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Formation of Intestinal Atresias in the *Fgfr2IIIb*^{−/−} Mice is not Associated with Defects in Notochord Development or Alterations in *Shh* Expression

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Abstract

Purpose—The etiology of intestinal atresia remains elusive but has been ascribed to a number of possible events including *in utero* vascular accidents, failure of recanalization of the intestinal lumen and mechanical compression. Another such event that has been postulated to be a cause in atresia formation is disruption in notochord development. This hypothesis arose from clinical observations of notochord abnormalities in patients with intestinal atresias as well as abnormal notochord development observed in a pharmacological animal model of intestinal atresia. Atresias in this model result from *in utero* exposure to Adriamycin, wherein notochord defects were noted in up to 80% of embryos that manifested intestinal atresias. Embryos with notochord abnormalities were observed to have ectopic expression of *Sonic Hedgehog* (*Shh*) which in turn was postulated to be causative in atresia formation. We were interested in determining whether disruptions in notochord development or *Shh* expression occurred in an established genetic model of intestinal atresia and utilized the *Fibroblast Growth Factor Receptor 2IIIb* homozygous mutant (*Fgfr2IIIb*^{−/−}) mouse model. These embryos develop colonic atresias (100% penetrance) and duodenal atresias (42% penetrance).

Methods—Wild-type and *Fgfr2IIIb*^{−/−} mouse embryos were harvested at E10.5, E11.5, E12.5 and E13.5. Whole mount *in situ* hybridization was performed on E10.5 embryos for *Shh*. Embryos at each time point were harvested and sectioned for H&E staining. Sections were photographed specifically for the notochord and resulting images reconstructed in 3-D using Amira software. Colons were isolated from wild-type and *Fgfr2IIIb*^{−/−} embryos at E10.5, then cultured for 48 hours in matrigel with FGF10 in the presence or absence of exogenous SHH protein. Explants were harvested, fixed in formalin and photographed.

Results—*Fgfr2IIIb*^{−/−} mouse embryos exhibit no disruptions in *Shh* expression at E10.5, when the first events in atresia formation are known to occur. Three-dimensional reconstructions failed to demonstrate any anatomical disruptions in the notochord by discontinuity or excessive branching. Culture of wild-type intestines in the presence of Shh failed to induce atresia formation in either the duodenum or colon. Cultured *Fgfr2IIIb*^{−/−} intestines developed atresias of the colon in either the presence, or absence, of Shh protein.

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Conclusions—Although disruptions in notochord development can be associated with intestinal atresia formation, in the *Fgfr2IIIb*^{-/-} genetic animal model neither disruptions in notochord development nor the presence of exogenous Shh protein are causative in the formation of these defects.

Introduction

The etiology of intestinal atresia remains elusive. Possible causative events include *in utero* vascular accidents (1), failure of recanalization of the intestinal lumen (2) and mechanical compression (1, 3). Another postulated etiology of atresia formation is disruption in notochord development (4). Observations of occasional vertebral abnormalities in patients with intestinal atresias and a pharmacological animal model of intestinal atresia form the basis of this hypothesis (4, 5).

Atresias in the pharmacological rat model result from *in utero* exposure to Adriamycin, an anti-neoplastic agent known to interfere with cellular growth. Notochord defects were noted in up to 78% of embryos that manifested intestinal atresias in the Adriamycin Rat Model (ARM) (6). Embryos in this model system frequently have profound segmental defects, notochord branching, and encroachment of the branched notochord on the developing gut, aorta, and kidney. Regions of ectopic notochord branching are estimated to have greater mass and proportionately increased *Sonic Hedgehog* (*Shh*) signaling, suggesting that increased notochord proximity in the affected ARM embryos results in ectopic *Shh* signaling (7). The Shh protein is expressed by the developing notochord as a morphogen, responsible for differentiation by both contact-dependent and diffusible mechanisms (5, 8). Adriamycin-dependent notochord disruption is thought to transmit incorrect patterning instructions through ectopic *Shh* expression and abnormal gradients of the signaling protein (5).

Notochord branching defects with encroachment on various neural crest-derived structures have also been described in the *Patch* genetic mouse embryo model (9). Like the Adriamycin rat model, the phenotype of *Patch* model embryos can include notochord disruption and anomalous *Shh* signaling in the developing foregut. In contrast to the Adriamycin model, intestinal atresias are not described in the *Patch* model. Furthermore, the research indicated that aberrant extra cellular matrix composition may be inhibiting neural crest cell migration and impacting coordinated foregut-notochord-somite signaling. Human notochord branching defects, such as Split Notochord Syndrome, are rare and intestinal tract involvement is theorized to be due to compression of the intestinal tube, as opposed to aberrant extracellular signaling during a critical phase of intestinal development (10).

We were interested in determining if disruptions in notochord development or *Shh* expression occurred in an established genetic model of intestinal atresia: the *Fibroblast Growth Factor Receptor 2IIIb* homozygous null (*Fgfr2IIIb*^{-/-}) mouse model. *Fgfr2IIIb*^{-/-} embryos develop colonic atresias (100% penetrance) and duodenal atresias (38–42% penetrance) (11, 12). The *Fgfr2IIIb* null defects include a host of skeletal, skin, and visceral abnormalities arising from the inability of *Fibroblast Growth Factor 10* (*FGF10*), expressed in the mesenchyme, to activate its cognate receptor, *Fgfr2IIIb*, in the epithelium (13); in the developing intestine, this was initially described in the budding cecum (14). Colonic atresia formation in *Fgfr2IIIb*^{-/-} embryos includes an increase in endodermal cell death and reduction in endodermal proliferation, events that precede formation. Both endodermal events are first observed at embryonic day (E) 10.5 and involution of the atretic intestine segment is complete by E13.5, this is observed in colonic and duodenal atresias (15, 16, 17). Notochord disruption and ectopic signaling of *Shh* have not been examined in this genetic

model of intestinal atresia. We examined *Fgfr2IIIb*^{-/-} embryos for these two events during the temporal window of atresia formation.

Materials and Methods

Animals

IACUC approval for these studies was obtained from the University of Wisconsin School of Medicine and Public health (P.F.N. protocol # M02258). All animals were maintained in a clean facility with *ad libitum* access to fresh food and water under a 12-hour alternating light/dark cycle.

Generation of mutant fetuses

The use of *HprtCre* technology (18) to efficiently generate *Fgfr2IIIb*^{-/-} embryos has been described previously (12). *Fgfr2IIIb*^{-/-} embryos were also generated through traditional heterozygous breedings with *Fgfr2IIIb* ^{+/-} parents. Primer sets for genotyping have been described previously (13).

Section *in situ* hybridization

Wild-type embryos were harvested at E11.5 or E13.5 into cold phosphate buffered saline (PBS) and fixed overnight (O/N) in Bouin's Fixative at 4°C. Tissues were dehydrated to 100% Ethanol through a series of escalating Ethanol/PBS steps and processed for paraffin sectioning at 10-micrometer (µm) thickness. Section *in situs* were performed at 60°C with antisense probes for *Fgfr2* (19) or *Shh* (20) as described previously (19). Photomicrographs were taken at 40X using a standard light microscope (Olympus BX-41, Tokyo, Japan).

Whole mount *in situ* hybridization

Fgfr2IIIb^{-/-} embryos and their normal littermate controls were harvested at E10.5 into cold PBS and fixed O/N in 4% Paraformaldehyde (PFA) at 4°C. Fixed samples were dissected, dehydrated to 100% Methanol through a series of escalating Methanol/PBS-Tween steps and stored at -20°C. The hybridization process included incubation at 68°C with *Shh* antisense probes (20). Photographs were taken under a dissecting light microscope.

Histology

Fgfr2IIIb^{-/-} embryos and their normal littermate controls were harvested at E10.5, E11.5, E12.5, and E13.5, fixed O/N in Bouin's Fixative at 4°C, dehydrated through a series of escalating Ethanol/PBS steps, embedded into paraffin, and sectioned at 7 µm thickness. Sections were floated onto slides in a 50°C water bath, de-waxed and stained for Hematoxylin and Eosin (H&E). Photomicrographs were taken at 40X using a standard light microscope (Olympus BX-41, Tokyo, Japan).

3-dimensional reconstructions

Photomicrographs of sequential H&E -stained sections were uploaded into the Amira™ Program (Visage Imaging, San Diego, CA). Images were aligned with the "Auto Align" tool, labeled using the "Segmentation Editor", and a 3-D structure was configured using the "Surface Generator" tool. External contour of the embryo, extent of the notochord, and external surfaces of the intestines were delineated. A final rendering was generated as a JPEG file at 600 pixels per-square-inch and imported into Adobe Photoshop.

Organ culture

Fgfr2IIIb^{-/-} embryos and normal littermate controls were harvested at E10.5 and developing intestinal tracts were isolated. Tracts were cultured in Matrigel (BD Biosciences, Bedford, MA) in the presence of 100 µg/ml of FGF10 (PeproTech, Rocky Hill, NJ). Additional normal embryo tracts were cultured in Matrigel in 100 µg/ml of FGF10 + 100 µg/ml of SHH (R&D Systems, Minneapolis, MN). Organ culture was conducted 3 times following previously published protocols (14) with a total of 4 mutant and 12 control tracts.

E-Cadherin staining

Cultured intestinal tracts were harvested at E12.5 and fixed in 4% PFA O/N at 4°C, dehydrated through escalating Methanol/PBST steps, and embedded into Paraffin. Sections were generated at 7 µm, floated onto slides, dried O/N at 37 °C, deparaffinized and rehydrated. Antigen retrieval was by HIER-citrate at pH 6.0 using a decloaking chamber at 100°C for 20 min. Next, sections were blocked with 10% Bovine serum albumin for 60 min and incubated with rabbit monoclonal anti-E-cadherin antibody (Cell Signaling Technology cat# 3195, Beverly, MA) at 1:50 dilution O/N at 4°C. Endogenous peroxidase was quenched with 1.0% hydrogen peroxide in Methanol for 10 min, then sections incubated with goat anti-rabbit HRP polymer (Vector Laboratories, Burlingame, CA) for 30 min at RT. Sections were washed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) for 2 min and counterstained with hematoxylin.

Results

Fgfr2 is not expressed in the mouse notochord

Normal expression patterns of *Fgfr2* in the notochord of wild-type mouse embryos have not been previously reported. The *Fgfr2* gene gives rise to two receptor isoforms through the process of alternative splicing: *Fgfr2IIIb* and *Fgfr2IIIc*. We examined expression of *Fgfr2* using a probe that detects both isoforms (14). Section *in situ hybridization* was performed on 10µm transverse paraffin-embedded sections from wild-type embryos taken at E11.5 and E13.5. No *Fgfr2* expression was detected in the notochord at these time points (Figure 1A, Green arrow). In contrast, expression of *Shh* is very robust in the notochord at E13.5 (Figure 1B, Green arrow). This led us to hypothesize that the mutation of *Fgfr2IIIb*, although causative in atresia formation, would not result in defects in either notochord development or in aberrant *Shh* expression.

Notochord structure in *Fgfr2IIIb* mutant embryos

Abnormal branching of the notochord has been observed in the Adriamycin Rat model of intestinal atresia formation. To determine whether this was occurring in *Fgfr2IIIb*^{-/-} embryos during the developmental window when intestinal atresias form, photomicrographs of transverse 7µm H&E-stained notochord sections were taken of normal control and *Fgfr2IIIb*^{-/-} mutant embryos at E10.5, E11.5, E12.5 and E13.5. The resulting photomicrographs were constructed into 3-D renderings using Amira™ imaging software. At all time points examined (E10.5 to E13.5), there were no discernible differences in the structure of the notochord between normal littermate controls and mutant embryos (Figure 2). Of note, involution of the atretic segment of the duodenum is observed at E12.5 and E13.5 when the notochord appears completely intact.

Shh is expressed normally in *Fgfr2IIIb* mutant embryo notochord

Ectopic expression of *Shh* is reported in the Adriamycin model of intestinal atresia and purported to be a causative factor of atresia formation. This ectopic *Shh* expression is hypothesized to be proportionally higher in regions proximal to the branched notochord.

However, it is possible that excessive levels of Shh expression in the notochord could occur in the absence of aberrant notochord development. To test this, we performed whole mount *in situ* on *Fgfr2IIIb*^{-/-} and littermate control embryos at E10.5. We observed that *Fgfr2IIIb*^{-/-} mutant embryos and normal littermate controls exhibited equivalent patterns of *Shh* expression by whole mount *in situ* hybridization at this time point: the initial stage of atresia formation with increased endodermal apoptosis and decreased endodermal proliferation (Figure 3).

Exogenous Shh does not induce atresia formation in cultured wild-type mouse intestine

Although no alterations in *Shh* signaling were observed in the *Fgfr2IIIb* mutants, we wanted to determine whether elevated levels of exogenous Shh could cause atresia formation in a tissue culture model of intestinal atresia. Intestinal explants were isolated from wild-type embryos at E10.5 and cultured in Matrigel in the presence of FGF10 or FGF10 + Shh protein for 48 hours. The addition of exogenous Shh signaling protein did not result in atresia formation (Figure 4). In fact, the endoderm of the cecum and colon are readily identifiable in the whole mount photomicrograph after the 48 hour culture. On transverse section, the endodermal tube of these samples remains intact and stains positively for *E-cadherin*, an epithelial marker. By contrast, explants of *Fgfr2IIIb*^{-/-} mutants demonstrated near-complete loss of the colon. Transverse histological sections of *Fgfr2IIIb*^{-/-} mutants demonstrate loss of the colonic endoderm, indicative of atresia formation. The results indicate that the presence of elevated/exogenous Shh protein does not cause atresia formation in cultured wild-type embryonic colons.

Discussion

We set out to determine if 1) notochord disruptions are present and 2) whether ectopic expression of *Shh* occurs in the *Fgfr2IIIb*^{-/-} model of intestinal atresia. Such a finding would provide additional supporting evidence for this unique hypothesis on atresia formation. Interestingly, we observed that the gene of interest in this model (*Fgfr2IIIb*) is not expressed in the notochord of wild-type embryos during the temporal window during which intestinal atresias are forming. Furthermore, mutation of both copies of *Fgfr2IIIb*, although causative in atresia formation, is not associated with disruptions in notochord anatomy or ectopic *Shh* expression during intestinal atresia formation. Finally, ectopic, exogenous SHH protein did not induce atresia in wild-type intestinal explants. These findings do not support notochord disruptions, such as branching or encroachment, or ectopic expression of *Shh* as causative in the formation of atresias in this genetic animal model.

In general, the mechanisms by which the teratogenic or anti-teratogenic actions of anthracyclines remain poorly understood. Additionally, the exact mechanism by which Adriamycin gives rise to notochord disruptions is also unclear. Embryos from the Adriamycin model frequently exhibit defects of the notochord, gut, and aorta that co-localize segmentally within the embryo. This observation gives significant weight to a series of articles that implicate notochord hypertrophy and increased fibronectin production as causative factors unique to the animal model (7, 21–23). Interestingly, notochord hypertrophy in this model is described as maximal at E11.0 after Adriamycin administration (7), aligning temporally with a critical window of intestinal development (11, 12, 15, 16). Further, the anatomical overlap in the notochord, gut, and aorta defects suggests that Adriamycin disrupts the development of multiple structures arising from one or two somites along the axial plane of the embryo. The Adriamycin model implicates *Shh* as a mediator between notochord disruptions and somite development, thus affecting the subsequent somite-derived structures (24). A precise understanding of how critical signaling pathways are affected by anthracyclines is essential to understand exactly how Adriamycin generates atresias.

Whether ectopic expression of *Shh* or downstream members of the *Shh* pathway are causative in atresia formation is uncertain. Based on evidence from *Shh*^{-/-} embryos (25), a lack of *Shh* expression results in mucosal webs (a variant of type I atresias) within the duodenal lumen. This line of evidence raises the question of the significance of ectopic *Shh* mRNA expression in the Adriamycin model.

Assuming that in genetic models intestinal atresias are not due to notochord disruptions, how do they arise? There is an increasing amount of evidence that atresias are a result of a defect in endodermal development or disruption in mesodermal-endodermal signaling. The *Fgfr2IIIb*^{-/-} and *Fgf10*^{-/-} mouse models provide supporting evidence for such a mechanism. Within the developing intestine, *Fgfr2IIIb*, a membrane-bound tyrosine kinase receptor, is thought to be expressed exclusively in the endoderm (14). Thus, loss of either the mesodermally expressed *Fgf10* ligand or its endodermal target, *Fgfr2IIIb*, appears to result in atresia formation. Early developmental interruption of the crucial *Fgf10-Fgfr2IIIb* signaling cascade is followed by dramatic segmental increases in endodermal cell death and decreased endodermal proliferation, with involution of the affected intestine segment occurring 2 days later in the mouse model of colonic atresia (11, 12, and 15). Collectively, the data suggest that a defect in endoderm development is the basis for atresia formation in both *Fgfr2IIIb*^{-/-} and *Fgf10* models. Speculation remains as to whether such a mechanism is at work during human atresia formation. Studying homozygous mutation of *Fgfr2IIIb* in larger animal models, such as pigs or monkeys, would help to elucidate if the cellular events seen in the mouse model are likely to be recapitulated in humans.

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Figure 1.

Figure 1A. Section *in situ* hybridization of an E11.5 wild-type mouse embryo for *Fgfr2*. Green arrow indicates notochord. LB-lung bud. Eso-Esophagus.

1B. Section *in situ* hybridization of an E13.5 wild-type mouse embryo for *Shh*. Green arrow indicates notochord. LB-lung bud. Eso-Esophagus.

Figure 2.

Three dimensional reconstructions of intestinal tube (yellow) and notochord (red) between E10.5 and E13.5 in wild-type (A–D) and *Fgfr2IIIb*^{-/-} embryos (E–H). Green arrow in F–H indicates area where atresia is forming in the *Fgfr2IIIb*^{-/-} mutants. Measure bars indicate 500 μ m.

Figure 3. Whole mount *in situ* hybridization for *Shh* in (A) normal littermate controls and (B) *Fgfr2IIIb*^{-/-} mutants at E 10.5. Green arrow indicates notochord.

Figure 4.

Whole mount and section photomicrographs of *Fgfr2IIIb*^{-/-} and normal colons between E10.5 and E12.5. *Fgfr2IIIb*^{-/-} colons cultured in the presence of Fgf10 at (A) E10.5, (B) 11.5 and (C) 12.5. White line in (C) indicates plane of section in photomicrograph shown in (D). Normal colons cultured in the presence of Fgf10 at (E) E10.5, (F) 11.5 and (G) 12.5. White line in (G) indicates plane of section in photomicrograph in (H). Normal colons cultured in the presence of Fgf10 and Shh at (I) E10.5, (J) 11.5 and (K) 12.5. White line in (K) indicates plane of section in section photomicrograph in (L). Green arrows indicates Cecum, Yellow arrows indicate colon. Orange arrows indicate lumen and endodermal tube in *E-Cadherin* stained sections of samples at E12.5.