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Structural and transcriptomic response to antenatal corticosteroids in an *Erk3*-null mouse model of respiratory distress

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Abstract

BACKGROUND—Neonatal respiratory distress syndrome in preterm infants is a leading cause of neonatal death. Pulmonary insufficiency-related infant mortality rates have improved with antenatal glucocorticoid treatment and neonatal surfactant replacement. However, the mechanism of glucocorticoid-promoted fetal lung maturation is not understood fully, despite decades of clinical use. We previously have shown that genetic deletion of *Erk3* in mice results in growth restriction, cyanosis, and early neonatal lethality because of pulmonary immaturity and respiratory distress. Recently, we demonstrated that the addition of postnatal surfactant administration to antenatal dexamethasone treatment resulted in enhanced survival of neonatal *Erk3*-null mice.

OBJECTIVE—To better understand the molecular underpinnings of corticosteroid-mediated lung maturation, we used high-throughput transcriptomic and high-resolution morphologic analysis of the murine fetal lung. We sought to examine the alterations in fetal lung structure and function that are associated with neonatal respiratory distress and antenatal glucocorticoid treatment.

STUDY DESIGN—Dexamethasone (0.4 mg/kg) or saline solution was administered to pregnant dams on embryonic days 16.5 and 17.5. Fetal lungs were collected and analyzed by microCT and RNA-seq for differential gene expression and pathway interactions with genotype and treatment. Results from transcriptomic analysis guided further investigation of candidate genes with the use of immunostaining in murine and human fetal lung tissue.

RESULTS—*Erk3*^{−/−} mice exhibited atelectasis with decreased overall porosity and saccular space relative to wild type, which was ameliorated by glucocorticoid treatment. Of 596 differentially

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expressed genes ($q < 0.05$) that were detected by RNA-seq, pathway analysis revealed 36 genes ($q < 0.05$) interacting with dexamethasone, several with roles in lung development, which included corticotropin-releasing hormone and surfactant protein B. Corticotropin-releasing hormone protein was detected in wild-type and *Erk3*^{-/-} lungs at E14.5, with significantly temporally altered expression through embryonic day 18.5. Antenatal dexamethasone attenuated corticotropin-releasing hormone at embryonic day 18.5 in both wild-type and *Erk3*^{-/-} lungs (0.56-fold and 0.67-fold; $P < .001$). Wild type mice responded to glucocorticoid administration with increased pulmonary surfactant protein B ($P = .003$). In contrast, dexamethasone treatment in *Erk3*^{-/-} mice resulted in decreased surfactant protein B ($P = .012$). In human validation studies, we confirmed that corticotropin-releasing hormone protein is present in the fetal lung at 18 weeks of gestation and increases in expression with progression towards viability (22 weeks of gestation; $P < .01$).

CONCLUSION—Characterization of whole transcriptome gene expression revealed glucocorticoid-mediated regulation of corticotropin-releasing hormone and surfactant protein B via *Erk3*-independent and -dependent mechanisms, respectively. We demonstrated for the first time the expression and temporal regulation of corticotropin-releasing hormone protein in midtrimester human fetal lung. This unique model allows the effects of corticosteroids on fetal pulmonary morphologic condition to be distinguished from functional gene pathway regulation. These findings implicate *Erk3* as a potentially important molecular mediator of antenatal glucocorticoid action in promoting surfactant protein production in the preterm neonatal lung and expanding our understanding of key mechanisms of clinical therapy to improve neonatal survival.

Keywords

antenatal glucocorticoid; CRH; ERK3; fetal lung maturation; SFTPB

Since the initial observations of Liggins in 1969 that demonstrated dexamethasone-induced inflation of fetal lungs in premature lambs, antenatal glucocorticoids have come to serve as the mainstay of clinical management of preterm birth for the promotion of fetal lung maturation.¹⁻³ This practice is now a standard of care in expected preterm birth at >34 weeks of gestation. Surprisingly, despite widespread use, documented clinical efficacy, and focused study,⁴⁻⁹ the molecular mechanism by which antenatal steroids function is not understood fully, which underscores our studies herein. Although guidelines on administration recommend a single dose of antenatal glucocorticoids, the most effective dosing regimen remains highly controversial.^{10,11} A better understanding of the molecular mechanism by which this prevalent and lifesaving therapy manifests functionally is expected to inform clinical decisions regarding its optimal use.

To investigate the mechanisms behind pulmonary insufficiency, we used our previously described¹² *Erk3* (*Mapk6*) knockout mouse, which are created by targeted disruption of the encoding gene. ERK3 is an atypical mitogen-activated protein kinase with relatively uncharacterized upstream regulators,^{13,14} which have been shown to accumulate during terminal cellular differentiation.^{15,16} In vivo analysis further supports a role for *Erk3* in regulation of cellular differentiation in the lungs.¹² *Erk3*^{-/-} mice display growth restriction and reduced organ weight without gross abnormalities in organ structure¹² yet exhibit uniform neonatal lethality because of respiratory distress with the major hallmarks of lethal pulmonary immaturity.¹² Histologic evaluation of *Erk3*^{-/-} fetal lungs revealed normal

branching morphology with impaired saccular development, which suggests that, although overall organogenesis of the lungs is preserved, lung maturation is impaired.¹² Ultrastructural analyses revealed that *Erk3*^{-/-} type II alveolar cells contain abundant glycogen granules and attenuated villi when compared with wild type controls, which is consistent with incomplete differentiation.¹²

In our characterization of the model, we tested whether the neonatal mortality rate of *Erk3*^{-/-} mice was due to potentially clinically modifiable pulmonary immaturity through transplacental administration of the glucocorticoid dexamethasone. Treatment of pregnant mice at embryonic days 16.5 and 17.5 (during the early saccular stage of fetal lung development) significantly improved the immature histologic architecture of *Erk3*^{-/-} lungs, which was demonstrated by the restoration of pulmonary airspace and reduction of the number of immature glycogen-containing cells.¹² Interestingly, despite antenatal dexamethasone treatment *Erk3*^{-/-} pups remained cyanotic, dying in the early neonatal interval.¹² Further investigation, however, revealed that the administration of exogenous surfactant improved offspring survival,¹⁷ which suggests a deficiency in surfactant production similar to that seen in clinical neonatal respiratory distress syndrome (RDS). Taken together, the *Erk3*^{-/-} model recapitulates morbidity and death observed in the preterm infant and allows examination of the effects of dexamethasone on lung maturity distinct from increased pulmonary surfactant. Having recently demonstrated that surfactant replacement in combination with steroid treatment rescued the lethal postnatal pulmonary defect associated with *Erk3* loss,¹⁷ we sought to expand our studies into the antenatal period to identify physiologically and clinically relevant molecular mechanisms by which glucocorticoids mediate fetal lung maturation.

Materials and Methods

Animal husbandry and dexamethasone administration

Animals were housed under pathogenfree conditions according to the procedures and protocols approved by the Institutional Animal Care and Use Committees of both Montreal University and Baylor College of Medicine (AN-4826). Mice were bred by intercrossing heterozygous *Erk3* mice, where the presence of a vaginal plug indicated the beginning of gestation (E0.5). On embryonic days 14.5, 16.5 or 18.5 pregnant females were killed by CO₂ euthanasia, and fetuses were removed by cesarean delivery and processed for further analysis. Genotype was determined by polymerase chain reaction (PCR), as previously described.¹² Dexamethasone (0.4 mg/kg; Sandoz, Princeton, NJ) or saline solution was administered intramuscularly to pregnant females on embryonic days 16.5 and 17.5.

RNA sequencing and analysis

High integrity (>8.5 Agilent Bioanalyzer; Agilent Technologies, Santa Clara, CA) coding 3' polyadenylated messenger RNA (mRNA) was extracted from lungs of E18.5 mice (n = 7) and purified with the Dynabeads mRNA Purification Kit (Invitrogen, Carlsbad, CA). Ambion RNA Fragmentation Reagents (Ambion, Inc., Foster City, CA) were used to fragment mRNA. Double-stranded complementary DNA (cDNA) was made with the use of the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) and

random hexamer primers (Invitrogen; 50 ng/μL). DNA sequencing libraries were generated with cDNA according to manufacturer's protocol. Cluster generation and sequencing was performed on the Illumina cBot station and Illumina HiSeq 2000 (Illumina, San Diego, CA) producing 114,868,849 60bp single end reads (Supplemental Table 1; Supplemental Figure 1). Reads were aligned to the mouse genome assembly (NCBI37/UCSC mm9) with Tophat2,¹⁸ and differentially expressed genes were identified with the intersection of Cuffdiff^{19,20} and NOISeq.²¹ Programs were run with standard settings and corrected for multiple testing with false discovery rate. Gene pathway interactions were determined with Ingenuity Pathway Analysis (IPA) software (QIAGEN, Redwood City, CA) and DAVID Gene Ontology.²²

Quantitative real time-PCR (RT-PCR)

Total RNA was isolated from E18.5 lung tissue (n = 12) and purified with the RNeasy kit (Qiagen, Valencia, CA). RNA was reverse transcribed with the High Capacity cDNA Archive Kit with random primers (Applied Biosystems, Foster City, CA). RT-PCR was performed using commercially available sense and antisense oligonucleotides (Applied Biosystems), TaqMan PCR Universal Master Mix (Applied Biosystems), and gene specific TaqMan probes. The Applied Biosystems PRISM 7900HT Sequence Detection System (Applied Biosystems) was used to detect amplification level. Glyceraldehyde-3-phosphate dehydrogenase was used as endogenous control. Relative gene expression was quantitated by the 2^{-CT} method.²³ The genes studied were corticotropin-releasing hormone (*Crh*), corticotropin-releasing hormone receptor 1 (*Crhr1*), corticotropin-releasing hormone receptor 2 (*Crhr2*), insulin-like growth factor 1 (*Igf1*), insulin-like growth factor 2 (*Igf2*), surfactant protein A1 (*Sftpa1*), surfactant protein B (*Sftpb*), surfactant protein C (*Sftpc*), short stature homeobox 2 (*Shox2*), thyrotropin-releasing hormone (*Trh*), and urocortin 2 (*Ucn2*).

Histology, immunohistochemistry, and analysis

Human tissue sample collection and experimental protocol was reviewed by the Institutional Review Board (IRB) of Baylor College of Medicine; the determination was that the thoroughly deidentified samples were exempt from IRB approval for use. All samples were obtained initially by Dr Susan Guttentag under IRB approval from The Children's Hospital of Philadelphia and released to Baylor College of Medicine with a materials transfer agreement. The fetal tissue was from previable neonates and included only tissue from fetuses with no known anomalies or exposure to antenatal steroids.

Tissues from human and murine fetuses (n = 5) were fixed overnight in 10% formalin and embedded in paraffin. Tissue sections were stained with the use of a conventional hematoxylineosin protocol. Immunostaining was performed by incubating slides with primary antibody overnight at 4°C, then secondary antibody for 15 minutes, followed by horseradish peroxidase label reagent. Stable DAB Plus (Diagnostic Biosystems, Pleasanton, CA) chromogen was applied for 5 minutes, followed by a Harris hematoxylin counterstain (Fisher Scientific, Pittsburgh, PA) for 15–30 seconds. Primary antibodies used were CRH rabbit polyclonal (1:400; Abcam, Cambridge, MA), surfactant protein A rabbit polyclonal (1:12000; Millipore, Temecula, CA), surfactant protein B (SFTPB) rabbit polyclonal

(1:2000; Millipore), and surfactant protein C (SFPTC) rabbit polyclonal (1:4000; Millipore). Negative controls and nonspecific antibodies were included in each immunostaining procedure. Immunostained slides of fetal lungs ($n = 5$) were examined by 2 blinded reviewers. For each slide, 5 random high-power fields were graded with a 0–5 scale, where 0 indicated the absence of positive staining and 5 indicated intense and diffuse positive staining. Inter-reviewer reliability was 100%. Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD) was also used to analyze 10 random high-power fields with a grading scale from 0–5 to identify the extent of positive staining (area counts) per high-power fields. The mean grade of each slide was calculated and compared across treated and untreated groups with the use of the independent sample t -test after an equal variance test was performed.

Micro computed tomography (microCT)

Fetal tissues ($n = 3$) were immersed in cold 4% paraformaldehyde for 24 hours then immersed in a hydrogel stabilization solution that was prepared as described²⁴ for 3 days at 4°C followed by polymerization at 37°C for 3 hours to preserve tissue structure and conformation. Before imaging, samples were immersed in 0.1N iodine overnight and embedded in 1% agarose, then imaged on the SkyScan 1272 MicroCT (Bruker, Billerica, MA) with the following parameters: 70 kV, 142 μ A, 0.5-mm aluminum filter, 2.161 second exposure, 180-degree rotation, and 0.2-degree step angle, which resulted in an isotropic voxel size of 3 μ m at 3k total resolution. Scans were reconstructed with SkyScan NRecon (Bruker) and processed with Bruker CT-Analyser by performing binary thresholding of scans with despeckling (<25 voxels) in 3-dimensional space and sweeping for largest object. Volumes of interest (VOI) were defined by manual delineation of lung lobes followed by adaptive boundary definition and ImageJ (National Institutes of Health, Bethesda, MD) was used for semi-automated segmentation of the VOI. The CTAn 3-D analysis plugin (Bruker, Billerica, MA) was used to determine total surface area and volume, density, object porosity^{25,26} (volume of all open plus closed pores as a percent of the total VOI volume), and structural thickness (with a sphere-fitting measurement). Saccular space was determined by the calculation of total lung volume minus tissue volume and conducting airways. Three-dimensional reconstructions were visualized with CTVox (Bruker).

Statistical analysis

Results are given as means $\pm$ standard deviation. Statistical analysis was performed with 1-way analysis of variance and the Student t tests, and graded histologic data were analyzed with Kruskal-Wallis and Mann—Whitney U tests. Correction for multiple testing used Tukey tests or false discovery rate (FDR) within each experiment with statistical significance accepted at a probability value of less than .05 with the use of the software packages SPSS (IBM Corp., Armonk, NY) and R (R Foundation for Statistical Computing, Vienna, Austria). All statistical analyses for expanded whole transcriptome analysis were performed as described earlier.^{18,19,21,27,28}

Results

Pulmonary immaturity and structural defects

Consistent with our previous reports,^{12,17} *Erk3*^{-/-} mice exhibited early postnatal death with signs of respiratory distress. To assess the impact of *Erk3* loss on in situ structure at a dimensional level, high-resolution microCT scans were performed on whole lungs from each group. *Erk3*^{-/-} lungs were smaller in total volume compared with those of wild-type mice, and exhibited areas of atelectasis (Figure 1, A). Lung composition as determined by comparison of voxel greyscale value revealed higher pulmonary density in *Erk3*^{-/-} mice that were treated with saline solution, which is indicative of decreased airspace consistent with human neonatal respiratory distress syndrome. Treatment of *Erk3*^{-/-} mice with dexamethasone reversed the morphologic defects and resulted in decreased lung density (Figure 1, B), increased saccular space, and increased porosity (Supplemental Videos 1–4).

Transcriptomic, pathway, and gene ontology analysis

Differential gene expression testing of whole transcriptome sequences generated from E18.5 wild type and *Erk3*^{-/-} lungs treated with dexamethasone or saline solution confirmed ablation of *Erk3* (*Mapk6*; Supplemental Tables 2 and 3) in *Erk3*^{-/-} mice, regardless of treatment ($q < 0.05$; Supplemental Figure 2). *Igf2* was downregulated with *Erk3* loss ($q = 9.35 \times 10^{-4}$; Figure 2, A), which was consistent with our previous description of IGF-2—related intrauterine growth restriction (IUGR) in *Erk3*^{-/-} mice.¹² Additionally, dexamethasone treatment in wild-type mice resulted in *Igf2* downregulation. These results validated the reliability of our expression analysis in detecting genes with known dysregulation in *Erk3*^{-/-} mice.

Pairwise comparisons (Supplemental Tables 2–7) revealed 596 genes differentially expressed in steroid-treated mice compared with controls ($q < 0.05$; Supplemental Table 8). Ingenuity Pathway Analysis of these genes identified 36 genes that were interacting with dexamethasone treatment ($q < 0.05$; Supplemental Table 9). Given the well-established role of the surfactant proteins in lung development and function, we examined their interactions with differentially expressed genes using Ingenuity Pathway Analysis (Figure 2, B). This revealed interactions between *Igf2*, *Sftpb* and *Sftpc* and between dexamethasone and *Sftpa1* and *Sftpb*. Based on the significant interactions that were detected on this unbiased analysis platform, we further characterized these genes.

Gene ontology classification implicated 3 as being involved in lung development: *Crh* (lung development), *Id1* (lung development, lung morphogenesis, lung vasculature development), and *Pdgfra* (lung development, lung alveolus development). *Igf2* was also downregulated by dexamethasone in wild-type mice and in both *Erk3*^{-/-} treatment groups. *Id1*, although showing significant differential expression in comparisons of saline solution to dexamethasone, also significantly differed ($q = 7.05 \times 10^{-4}$) between wild-type and *Erk3*^{-/-} mice treated with dexamethasone (Supplemental Figure 2), which suggested *Erk3* regulation of its corticosteroid response. Of the interacting genes, *Crh* was identified uniquely as being differentially expressed in both wild type and *Erk3*^{-/-} mice in response to glucocorticoid treatment ($q < 0.05$; Figure 2, A). This downregulation of *Crh*, along with their recognized

relationship with glucocorticoids, indicated a likely biologic and clinical significance, which prompted us to further investigate *Crh* in our model.

***Erk3*-independent glucocorticoid-mediated attenuation of fetal CRH**

We detected pulmonary CRH protein expression at embryonic day 14.5 in both wild-type and *Erk3*^{-/-} lungs by IHC (Figure 3, A). Evaluation of fetal lungs at subsequent time points demonstrated increased CRH staining at embryonic day 16.5, with attenuation at embryonic day 18.5 as gestational advanced toward term (Figure 3, A). Moreover, by embryonic day 18.5, dexamethasone administration resulted in further reduction of CRH compared with saline solution in both wild-type and *Erk3*^{-/-} mice ($P < .001$; Figure 3, B). Quantitative RT-PCR confirmed the downregulation of *Crh* identified by RNA-seq in embryonic day 18.5 lungs after antenatal dexamethasone treatment ($P < .05$; Figure 3, C). Overall, these results denote a progressive pattern of CRH downregulation in the lung as the fetus approaches term gestation, which is accelerated by glucocorticoid administration.

Validated CRH production in the human fetal lung

To validate our findings and determine whether the regulation of CRH that was detected in the murine lung could be observed in humans, we examined mid-trimester human fetal tissue for the presence of CRH. At 18 weeks of gestation, CRH was present in both epithelial and interstitial lung cells; pulmonary CRH protein expression significantly increased with advancing gestational age ($P < .01$; Figure 4), which approaches the threshold of viability ex utero (22–23 weeks of gestation).

***Erk3*-dependent glucocorticoid induction of surfactant protein B production**

Gene pathway interaction analyses directed additional examination of surfactant response to glucocorticoids in our model. Dexamethasone treatment induced alterations in pulmonary *Sftpa1* (Supplemental Figure 3, A) and *Sftpc* (Supplemental Figure 3, B) mRNA levels of both wild-type and *Erk3*^{-/-} mice as determined by quantitative RT-PCR; however, no significant changes in protein were detected by immunohistochemistry (IHC; Supplemental Figure 3, C and D). The administration of antenatal dexamethasone to wild-type mice increased *Sftpb* levels as measured by immunostaining ($P = .003$; Figure 5, A and B) and quantitative RT-PCR (Figure 5, C). Interestingly, in saline solution—treated animals, *Erk3* loss was associated with *Sftpb* mRNA upregulation but decreased protein as determined by IHC and western blot (WB; Supplemental Figure 3, E). Similarly, in *Erk3*^{-/-} lungs, quantitative RT-PCR showed a significant increase in *Sftpb* mRNA after dexamethasone treatment (Figure 5, C); IHC ($P = .012$; Figure 5, B) showed a decrease in SFTPB protein, which suggests a posttranscriptional role of ERK3 in regulating SFTPB protein levels.

Comment

Fetal and neonatal lung maturation is thought to be modulated by several intrinsic and extrinsic factors. However, insight regarding the expression, localization, and regulation of important molecular mediators of lung maturation is limited. Our murine *Erk3*^{-/-} model functionally, anatomically, and physiologically recapitulates neonatal respiratory distress and its related morbidity and mortality rates in the human preterm fetus, which allows a study of

the morphologic and functional response of the fetal lung to antenatal glucocorticoid administration.

There are a number of unique strengths to our study. First, high resolution microCT scans of the fetal lung enabled detailed structural characterization of neonatal respiratory distress syndrome-associated alterations and glucocorticoid rescue in the entire lung, which previously had been limited to histologic observations.^{6,13} In addition to confirming our previous reports of decreased *Erk3*^{-/-} total lung volume, we observed increased lung porosity and airspace with dexamethasone treatment, which highlighted our model's similarity to the human neonatal clinical condition of respiratory distress and its therapeutic prevention.

A second strength to our study is the application of whole transcriptomic analysis with the use of shotgun RNA sequencing. Transcriptomic and pathways analysis of alterations in gene expression that is associated with *Erk3* loss and antenatal glucocorticoid treatment identified several clinically relevant genes of interest, which included *Igf2*, *Crh*, and *Sftpb*. *Igf2* is an important regulator of fetal growth,²⁹ and *Igf2* knockout mice exhibit IUGR with delayed lung development that is abrogated by corticosterone treatment.³⁰ In our model, *Erk3*^{-/-} mice also experience fetal growth restriction with decreased serum IGF-2 levels.¹² Here we show that, in wild-type mice, dexamethasone administration resulted in *Igf2* downregulation to levels seen in knockout mice. These data may implicate *Igf2* in the reduction in birthweight that is associated with repeated doses of corticosteroids.³¹

A third strength to our study is the rendered molecular insights that were yielded, which collectively extend previous observations of other investigators who described pulmonary *Crh* expression.³²⁻³⁵ In a series of elegant experiments several years ago, Muglia et al³⁶ reported on their generation of *Crh* knockout mice that revealed a fetal glucocorticoid requirement for lung maturation but concluded that it was due to the absence of CRH neuropeptide production in the brain. Further analysis of their *Crh*-deficient mice revealed that administration of antenatal corticosterone beginning at day 12 of gestation completely reversed the abnormal lung architecture and increased *Sftpb* mRNA levels at embryonic day 18.5.³⁷ These studies suggested a second corticosteroid-responsive molecular target that was necessary for the promotion of functional fetal lung maturation that, before our investigation of *Erk3*-deficient mice, has eluded the field. In the current study, we demonstrate temporal regulation of intrinsic CRH protein expression in the murine fetal lung by dexamethasone, which matches reports on the ontogeny of pulmonary *Crh* in other mammals.^{33,34} The increase in *Crh* mRNA and protein with *Erk3* loss, independent of in utero glucocorticoid treatment, suggests an important function in modulating CRH function in the developing lung. Moreover, in the mouse, there is subsequent attenuation of CRH expression both close to term and with antenatal glucocorticoid production. Although such experiments could not be capitulated in healthy human preterm and term subjects, our collective molecular and IHC findings would suggest that intrinsic pulmonary CRH production is at least temporally related to SFTPB production and functional lung maturity. This is further consistent with the findings of others in genetic mouse models.^{36,37} To our knowledge, ours is the first study to demonstrate the expression and ontogeny of CRH protein in the human fetal lung.

A fourth strength of our study is its potential for translational significance. Pulmonary surfactant is composed of phospholipids and small hydrophobic proteins that are necessary to reduce surface tension and prevent atelectasis;³⁸ mutations in these genes or their regulators³⁹ result in respiratory distress and intractable respiratory failure.^{40–42} The introduction of intratracheal exogenous surfactant replacement has improved RDS-related preterm neonatal mortality rates greatly.⁴³ In humans and wild-type mice, corticosteroid administration has long been shown to enhance surfactant protein production.³ Interestingly, *Erk3*^{-/-} mice do not exhibit this response despite increased *Sftpb* mRNA, which suggests that *Erk3* loss may result in impaired posttranscriptional regulation of SFTPB and its induction by glucocorticoids. The failure of dexamethasone to induce SFTPB in *Erk3*^{-/-} mice explains the requirement of exogenous surfactant replacement therapy for neonatal survival in this model¹⁷ and reveals the importance of *Erk3* in the mediation of glucocorticoid induction of pulmonary maturity in the developing fetus through an *Erk3*-dependent pathway. Indeed, the persistent neonatal lethality, despite corticosteroid administration, distinguishes *Erk3* loss from *Crh* deficiency and allows for in-depth investigation of prenatal surfactant expression and regulation.

Potential limitations to our study include the presentation of IUGR in homozygous *Erk3* knockout pups.^{12,17} Growth restriction is not characteristic of all newborn infants exhibiting RDS, and there is conflicting clinical evidence on the extent to which fetal growth restriction increases the risk of neonatal RDS in humans.^{44–47} The growth restriction phenotype in *Erk3*^{-/-} pups may have an impact on the molecular mechanisms of lung maturation that were detected in this study, potentially limiting its applicability to the subset of neonates with RDS who also experienced IUGR. Another possible limitation is in the incomplete comparison of temporal gene regulation and glucocorticoid response between our murine model and previable human fetal tissue not exposed to steroids. However, because of differences in timing of pulmonary maturation, our studies in the near term mouse lung are applicable readily to periviable human fetuses likely to be exposed to glucocorticoids. By integrating findings in genetic models of disease with those in human fetal tissue, we achieve a greater understanding of the complex molecular interactions that mediate pulmonary immaturity and their clinical interventions and uncover promising directions for future research using other models of lung function.

Clinical trials that have compared antenatal corticosteroid dosing schedules have shown decreased severity of pulmonary disease with repeated doses but were halted before completion because of the observation of reduced birthweight and increase in small-for-gestational-age incidence.⁴⁸ Limited data on glucocorticoid administration during the periviable period⁴⁹ (20–26 weeks of gestation) have shown benefit to preterm infants who are born as early as 22 weeks of gestation.^{50,51} Additionally, antenatal steroids are used for in utero therapy for congenital virilizing adrenal hyperplasia because of 21-hydroxylase deficiency⁵² and the treatment of fetal heart block that is associated with maternal autoimmune disease.⁵³ Because the timing of steroid administration differs between interventions, minimizing side-effects of antenatal glucocorticoid administration is dependent on the identification of the underlying molecular pathways and pathophysiologic-mediating adverse effects. Our initial observations that were related to the use of steroids late in pregnancy may lead to dosage and timing optimization of clinical interventions in

scenarios in which steroids are used in the first and second trimester or extended beyond 34 weeks of gestation in the third trimester. Moreover, our novel demonstration of CRH in the human fetal lung, with temporal modulation of expression toward the threshold of viability, may have strong translational significance in the understanding of preterm parturition and neonatal survival and the identification of future targets for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. Micro computed tomography analysis of fetal mouse lungs

A, Representative images of the right lobe show normal pulmonary structure in wild-type lungs and atelectasis in lungs of *Erk3*^{-/-} mice that were treated with saline solution. Gross structural defects were reversed by dexamethasone treatment. **B**, Histogram of voxel density from micro computed tomography scans. Higher greyscale values indicate greater density. Lungs from *Erk3*^{-/-} mice exhibited higher relative density because of lower total airspace and collapse of lung structures. Treatment with dexamethasone partially reversed this phenotype.

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FIGURE 2. Differential gene expression and pathway interactions detected in fetal lungs

A, Differential expression of *Crh* and *Igf2* as detected by RNA-seq. Expression estimates (fragments per kilobase of transcript per million mapped reads, FPKM) for *Crh* and *Igf2* across each sample group with FDR-corrected q-values for significant expression differences in comparisons between saline solution— and dexamethasone-treated groups. Error bars represent cross-replicate variability and measurement uncertainty, as estimated by the Cuffdiff statistical model. **B**, Ingenuity Pathway Analysis interactions. *Crh*, *Id1*, *Pdgfa*, and *Igf2* are expressed differentially in saline solution— vs dexamethasone-treated lungs. All but

Igf2 were identified by DAVID Gene Ontology as being involved in lung development. Surfactant proteins were included in the Ingenuity Pathway Analysis because of their established role in lung function and development.

CRH, corticotropin-releasing hormone; *Dex*, dexamethasone; *FPKM*, fragments per kilobase of transcript per million mapped reads; *ID1*, Inhibitor Of DNA Binding 1; *IGF2*, insulin-like growth factor 2; *PDGFA*, platelet-derived growth factor alpha; *SFTPA1*, surfactant protein A1; *SFTPA2*, surfactant protein A2; *SFTPB*, surfactant protein B; *SFTPC*, surfactant protein C.

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FIGURE 3. *Crh* gene expression in lung tissue of wild type and *Erk3^{-/-}* mice
 Evaluation of corticotropin-releasing hormone expression in embryonic day 14.5, 16.5, or 18.5 fetuses that were exposed to transplacental administration of dexamethasone or saline solution at embryonic days 16.5 and 17.5 of gestation. **A**, Immunohistochemistry photomicrographs of lung sections stained for corticotropin-releasing hormone. (Original magnification, $\times 40$). **B**, Quantitation of staining in lung sections ($n = 5$) illustrates corticotropin-releasing hormone down-regulation with dexamethasone treatment (*Erk3^{+/+}* [P

< .001] and *Erk3*^{-/-} [*P* < .001]). C, *Crh* expression relative to saline solution—treated *Erk3*^{+/+} mice by quantitative RT-PCR.

CRH, corticotropin-releasing hormone; *Dex*, dexamethasone; *E*, embryonic day; *IHC*, immunohistochemistry; *mRNA*, messenger RNA.

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FIGURE 4. Protein expression of corticotropin-releasing hormone in human fetal lung

A, Corticotropin-releasing hormone staining demonstrated for the first time that corticotropin-releasing hormone is expressed broadly in human lungs through the mid trimester in both epithelial and interstitial cells. (Original magnification, $\times 40$.) **B**, Protein expression of corticotropin-releasing hormone was evaluated by immunohistochemistry on archived human fetal lung specimens ($n = 5$). Corticotropin-releasing hormone increased as gestational age advanced from 18–22 weeks, with a significant increased expression from 18–22 weeks ($P < .0001$), 19–22 weeks ($P < .001$), and 20–22 weeks ($P < .001$). *Two asterisks* indicate $P .001$ vs 22 weeks; *3 asterisks* indicate $P .0001$ vs 22 weeks.

CRH, corticotropin-releasing hormone; IHC, immunohistochemistry.

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FIGURE 5. Dexamethasone-induced increase in surfactant protein B is modulated by ERK3 signaling

Pregnant female mice were treated with dexamethasone or saline solution at embryonic days 16.5 and 17.5 of gestation. Fetuses were collected by cesarean delivery at embryonic day 18.5. **A**, Immunohistochemistry images of lung sections that were stained for surfactant protein. (Original magnification, $\times 40$.) **B**, Quantitation of staining in lung sections ($n = 5$) illustrates increased surfactant protein B production with dexamethasone treatment in wild type mice ($P = .003$) but not in *Erk3*^{-/-} mice, which show decreased expression ($P = .012$).

C, *Sftpb* expression relative to saline solution—treated *Erk3^{+/+}* mice by quantitative RT-PCR.

Dex, dexamethasone; *IHC*, ••••; *mRNA*, messenger RNA; *SFTPb*, surfactant protein B.

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