Online Supplemental Materials:

rhIGF-1/BP3 Preserves Lung Growth and Prevents Pulmonary Hypertension in Experimental BPD

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Detailed Methods:

General Approach: Based on strong epidemiologic and clinical data demonstrating the critical role of antenatal determinants in the pathogenesis of BPD in preterm infants[1,2], we studied the impact of rhIGF-1/BP3 therapy on lung alveolar and vascular growth and lung function. In this study, we used rodent models of chorioamnionitis (CA) as induced by intra-amniotic endotoxin (ETX) injection and preeclampsia (PE), as mimicked by sFlt-1 injection, as previously described.[3-5] (Figure 1) To further determine whether rhIGF-1/BP3 therapy could improve lung structure and function induced by postnatal hyperoxia exposure, we also studied the effects of rhIGF-1/BP3 therapy in a traditional and long-standing model over the past decades from the era of the "old BPD."[6,7]

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver's altitude (1600m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 week before giving birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA)

Study Design:

Experimental Preeclampsia due to Intra-amniotic sFlt-1 Administration.

Details regarding methods used to develop the approach to establish this animal model of PE have been previously published.[3,4] As illustrated in Figure 1, pregnant rats were prepared for receiving

intra-amniotic injections at 20 days gestation (term: 22 days). The timing of injection during the late canalicular stage of lung development in the rat was selected to parallel similar stage of human lung development in 24-26 week premature newborns, who are at the highest risk for BPD. After premedication with buprenorphine (0.01-0.05 mg/kg, subcutaneous injection), laparotomy was preformed in pregnant rats under general anesthesia with 1-2% isoflurane inhalation via face mask. (Anesthesia machine: Matrix by Midmark, model VIP3000.) During anesthesia and laparotomy, pregnant rats were kept on a heating pad to prevent hypothermia.

Pregnant rats were randomly assigned to saline (control) or sFlt-1 treatment. The control group received 50 μl of normal saline per amniotic sac and the sFlt-1 group received 1 μg of recombinant human sFlt-1-Fc (R&D Systems, Minneapolis, Minnesota) diluted to 50 μl with normal saline per sac. The dose of intra-amniotic sFlt-1 was based on data obtained from past studies in which sFlt-1 at lower doses (0.125-0.25 μg/sac) failed to induce sustained abnormalities of lung structure at 14 days of age. Under aseptic conditions, a midline abdominal incision of 3-4 cm in length was made to expose the amniotic sacs for intra-amniotic injections. The amniotic sac closest to the right ovary was first identified and injected, and then in a counterclockwise sequence, each sac was identified and injected with a maximum of 10 sacs injected per dam. Limiting sFlt-1 injections to 10 sacs per pregnant rat was to utilized to maintain a consistent total dose of sFlt-1 in the individual dams. The total number of amniotic scas in each mother rat was examined and recorded during laparotomy. The abdominal incision was closed with nylon sutures. Bupivacaine (1-2 mg/kg, intramuscular injection) was applied over the incision wound for postoperative pain control. Pregnant rats were monitored closely to ensure arousal within 10 minutes after surgery and rats were placed back to the cages and were monitored for activity, ability to drink and eat and for signs of bleeding or infection.

Cesarean section delivery and postnatal treatment: Two days after intra-amniotic injections, cesarean section was performed on pregnant rats under general anesthesia with isoflurane inhalation. The fetus in the amniotic sac closest to the right ovary was first delivered, which was followed by delivery of the rest of the fetuses in a counterclockwise sequence to enable identification of fetuses that were treated with saline or sFlt-1. The total number of amniotic sacs in each mother rat was further verified at the time of delivery. All rat pups in the injected amniotic sacs were delivered within 5 minutes after onset of anesthesia. Maternal rats were euthanized with pentobarbital sodium. Newborn rats were immediately placed on a heating pad to avoid hypothermia and dried manually with gauze sponges. Pups received no supplemental oxygen or artificial ventilation at birth. Within 30 minutes after birth, the pups were weighed and placed with foster mother rats in regular cages. For the first 24 hr of life, the newborn pups were monitored closely for mortality or signs of respiratory distress. All rats were maintained in room air conditions without supplemental oxygen.

Pups were weighed and randomized to daily treatment with IGF-1/IGFBP3 at one of 4 doses (0.02, 0.2, 2, 20 mg/kg) or saline (control) by intraperitoneal injection. At 14 days of age, rat lungs were harvested for physiologic and histological assessments, which included assessment of lung mechanics, inflation of lungs for morphometric analysis, collecting and freezing lung tissue for western blot analyses and heart collections for later assessment of right ventricular hypertrophy (RVH). 10 – 15 rats were studied in each group for each measurement at each time point. Survival and respiratory status of the infant rats were monitored and recorded daily from birth throughout the study period.

Experimental Chorioamnionitis due to Intra-amniotic ETX Administration:

The approach used to establish this model for chorioamnionitis (CA) has been previously published[3,5] and follows the same protocol as described above for the pre-eclampsia (PE) model (Figure 1; see above). Briefly, antenatal injections were performed by direct visualization through a maternal laparotomy at 20 days gestational age (term, E22). Pups received either ETX (10 ug/ml) or saline (control) and were delivered by cesarean-section on E22. Pups were weighed and randomized to daily treatment with rhIGF-1/BP3 at one of 4 doses (0.02, 0.2, 2, 20 mg/kg) intraperitoneal or saline (control). As descried above, animals were killed at 14 days for study endpoints, including measurements of lung mechanics, lung inflation (for histology and morphometric analysis); cardiac weights (to assess right RVH as a metric for pulmonary hypertension). Additionally, to determine whether brief rhIGF-1/BP3 treatment would be as effective as more prolonged therapy, we randomized 12 pups for rhIGF-1/BP3 treatment at a dose of 20 mg/kg daily for only 3 days, which were then killed at 14 days of age for studies of lung structure (as described above).

Experimental Model of BPD Induced by Postnatal Hyperoxia Exposure:

To determine the effects of rhIGF-1/BP3 in a traditional postnatal model of BPD, we studied the effects of IGF-1/P3 therapy in rat pups exposed to prolonged hyperoxia, as previously described.[8] Neonatal rat pups were exposed to high oxygen tension (FiO_2, 0.90) from days 1 through 14 of postnatal life. Animals received daily intraperitoneal injections of rhIGF-1/BP3 (0.2 mg/kg daily) or saline (control) for 14 days. Animals were studied at day 14 to assess lung function by Flexivent measurements and then killed. Lungs and hearts were collected for histology with morphometric analysis and assessment of RVH, respectively. We further compared the effects of short (3 day)

versus prolonged (14 day) therapy on lung structure at 2 weeks of age. In these studies, animals were maintained in hyperoxia throughout the entire 2 week study period.

Study Measurements

Lung Function: Lung function was determined in 14 day old pups with the flexiVent system (Flexivent; SCIREQ, Montreal, QC, Canada), which measured the *in vivo* respiratory relationships, flow volumes, resistances, and compliances, in compliance with standard methods from the manufacturer. Fourteen-day-old rats were anesthetized by intraperitoneal injection with ketamine at a dose of 80 mg/kg (20 mg/ml; Ketaset; Zoetis Inc, Kalamazoo MI) and xylazine at a dose of 10 mg/kg (20 mg/ml; AnaSed Injection; AKORN Animal Health, Lake Forest IL) and pancuronium (1 mg/ml at a dose of 0.01 ml/g) to avoid spontaneous breathing and attached to the mechanical system via tracheotomy. Two-thirds of the dose was given before tracheostomy and cannulation, and the remaining was given when rats were placed on mechanical ventilation. Input weight was provided for progressive ventilation patterns at tidal volumes of 10 cc/kg with frequency 150 breaths/minute at I:E 67% with peak pressures 30 cm H2O between prolonged and stepwise inspiration/expirations, rapid shallow breathing, and sinusoidal breathing patterns. Wave pattern iterations interrupted with spontaneous breathing or software alerts were excluded from the trial, patterns were performed sequentially for a maximum of 6 occasions, and means over at least 3-6 non-failed attempts were used for each animal. Used coefficients of determination were all greater than 0.95. Lung system variables of compliance and resistance were measured.

Tissue for histological analysis. Animals were killed with intraperitoneal pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated with 4% paraformaldehyde and maintained at 20 cm H_2O pressure for 60 min. A ligature was tightened around the trachea to

maintain pressure, and the tracheal cannula was removed. Lungs were immersed in 4% paraformaldehyde at room temperature for overnight fixation. A 2-mm thick transverse section was taken from the mid-plane of right lower lobe and left lobe of the fixed lungs per animal, respectively, to process and embed in paraffin wax.

Immunohistochemistry. Slides with 5-μm paraffin sections were stained with hematoxylin and eosin for assessment of alveolar structure and with von Willebrand Factor (vWF), an endothelial cell-specific marker, for assessment of vascular density.

Morphometric analysis. Radial alveolar counts (RAC) and pulmonary vessel density were determined by standard morphometric techniques,[9,10] In each pup, at least 5 measurements were obtained for RAC, at least 10 images were processed for computer-assisted image analysis of alveolar structure, and at least 10 pulmonary vessels were measured for pulmonary vessel density and vascular wall thickness. Alveolarization was assessed by standard radial alveolar count methods (RAC) as previously described.[9,10] Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective tissues or septum or pleura, and the number of septae intersected by this line was counted. Pulmonary vessel density was determined by counting vWF-stained vessels with external diameter at 50 μm or less per high-power field. The fields containing large airways or vessels with external diameter greater than 50 μm were avoided.

Indices of right ventricular hypertrophy (RVH) and left ventricular hypertrophy (LVH). The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed. The ratios of RV to LV+S weights and RV/body weights were determined to evaluate RVH.

Western blot analysis. Frozen lung samples were homogenized in ice-cold radioimmunoprecipitation buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 10 mg/ml PMSF) with protease inhibitor (Pierce, Rockford IL) and phosphatase inhibitor cocktail (Calbiochem, Gibbstown, NJ). Samples were centrifuged at 1,500 *g* for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined using bovine serum albumin as the standard. Briefly, 25 μg of protein sample per lane was resolved by SDS-PAGE, and proteins from the gel were transferred to PVDF membrane. Blots were blocked for 1 h in 5% nonfat dry milk in TBS with 0.1% Tween 20 TBST. These blots were incubated overnight at 4°C with mouse anti-human polyclonal eNOS (BD610297, BD Biosciences) or rabbit anti-human polyclonal IGF-1 (8917SC, Cell Signaling) in 5% nonfat dry milk in PBS with 0.1% Tween 20. Blots were washed with TBST and incubated with goat anti-mouse (170-6516 BioRad) or goat anti-rabbit (170-6515 BioRad) secondary antibodies for 2 h at room temperature. Blots were washed with TBST, developed with ECL-Plus (RPN2232 Amersham), images captured with BioRad Chemi-Doc XRS+ and analyzed with BioRad Imagelab Software. Blots were stripped of primary antibody and re-probed with housekeeping protein mouse anti-human b-actin (A1978 Sigma Aldrich) for 1 h room temp, washed with TBST, incubated with goat anti-mouse (170-6516 BioRad) secondary antibody for 1 h at room temp. Blots were washed with TBST, developed with ECL-Plus (RPN2232 Amersham), images captured with BioRad Chemi-Doc XRS+ and analyzed with BioRad Imagelab Software.

Studies of isolated Neonatal Lung Endothelial Cells (LEC) and Alveolar Type 2 Cells (AT2C):

Lung Endothelial Cell (LEC) studies: Newborn rat pups were euthanized, lungs perfused with heparinized saline, excised, heart and trachea removed and placed in sterile EBM-2 10%FBS media. Lung tissue was mechanically minced into 1mm^3 sections and placed in type-I collagenase

(0.5mg/ml) at 37°C for 30 minutes at 200-250rpm. Tissue was further disrupted by repeated manual trituration, then strained through 70mm sterile filter to remove undigested tissue. To purify LEC, lung cell suspension was incubated with anti-rat CD31 conjugated magnetic beads for 30 minutes. Following magnetic bead separation, cells were plated on tissue culture plastic dishes coated with 0.2% gelatin in PBS in EBM-2 10% FBS media. LEC phenotype was confirmed by cobblestone morphology, positive immunostaining for CD31, eNOS, KDR and negative staining for aSM-actin and desmin. Cells were used at passage 1-3 for all studies.

AT2 cell studies: Neonatal lungs were collected from newborn rat pups. Pulmonary circulation was flushed with heparinized saline to remove blood, excised, heart and trachea were removed from lungs. Distal lung was further minced to 1-2mm^3 sections and incubated with 0.1% collagenase and 0.025% trypsin for 30 min at 37C. Trypsin inhibitor solution was added to lung digest and then subjected to 60 sec of low speed homogenization. Lung suspension was filtered through a 10um nitex filter to remove undigested tissue. Cells were centrifuged at 300rcf for 10 min, resuspended in wash media and plated on IgG coated dishes for 1 hr. at 37C. Non-adherent cells were recovered, centrifuged and plated in DMEM/F12 media supplemented with 10% FBS at a density of 1x10^5 cells/well and allowed to adhere overnight. Cells were plated in DMEM/F12 media supplemented with 10% FBS for 24 hrs to allow cells to adhere. Cells were washed with PBS and DMEM 2.5% FBS or Conditioned media was added, placed in room air or 50% O_2 and media was changed daily. After 4 days of treatment AT2 cells were washed twice with PBS and incubated with 0.25% trypsin until all cells were detached. Cells were assayed for viability using trypan blue exclusion and counted on a hemocytometer (viability >90%).

RNA isolation and Microarray analysis

RNA isolation: The whole lungs of offspring from IA ETX exposed pups and control pups were harvested on day 0 of life (n=4 for each group). Animals used for lung RNA isolation were not used for any other study endpoint. Tissue was homogenized on ice in TRIzol (Invitrogen) using a PowerGen 500 homogenizer (Fisher Scientific). Total RNA was extracted using the miRNeasy Mini Kit per manufacturer's instructions (Qiagen). Following isolation, total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Microarray Analysis*:* RNA from these samples were analyzed using Affymetric microarrays (Clariom D Rat Transcriptome Array 1.0) using standard procedures. Data were RMA normalized and differential gene expression was determined using Significance Analysis of Microarrays in BRB Array Tools v 4.5.1. and the following settings: exclude genes with log-ratio variation < 50%; 500 permutations; FDR = 5%, and 90% false negative rate (type II error). 11066 probesets met the exclusion criteria. Following statistical analysis, probesets that represented duplicate analysis of genes were removed to create the final results. Enrichment of molecular pathway changes were assessed for differentially expressed probesets using MetaCore (Clarivate Analytics, Philadelphia, PA). The microarray reported gene expression intensity was converted from the log2 scale of the RMA signal for reporting in figures and tables. The array data and a list of differentially expressed genes are available in the GEO database as series number GSE136736.

Statistical Analyses: Statistical analysis was performed with the GraphPad Prism 8.0 software package (GraphPad Software, San Diego, CA). Statistical comparisons were made between groups using t-test or analysis of variance with Kruskal-Wallis/Dunns post-hoc analysis for significance.

$p < 0.05$ was considered significant. Data are presented as interquartile range (IQR) with min-max data points.

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