nature publishing group

Administration of antenatal glucocorticoids and postnatal surfactant ameliorates respiratory distress syndrome–associated neonatal lethality in *Erk3*$^{-/-}$ mouse pups

Milenka Cuevas Guaman[1], Elena Sbrana[2], Cynthia Shope[3], Lori Showalter[3], Min Hu[3], Sylvain Meloche[4] and Kjersti Aagaard[3,5]

BACKGROUND: Respiratory distress syndrome (RDS) persists as a prevalent cause of infant morbidity and mortality. We have previously demonstrated that deletion of *Erk3* results in pulmonary immaturity and neonatal lethality. Using RNA sequencing, we identified corticotrophin releasing hormone (CRH) and surfactant protein B (SFTPB) as potential molecular mediators of *Erk3*-dependent lung maturation. In this study, we characterized the impact of antenatal glucocorticoids and postnatal surfactant on neonatal survival of *Erk3* null mice.

METHODS: In a double crossover design, we administered dexamethasone (dex) or saline to pregnant dams during the saccular stage of lung development, followed by postnatal surfactant or saline via inhalation intubation. Survival was recorded, and detailed lung histological analysis and staining for CRH and SFTPB protein expression were performed.

RESULTS: Without treatment, *Erk3* null pups die within 6 h of birth with reduced aerated space, impaired thinning of the alveolar septa, and abundant glycogen stores, as described in human RDS. The administration of dex and surfactant improved RDS-associated lethality of *Erk3*$^{-/-}$ pups and partially restored functional fetal lung maturation by accelerating the downregulation of pulmonary CRH and partially rescuing the production of SFTPB.

CONCLUSION: These findings emphasize that Erk3 is integral to terminal differentiation of type II cells, SFTPB production, and fetal pulmonary maturity.

Erk3 is a member of the MAP kinase family of serine/threonine kinases that play a key role in transducing environmental stimuli into a wide range of intracellular responses (1,2). The expression of *Erk3* is temporally regulated during embryonic development, increasing at the time of early organogenesis and declining after birth (3–5). The highest expression of *Erk3* is found in specific regions of the brain, lungs, skeletal muscle, and gastrointestinal tract (3,6,7). In the lung, *Erk3* is restricted to the distal lung epithelium during the pseudoglandular phase but shifted to the proximal airways during the saccular stage (8).

Targeted disruption of the *Erk3* gene in mice results in intrauterine growth restriction and lung immaturity with subsequent lethality (7). Histological and morphogenic characterization indicate that the type II pneumocytes of *Erk3*$^{-/-}$ mice differentiate to the stage of being able to synthesize surfactant-associated proteins but cannot complete full functional development (as demonstrated by abundant intracellular glycogen with early neonatal lethality from respiratory distress syndrome (RDS)) (7). We have recently extended these studies at the whole transcriptome level. RNA sequencing analysis of the lungs of wild-type and *Erk3*$^{-/-}$ mice revealed differential expression of genes related to glucocorticoid-induced lung maturation pathway. Specifically, we have observed downregulation of corticotrophin releasing hormone (CRH) in dexamethasone (dex)-treated lungs compared with saline-treated lungs. This is temporally associated with upregulation of surfactant B production, which is *Erk3* dependent.

The purpose of the current study was to better define the role of *Erk3* in lung maturation and in regulation of CRH and surfactant protein B (SFTPB) expression and to investigate the potential role of antenatal glucocorticoid therapy and exogenous surfactant in preventing severe respiratory distress and early death in newborn *Erk3* null pups.

RESULTS

Demonstrated Impact of Antenatal Dex and Postnatal Surfactant on Neonatal Survival

Three groups of mice were analyzed in this double crossover study (**Figure 1**). Group 1 received one dose of surfactant or saline and was followed up for a period of 24 h (neonatal demise or killing for histology at 24 h); group 2 similarly received one dose of surfactant or saline but was followed up for up to 72 h; and group 3 received three doses of surfactant or saline and was followed up to 1 wk of life. Group 1 showed a mean survival increase from 6 ± 0 to 16.2 ± 2.4 h among mice receiving

[1]Division of Neonatology, Department of Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas; [2]Department of Pathology, University of Texas Medical Branch-Galveston, Galveston, Texas; [3]Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas; [4]Institut de Recherche en Immunologie et Cancérologie, Université de Montreal, Montréal, Québec, Canada; [5]Department of Molecular and Cell Biology, Baylor College of Medicine, Houston, Texas. Correspondence: Kjersti Aagaard (aagaardt@bcm.tmc.edu)

Received 25 September 2013; accepted 3 January 2014; advance online publication 14 May 2014. doi:10.1038/pr.2014.54

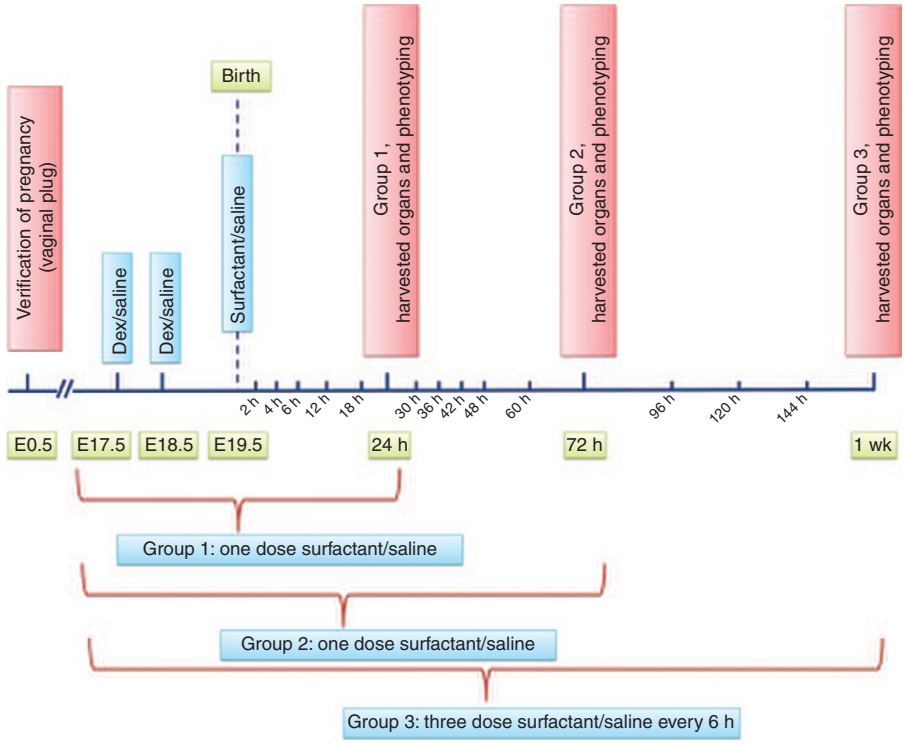

Figure 1. Timeline schematic of the double crossover design showing important events and defining the three groups. In the time line, the green squares demark the time point for interventions. With the verification of pregnancy indicated by the presence of vaginal plug on E0.5, timing of antenatal administration of dex/saline as thereafter designated. Birth uniformly occurred on E19–19.5, and deliveries were attended in order for the pups to be weighed, marked, and precise timing for rate of live birth survival to be calculated. The initial dose of surfactant or saline control was administered at the time of birth. Ongoing direct investigator observation and identification of neonatal demise vs. survival occurred at the indicated time points (black lettering). As noted, this occurred every 2 h for the first 6 h of life, then at 6-h intervals through 48 h. The light blue squares list each group definition. In all groups, dams received dex or saline then newborn pups of group 1 and 2 received one dose of surfactant, but in group 1, organs were harvested at 24 h and in group 2, organs were harvested at 72 h. In contrast, group 3 received three doses of surfactant/saline every 12 h, and organs were harvested at 1 wk. Rate of survival was calculated at 24 h (group 1), 72 h (group 2), and 7 d (group 3). Genotyping occurred at the time of neonatal killing, thus investigators were blinded to genotyping during the course of the experiment. dex, dexamethasone; E, embryonic day.

dex/surfactant vs. saline/saline, with 57% surviving to 24 h (**Figure 2a**). When the observation period was extended to 72 h, there was a significant difference in mean survival with lengthening from 4.3 ± 0.7 to 28.1 ± 4.4 h ($P = 0.01$), with 38% of pups surviving to 72 h (**Figure 2b**). We therefore reasoned that the initial benefit of surfactant may be lost after 24 h and next tested whether repeat dosing would improve the interval to survival. The mean survival improved from 1.5 ± 0.5 to 59.5 ± 21.6 h ($P < 0.01$), with 35% of animals surviving to 1 wk without evidence of RDS in this group (**Figure 2c**). In all three groups, survival of both *Erk3*$^{+/+}$ (**Figure 2d**) and *Erk3*$^{+/-}$ (**Figure 2e**) littermates were not altered by drug or control treatment, and there was no significant difference among homozygous and heterozygote littermates.

Detailed Histological Analysis of the Lungs

To assess the cellular impact of glucocorticoid and surfactant therapies, dams were treated with dex or saline at embryonic day (E) 17.5 and E18.5 of gestation, and *Erk3*$^{-/-}$ pups (identified by rapid genotyping) received either surfactant or saline at birth. Lungs were harvested at 3 h of life and processed for histology. Following strict histology guidelines (9), we observed that *Erk3*$^{-/-}$ mice receiving antenatal dex and postnatal surfactant administration demonstrate increased aerated lung area when compared to saline or dex alone ($P \leq 0.05$; **Figure 3b**) with thinner septal membranes ($P < 0.01$; **Figure 3c**). Dex alone and the combination of dex and surfactant were equally efficacious in reducing functionally immature glycogen laden pneumocytes ($P < 0.01$). Dex alone did not significantly alter neonatal survival relative to combination therapy (**Figure 2a**,**b**), likely due to a hydration effect by the administration of saline at birth. These results suggest that *Erk3* null pups are dying in early neonatal period secondary to a condition histologically and functionally analogous to RDS in humans.

Immunohistochemistry Analysis

Using a double crossover design, we administered antenatal dex or saline at E17.5 or E18.5. At birth, newborn pups of *Erk3*$^{-/-}$ and *Erk3*$^{+/+}$ genotypes received either surfactant or saline, and lung sections were obtained at 3 or 6 h of life. By immunohistochemistry (IHC) analysis, *Erk3*$^{-/-}$ pups treated with antenatal dex and postnatal surfactant demonstrated persistent attenuation of CRH expression (**Figure 4a**) when compared with controls (saline/saline; $P < 0.001$) and pups treated with dex alone ($P < 0.05$; **Figure 4b**). Moreover,

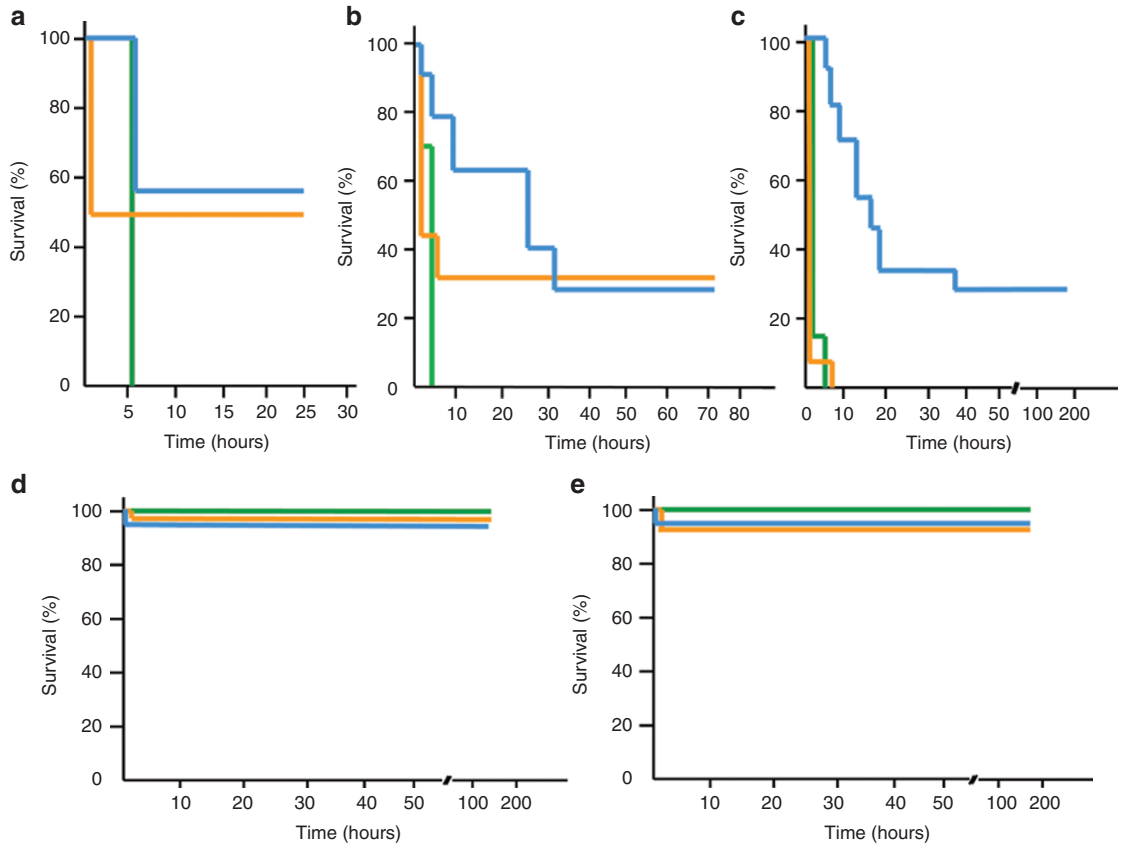

Figure 2. Enhanced free-RDS survival of *Erk3* knockout with dexamethasone (dex)/surfactant administration. Survival curves where y-axis represents survival in percentages, and x-axis represents time in hours, green represents saline/saline, orange is dex/saline, and blue is dex/surfactant. (**a**) Group 1 (killed at 24 h; $n = 16$): $Erk3^{-/-}$ mice that received antenatal dex or saline and one dose of postnatal surfactant or saline were compared with mice that received saline/saline and observed up to 24 h. Mean survival lengthened from 6 ± 0 to 16.2 ± 2.4 h with 57% surviving to 24 h. (**b**) Group 2 (killed at 72 h; $n = 26$): $Erk3^{-/-}$ mice that received antenatal dex or saline and one dose of postnatal surfactant or saline were compared with mice that received saline/saline and observed up to 72 h. Mean survival lengthened from 4.3 ± 0.7 to 28.1 ± 4.4 h ($P = 0.01$) with 38% surviving to 72 h. (**c**) Group 3 (killed at 7 d; $n = 30$): $Erk3^{-/-}$ mice that received antenatal dex or saline and one dose of postnatal surfactant or saline were compared with mice that received saline/saline and observed up to 7 d. Mean survival improved from 1.5 ± 0.5 to 59.5 ± 21.6 h ($P < 0.01$) with 35% surviving to 1 wk without evidence of RDS. (**d**) Groups 1, 2, and 3 ($n = 30$): $Erk3^{+/+}$ littermates were observed with <5% mortality observed on all groups. (**e**) Groups 1, 2, and 3 ($n = 30$): $Erk3^{+/-}$ heterozygote littermates. Less than 8% mortality observed for all groups. There was no significant difference in survival among $Erk3^{+/+}$ and $Erk3^{+/-}$ littermates under all treatment conditions. RDS, respiratory distress syndrome.

administration of antenatal dex and postnatal surfactant in $Erk3^{-/-}$ pups persistently demonstrated attenuation of CRH up to 6 h of life when compared with controls (saline/saline; $P < 0.01$; **Figure 5a**,**b**).

Dex- and surfactant-treated $Erk3^{-/-}$ pups demonstrated increased SFTPB production when compared with pups treated with antenatal dex alone ($P < 0.001$) or saline alone ($P = 0.025$; **Figure 4d**). The increase in SFTPB production continued up to 6 h of life ($P = 0.006$; **Figure 5c**,**d**). Functional and histological findings significantly correlated with SFTPB expression *in situ* (**Figure 4c**).

DISCUSSION

We have developed a novel murine model to study the molecular mechanisms underlying functional pulmonary maturation and neonatal RDS. We have demonstrated that administration of antenatal glucocorticoids (dex) and postnatal surfactant significantly improve neonatal RDS and survival in an *Erk3* null murine model.

These studies have necessitated several technical innovations which are of likely interest to translational investigators, including neonatal drug inhalation. In a pilot study to determine the best method of surfactant administration, we initially tested three distinct techniques. The only published literature of drug instillation techniques in mice used significantly larger juvenile or adult animals (10–12). Intubation and tracheostomy proved infeasible due to the newborn pups' small size. Drug administration itself could also be fatal. We initially attempted to modify the tracheal installation technique used in adult mice to our pups (13,14) using a syringe with a 26 gauge needle to inject surfactant through the neck directly into the trachea, but found that mortality was high with this technique. The use of nasal instillation, wherein surfactant or saline is administered through the nares, was similarly unsuccessful. Administration time was 60 min for half of the dose, and the pups became hypothermic, and many died during the treatment. Finally, we modified the inhalation intubation technique (15,16). Using a micropipette, we placed the semi-flexible

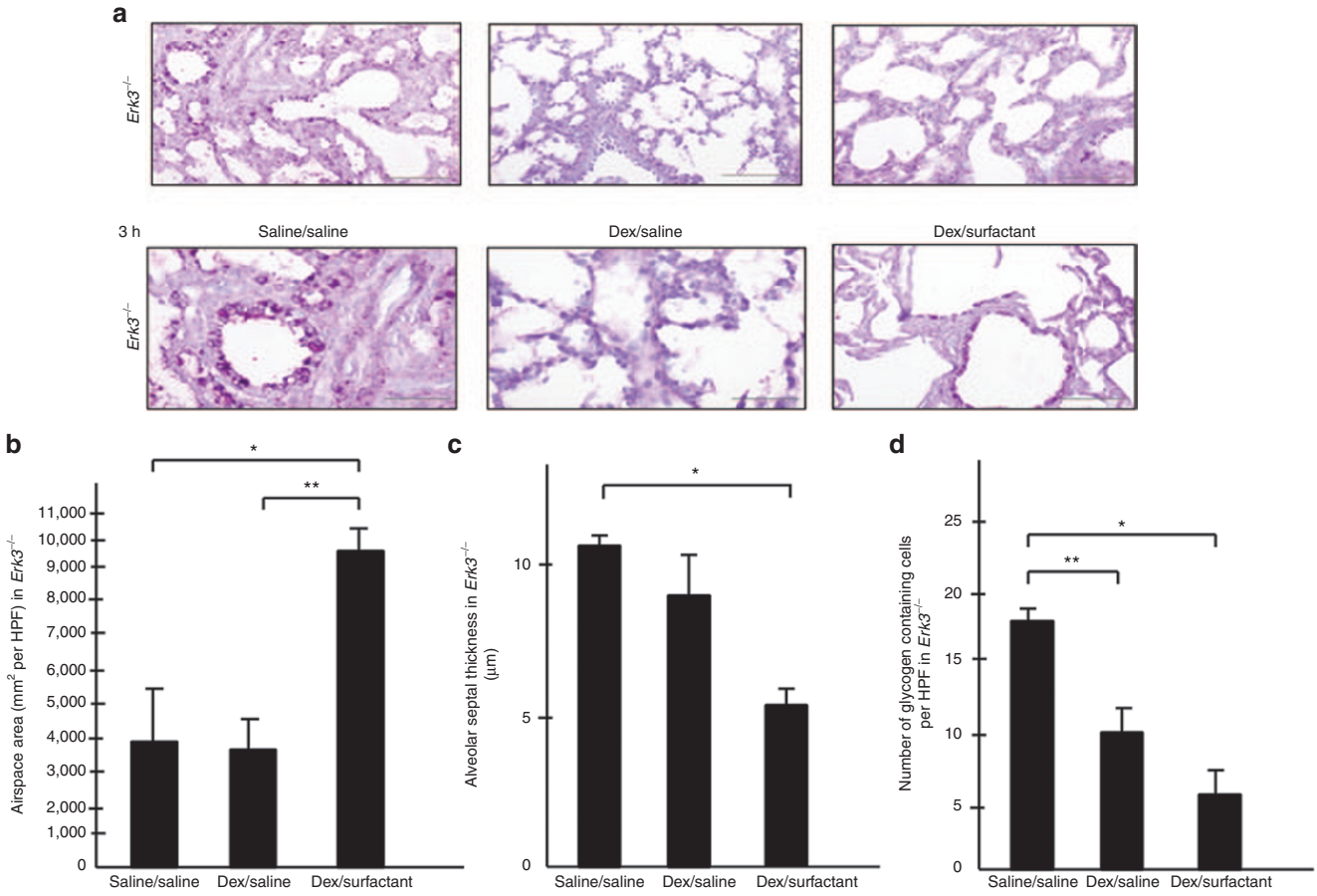

Figure 3. *Erk3*$^{-/-}$ pups demonstrate histological and morphologic classical hallmarks of respiratory distress syndrome (RDS) at 3 h of life. (**a**) Pregnant females from *Erk3* intercrosses were treated with dexamethasone (dex) or saline as described. To avoid potential artifact from end-stage RDS, neonates were killed at 3 h postnatally. Lung sections were stained with periodic acid–Schiff staining. Magnification: top row, ×20 and bottom row, ×40. Histological sections demonstrative of five littermates in each treatment group. (**b**) Airspace quantification (dissector and section area) in lung sections from *Erk3*$^{-/-}$ ($n = 5$) as described for panel **a**. Antenatal dex combined with postnatal surfactant administration in *Erk3*$^{-/-}$ mice increased aerated lung area when compared with saline alone, or in combination with dex alone (panel **b**; $^{*}P = 0.05$; $^{**}P = 0.03$) alongside thinner septal membranes (panel **c**; $^{*}P = 0.01$). Dex alone and the combination of dex and surfactant was equally efficacious in reducing histological immature glycogen laden pneumocytes (panel **d**; $^{**}P = 0.02$; $^{*}P = 0.01$). HPF, high-power field.

blunted pipette tip as close to the trachea as possible through the pups' open mouths and administered the solution (saline or surfactant). We found that if the administered solution was delivered to the lungs, bubbles would arise from the nares. To further confirm pulmonary surfactant delivery, we stained the saline or surfactant solution with indigo carmine and were able to visualize the color at the level of the lungs through the pups' skin. Although we found that the inhalation intubation technique is reproducible, we cannot verify that all of the dosage reached the lungs. In published studies using this technique in adult mice, ~85% of drug enters the lungs (15). These results are comparable to intratracheal injection (15,17).

We used Survanta, a pulmonary surfactant suspension from natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and surfactant-associated proteins SFTPB and SFTPC. It does not contain SFTPA (18). We chose this solution because it has the lowest concentration of SFTPB. Survanta contains 25 mg phospholipids per ml (18,19). Current published studies reference surfactant dosages from 50 to 200 mg/kg in adult mice, and there is no significant difference in dose response (13,20,21). We therefore selected the lowest recommended dose, 50 mg/kg of the phospholipid component, to administer the lowest possible volume (22), as it is less traumatic to the pups. Similarly, we modified the administration period of antenatal glucocorticoid from E16.5 and E17.5 used in previous studies (7) to E17.5 and E18.5 to more closely correspond with the saccular stage of lung development. This is the same stage when glucocorticoids are used in human development to improve lung maturity for preterm infants (23–25).

We have previously demonstrated uniform neonatal lethality due to lung immaturity by 6 h of life among *Erk3*$^{-/-}$ pups (7). With the introduction of surfactant among all groups of Erk3 null pups, neonatal surfactant with antenatal dex resulted in enhanced survival. Our observation of partial phenotypic abrogation with antenatal glucocorticoid administration and postnatal surfactant administration provides strong evidence for an *Erk3* pathway of type II pneumocyte differentiation and maturation.

Histological analysis of *Erk3*-deficient lungs revealed that overall organogenesis of the lungs was preserved, and there

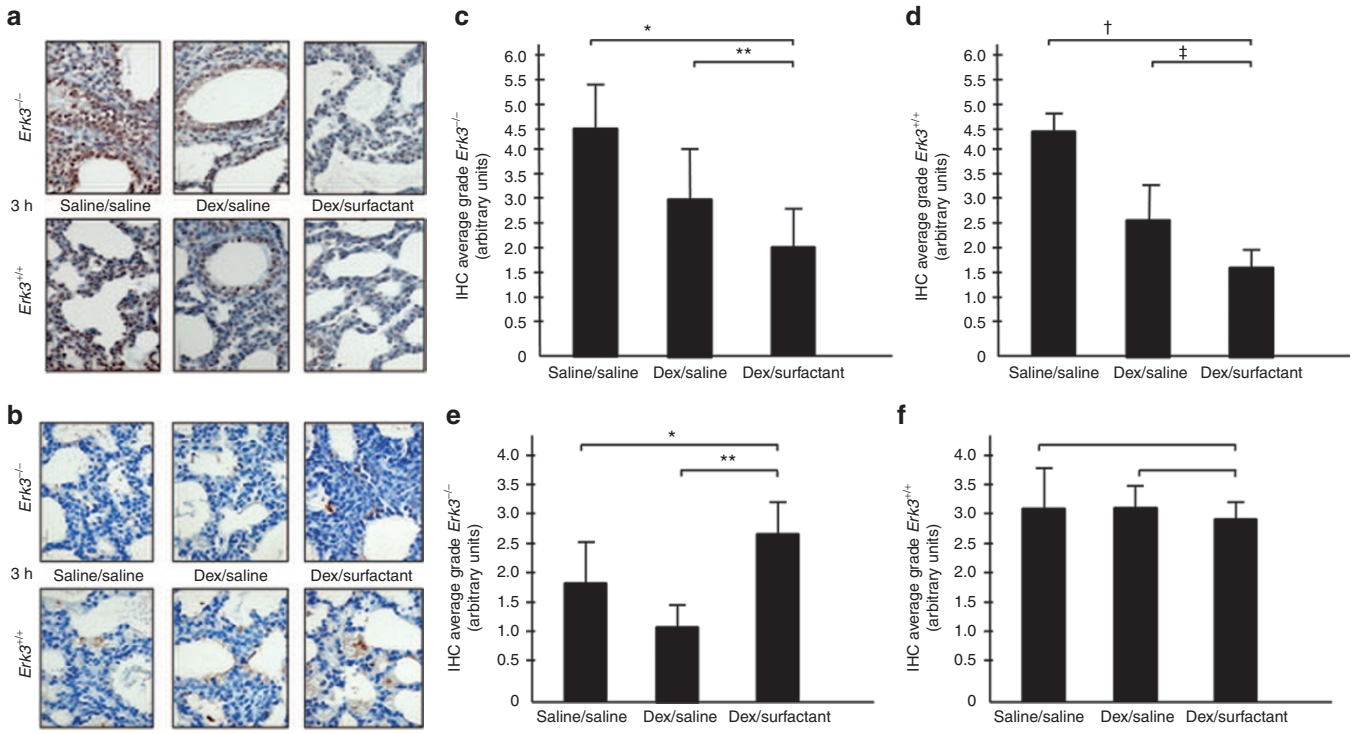

Figure 4. Antenatal dexamethasone (dex) accelerated corticotrophin releasing hormone (CRH) downregulation in both Erk3$^{+/+}$ and Erk3$^{-/-}$, and postnatal surfactant rescued surfactant protein B (SFTPB) production among Erk3$^{-/-}$ pups at 3 h of life. Dams from Erk3 intercrosses were treated with dex or saline, and newborn pups were administered surfactant or saline. Lungs were harvested at 3 h of life. (**a**) IHC of lung sections stained with DAB chromagen for CRH. Magnification: ×40. (**b**) IHC of lung sections stained with DAB chromagen for SFTPB. Magnification: ×40. (**c**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{-/-}$ pups ($n = 5$). Significant attenuation of CRH with saline (*$P < 0.001$) or dex alone (**$P < 0.05$) compared with dex and surfactant. (**d**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{+/+}$ pups ($n = 5$). Significant attenuation of CRH with saline ($^{\dagger}P < 0.05$) or dex alone ($^{\ddagger}P < 0.05$) compared with dex and surfactant. (**e**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{-/-}$ pups ($n = 5$). Significant rescue of postnatal SFTPB production relative to antenatal dex (**$P < 0.001$) or saline (*$P = 0.025$). (**f**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{+/+}$ pups ($n = 5$). No significant difference in SFTPB expression. IHC, immunohistochemistry.

were no obvious defects in branching morphogenesis (7). We assessed the differentiation of type II pneumocytes by analyzing the expression of pulmonary SFTPC and measuring the content of cytoplasmic glycogen, which serves as a substrate for surfactant phospholipids (26), in lungs from E18.5 embryos. Immunoreactive SFTPC was not altered in *Erk3*$^{-/-}$ mice. Ultrastructural analysis by electron microscopy revealed that type II pneumocytes from *Erk3* null mutant mice contain abundant glycogen granules (qualitative and quantitative) as compared with controls. Antenatal glucocorticoids partially rescued type II pneumocyte differentiation restoring glycogen levels to those of normal wild-type mice. Nevertheless, they had no effect on neonatal morbidity nor did they rescue *Erk3*-mediated modification of fetal growth potential. We have further characterized lung histology of *Erk3* null mice demonstrating that their lethality is related to the classic histology described in RDS in humans. The replacement of surfactant in this mouse model of RDS extended survival, restored the glycogen content of type II pneumocytes to normal levels, and improved their architecture. Although the surfactant effect on survival is lost after 24 h, these findings suggest that *Erk3* is integral to terminal differentiation of type II cells, SFTPB production, and fetal pulmonary maturity.

The association of the attenuation of pulmonary CRH by administration of glucocorticoids is consistent with data from an unrelated mouse model of CRH deficiency (27), suggesting a fetal glucocorticoid requirement for lung maturation (28). Postnatally, the CRH knockout mice demonstrated normal growth and longevity, suggesting that the major role of glucocorticoid is during fetal rather than postnatal life. The attenuation of pulmonary CRH in our *Erk3* null mice combined with published data in humans showing high levels of CRH in maternal serum correlating with fetal lung maturity suggests the existence of a feedback loop between CRH and fetal lung maturation. Moreover, it indicates that CRH is a significant molecular mediator of pulmonary maturation.

CRH has been put forward as a key neuroendocrine modulator of pregnancy and parturition (29–33). During midgestation, there is a rapid increase in the concentration of CRH in the maternal plasma (34). This occurs alongside production of surfactant in the fetal human lung beginning after week 24 of gestation, which corresponds to the saccular stage of lung development and the earliest consistently documented threshold of viability. The second peak in CRH concentration occurs during the last few weeks of pregnancy (29). In groups matched by gestational age in proximity to term, larger mean values of CRH are observed among neonates in which

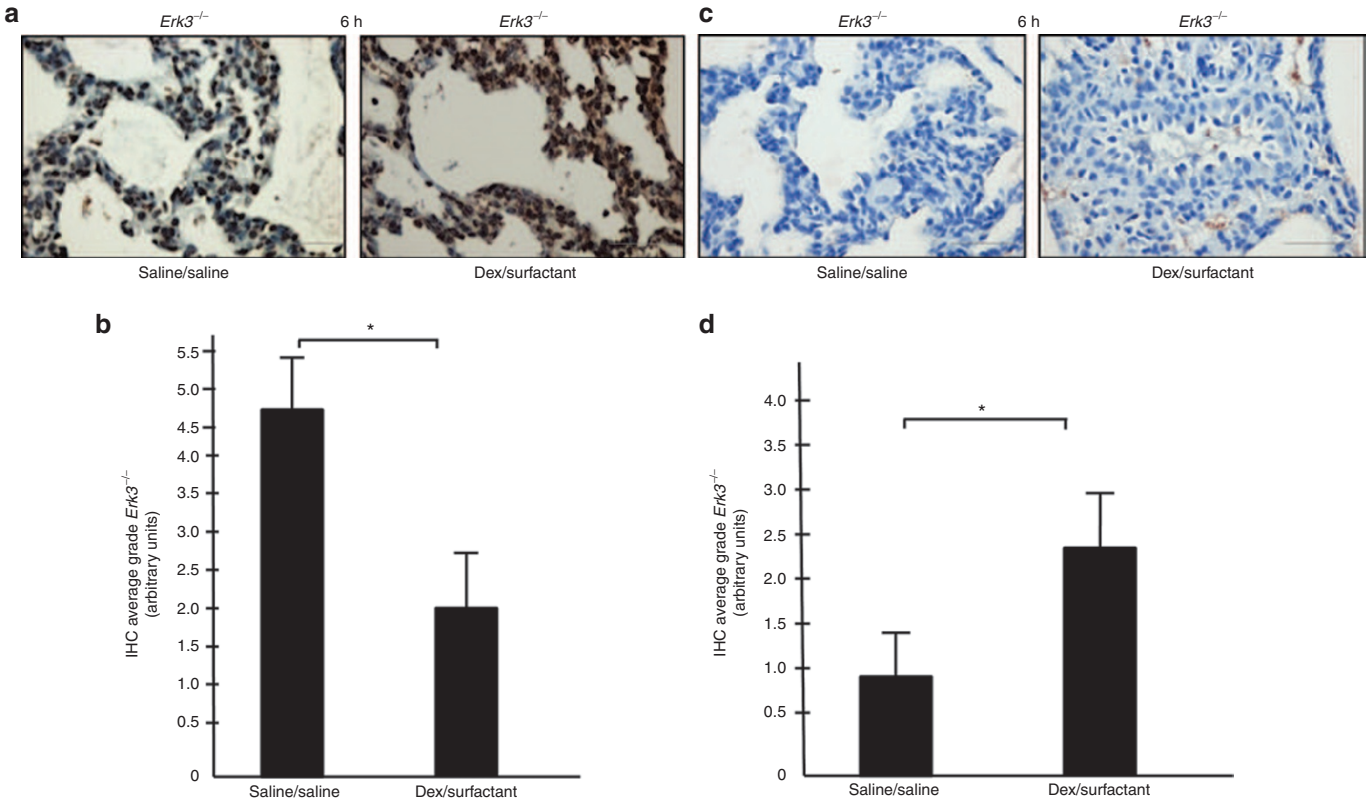

Figure 5. Antenatal dexamethasone and postnatal surfactant continue to downregulate CRH in Erk3 knockout mice, and postnatal surfactant rescued SFTPB production at 6 h of life. Pregnant females from Erk3 intercrosses were treated with dexamethasone or saline alongside newborn pups that postnatally received surfactant or saline. Lungs were harvested at 6 h of life. (**a**) Immunohistochemistry photomicrographs of lung sections were stained with DAB chromagen for CRH. Magnification: ×40. (**b**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{-/-}$ pups ($n = 5$). Significant attenuation of CRH is observed (*$P < 0.001$). (**c**) Immunohistochemistry photomicrographs of lung sections were stained with DAB chromagen for SFTPB. Magnification: ×40. (**d**) Quantitation of DAB chromagen staining in lung sections from Erk3$^{-/-}$ pups ($n = 5$). Significant increased SFTPB production relative to saline (*$P = 0.006$) at 6 h of life. CRH, corticotrophin releasing hormone; SFTPB, surfactant protein B.

the lecithin/sphingomyelin ratio was greater than two or the phosphatidylglycerol test for lung maturity was positive (35). These could explain a possible relationship between the peaks in CRH in human maternal plasma with the attenuation of CRH in the lungs that we find in our murine model. Further work is needed to discover what cells are producing or expressing CRH at the level of the lung and to continue to discover other molecular mediators of glucocorticoid-induced lung maturation.

In sum, we have demonstrated that neonatal surfactant combined with antenatal glucocorticoids enhances neonatal RDS-free survival in a murine model of *Erk3*-dependent pulmonary maturation and neonatal survival. Treatment with antenatal dex partially restored functional fetal lung maturation by accelerating the downregulation of pulmonary CRH (*Erk3* indirect or independent) and administration of postnatal surfactant partially to rescued endogenous production of SFTPB (*Erk3* dependent). Collectively, our results suggest a functional role to the elusive atypical MAP kinase Erk3, demonstrating its essential function in terminal functional differentiation of type II pneumocytes and SFTPB production (**Figure 6**).

To date, no treatment prevents RDS, and therapy is both costly and of limited benefit. Thus, there is a need to explore additional pathways to facilitate new therapeutic developments in this arena. Ongoing work to further elucidate the relationship of Erk3 with the development of RDS and SFTPB is one pathway which may lead to these developments. It is also unclear to what extent regulatory networks involved in type II cell maturation are involved in alveolar repair and reconstitution of surfactant homeostasis (i.e., bronchopulmonary dysplasia). Our described work herein will lay the foundation for these and future studies.

METHODS

Animal Husbandry and Survival Analysis

Animals were housed under pathogen-free conditions according to the procedures and protocols approved by the Institutional Review Board at the Baylor College of Medicine. For breeding, heterozygous males and females were intercrossed in mixed C57BL/6×129/Sv background. The presence of a vaginal plug indicated the beginning of gestation (E0.5).

Pups were attended at the time of delivery, and weighed at birth; total litter counts and pup weights were recorded and pups were marked. In a double crossover design, we subcutaneously administered 0.4 mg/kg prenatal dex (Sandoz, Princeton, NJ) or saline at E17.5 and E18.5, alongside phospholipids-enriched surfactant (Survanta (beractant) 25 mg/ml suspension; Abbott Nutrition, Columbus, OH) or saline via inhalation intubation at birth. Confirmation of pulmonary Survanta receipt was visualized by confirming indigo carmine costain at the level of the lungs, and neonatal survival with successful feeding was confirmed by the presence or absence of a milk spot.

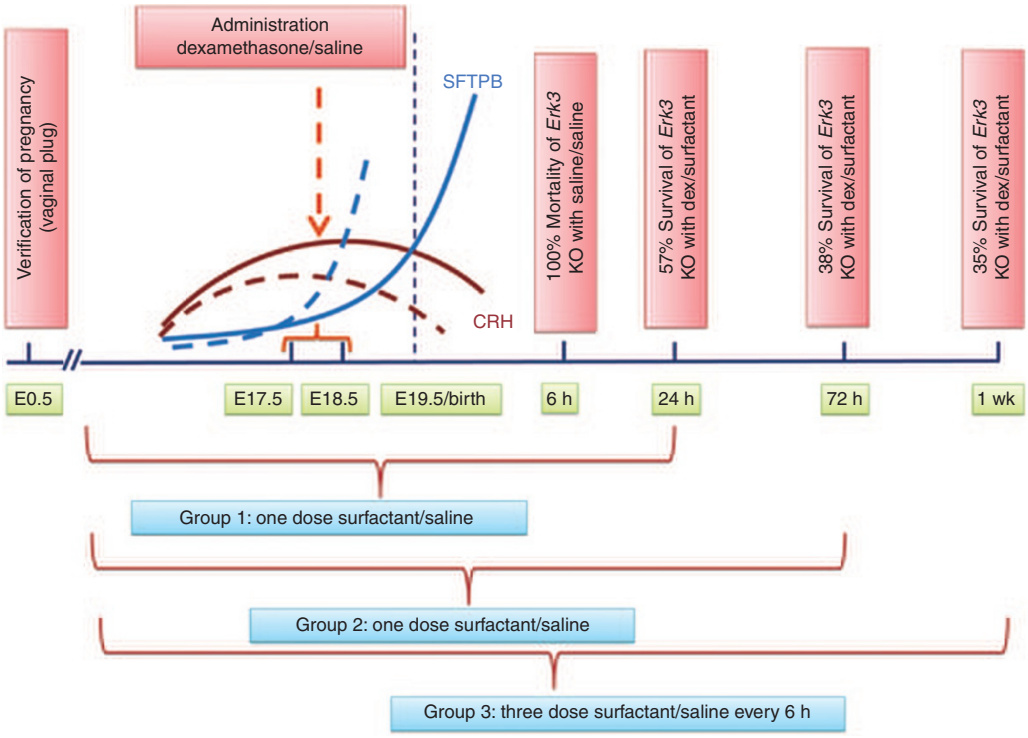

Figure 6. Administration of antenatal glucocorticoids (dex) and postnatal surfactant significantly improve neonatal RDS-free survival in an *Erk3* null murine model. Red and blue solid lines represent endogenous pulmonary CRH and SFTPB protein expression in lungs of *Erk3* null newborn pups that received control (saline) treatments. Dashed lines represent CRH and SFTPB levels when antenatal dexamethasone and postnatal surfactant are administered, which in turn leads to significantly improved neonatal survival as explained on the light pink squares. CRH, corticotrophin releasing hormone; KO, knockout; RDS, respiratory distress syndrome; SFTPB, surfactant protein B.

There were 3 experimental groups; each group contained 16 littermates with an average of 6 to 8 pups per litter. Group 1 received one dose of surfactant; lungs, brain, heart, and liver were harvested and phenotype analysis was performed at 24 h; survival was calculated as the number alive pups at 24 h. Group 2 received one dose of surfactant, and organ harvest and phenotype were performed at 72 h; survival was calculated as the number alive pups at 72 h. Group 3 received three doses of surfactant every 12 h and organ harvest and phenotype were performed at 7 d. Survival was recorded every 2 h for the first 6 h, every 6 h up to 48 h, every 12 h until 72 h, and every 24 h until 7 d (**Figure 1**).

Administration of Surfactant

Inhalation intubation technique. Pups were placed in 80% supine position and braced at the level of the scapula. A soft blunt-end micropipette tip was introduced into their open mouths and placed as proximal to the trachea as possible. Either saline or surfactant at 50 mg/kg of the phospholipid component was given. Survanta contains 25 mg of the phospholipid component per 1 ml, so the volume averaged between 2 and 3 µl depending upon pup weights and was rapidly administered over 30 s. For the administration, the entire litter was removed briefly from the cage at once and returned after all pups received the solution. Confirmation of pulmonary Survanta intake was visualized by mixing surfactant with indigo carmine 0.08% (Akron, Lake Forest, IL) in a 10:1 dilution to enable identification of surfactant receipt as a grossly visible blue stain at the level of the thorax or subscapular muscle, through the thin neonatal skin.

Detailed Lung Histology

Lungs were harvested at detailed time intervals, then fixed, and sectioned via systematic uniform random sampling (9,36,37). Each slide was examined for quantitative morphometric of periodic acid–Schiff (glycogen laden) staining (38), airspace quantification (dissector and section area) (9,39), and septal thickness (Image ProPlus; Media Cybernetics, Rockville, MD) (9,38).

Immunohistochemistry

Protein IHC was performed on postnatal lung sections harvested at indicated time intervals. Primary antibodies employed were CRH rabbit polyclonal (Abcam, Cambridge, MA) and SFTPB rabbit polyclonal (Millipore, Temecula, CA) antibodies. Unstained paraffin sections were deparaffinized in four changes of xylene for 5 min each and rehydrated through a series of graded alcohols with a final rinse in distilled water. Endogenous peroxidase was quenched by soaking sections in two solutions of 3% H_2O_2 in methanol for 10 min at room temperature. Prior to staining, antigen retrieval was performed to facilitate antibody binding to antigen. Slides were incubated at 99 °C under pressure for 30 min in either Tris-EDTA buffer, pH 8.0 (Sigma-Aldrich, St Louis, MO). No antigen retrieval step was required for staining with anti-SFTPB. Slides were then allowed to cool down for 10 min in the same solution, rinsed in three changes of distilled water, and placed in Tris-buffered saline with Tween 20, pH 7.4 (Signet Pathology Systems, Dedham, MA) for 5 min to decrease surface tension and facilitate coating by the subsequent reagents. The PolyVue HRP/DAB nonbiotin polymer detection system (Diagnostic Biosystems, Pleasanton, CA) was used in the immunostaining protocols for CRH, while the Rabbit-on-rodent Detection System (Biocare Medical, Concord, CA) was used for SFTPB. Incubations occurred at room temperature unless otherwise specified, and for each step, the sections were coated with 200 µl of reagent. Tris-buffered saline with Tween 20, pH 7.4, was used to rinse the sections between each of the IHC steps. Background Sniper solution (Biocare Medical) was used to block nonspecific staining for 10 min at room temperature. The primary antibody was diluted using renaissance antibody diluents (Biocare Medical), at a dilution of 1:400 for CRH, and 1:2,000 for SFTPB. Slides were incubated with the primary antibody solution overnight at 4 °C. Sections were then incubated in the universal secondary antibody provided with the kit for 15 min, followed by the horseradish peroxidase label reagent. Afterwards, Stable DAB Plus (Diagnostic Biosystems) was applied for 5 min as chromagen. The slides were rinsed in distilled water and manually counterstained

with Harris Hematoxylin (Fisher Scientific, Pittsburgh, PA) for 15–30 s and rinsed in distilled water. Coverslips were then applied to each slide using synthetic glass and permount mounting media. Negative controls and nonspecific antibodies were included in each immunostaining procedure, and both were negative.

SFTPB. Immunostained slides were examined by reviewers masked to whether the tissue originated from an animal exposed to corticosteroid treatment or not. For each slide examined, five random high-power fields were graded using a 0 to 3 scale, where 0 indicated the absence of positive staining and 3 indicated intense and diffuse positive staining.

CRH. Immunostained slides were examined by reviewers masked to whether the tissue originated from an animal exposed to corticosteroid treatment or not. For each slide examined, five random high-power fields were graded using a 0 to 5 scale, where 0 indicated the absence of positive staining and 5 indicated intense and diffuse positive staining. The location of positive staining areas was also recorded.

Statistical Analysis

Survival analysis. The data was analyzed with Kaplan–Meier survival analysis using Sigma Plot 11 with minimal significance designated at $P < 0.05$.

Lung histology analysis. Data was analyzed with ANOVA or an independent samples *t*-test as appropriate using SPSS V 11.5 with minimal significance designated at $P < 0.05$ ($n = 5$).

IHC analysis. The average of all grades was calculated for each slide, and IHC grades of were compared across the treated and untreated groups using the independent sample *t*-test after the equal variance test was performed. The statistical software package SPSS v 11.5 with minimal significance designated at $P < 0.05$ ($n = 5$) was employed.

STATEMENT OF FINANCIAL SUPPORT

This work was supported by the US National Institutes of Health (NIH; Bethesda, MD) Director New Innovator Pioneer Award (K.A.) and National Institute of Child Health and Human Development (NICHD) March of Dimes Reproductive Scientist Development Program (K.A.).

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure: No financial ties to products in the study or potential/perceived conflicts of interest.

REFERENCES

1. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–83.
2. Coulombe P, Meloche S. Atypical mitogen-activated protein kinases: structure, regulation and functions. Biochimica et Biophysica Acta 2007;8:7.
3. Turgeon B, Saba-El-Leil MK, Meloche S. Cloning and characterization of mouse extracellular-signal-regulated protein kinase 3 as a unique gene product of 100 kDa. Biochem J 2000;346:Pt 1:169–75.
4. Aberg E, Perander M, Johansen B, et al. Regulation of MAPK-activated protein kinase 5 activity and subcellular localization by the atypical MAPK ERK4/MAPK4. J Biol Chem 2006;281:35499–510.
5. Julien C, Coulombe P, Meloche S. Nuclear export of ERK3 by a CRM1-dependent mechanism regulates its inhibitory action on cell cycle progression. J Biol Chem 2003;278:42615–24.
6. Schumacher S, Laass K, Kant S, et al. Scaffolding by ERK3 regulates MK5 in development. EMBO J 2004;23:4770–9.
7. Klinger S, Turgeon B, Lévesque K, Wood GA, Aagaard-Tillery KM, Meloche S. Loss of Erk3 function in mice leads to intrauterine growth restriction, pulmonary immaturity, and neonatal lethality. Proc Natl Acad Sci USA 2009;106:16710–5.
8. Kling DE, Brandon KL, Sollinger CA, et al. Distribution of ERK1/2 and ERK3 during normal rat fetal lung development. Anat Embryol (Berl) 2006;211:139–53.
9. Hsia CC, Hyde DM, Ochs M, Weibel ER; ATS/ERS Joint Task Force on Quantitative Assessment of Lung Structure. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. Am J Respir Crit Care Med 2010;181:394–418.
10. Rasaiah VP, Malloy JL, Lewis JF, Veldhuizen RA. Early surfactant administration protects against lung dysfunction in a mouse model of ARDS. Am J Physiol Lung Cell Mol Physiol 2003;284:L783–90.
11. Iwasaki T, Tamura S, Kumasaka T, et al. Exacerbation of influenzavirus pneumonia by intranasal administration of surfactant in a mouse model. Arch Virol 1999;144:675–85.
12. Fukushi M, Yamashita M, Miyoshi-Akiyama T, Kubo S, Yamamoto K, Kudo K. Laninamivir octanoate and artificial surfactant combination therapy significantly increases survival of mice infected with lethal influenza H1N1 Virus. PLoS One 2012;7:e42419.
13. Mikawa K, Nishina K, Takao Y, Obara H. Intratracheal application of recombinant surfactant protein-C surfactant to rabbits attenuates acute lung injury induced by intratracheal acidified infant formula. Anesth Analg 2004;98:1273–9, table of contents.
14. Yap KL. A method for intratracheal inoculation of mice. Lab Anim 1982;16:143–5.
15. Yu H, Zeidan YH, Wu BX, et al. Defective acid sphingomyelinase pathway with Pseudomonas aeruginosa infection in cystic fibrosis. Am J Respir Cell Mol Biol 2009;41:367–75.
16. Revelli DA, Boylan JA, Gherardini FC. A non-invasive intratracheal inoculation method for the study of pulmonary melioidosis. Front Cell Infect Microbiol 2012;2:164.
17. Driscoll KE, Costa DL, Hatch G, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicol Sci 2000;55:24–35.
18. Blanco O, Pérez-Gil J. Biochemical and pharmacological differences between preparations of exogenous natural surfactant used to treat respiratory distress syndrome: role of the different components in an efficient pulmonary surfactant. Eur J Pharmacol 2007;568:1–15.
19. Rüdiger M, Tölle A, Meier W, Rüstow B. Naturally derived commercial surfactants differ in composition of surfactant lipids and in surface viscosity. Am J Physiol Lung Cell Mol Physiol 2005;288:L379–83.
20. Kramer BW, Jobe AH, Ikegami M. Exogenous surfactant changes the phenotype of alveolar macrophages in mice. Am J Physiol Lung Cell Mol Physiol 2001;280:L689–94.
21. Salez L, Balloy V, van Rooijen N, et al. Surfactant protein A suppresses lipopolysaccharide-induced IL-10 production by murine macrophages. J Immunol 2001;166:6376–82.
22. Starcher B, Williams I. A method for intratracheal instillation of endotoxin into the lungs of mice. Lab Anim 1989;23:234–40.
23. Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol 2001;32:76–91.
24. Grier DG, Halliday HL. Effects of glucocorticoids on fetal and neonatal lung development. Treat Respir Med 2004;3:295–306.
25. Jobe AH. Glucocorticoids, inflammation and the perinatal lung. Semin Neonatol 2001;6:331–42.
26. Ridsdale R, Post M. Surfactant lipid synthesis and lamellar body formation in glycogen-laden type II cells. Am J Physiol Lung Cell Mol Physiol 2004;287:L743–51.
27. Muglia LJ, Jenkins NA, Gilbert DJ, Copeland NG, Majzoub JA. Expression of the mouse corticotropin-releasing hormone gene *in vivo* and targeted inactivation in embryonic stem cells. J Clin Invest 1994;93:2066–72.
28. Muglia L, Jacobson L, Dikkes P, Majzoub JA. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 1995;373:427–32.
29. Petraglia F, Imperatore A, Challis JR. Neuroendocrine mechanisms in pregnancy and parturition. Endocr Rev 2010;31:783–816.
30. Reis FM, Fadalti M, Florio P, Petraglia F. Putative role of placental corticotropin-releasing factor in the mechanisms of human parturition. J Soc Gynecol Investig 1999;6:109–19.
31. Smith R, Nicholson RC. Corticotrophin releasing hormone and the timing of birth. Front Biosci 2007;12:912–8.

32. McLean M, Smith R. Corticotrophin-releasing hormone and human parturition. Reproduction 2001;121:493–501.
33. Smith R. Alterations in the hypothalamic pituitary adrenal axis during pregnancy and the placental clock that determines the length of parturition. J Reprod Immunol 1998;39:215–20.
34. Siler-Khodr TM, Forthman G, Khodr C, Matyszczyk S, Khodr Z, Khodr G. Maternal serum corticotropin-releasing hormone at midgestation in Hispanic and white women. Obstet Gynecol 2003;101:557–64.
35. Laatikainen TJ, Räisänen IJ, Salminen KR. Corticotropin-releasing hormone in amniotic fluid during gestation and labor and in relation to fetal lung maturation. Am J Obstet Gynecol 1988;159:891–5.
36. Weibel ER, Hsia CC, Ochs M. How much is there really? Why stereology is essential in lung morphometry. J Appl Physiol (1985) 2007;102:459–67.
37. Brusasco V, Dinh-Xuan AT. Stereology: a bridge to a better understanding of lung structure and function. Eur Respir J 2010;35:477–8.
38. Ikeda H, Shiojima I, Oka T, et al. Increased Akt-mTOR signaling in lung epithelium is associated with respiratory distress syndrome in mice. Mol Cell Biol 2011;31:1054–65.
39. Compernolle V, Brusselmans K, Acker T, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med 2002;8:702–10.