Defective parasympathetic innervation is associated with airway branching abnormalities in experimental CDH

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Submitted 16 October 2014; accepted in final form 30 April 2015

Rhodes J, Saxena D, Zhang G, Gittes GK, Potoka DA. Defective parasympathetic innervation is associated with airway branching abnormalities in experimental CDH. Am J Physiol Lung Cell Mol Physiol 309: L168–L174, 2015. First published May 1, 2015; doi:10.1152/ajplung.00299.2014.—Developmental mechanisms leading to lung hypoplasia in congenital diaphragmatic hernia (CDH) remain poorly defined. Pulmonary innervation is defective in the human disease and in the rodent models of CDH. We hypothesize that defective parasympathetic innervation may contribute to airway branching abnormalities and, therefore, lung hypoplasia, during lung development in CDH. The murine nitrofen model of CDH was utilized to study the effect of the cholinergic agonist carbachol on embryonic day 11.5 (E11.5) lung explant cultures. Airway branching and contractions were quantified. In a subset of experiments, verapamil was added to inhibit airway contractions. Sox9 immunostaining and 5-bromo-2-deoxyuridine incorporation were used to identify and quantify the number and proliferation of distal airway epithelial progenitor cells. Intra-amniotic injections were used to determine the in vivo effect of carbachol. Airway branching and airway contractions were significantly decreased in nitrofen-treated lungs compared with controls. Carbachol resulted in increased airway contractions and branching in nitrofen-treated lungs. Nitrofen-treated lungs exhibited an increased number of proliferating Sox9-positive distal epithelial progenitor cells, which were decreased and normalized by treatment with carbachol. Verapamil inhibited the carbachol-induced airway contractions in nitrofen-treated lungs but had no effect on the carbachol-induced increase in airway branching, suggesting a direct carbachol effect independent of airway contractions. In vivo treatment of nitrofen-treated embryos via amniotic injection of carbachol at E10.5 resulted in modest increases in lung size and branching at E17.5. These results suggest that defective parasympathetic innervation may contribute to airway branching abnormalities in CDH.

congenital diaphragmatic hernia; pulmonary innervation; lung development; nitrofen model

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a birth defect associated with high rates of mortality and significant morbidity in survivors due to lung hypoplasia and pulmonary hypertension (6, 21, 23). The lungs in CDH exhibit developmental defects in airway branching morphogenesis, resulting in lung hypoplasia, and pulmonary vascular development, resulting in decreased pulmonary arteriolar density and increased muscularization of small intrapulmonary arteries (8, 9, 12, 15, 16, 24). The etiology and underlying molecular mechanisms responsible for abnormal lung development in CDH remain incompletely understood.

There is increasing evidence that pulmonary innervation is impaired in human CDH and in the rodent nitrofen model of CDH (14, 17, 18). Lath et al. (14) reported less complex innervation of the central and peripheral airways and pulmonary vasculature in autopsy specimens of lungs from infants with CDH than controls. Similar defects of innervation are seen during lung development in the mouse nitrofen model of CDH. In addition, Lath et al. reported relatively decreased expression of parasympathetic markers and increased expression of sympathetic markers in nitrofen-treated embryonic lungs, suggesting an imbalance of autonomic innervation in CDH. Similarly, Pederiva et al. (19) described deficient airway innervation in nitrofen-treated embryonic lungs and further demonstrated reduced airway peristaltic contractions in these lung explants.

The role of defective pulmonary innervation in the specific lung developmental defects in CDH is unknown. Pulmonary innervation is closely associated with development of airway smooth muscle (3). After investment of the developing airways with smooth muscle, rhythmic peristaltic contractions of the airways propel secreted lung fluid distally and promote airway branching through increased distal airway pressure (10, 11, 22). Although a large component of rhythmic contractions of the airways seems to be intrinsic to the airway smooth muscle cells, it is likely that pulmonary innervation modulates this process. Furthermore, pulmonary innervation during development may influence airway branching through direct effects on an airway epithelial progenitor cell population within the terminal bud (20). In a system analogous to lung airway branching morphogenesis, parasympathetic innervation has recently been shown to be necessary to maintain an epithelial progenitor cell population during salivary gland development (13). However, a similar effect of parasympathetic innervation on branching morphogenesis in lung development has not been demonstrated.

We hypothesize that defective parasympathetic innervation may contribute to airway branching abnormalities and, therefore, lung hypoplasia, during lung development in CDH, through reduction of rhythmic peristaltic airway contractions or direct effects of innervation on airway epithelial progenitor cells. To address this hypothesis, we utilize the murine nitrofen model of CDH. We demonstrate increased airway branching of nitrofen-treated embryonic lungs following treatment with carbachol, a cholinergic agonist, and show that the carbachol effect is at least partially independent of airway contractions.

METHODS

Animals. Time-mated CD-1 mice were obtained from Charles River. Experiments were performed in accordance with guidelines established by the University of Pittsburgh Animal Care and Use Committee, which approved the study.

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Tissue culture. Timed-pregnant CD-1 mice were gavage-fed 25 mg of nitrofen dissolved in 0.5 ml of olive oil (nitrofen group) or 0.5 ml of olive oil alone (control) on gestational (embryonic) day 8.5 (E8.5), as previously described (4, 5). On E11.5, pregnant females were euthanized by CO2 asphyxiation, and embryos were extracted for isolation of lung tissues from nitrofen- and oil-treated animals. A dissecting microscope and microsurgical instruments were used to isolate the lungs. The lungs were individually placed into the center of 0.4-μm cell culture inserts (Millicell, Millipore) that had been set inside 24-well tissue culture plates (Fisher Scientific), each containing 400 μl of culture medium. Tissue culture was performed in an atmosphere of 5% CO2 at 37°C for 72 h with replacement of media every 24 h. Daily photographs of each tissue were obtained for assessment of airway branching and terminal bud quantification. After 72 h of culture, 5-bromo-2-deoxyuridine (BrDU; Sigma) was added to the culture medium to obtain a final concentration of 325 mM, and cultures remained in a dark incubator for 1 h for labeling of proliferating cells. Lungs were then fixed in 4% paraformaldehyde overnight at 4°C and processed for cryosectioning and immunohistochemistry.

In a subset of lungs, videos were obtained at 72 h for quantification of airway contractions. Culture conditions and media preparation. Nitrofen- and oil-treated embryonic lung tissues were cultured under four different culture media conditions, with replacement of media every 24 h. The standard medium consisted of DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin (Gibco), and 1× glutamate (Gibco). For the treatment groups, lungs were cultured in standard medium with the addition of 25 nM carbamoylcholine chloride (carbachol; Sigma) with or without 100 μM verapamil hydrochloride.

Antibodies. The following antibodies were used for immunofluorescence: goat anti-E-cadherin (1:200 dilution; R & D Systems, Minneapolis, MN), rabbit anti-Sox9 (1:5,000 dilution; Millipore, Billerica, MA), rat anti-BrdU (1:100 dilution; Abcam, Cambridge, MA), rat anti-CD31 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit muscarinic acetylcholine receptor M3 (1:1,000 dilution; Thermo Fisher Scientific, Waltham, MA), donkey anti-rabbit biotin (1:500 dilution; Jackson ImmunoResearch), donkey anti-rat Cy2 (1:300 dilution; Jackson ImmunoResearch), and donkey anti-goat Cy2 (1:300 dilution; Jackson ImmunoResearch).

Immunohistochemistry. Cryoembedded 72-h lung culture tissues were sectioned at 6 μm, attached serially to Superfrost Plus glass slides (Fisher Scientific), and stained for the antibodies listed above. Immunofluorescent staining was imaged using a Zeiss Imager Z.1 microscope with AxioVision software. Photographs of intact whole lung tissue sections that had been stained for Sox9, BrDU, and E-cadherin were obtained and utilized to quantify the number of BrDU+/Sox9+ and BrDU+/Sox9+ cells occupying the single layer of epithelium constituting the terminal buds. For antigen retrieval, slides were exposed to 10 mM sodium citrate buffer for 20 min within a plastic coplin jar set inside an electric Oster steamer and allowed to cool to room temperature prior to immunostaining.

Lung culture real-time video acquisition. An Olympus SZX12 inverted microscope and SPOT Advanced software were utilized to acquire videos of lungs cultured for 72 h and record contractions of the main bronchi. Images of the tissue were captured every 0.5 s for a total of 480 images accumulated over 4 min of real-time viewing of the tissues. Images were stitched together to construct a single video for each lung explant, and the number of main bronchial contractions was counted over the length of the entire video.

Intra-amniotic carbachol injection. Timed-pregnant CD-1 mice were gavage-fed 25 mg of nitrofen in 1 ml of olive oil or olive oil alone on E8.5, as described above. On E10.5, mice were placed under inhaled isoflurane anesthesia, and the uterus was accessed via a midline laparotomy. A single intra-amniotic injection of 5 μl of 625 nM carbachol solution in PBS (to achieve 25 nM concentration within the expected late-gestation amniotic fluid volume of 125 μl) or PBS vehicle was performed under ultrasound biomicroscopy guidance. Mice were euthanized at E17.5, and embryonic lungs were harvested for determination of lung size and weight and sectioning for hematoxylin-eosin staining.

Quantitative and statistical analysis. Numeric data are expressed as means ± SE. ANOVA was used to determine statistically significant differences among means. When ANOVA identified significant differences, post hoc analysis by Tukey’s honestly significant difference test was used to determine significant differences between groups. Statistical significance was defined as P < 0.05.

RESULTS

To determine whether a defect in parasympathetic innervation may contribute to airway branching abnormalities in CDH, we added the cholinergic agonist carbachol to explants of control and nitrofen-treated embryonic lungs in organ culture. Lungs from control or nitrofen-treated E11.5 embryos were harvested and cultured for 3 days in the presence of 25 nM carbachol or vehicle control (Fig. 1A). Nitrofen-treated embryonic lungs exhibited decreased branching, as determined by terminal bud count at 72 h, compared with controls (22.5 ± 0.68 vs. 33.4 ± 1.98; Fig. 1B). Addition of carbachol to nitrofen-treated embryonic lung explants resulted in an increase in the number of terminal buds at 72 h (27.4 ± 1.12), a ~20% increase in branching compared with untreated nitrofen lungs (Fig. 1B).

Since increased airway branching associated with carbachol treatment may be a result of increased rhythmic peristaltic airway contractions, we used time-lapse videos of lung explants at 72 h to count airway contractions. In vehicle-treated explants, airway contractions were reduced ~50% in nitrofen-treated lungs compared with controls (Fig. 1C). Addition of carbachol led to a modest increase in the number of rhythmic airway contractions in control oil-fed embryonic lungs and a significant, approximately threefold increase in airway contractions in nitrofen-treated embryonic lungs (Fig. 1C). These results suggest that parasympathetic innervation during lung development may promote airway branching by enhancing airway smooth muscle contraction.

We hypothesized that parasympathetic innervation, or carbachol administration in lung explants, may promote airway branching by altering the balance between proliferation and differentiation of airway epithelial progenitor cells within the terminal buds. These cells express a number of markers, including Sox9 (20). We therefore used Sox9 immunofluorescent staining to quantify this cell population within control or nitrofen-treated embryonic lung explants treated with carbachol. Addition of carbachol did not significantly affect the number of Sox9-positive cells in oil-fed control lung explants. Compared with control lungs, there was a trend toward an increased number of Sox9-positive cells within the terminal buds of E10.5 nitrofen-treated embryonic lung explants cultured for 72 h and normalization of the number of Sox9-positive cells within terminal buds of nitrofen-treated lung explants following addition of carbachol (Fig. 2, A and B). However, there was a significant increase in the number of proliferating Sox9-positive cells within the terminal buds of nitrofen-treated embryonic lungs, as determined by BrDU incorporation, compared with oil-fed control lungs (Fig. 2C). The number of proliferating Sox9-positive cells was slightly reduced by addition of carbachol to control lung explants, as
determined by BrdU incorporation, but was more significantly reduced in nitrofen-treated lung explants (Fig. 2C). Thus, in nitrofen-treated embryonic lung explants, a defect in airway epithelial progenitor cells, seen as an increased number and overproliferation of these cells within the lung buds, occurs and can be corrected with exogenous carbachol.

The effects of carbachol on lung explant airway branching and the Sox9-positive airway epithelial progenitor cell population could be secondary, through increased airway peristaltic contractions, or primary, through a direct effect on the Sox9-positive cells. To distinguish between these possibilities, we performed explant cultures of control or nitrofen-treated embryonic lungs with or without carbachol and with or without the calcium channel blocker verapamil to directly inhibit airway contractions (7). Lungs harvested at E10.5 were cultured for 72 h with the addition of 25 nM carbachol alone, 100 μM verapamil alone, or carbachol + verapamil. Representative time-lapse videos used to quantify airway contractions are shown for each nitrofen-treated group in Supplemental Material for this article, available online at the Journal website. As with addition of carbachol alone, airway branching was not significantly affected by the various treatments in embryonic lungs from oil-fed controls (data not shown). In nitrofen-treated embryonic lungs, carbachol alone resulted in a significant increase in airway contractions compared with no treatment (Fig. 3A), while verapamil alone slightly reduced airway contractions below baseline but did significantly reduce airway contractions in the carbachol + verapamil group compared with carbachol alone, essentially negating the carbachol-induced increase in airway contractions (Fig. 3A). Addition of verapamil + carbachol did not reduce airway branching compared with carbachol alone, despite this inhibition of airway contractions (Fig. 3B). This suggests a potential direct effect of carbachol on airway branching in lung explants, possibly through effects on airway epithelial progenitor cells. We therefore performed immunofluorescent staining for the M3 muscarinic receptor in control and nitrofen-treated embryonic lungs. These results show epithelial expression of muscarinic recep-

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Fig. 1. Effect of carbachol on lung explant airway branching and airway contractions. A and B: control or nitrofen-treated embryonic lungs were harvested at embryonic day 11.5 (E11.5) and placed into organ culture for 72 h with or without addition of 25 nM carbachol to the culture medium. Airway branching was determined by photomicroscopy (A) and determination of terminal bud counts in lung explants after 72 h of culture (B). Addition of carbachol did not result in increased airway branching in control lungs but did result in increased airway branching in nitrofen-treated lungs at 72 h. Values are means ± SE; n = 14–23 explants per group pooled from 3 separate experiments. C: airway contractions in explants after 72 h of culture with or without carbachol was determined by counting contractions within the main stem bronchi during 4-min time-lapse videos of lung explants. Nitrofen-treated lung explants exhibited reduced airway contraction compared with oil-fed control lungs. Addition of carbachol led to a significant increase in airway contractions in nitrofen-treated embryonic lung explants. Values are means ± SE; n = 12–14 explants per group pooled from 3 separate experiments. *P < 0.05 vs. oil control. #P < 0.05 vs. nitrofen control.
tors within the epithelium of the terminal buds at E14.5 (Fig. 4). A similar staining pattern was seen at E16.5 (data not shown). No obvious difference in the distribution or the amount of expression was noted between the control and nitrofen-treated embryonic lungs.

To determine whether carbachol may affect airway branching in vivo, we injected carbachol intra-amniotically into control and nitrofen-treated pregnant mice. Intra-amniotic injection of carbachol (to achieve an estimated final concentration of 25 nM within the amniotic fluid) or vehicle alone was

Fig. 2. Effect of carbachol on terminal bud Sox9-positive cell number and proliferation. A: number and proliferation of Sox9-positive epithelial progenitor cells within terminal buds assessed by immunofluorescent staining of E11.5 oil-fed and nitrofen-treated lung explants cultured for 72 h with and without carbachol following 1 h of exposure to 5-bromo-2-deoxyuridine (BrdU). B: quantification of the total number of Sox-9 positive cells per terminal bud did not show a significant difference by ANOVA; however, there was a trend toward an increased number of Sox9-positive cells in nitrofen-treated lungs vs. oil-fed controls and normalization of the number of Sox9-positive cells in nitrofen-treated lungs with addition of carbachol. C: nitrofen-treated embryonic lungs did exhibit an increased number of BrdU-positive, Sox9-positive cells per terminal bud compared with oil-fed controls, and addition of carbachol resulted in a significant decrease and normalization of the number of proliferating Sox9-positive cells in nitrofen-treated embryonic lungs. Values are means ± SE; n = 10–12 terminal buds per group.

Fig. 3. Effect of inhibition of airway contractions on airway branching in nitrofen-treated lung explants. E11.5 nitrofen-treated lung explants were cultured for 72 h with or without carbachol and verapamil to inhibit airway contractions. A: number of airway contractions in explants after 72 h of culture with or without carbachol was determined by counting contractions within the main stem bronchi during 4-min time-lapse videos of lung explants. Addition of carbachol resulted in a significant increase in airway contractions compared with vehicle, and addition of verapamil effectively reduced airway contractions in carbachol-treated lungs to baseline, thus negating the carbachol effect on airway branching. *P < 0.05 vs. nitrofen, nitrofen + verapamil, and nitrofen + carbachol + verapamil. B: addition of verapamil did not affect the trend toward increased terminal bud count in nitrofen-fed lungs treated with carbachol. Values are means ± SE; n = 12 explants per group pooled from 3 separate experiments.
performed at E10.5, and embryonic lungs were harvested at E17.5. There was no significant difference in lung size or weight in oil-fed controls with or without intra-amniotic injection of carbachol (Fig. 5A). Intra-amniotic injection of carbachol did result in a modest increase in lung size in the nitrofen-treated group, as determined by gross appearance and left lobe lung weight (Fig. 5, A and B). Furthermore, hematoxylin-eosin staining suggests increased airway branching complexity with intra-amniotic injection of carbachol in the nitrofen group compared with control (vehicle) injection (Fig. 5C).

**DISCUSSION**

CDH is associated with abnormal lung development, resulting in significant mortality and morbidity as a result of lung hypoplasia and pulmonary vascular defects that contribute to pulmonary hypertension (8, 9, 12, 15, 16, 24). The underlying mechanisms that contribute to these defects in lung development are not fully understood and are being investigated in a number of animal models of the disease. We utilize the nitrofen model of CDH, which closely mimics the human disease in terms of lung hypoplasia, pulmonary vascular abnormalities, and posterior-lateral diaphragmatic defects (4, 5). The lung hypoplasia and pulmonary vascular abnormalities are seen independent of the presence of the diaphragmatic defect in this model.

Pulmonary innervation has been shown to be abnormal in human CDH and in the mouse and rat nitrofen models (14, 17, 18). Our laboratory has shown less complex innervation of both the central and peripheral airways and vasculature in human autopsy specimens of lungs from infants with CDH compared with controls (14). Furthermore, we have shown that these differences occur developmentally in the mouse nitrofen model, in which we demonstrate a similar less complex innervation starting early in gestation. Additionally, there is relatively decreased expression of parasympathetic markers and increased expression of sympathetic markers in nitrofen-treated embryonic lungs compared with controls, suggesting an imbalance of autonomic innervation prenatally. Pederiva et al. (19) demonstrated deficient airway innervation and reduced airway peristaltic contractions in nitrofen-treated embryonic lung explants compared with controls.

The role of pulmonary neural development in normal lung development and the role of defective pulmonary neural development in the characteristic developmental defects and postnatal physiological derangements in CDH are largely unexplored. The embryonic lungs receive parasympathetic (vagal) and sympathetic extrinsic innervation, while the intrinsic innervation of the lung is derived from neural crest cells (2, 3). Multiple studies have shown that functional innervation of the embryonic lung begins in early stages of lung development. For example, branches from the vagus nerve to the developing trachea and lung buds are evident as early as E11.5 in mice (25). Subsequently, there is formation of a complex peribronchial neural plexus extending to the distal airways. In addition, neurotransmitters, including acetylcholine, and neuropeptides...
are present in early stages of lung development, although the functional significance of this expression in early lung development is not clear (1, 26). Since pulmonary innervation is closely associated with the development of airway smooth muscle, it is possible that innervation may modulate airway rhythmic contractions, which are thought to promote airway branching by causing rhythmic increases in pressure within the distal airways (3, 10, 11, 22). It is also possible that pulmonary innervation could influence lung development by direct effects on the epithelium that influence proliferation, differentiation, and/or response to branching signals or by effects on the pulmonary vasculature and blood flow.

We demonstrate that carbachol treatment can increase airway branching in nitrofen-treated embryonic lung explants. As previously shown by Pederiva et al. (19), we also demonstrate a reduction in rhythmic peristaltic airway contractions in nitrofen-treated embryonic lungs compared with control lungs. Addition of carbachol results in increased airway contractions in the nitrofen group. A similar effect was shown previously with the addition of retinoic acid to nitrofen-treated lung explants (19). Nitrofen inhibits retinoid signaling; therefore, retinoic acid may essentially block the nitrofen effect. We show that airway peristaltic contractions and branching can be at least partially corrected in the nitrofen model pharmacologically with carbachol, presumably independent of effects on the retinoid signaling pathway.

Addition of carbachol normalizes the number of total and proliferating Sox9-positive cells in the terminal buds of nitrofen-treated embryonic lungs, suggesting that carbachol acts via effects on airway epithelial progenitor cells. This effect could potentially be secondary, through the mechanical effects of increased airway contractions and increased intraluminal airway pressure. However, the fact that verapamil could block the carbachol-induced increase in airway contractions but did not affect the increased airway branching induced by carbachol suggests that carbachol has a direct effect on airway epithelial progenitor cells. This direct effect, supported by the expression of M₃ muscarinic receptors in the developing lung epithelium, may occur through modulation of the balance of progenitor cell proliferation and differentiation, or possibly by an effect on the response of distal airway epithelial cells to mesenchymal branching signals, although the exact effect remains to be determined. Knox et al. (13) showed that parasympathetic innervation maintains epithelial progenitor cells in salivary gland development. In contrast, our results show that parasympathetic innervation, i.e., addition of carbachol, causes de-

Fig. 5. In vivo treatment with carbachol via intra-amniotic (IA) injection. Intra-amniotic injection of carbachol (to achieve an estimated final concentration of 25 nM within the amniotic fluid) or vehicle alone (control) was performed at E10.5, and lungs were harvested at E17.5. A and B: there was no significant difference in lung size or weight between oil-fed control lungs treated with intra-amniotic carbachol and those injected with vehicle. Intra-amniotic carbachol injection did result in a modest, but significant, increase in lung size compared with intra-amniotic vehicle injection in the nitrofen-treated group as determined by left lobe lung weight (A) and gross appearance (B). Values are means ± SE; n = 5–10 lungs per group pooled from 3 separate experiments. *P < 0.05 vs. oil-fed control + control (vehicle). #P < 0.05 vs. nitrofen + control (vehicle). C–E: hematoxylin-eosin staining suggests increased airway branching with intra-amniotic injection of carbachol into the nitrofen group (nitrofen:IA carbachol) compared with the control group (nitrofen:control).
creased airway epithelial proliferation in nitrofen-treated embryonic lungs. This result suggests overproliferation or over-accumulation of airway epithelial progenitor cells within the terminal buds in the nitrofen model, perhaps indicating that these cells are not able to respond to branching signals or differentiate normally and that carbachol acts to correct this disturbance.

The expression of muscarinic receptors within the embryonic lung epithelium suggests that parasympathetic innervation may play a role similar to exogenous carbachol in vivo. As noted above, we previously demonstrated a relative decrease in parasympathetic innervation in nitrofen-treated embryonic lungs (14). The expression of epithelial muscarinic receptors, however, does not appear to differ between control and nitrofen-treated embryonic lungs. In vivo treatment of embryos exposed to nitrofen with an intra-amniotic injection of carbachol at E10.5 resulted in increased lung size and weight, as well as improvement in airway branching at E17.5 compared with vehicle-treated controls. Intra-amniotic injection was chosen here, because the amniotic fluid likely serves as a reservoir for the drug that results in prolonged exposure to the embryo, without significant metabolism by the embryo. These in utero results suggest that parasympathetic innervation can affect airway epithelial progenitor cells and promote airway branching in vivo, although further experiments are required to determine the optimal conditions and underlying mechanisms of this in vivo effect. These effects also raise the possibility of a therapeutic potential for amniotic injection of cholinergic agonists. However, our injections occurred at a comparable stage of lung development prior to typical prenatal diagnosis by ultrasound in humans, and potential maternal and fetal effects of the treatment need to be determined.

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GRANTS

This study was supported by National Institute of Child Health and Human Development Grant 1K08 HD-061599 (to D. A. Potoka).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.R., D.S., G.Z., G.K.G., and D.A.P. analyzed the data; J.R. drafted the manuscript; J.R., D.S., G.Z., and D.A.P. edited and revised the manuscript; D.A.P. prepared the figures. G.Z., G.K.G., and D.A.P. edited and revised the manuscript; D.A.P. prepared the figures. G.K.G., and D.A.P. interpreted the results of the experiments; G.K.G. and D.A.P. approved the final version of the manuscript; J.R., D.S., G.Z., and D.A.P. analyzed the data; J.R. drafted the manuscript; J.R., D.S., G.Z., and D.A.P. interpreted the results of the experiments; G.K.G. and D.A.P. edited and revised the manuscript; D.A.P. prepared the figures.

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