<td>Ascorbyl/peroxide (infused)</td>
<td>Guinea pig</td>
<td></td>
<td>Increased oxidative stress</td>
<td>Destruction of alveoli (95)</td>
<td></td>
</tr>
<tr>
<td>Aspirin (40 mg/kg daily, R/ND)</td>
<td>Mouse C3H/HeN</td>
<td>85% O₂; P1-P14</td>
<td>Cytosine-oxygenase-2 inhibitor</td>
<td>No improvement in alveolarization (50)</td>
<td></td>
</tr>
<tr>
<td>ATRA (5 mg/kg daily, ip)</td>
<td>Rat S-D</td>
<td>Nitroen E9.5</td>
<td>Retinoic acid (primary) Increased lepitin signaling</td>
<td>Improved alveolarization (109)</td>
<td></td>
</tr>
<tr>
<td>BAPN (15 mg/kg daily, ip)</td>
<td>Mouse C57BL/6</td>
<td>85% O₂; P1-P10/P20</td>
<td>Lysyl oxidase inhibitor Altered ECM morphology</td>
<td>Worsened alveolarization (238)</td>
<td></td>
</tr>
<tr>
<td>BAPN (dose indeterminate)</td>
<td>Mouse C57BL/6</td>
<td>85% O₂; P1-P10</td>
<td>Lysyl oxidase inhibitor Altered ECM morphology</td>
<td>Improved alveolarization (212)</td>
<td></td>
</tr>
<tr>
<td>BAY 11-7082 (10 mg/kg once, ip)</td>
<td>Mouse C57BL/6</td>
<td>LPS</td>
<td>NF-kB pathway inhibitor</td>
<td>Improved MIP-2 levels (144)</td>
<td></td>
</tr>
<tr>
<td>Benzoyl/pyrene (25 mg/kg, ip, E18/19/20)</td>
<td>Rat Fisher 344</td>
<td>90% O₂; P1-P14</td>
<td>Increased oxidative stress</td>
<td>Worsened alveolarization (331)</td>
<td></td>
</tr>
<tr>
<td>Caffeine (10 mg/kg daily, ip)</td>
<td>Mouse FVB/N</td>
<td>80% O₂; P1-P8</td>
<td>Increased levels of isoprostanes</td>
<td>Increased inflammation (83)</td>
<td></td>
</tr>
<tr>
<td>Caffeine (10 mg/kg, once at P6)</td>
<td>Rat Wistar</td>
<td>80% O₂; P6-P8</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization (362)</td>
<td></td>
</tr>
<tr>
<td>Carbon monoxide (250 ppm, continuous)</td>
<td>Mouse C57BL/6</td>
<td>75% O₂; P3-P21</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization (23)</td>
<td></td>
</tr>
<tr>
<td>Celecoxib (5 mg/kg daily, R/ND)</td>
<td>Mouse C3H/HeN</td>
<td>85% O₂; P1-P14</td>
<td>Cyclooxygenase-2 inhibitor</td>
<td>No improvement in alveolarization (50)</td>
<td></td>
</tr>
<tr>
<td>Curcumin (5 mg/kg daily, ip)</td>
<td>Rat S-D</td>
<td>95% O₂; P1-P5</td>
<td>Attenuated TGF-β signaling Blunted ECM deposition</td>
<td>Improved alveolarization (305)</td>
<td></td>
</tr>
<tr>
<td>cyclic (Ang) 1-7 (10–50 μg/kg daily, sc)</td>
<td>Rat Wistar</td>
<td>100% O₂; P1-P10</td>
<td>MAS oncogene receptor agonist</td>
<td>Suppressed inflammation (352)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine (15 mg/kg daily, sc)</td>
<td>Rat S-D</td>
<td>60% O₂; P1-P14</td>
<td>Not reported</td>
<td>No improvement in alveolarization (285)</td>
<td></td>
</tr>
<tr>
<td>DFU (10 μg/g daily, ip)</td>
<td>Rat S-D</td>
<td>60% O₂; P1-P14</td>
<td>CoS2 inhibitor</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization (220)</td>
</tr>
<tr>
<td>DHA (diet supplement)</td>
<td>Mouse C3H/HeN</td>
<td>85% O₂; P1-P14</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>(345)</td>
</tr>
<tr>
<td>GYY4137 (50 mg/kg daily, ip)</td>
<td>Mouse C57BL/6</td>
<td>LPS + 85% O₂; P1-P14</td>
<td>H₂S donor</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
</tr>
<tr>
<td>GYY4137 (37 mg/kg daily, ip)</td>
<td>Rat S-D</td>
<td>90% O₂; P1-P14</td>
<td>H₂S donor Blunted PASMC proliferation</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (343)</td>
</tr>
<tr>
<td>5-HT (12–20 μg infused)</td>
<td>Sheep C-R</td>
<td>Fetal d.a.constriction</td>
<td>5-HT3 receptor agonist β-catenin inhibitor Blunted PASMC proliferation Blunted ECM production</td>
<td>Increased PVR (85)</td>
<td>Improved alveolarization</td>
</tr>
<tr>
<td>ICG001 (10 mg/kg daily, ip)</td>
<td>Rat S-D</td>
<td>90% O₂; P2-P14</td>
<td>IL-1 receptor antagonist</td>
<td>Improved alveolarization (257)</td>
<td></td>
</tr>
<tr>
<td>IL-1Ra (10 mg/kg daily, sc)</td>
<td>Mouse C57BL/6</td>
<td>LPS + 65% or 85% O₂ (to P28)</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (276)</td>
</tr>
<tr>
<td>α-NOS (10 μg/g, ip, E18/19/20)</td>
<td>Rat S-D</td>
<td>80% O₂; P1-P7</td>
<td>Increased neutrophil expression</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (89)</td>
</tr>
<tr>
<td>Ketanserin (20 mg infused)</td>
<td>Mouse C3H/HeN</td>
<td>100% O₂; P1-P10</td>
<td>5-HT3 receptor antagonist</td>
<td>Decreased PVR (85)</td>
<td>Reduced edema (295)</td>
</tr>
<tr>
<td>α-Clotho (60 pmol daily, ip)</td>
<td>Mouse AKR/J</td>
<td>Chronic smoke exposure</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (153)</td>
</tr>
<tr>
<td>LA-1 (1 mg/kg b.i.d., ip)</td>
<td>Mouse AKR/J</td>
<td>Chronic smoke exposure</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (275)</td>
</tr>
<tr>
<td>Lipoxin A₄ (2 ng/g, ip, on P13/609)</td>
<td>Mouse C57BL/6</td>
<td>100% O₂; P1-P10</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>Normalized septal thickness (217)</td>
</tr>
<tr>
<td>Liver growth factor (1.7 μg b.w., ip)</td>
<td>Mouse AKR/J</td>
<td>Chronic smoke exposure</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (275)</td>
</tr>
<tr>
<td>Lysyl oxidase inhibitor</td>
<td>Rat S-D</td>
<td>90% O₂; P1-P14</td>
<td>Increased MIP-2 levels</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (14)</td>
</tr>
<tr>
<td>Metformin (25-100 mg/kg daily, sc)</td>
<td>Rat Wistar</td>
<td>100% O₂; P1-P10</td>
<td>Suppressed inflammation</td>
<td>Improved septal thickness</td>
<td>Improved alveolarization (68)</td>
</tr>
<tr>
<td>MIP20 (0.4 mg/g daily, P1-P4, id, P5-P14, ip)</td>
<td>Mouse C57BL/6</td>
<td>100% O₂; P1-P4</td>
<td>MIP agonist Inflammation</td>
<td>Improved alveolarization (321)</td>
<td></td>
</tr>
<tr>
<td>MIP98 (0.4 mg/g daily, P1-P4, id, P5-P14, ip)</td>
<td>Mouse C57BL/6</td>
<td>100% O₂; P1-P4</td>
<td>MIP agonist</td>
<td>Improved alveolarization</td>
<td>Improved vascular structure (321)</td>
</tr>
</tbody>
</table>
lung maturation is exciting, but reports on caffeine toxicity raised concerns about dose (338), and the observed impact of early vs. late administration of caffeine to infants (272) highlights the need for optimization of treatment protocols. Some recent evidence suggests that caffeine may also exert an effect on TGF-β-driven epithelial-to-mesenchymal transition of lung epithelial cells (100) and may influence steroid-mediated surfactant protein B expression (99); however, the relevance of this observation to lung alveolarization has not yet been established.

The potential application of vitamin A and vitamin A analogs to promote lung maturation continues to receive attention because retinoid signaling is essential for proper diaphragm and lung development. Costa and coworkers (76) have added ideas to the ongoing debate about the use of and role of retinoids in lung development and lung structural homeostasis that started in 1927 with Harry Goldblatt and Maria Benishek’s original observations that vitamin A deficiency causes lung metaplasia (119). It appears that we have some way to go yet.

Cell Therapy to Promote Alveolarization

The applicability of stem cells to drive the repair of damaged lungs and the regeneration of lung tissue to promote that repair are currently a hot topic in pulmonary research (131, 141, 174, 241), including BPD (265). Some strides forward have been made in the validation of cell therapy as a viable route to restore and promote lung maturation in experimental BPD. Key recent observations include the report of Alphonse and coworkers (19), who demonstrated that, after exposure to hyperoxia in vitro, endothelial colony-forming cells (ECFCs) from human fetal lungs exhibited blunted proliferative and vessel-formation activity, as did ECFCs from hyperoxia-treated rat lungs (95% O₂, from P1 to P14). Intraocular

<table>
<thead>
<tr>
<th>Drug (dose and frequency)</th>
<th>Species and Strain</th>
<th>Injury Stimulus</th>
<th>Molecular/Pathway Targets</th>
<th>Lung Pathological Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitoTEMPO (0.7 μg/g daily, sc)</td>
<td>Mouse C57BL/6</td>
<td>75% O₂; 72 h at P0 or P4</td>
<td>Not reported</td>
<td>Improved alveolarization</td>
<td>(81)</td>
</tr>
<tr>
<td>MutTBAp (1-10 mg/kg, ip, P1/2/3)</td>
<td>Rat S-D</td>
<td>50% O₂; Inter. 12% O₂; P1-P14</td>
<td>Superoxide dismutase mimetic</td>
<td>Improved alveolarization</td>
<td>(62)</td>
</tr>
<tr>
<td>PD123319 (0.5-2.0 mg/kg daily, ip)</td>
<td>Rat Wistar</td>
<td>100% O₂; P1-P10</td>
<td>ATII type 2 receptor antagonist</td>
<td>Improved septal thickness</td>
<td>(353)</td>
</tr>
<tr>
<td>Pioglitazone (3 mg/kg, nebulized)</td>
<td>Rat S-D</td>
<td>95% O₂; P1-P3</td>
<td>PPAR-γ agonist</td>
<td>Improved alveolarization</td>
<td>(240)</td>
</tr>
<tr>
<td>Resolvin D1 (2 ng/g, ip, P1/3/6/9)</td>
<td>Mouse C57BL/6</td>
<td>100% O₂; P1-P10</td>
<td>Suppressed inflammation</td>
<td>Normalized septal thickness</td>
<td>(217)</td>
</tr>
<tr>
<td>Sildenafil (100 mg/g daily, ip)</td>
<td>Rat S-D</td>
<td>LPS + 80% O₂; P1-P7</td>
<td>PPAR-γ agonist</td>
<td>Improved alveolarization</td>
<td>(187)</td>
</tr>
<tr>
<td>Sertraline (10 mg infused)</td>
<td>Sheep C-R</td>
<td>Fetal d.a. constriction</td>
<td>Serotonin reuptake inhibitor</td>
<td>Improved alveolarization</td>
<td>(270)</td>
</tr>
<tr>
<td>Sildenafil (100 μg/g daily, ip)</td>
<td>Mouse C57BL/6</td>
<td>80% O₂; P1-P28</td>
<td>Increased PI3K/Akt/mTOR axis</td>
<td>Improved alveolarization</td>
<td>(336)</td>
</tr>
<tr>
<td>VARA (oral on alternate days)</td>
<td>Mouse C57BL/6</td>
<td>85% O₂; P1-P14</td>
<td>Retinoic acid pathway (primary)</td>
<td>Improved alveolarization</td>
<td>(156)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Rat S-D</td>
<td>Endotoxin at E20 (intra amniotic)</td>
<td>ROCK inhibitor</td>
<td>Improved alveolarization</td>
<td>(186)</td>
</tr>
<tr>
<td>Y-27632 (10 mg/kg daily, ip)</td>
<td>Rat S-D</td>
<td>Bleomycin</td>
<td>Suppressed inflammation</td>
<td>Improved alveolarization</td>
<td>(85)</td>
</tr>
</tbody>
</table>
administration of human umbilical cord blood-derived ECFCs after hyperoxic lung injury in Rag1−/− mouse pups (85% O2, from P4 to P14) restored lung function and also restored alveolarization (assessed by MLI) and lung vascular growth and blunted pulmonary hypertension. The low level of engraftment of ECFCs, together with protective effects of ECFC-conditioned medium, prompted the investigators to suggest that a paracrine effect conferred the restorative effects of ECFCs (110). Notably, 10 mo after ECFC administration, the improvement in lung structure persisted. The same group of investigators reported the beneficial impact of human umbilical cord-derived perivascular cells and mesenchymal stem cells (MSCs) delivered by the intratracheal route in the rat hyperoxia model, in which alveolarization (assessed by MLI) was rescued both in the short and long term (6 mo) (278). In a similar study, Sutsko and coworkers (324) compared whether intratracheal administration of either MSCs or conditioned medium from MSCs influenced lung development in the hyperoxia rat model (90% O2, between P2 and P16). Both MSC and MSC-conditioned medium blunted the impact of hyperoxia on alveolar development (assessed by MLI) and lung vessel density. Along these lines, Baker and coworkers (29) applied conditioned medium from late-outgrowth colony-forming cells, a type of endothelial progenitor cell, in a bleomycin-based rat model of...
BPD. Although application of conditioned medium blunted heart remodeling (assessed by Fulton index) provoked in the model, no impact on alveolarization (assessed by RAC) of rat pup lungs was noted, leading these authors to criticize the potential benefit of conditioned medium administration in this model. Why the study of Baker and coworkers (29) could not demonstrate a beneficial impact of the intervention on alveolarization is not clear but may be related to the use of late-outgrowth colony-forming cells or a different model (bleomycin), compared with the other three studies discussed above.

Along similar lines, Ahn and coworkers (11) evaluated the relative efficiency of human umbilical cord blood-derived MSCs, human adipose tissue-derived MSCs, and human umbilical cord blood mononuclear cells to protect against blunted alveolar development induced by hyperoxia (90% O2 for 14 days) in rat pups. The investigators assessed that the human umbilical cord blood-derived MSCs were most efficient at promoting improved lung maturation in the background of hyperoxia, which was correlated with increased VEGF and hepatocyte growth factor production. The investigators followed up with a timing-of-administration study (64) and revealed that the application of umbilical cord blood-derived MSCs was only beneficial when applied at P3 but not P10 (90% O2, from P1 to P14, followed by 60% O2 from P14 to P21). In another cord blood study, Mao and coworkers (216) applied cord blood-derived CD34+ cells that had been expanded in vitro, via the intranasal route, to mice in an FVB/N exposed to 85% O2 from P1 to P14 improved alveolarization (assessed by MLI) (349). 

MSCs, human adipose tissue-derived MSCs, and human umbilical cord blood-derived MSCs have recently been successfully demonstrated in preterm infants in a phase I clinical trial (NCT01297205) (63). The results of a follow-up phase II clinical trial to assess therapeutic efficacy (NCT01828957), as well as a long-term follow-up safety assessment study of the MSC transplant recipients (NCT01897987) (10), are eagerly awaited.

### Synthesis and Concluding Remarks

It is clear that many developments in our understanding of alveolarization and the pathogenesis of BPD have been made since the beginning of 2013. It is also clear that many open questions remain, and new questions have been asked. Notable advances in methodology used to study lung development have been made, including the development of new tissue fixation and embedding protocols and stereology approaches for the unbiased quantification of lung structure. Further implementation of these methodologies, moving from RAC or MLI to stereology measurements, would represent an important advance in how we study lung structure although concerns about time and cost are likely to remain barriers to the widespread implementation of these approaches in the foreseeable future.

This Perspectives article has also highlighted the tremendous heterogeneity of animal models to study arrested alveolarization. The hyperoxia-based approaches in particular are problematic, given the current range of oxygen levels employed, together with variable periods of oxygen exposure, the combination of a second hit and a period of recovery under room air conditions, and posthyperoxia exposure. It appears that there remains scope for a systematic study, using state-of-the-art stereological approaches, to clarify whether an optimal injurious oxygen concentration and window of exposure exist. Along these lines, it would be important to strike a balance between the amount of oxygen employed as an injurious stimulus and the degree of damage to the developing lung that can be quantified. This is particularly important in pharmacological or transgenic animal studies in which a potentially important subtle effect of a pharmacological or genetic intervention on the structural development of an injured lung may be missed because the injurious stimulus was unnecessarily severe. In this way, the potential subtle modulation of alveolarization by interventions at superoxia levels (≥85% O2) may not be noted in lung structural analyses. Concerns also persist about the sex of the experimental animals employed and the range of strains with variable responses to oxygen injury, which has resulted in opposite observations being made in ostensibly the same studies. Also related to mouse strains, there is increasing concern about the appropriateness of controls employed in transgenic mouse studies, particularly where identifying the correct stem cell type and route of administration, keeping in mind that a cocktail of multiple stem cell types may be the best approach, understanding how these cells promote lung maturation or correct blunted alveolarization, and clarifying whether stem cell application may have harmful long-term consequences, such as tumorigenesis. The considerable progress made over the past few years in applying stem cells in animal models that recapitulate the arrested alveolarization associated with clinical BPD has provided impetus for translation of this idea to the clinical setting. The safety and feasibility of transplanting allogeneic human umbilical cord blood-derived MSCs were most efficient at promoting improved lung maturation in the background of hyperoxia, which was correlated with increased VEGF and hepatocyte growth factor production. The investigators followed up with a timing-of-administration study (64) and revealed that the application of umbilical cord blood-derived MSCs was only beneficial when applied at P3 but not P10 (90% O2, from P1 to P14, followed by 60% O2 from P14 to P21). In another cord blood study, Mao and coworkers (216) applied cord blood-derived CD34+ cells that had been expanded in vitro, via the intranasal route, to mice in an FVB/N exposed to 85% O2 from P1 to P14 improved alveolarization (assessed by MLI) (349). 

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### Perspectives

**ADVANCES IN LUNG ALVEOLARIZATION AND BPD**

L1261

**Perspectives**

**AJP-Lung Cell Mol Physiol**

*doi:10.1152/ajplung.00268.2015*  *www.ajplung.org*
strains are maintained on mixed mouse strain backgrounds. It is clearly important that, in all experimental studies, the mouse strain, the backcrossing and background of the transgenic mice, and the sex of the mice should be clearly reported.

A large number of correlative studies have been reported since 2013, revealing expression changes in molecules of interest that correlate with arrested alveolarization. Demonstration of a causal role for these pathogenic candidates remains to be reported. Similarly, several transgenic mouse studies have causally implicated particular proteins or pathways in normal or arrested alveolarization, and the further delineation of these pathways to reveal new developmental mechanisms is eagerly awaited. New technologies (such as the clustered regularly interspaced short palindromic repeats/Cas9), which will massively ease genetic manipulation in vitro and in vivo, together with the continued production of new floxed mouse strains and driver lines, will open many doors for the further dissection of molecular pathways that drive normal and aberrant late lung development. Among these, the role of ECM remodeling, ECM cell cross talk, and cell-cell cross talk will no doubt feature in work that will emerge over the coming years, along with attention to the role of progenitor cells and distinct cell subpopulations and the regulation of alveolar epithelial cell plasticity, together with the mechanisms of disturbed vascular growth and homeostasis. It is clear that much challenging but exciting work lies ahead.

ACKNOWLEDGMENTS

The authors thank Dr. José Alberto Rodríguez Castillo (Morty Laboratory, Department of Lung Development and Remodelling, Max Planck Institute for Heart and Liver Research, Bad Nauheim, Germany) for outstanding assistance with the preparation of this Perspectives article.

GRANTS

This study was supported by the Max Planck Society (to D. Silva, C. Nardiello, A. Pozarska, and R. Morty), the German Research Foundation through grant Mo 1789/1 (to R. Morty), Excellence Cluster 147 “Cardio-Pulmonary System” (to R. Morty), the Federal Ministry of Higher Education, Research, and the Arts of the State of Hessen LOEWE Program (to R. Morty), and the German Center for Lung Research (Deutsches Zentrum für Lungenforschung; to D. Silva, C. Nardiello, A. Pozarska, and R. Morty).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.M.S., C.N., and A.P. prepared figures; D.M.S., C.N., A.P., and R.E.M. drafted manuscript; D.M.S., C.N., A.P., and R.E.M. edited and revised manuscript; D.M.S., C.N., A.P., and R.E.M. approved final version of manuscript.

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