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Exogenous Sonic hedgehog protein does not rescue cultured intestine from atresia formation

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ARTICLE INFO

Article history:
Received 7 January 2013
Received in revised form
23 October 2013
Accepted 27 November 2013
Available online 7 December 2013

Keywords:
SHH
Intestinal atresia
Expression
Foxf1
Fgfr2IIIb$^{-/-}$
Organ culture

ABSTRACT

Background: The mechanism of intestinal atresia formation remains undefined. Atresia in *fibroblast growth factor receptor 2IIIb* (*Fgfr2IIIb*$^{-/-}$) mutant mouse embryos is preceded by endodermal apoptosis and involution of the surrounding mesoderm. We have observed that involution of the atretic segment is preceded by the downregulation of Sonic hedgehog (SHH) in the endoderm, which is a critical organizer of the intestinal mesoderm. We hypothesized that supplementation of *Fgfr2IIIb*$^{-/-}$ intestinal tracts with exogenous SHH protein before atresia formation would prevent involution of the mesoderm and rescue normal intestinal development.

Methods: In situ hybridization was performed on control and *Fgfr2IIIb*$^{-/-}$ intestinal tracts for *Shh* or *forkhead box protein F1* (*FoxF1*) between embryonic (E) day 11.5 and E12.0. Control and *Fgfr2IIIb*$^{-/-}$ intestinal tracts were harvested at E10.5 and cultured in media supplemented with fibroblast growth factor (FGF) 10 + SHH, or FGF10 with a SHH-coated bead. *In situ* hybridization was performed at E12.5 for *Foxf1*.

Results: SHH and *Foxf1* expression were downregulated during intestinal atresia formation. Media containing exogenous FGF10 + SHH did not prevent colonic atresia formation (involution). A SHH protein point source bead did induce *Foxf1* expression in controls and mutants.

Conclusions: Shh and *Foxf1* expression are disrupted in atresia formation of distal colon, thereby serving as potential markers of atretic events. Application of exogenous SHH (in media supplement or as a point source bead) is sufficient to induce *Foxf1* expression, but insufficient to rescue development of distal colonic mesoderm in *Fgfr2IIIb*$^{-/-}$ mutant embryos. *Shh* signal disruption is not the critical mechanism by which loss of *Fgfr2IIIb* function results in atresia formation.

1. Introduction

The mechanism of intestinal atresia remains undefined. Homozygous mutation of *fibroblast growth factor receptor 2IIIb* (*Fgfr2IIIb*) early in intestinal development causes atresia formation in the colon of mice [1]. Atresia formation is preceded by endodermal apoptosis in the areas where the atresia will form [1,2]. *Fgfr2IIIb* expression is limited to the intestinal endoderm at the early stages of development [3] indicating that loss of the receptor in the endoderm

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0022-4804/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2013.11.1114

causes endodermal apoptosis, loss of intestinal endoderm, and involution of the affected segment of intestine as a result of a loss of instructive signal to the surrounding mesoderm.

Sonic hedgehog (SHH) is expressed in the early intestinal endoderm. It is a critical organizer of the intestine during development, instructing both radial and longitudinal growth [4,5]. Loss of *Shh* expression results in impaired organization of the intestinal mesoderm, although the intestinal tube remains continuous and does not form atresias. Tissue specific and spatial-temporal overlap is seen in the expression of *Shh* and *Fgfr2IIIb*, each performing a critical role in instructing intestinal development. Therefore, we hypothesized that after endodermal apoptosis, *Shh* signaling would be disrupted in the atretic precursor region (where the atresia will form).

We set out to investigate the role of *Shh* signaling pathway disruptions during atresia formation by examining expression patterns of *Shh* and its downstream mesodermal target, Foxf1 [6–8], by *in situ* hybridization before involution of the atretic segment. Furthermore, we tested whether the addition of exogenous SHH protein to cultured embryonic intestines before atresia formation would rescue mesodermal development, prevent involution of the intestinal tube, and halt intestinal atresia formation.

2. Materials and methods

2.1. Animals

Institutional Animal Care and Use Committees approval for these studies was obtained from the University of Wisconsin School of Medicine and Public health (P.F.N. protocol No. 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.

2.2. Generation of mutant fetuses

Fgfr2IIIb$^{-/-}$ mutant and *Fgfr2IIIb*$^{+/-}$ littermate control embryos were generated using the *Hprt-Cre* breeding strategy [9] as has been described previously [2].

2.3. Whole mount in situ hybridization

Fgfr2IIIb$^{+/-}$ and *Fgfr2IIIb*$^{-/-}$ embryos were harvested at embryonic day (E) 11.5 and E12.0 into cold phosphate buffered saline and fixed overnight in 4% paraformaldehyde at 4°C. Fixed samples were dissected and dehydrated to 100% MeOH through a series of escalating methanol/PBS-Tween steps and stored at −20°C. The *in situ* hybridization protocol has been published elsewhere [10] and included incubation with antisense riboprobes at 68°C for *Shh* and 70°C for *Foxf1* (constructs kindly provided by H. Hamada and Y. Saijoh). Photographs were taken using a dissecting light microscope.

2.4. Organ culture

For the media supplemented SHH protein experiments, *Fgfr2IIIb*$^{+/-}$ and *Fgfr2IIIb*$^{-/-}$ embryos were harvested at E10.5 and the developing intestinal tracts were isolated. Intestinal tracts were cultured in Matrigel (BD Biosciences, Bedford, MA) and allowed to polymerize at 37°C for 30 min within Millicell EZslide wells (Millipore, Billerica, MA). Matrigel embedded tracts were overlaid with a base media of DMEM/F-12 (HyClone, Logan, UT) containing L-Glutamine, penicillin/streptomycin, and fetal bovine serum. In this set of experiments, the base culture media was supplemented with fibroblast growth factor 10 (PeproTech, Rocky Hill, NJ) and SHH (R&D Systems, Minneapolis, MN) to a final concentration of 500 ng/mL each. Media-supplemented organ culture was conducted five times after previously published protocols [10], with 12 normal (*Fgfr2IIIb*$^{+/-}$) littermate controls and eight mutant (*Fgfr2IIIb*$^{-/-}$) tracts.

For the point source SHH protein experiments, E10.5 intestinal tracts were harvested as described previously. These three additional culture experiments included 17 littermate controls and nine mutant intestinal tracts. Intestinal explants were cultured in Matrigel with SHH-laden Affi-Gel beads (153–7302; Bio-Rad, Hercules, CA) at 80–150 μm diameter. Beads were incubated with approximately 50 μg/mL of SHH protein before being polymerized within Matrigel and overlaid with media containing 500 ng/mL of FGF10 within EZslide wells. Each well contained at least one control tract, one mutant tract and one SHH-laden bead. All organ cultures were maintained at 37°C and 5% CO_2 conditions. Media were

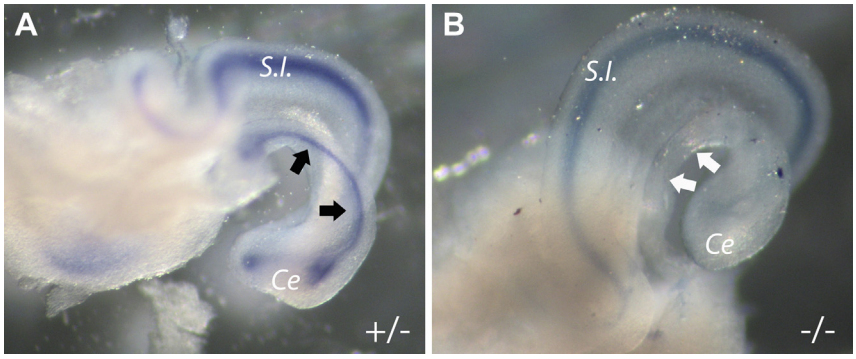

Fig. 1 – *Shh* Expression is absent in *Fgfr2IIIb*$^{-/-}$ colon at E11.5. Whole mount *in situ* hybridization for *Shh* in (A) control *Fgfr2IIIb*$^{+/-}$ and (B) mutant *Fgfr2IIIb*$^{-/-}$ colons. Presence of staining in control colon is indicated by black arrows, whereas absence of staining in mutant colon is indicated by white arrows. Ce = cecum; S.I. = small intestine.