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Retinaldehyde dehydrogenase 2 is Down-Regulated During Duodenal Atresia Formation in *Fgfr2IIIb-/-* Mice

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Background. **Homozygous null mutation of *fibroblast growth factor receptor 2* (*Fgfr2IIIb*) or its ligand *fibroblast growth factor 10* (*Fgf10*) results in duodenal atresia in mice. Mutations of either of these genes in humans cause Matthew-Wood syndrome and associated duodenal stenosis. Recently, mutations in the retinol-binding protein receptor gene *STRA6* were reported to be implicated in this syndrome as well. This suggests that the retinoic acid (RA) signaling pathway interacts with the Fgf10-Fgfr2IIIb signaling pathway during duodenal development. Accordingly, we hypothesized that *Fgfr2IIIb-/-* mouse embryos would exhibit disruptions in expression of *Raldh2,* the gene for the enzyme that regulates the final step in the conversion of vitamin A to the active form RA, during duodenal atresia formation.**

Materials and Methods. **_Fgfr2III -/-_ mice were generated from heterozygous breedings. Embryos were harvested between embryonic day (E) 11.0 to E 13.5 and genotyped by polymerase chain reaction (PCR). Duodenums were dissected out, fixed and photographed. Whole mount and section *in situs* were performed for *Raldh2*.**

Results. **_Fgfr2IIIb-/-_ embryos demonstrate subtle changes in the duodenal morphology by E11.5 with complete involution of the atretic precursor by E 13.5. *Raldh2* appears to be down-regulated as early as E 11.5 in the atretic precursor a full 2 days before this segment disappears.**

Conclusions. **In _Fgfr2IIIb-/-_ mouse embryos, a reduction of *Raldh2* expression is observed within the region that is forming the atresia. This is the first demonstration of such an event in this model. As in humans, these results implicate disruptions between Fgfr2IIIb receptor function and RA signaling in the formation of this defect and indicate that _Fgfr2IIIb-/-_ mouse embryos are a valid model for the study of the atretic spectrum of defects in human duodenal development.**

Key Words: **duodenal atresia; mouse; Fgfr2IIIb; Raldh2; retinoic acid; down-regulation; gene expression; Matthew-Wood syndrome.**

INTRODUCTION

During duodenal development in humans, the lumen of the proximal duodenum becomes occluded by an endodermal plug at Carnegie stage (CS) 17 and then begins to recanalize 2 d later at CS 18. The first description of these events was published by Tandler in 1900, and he went on to hypothesize that a failure of the recanalization step could result in duodenal atresia [1]. Recently, a genetic animal model for duodenal atresia has been described in which the defect arises from a deletion of the *IIIb* exon of the *fibroblast growth factor receptor 2* gene (*Fgfr2*). This results in the loss of the Fgfr2IIIb isoform of the receptor throughout the embryo during development [2]. The mutant embryos form atresias in the second portion of the duodenum similar to humans. The legitimacy of this model has been bolstered by the observation that mutations in the *fibroblast growth factor receptor 2* gene or the gene encoding its ligand *fibroblast growth factor 10* (*Fgf10*) result in duodenal stenosis in humans [3]. Interestingly, the morphogenesis of atresia in the animal model has not been described and signaling pathways downstream of Fgfr2IIIb have not been identified in the formation of this defect.

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Disruptions in the retinoic acid (RA) signaling pathway have been implicated in the formation of duodenal stenosis, a type of atretic defect in humans [4]. The RA signaling pathway interacts with Fgfr2IIIb signaling during lung and pancreas development in mice [5, 6]. Loss of *Raldh2* expression (a gene that encodes the enzyme that regulates the final step in the conversion of vitamin A to RA) impedes the generation of Fgf10 in the lung, thus, disrupting lung development [5]. In the pancreas, loss of *Raldh2* expression results in agenesis of the dorsal pancreatic bud [6]. Based on this evidence, we hypothesized that Fgfr2IIIb signaling would interact with the retinoic acid signaling pathway and we specifically predicted that loss of Fgfr2IIIb function would down-regulate *Raldh2* expression in the duodenum during atresia formation in *Fgfr2IIIb-/-* mouse embryos.

MATERIALS AND METHODS

Animals

Institutional Animal Care and Use Committee (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 access to fresh food and water and kept on a 12 h alternating light/dark cycle. *Fgfr2IIIb-/-* embryos [7] were generated through traditional heterozygous *Fgfr2IIIb+/-* breedings. Forty percent of the embryos developed a duodenal atresia as has been previously reported [2]. Litters were harvested between E 11.0 and E 13.5 into cold phosphate buffered saline (PBS). Genotyping was performed on yolk sacs as described previously [7]. Embryos were fixed overnight in 4% paraformadehyde (PFA) at 4°C, and intestines were dissected the following day under a stereoscopic microscope and photographed.

Whole Mount *In Situ* Hybridization

Samples were dehydrated through a series of escalating PBS-Tween methanol steps, then, stored overnight at −20°C. The following day, they were rehydrated into PBS-Tween, treated with hydrogen peroxide and proteinase K, and *in situ* hybridization was performed at 70°C for *Raldh2* with antisense probes [8]. Specimens were stained at 37°C, washed in PBS-Tween and fixed with 4% PFA. Photographs were taken under a dissecting light microscope.

Section *In Situ* Hybridization

Embryos were harvested into cold PBS at E 11.5 and fixed overnight in Bouin's fixative at 4°C. They were dehydrated through a series of escalating PBS/ethanol steps and embedded into paraffin. Sections were taken a 10 μm thickness and floated onto slides. Sections were de-waxed and rehydrated. Slides were treated proteinase K followed by acetic anhydride and triethanolamine, re-fixed with paraformaldehyde, and section *in situs* were performed at 60°C with antisense probes for *Raldh2*. Sections were stained overnight at 37°C, fixed with 4% PFA, and cover slipped with glycerol. Photographs were taken under a light microscope.

RESULTS

Morphogenesis of Duodenal Atresia in *Fgfr2IIIb-/-* Mouse Embryos

The timing of duodenal atresia formation in the *Fgfr2IIIb-/-* mouse model has not been previously described. The atresia forms in the second portion of the duodenum [2]. We examined this process in early development with standard stereoscopic microscopy on partially dissected whole mount specimens. We observed that the proximal duodenum of in *Fgfr2IIIb-/-* embryos appeared slightly narrowed at E 11.5 (Fig. 1B, black arrow) compared with the control (Fig. 1A). This is the equivalent of Carnegie stage (CS) 16 in a human (the stage just before the endodermal plug forms). By E 12.5 (CS 18- when the plug first begins to recanalize in humans), the midpoint of the proximal limb of the duodenum had narrowed more significantly (Fig. 1D, black arrow) in comparison to the control (Fig. 1C). By E 13.5, or CS 20, the midportion of the proximal duodenum was absent (Fig. 1F, black arrow) indicating that the atresia had completely formed. The results suggest that the timing of the changes to the duodenum leading up to atresia formation occur rather rapidly, beginning at the equivalent of CS 16 in the human and reaching completion around CS 20.

Expression of *Raldh2* in the Developing Intestine and Duodenum

Disruptions in retinoic acid signaling have been linked to duodenal stenosis in humans. We hypothesized that similar disruptions would occur during duodenal atresia formation in mice. Prior to testing this, we first characterized the expression pattern of *Raldh2* in normal mouse duodenum by whole mount *in situ* hybridization. We observed that at E 11.0 *Raldh2* was robustly expressed in the proximal intestine with diminishing intensity distally, and at this stage was altogether absent in the early cecum and colon (Fig. 2A). To determine the tissue specific expression within the intestine, we performed a section *in situ* hybridization at E 11.5. This demonstrated expression of *Radh2* within the duodenum was limited to the mesoderm and that the expression was of equal intensity both in the proximal and distal duodenum (Fig. 2B).

Expression of *Raldh2* During Duodenal Atresia Formation

To determine whether *Raldh2* expression was altered during duodenal atresia formation, we examined *Raldh2* expression in *Fgfr2IIIb-/-* embryos between E 11.5 and E 12.5 by *in situ* hybridization. We observed that at E 11.5, there appeared to less *Raldh2* expression in the proximal duodenum (Fig. 3B, black arrowheads) in comparison to the distal duodenum (Fig. 3B, white