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Impaired mesenchymal cell function in *Gata4* mutant mice leads to diaphragmatic hernias and primary lung defects

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Abstract

Congenital diaphragmatic hernia (CDH) is an often fatal birth defect that is commonly associated with pulmonary hypoplasia and cardiac malformations. Some investigators hypothesize that this constellation of defects results from genetic or environmental triggers that disrupt mesenchymal cell function in not only the primordial diaphragm but also the thoracic organs. The alternative hypothesis is that the displacement of the abdominal viscera in the chest secondarily perturbs the development of the heart and lungs. Recently, loss-of-function mutations in the gene encoding FOG-2, a transcriptional co-regulator, have been linked to CDH and pulmonary hypoplasia in humans and mice. Here we show that mutagenesis of the gene for GATA-4, a transcription factor known to functionally interact with FOG-2, predisposes inbred mice to a similar set of birth defects. Analysis of wild-type mouse embryos demonstrated co-expression of *Gata4* and *Fog2* in mesenchymal cells of the developing diaphragm, lungs, and heart. A significant fraction of C57Bl/6 mice heterozygous for a *Gata4* deletion mutation died within 1 day of birth. Developmental defects in the heterozygotes included midline diaphragmatic hernias, dilated distal airways, and cardiac malformations. Heterozygotes had any combination of these defects or none. In chimeric mice, $Gata4^{-/-}$ cells retained the capacity to contribute to cells in the diaphragmatic central tendon and lung mesenchyme, indicating that GATA-4 is not required for differentiation of these lineages. We conclude that GATA-4, like its co-regulator FOG-2, is required for proper mesenchymal cell function in the developing diaphragm, lungs, and heart.
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Keywords: Birth defect; Diaphragm; Pulmonary hypoplasia; Transcription factor

Introduction

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly that affects 1 per 3000 live births and accounts for 1–2% of infant mortality (Pober et al., 2005; Colvin et al., 2005; Yang et al., 2006). The hallmark of the disorder is a defect in the muscular or tendinous portion of the diaphragm. CDH is thought to result from abnormal embryonic development of the diaphragmatic substratum, but the molecular pathogenesis of this disorder is poorly understood (Greer et al., 2000b; Babiuk and Greer, 2002).

Primary defects in lung morphogenesis and cardiovascular malformations often accompany CDH in animal models and patients (Migliazza et al., 1999; Losty et al., 1999; Graziano, 2005). This association has given rise to the *mesenchymal hit hypothesis*, which posits that genetic or environmental triggers of CDH disrupt the function of mesenchymal cells in not only the primordial diaphragm but also the developing lungs and heart (Keijzer et al., 2000). A corollary, the *smooth muscle hypothesis*, proposes that disruption of mesenchymal progenitors of smooth muscle in the pulmonary vasculature and airways leads to pulmonary hypertension, airway hyperreactivity, and other pulmonary complications commonly encountered in pa-

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tients with CDH (Jesudason, 2006). Consequently, a major goal of CDH research is to identify genes critical for early mesenchymal cell function in the diaphragm, lungs, and cardiovascular system.

Recent studies, including analysis of recurring chromosomal rearrangements in patients with CDH (Lurie, 2003), suggest that transcription factor haploinsufficiency may cause or contribute to this disorder. Chromosome 8q22–23 abnormalities have been associated with CDH (Wilson et al., 1983; Temple et al., 1994; Howe et al., 1996), and mutation of one of the genes in this region, *FOG2*, has been shown to cause diaphragmatic defects and primary pulmonary hypoplasia in humans and mice (Ackerman et al., 2005). *FOG2* encodes a transcriptional co-regulator that is expressed by mesenchymal cells in the diaphragm, lung, and heart and by somatic cells in the testis (Svensson et al., 2000; Tevosian et al., 2000; Ketola et al., 2002; Ackerman et al., 2005). Another recurring chromosomal abnormality in CDH is microdeletion of 8p23.1 (Pecile et al., 1990; Faivre et al., 1998; Borys and Taxy, 2004; Shimokawa et al., 2005; Barber et al., 2005; López et al., 2006). One of the genes in the critical region of 8p23.1 is *GATA4*, which encodes a transcription factor that physically interacts with FOG-2 during morphogenesis of the heart and testis (Crispino et al., 2001; Tevosian et al., 2002). It is therefore plausible that *GATA4* haploinsufficiency contributes to the pathogenesis of CDH, particularly in patients with concomitant diaphragm and heart defects as loss-of-function mutations in *GATA4* have been linked to cardiac malformations in humans (Garg et al., 2003; Okubo et al., 2004; Nemer et al., 2006) and mice (Kuo et al., 1997; Molkentin et al., 1997; Crispino et al., 2001; Watt et al., 2004; Pu et al., 2004; Zeisberg et al., 2005; Xin et al., 2006).

Here we show that C57Bl/6 mice heterozygous for a mutant allele of *Gata4* are predisposed to CDH and primary lung abnormalities. We propose that GATA-4, working in concert with FOG-2 or other transcription factors, regulates mesenchymal cell function in the developing diaphragm and lungs. Our findings support the premise that GATA-4 mutation contributes to the pathogenesis of CDH and related developmental anomalies in man.

Materials and methods

Experimental mice

Procedures involving mice were approved by institutional committees for laboratory animal care and were conducted in accordance with NIH and EU guidelines for the care and use of experimental animals. C57Bl/6 and *Rosa26* (C57Bl/6, *Gpi-1*^{b}) (Friedrich and Soriano, 1991) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Gata4*$^{+/\Delta ex2}$ mice, which harbor a deletion in exon 2 of the gene, were generated and genotyped as described elsewhere (Pu et al., 2004; Zeisberg et al., 2005). These mice were backcrossed with C57Bl/6 mice for a minimum of 7 generations. *Nkx2–5*$^{+/-}$ mice were prepared and genotyped as described previously (Tanaka et al., 2002) and were backcrossed with C57BL/6 mice for a minimum of 12 generations. Chimeric mice were prepared by injection of XY$^{Gata4-/-}$ (CCE, 129/Sv//Ev, *Gpi-1*^{c}) ES cells (Soudais et al., 1995; Kuo et al., 1997) into *Rosa26* embryos as described (Narita et al., 1997a). These nullizygous ES cells harbor a *neomycin resistance* cassette in exon 2 of the gene. Chimeras were initially identified by GPI-1 isoenzyme analysis of tail tissue (Narita et al., 1997a). *XIST* RT-PCR analysis of tail or hind limb tissue was used to distinguish chimeras derived from XX versus XY host blastocysts (Natoli et al., 2004). Only chimeric mice derived from XY hosts were subjected to further analysis.

Tissue isolation and histological analyses

Late gestation fetuses were harvested, fixed overnight in 4% paraformaldehyde in PBS, and embedded in paraffin for routine histology. In some cases, pregnant females were injected intraperitoneally with 2 mg bromodeoxyuridine (BrdU) 15 h before embryo harvest. Alternatively, unfixed fetuses were embedded directly in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) for preparation of cryosections. Thoraces of newborn pups were isolated by decapitation and transection at the level of the liver. Skin and soft tissue were removed, and the intact thorax was fixed for 1–2 days in 4% paraformaldehyde+1% glutaraldehyde in PBS at 4°C. Diaphragm, heart, and lungs were then dissected and processed for morphometric and ultrastructural analyses. Paraffin sections (5–6 μm) were stained with hematoxylin and eosin (H&E) or with Masson's trichrome to visualize extracellular matrix (ECM).

To detect β-galactosidase (β-gal) expression, frozen tissue sections (10 μm) were fixed with 0.2% gluteraldehyde for 5 min, permeabilized with 100 mM potassium phosphate pH 7.4, 0.02% NP-40 and 0.01% sodium deoxycholate for 5–15 min, and then incubated in 0.5 mg/ml X-gal (Promega) with 10 mM $K_3[Fe(CN)_6]$, 10 mM $K_4[Fe(CN)_6]$, 100 mM potassium phosphate pH 7.4, 0.02% NP-40, and 0.01% sodium deoxycholate at 30°C overnight (Narita et al., 1997a). The sections were then counterstained with eosin.

Morphometric analysis

Air space size was estimated from the mean chord length of the airspace (Ray et al., 1997). Images of H&E-stained lung tissue from postnatal day 1 mice were acquired at 400× magnification using an Olympus BX60 microscope and a Zeiss Axiocam digital camera and superimposed on a grid (Ray et al., 1997). The length of the lines overlying air space air was measured using Scion® Image software and then averaged to obtain the mean chord length. Fields containing large airways and vessels were excluded from the chord length measurements. The number of bronchioles and arteries per mm^2 was quantified as described elsewhere (Mäki et al., 2005).

Electron microscopy

Excised tissue was processed for electron microscopy as described (Narita et al., 1997a). Briefly, paraformaldehyde/glutaraldehyde-fixed tissue was treated with OsO_4 and then embedded in resin. Sections (1 μm) were cut and stained with methylene blue for preliminary light microscopic evaluation. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then examined in a JEM 1010 transmission electron microscope.

Immunostaining

Paraformaldehyde-fixed, paraffin-embedded tissue sections were processed for immunoperoxidase staining using previously described methods (Bielinska et al., 2005; Jacobsen et al., 2005). The following primary antibodies were employed: (1) goat anti-mouse GATA-4 IgG (sc-1237, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:200 dilution; (2) goat anti-mouse FOG-2 (sc-9264, Santa Cruz Biotechnology, Inc.), 1:100 dilution; (3) rabbit anti-mouse smooth muscle α-actin (AnaSpec, Inc., San Jose, CA), 1:1000 dilution; (4) rabbit anti-mouse Clara cell secretory protein CC10 (sc-25555, Santa Cruz Biotechnology, Inc.), 1:100 dilution; (5) goat anti-mouse surfactant protein C (sc-7706, Santa Cruz Biotechnology, Inc.), 1:200 dilution; (6) goat anti-phosphohistone H3 (sc-12927, Santa Cruz Biotechnology, Inc.), 1:200 dilution; and (7) mouse anti-BrdU (sc-32323, Santa Cruz Biotechnology, Inc.), 1:200 dilution. Secondary antibodies employed for immunoperoxidase staining were: donkey anti-goat biotinylated IgG (Jackson Immunoresearch, West Grove, PA) 1:1000 dilution; donkey anti-mouse biotinylated IgG (Jackson Immunoresearch), 1:2000 dilution; goat anti-rabbit biotinylated IgG (NEF-813, NEN Life Science, Boston MA), 1:2000 dilution. The avidin–biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine (Sigma-Aldrich Corp.,

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