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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 pharmacologic 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 used 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 embryonic day (E) 10.5, 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 hematoxylin-eosin 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

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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.

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.

1. 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 pharmacologic animal model of intestinal atresia form the basis of this hypothesis [4,5].

Atresias in the pharmacologic rat model result from *in utero* exposure to Adriamycin, an antineoplastic 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 [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 Adriamycin rat model 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 extracellular 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]. Atresia formation in *Fgfr2IIIb*−/− embryos includes an increase in endodermal cell death and reduction in endodermal proliferation, events that precede involution of the affected segment. 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 both colonic and duodenal atresias [15–17]. 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.

2. Materials and methods

2.1. 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-h alternating light/dark cycle.

2.2. 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].

2.3. 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 40× magnification using a standard light microscope (BX-41; Olympus, Tokyo, Japan).