Background/Purpose: Anorectal malformations occur in 1 per 4,000 live births and represent a surgical challenge. Although critically important, the basic mechanisms of normal anorectal union are incompletely understood. Fgf10 signaling is known to serve a key role in mesenchymal/epithelial interactions in many organ systems including the gastrointestinal tract (GIT). The authors therefore hypothesized that Fgf10 signaling has a central role in normal anorectal development.

Methods: Fgf10 expression in wild-type (Wt) embryos was evaluated using whole-mount in situ hybridization. Wt and Fgf10<sup>−/−</sup> embryos were harvested from timed pregnant mothers at E12.5 through E17.5 and were analyzed for anorectal phenotype.

Results: Wt development of union between anorectal structures is completed between E12.5 and E13.5 with luminal communication between distal rectal epithelium and anus. Fgf10 is discretely expressed at E12.5 in the distal rectum. Fgf10<sup>−/−</sup> mutants show failure of union of the rectum and anus at an early stage (E13.5) and near term (E17.5).

Conclusions: Fgf10 is expressed in the rectum at the time when anorectal continuity is established, indicating a role in normal anorectal development. Fgf10 invalidation (Fgf10<sup>−/−</sup> mutant) results in a genetically reproducible anorectal malformation phenotype. Fgf10 function is critical for normal anorectal development.

INDEX WORDS: Anorectal malformation, fibroblast growth factor 10, gastrointestinal development.

Anorectal malformations occur in 1 per 4,000 live births and represent an immediate and long-term surgical challenge. Anorectal malformations represent a spectrum of anomalies from the very minor in covered anus to the more extreme cloaca. At birth, these patients present with absence of anorectal continuity on physical examination. After correction of the anomaly, problems such as constipation and incontinence can prove to be continuing challenges in management. The development of normal anorectal structures is a complex process requiring coordinated molecular signaling between mesenchymal and epithelial elements. Whereas animal models such as Sonic hedgehog (Shh), Gli2, Gli3 (2 zinc finger transcription factors) and all-trans retinoic acid (ATRA) have been studied for anorectal malformations, the understanding of normal development and pathologic variations remains incomplete. The role of specific growth factors, such as fibroblast growth factor 10 (Fgf10), on the development of this complex of structures has not been investigated. The fibroblast growth factor family of signaling molecules has at least 22 members involved in different aspects of organogenesis. Fgf10 has a clear role in the development of the lungs, mammary glands, and pancreas.

The current study seeks to evaluate the function of Fgf10 in the development of anorectal continuity. Expression of Fgf10 was evaluated using in situ hybridization to determine if Fgf10 is relevant at the time-points when anorectal continuity is being established. We also examined Fgf10<sup>−/−</sup> mutant embryos at an early stage of development (E13.5) to consider the role of Fgf10 in early formation of the anorectal structures. In addition, the Fgf10<sup>−/−</sup> mutants were evaluated at a later stage of development (E17.5), to consider the persistence of defects noted in early development, and to evaluate relevance in comparison with the anomalies found in clinical practice.

Materials and Methods

Mutant Embryos

Fgf10<sup>−/−</sup> mice were generated as previously described in the C57Bl/6 murine strain. C57Bl/6 (wild type [Wt]) littermates were used as controls. Embryos from Wt (E12.5, E13.5, and E17.5, n = 8 at each stage), Fgf10<sup>−/−</sup> (E13.5 and E17.5, n = 8 at each stage) were
evaluated. All embryos were the result of a timed mating; E12.5, E13.5, and E17.5 referring to embryonic day or day postconception 12.5, 13.5, and 17.5, respectively. Animals were used according to approved Institutional Animal Care & Use Protocol # 32-03.

Biological Samples

Embryos used for in situ hybridization were removed from the uteri at E12.5. The gastrointestinal tract (GIT) was isolated from the embryos and fixed in 4% Paraformaldehyde solution in phosphate-buffered saline (PBS) at 4°C for 2 hours. The samples then were washed twice in PBS for 5 minutes, stored overnight in 70% ethanol, and transferred to 100% ethanol until needed. Embryos used for sectioning were removed from the uteri and preserved in Methacarn (120 mL Methanol, 60 mL Chloroform, 20 mL Acetic Acid) for 2.5 hours (E13.5). Embryos at E13.5 were then incubated in embedding mix (gelatin, albumin, and sucrose) and solidified with the addition of 25% glutaraldehyde. Vibratome sagittal sections were generated at 20 μm. The sections were then mounted and photographed.

Embryos at E17.5 were removed from the uteri and photographed from a profile view as fresh samples. The tail of all samples was removed at the level of the perineum to allow for better visualization of the perineal structures and photographed from a landscape view. Once photographed, the pelvis was carefully dissected to maintain the integrity of the anorectal structures. The tissues were preserved in methacarn for 2 hours and placed in 100% ethanol until needed. These specimens then were embedded in paraffin and sectioned at 8 μm sagittal thickness using a microtome (Leica). The sections were mounted and processed using standard H & E staining, and photomicrographs were taken.

In Situ Hybridization

The murine cDNA used as templates for riboprobes was a 584 base-pair Fgf10 fragment previously described by Bellusci et al. The whole mount in situ hybridization was performed using the protocol described by Winnier et al to evaluate the expression of the Fgf10 gene in rectal structures. Photomicrographs then were taken of the colon and rectum.

RESULTS

Fgf10 is Expressed Early in Anorectal Development

Fgf10 expression in the GIT as detected by whole-mount in situ hybridization performed at E12.5 is shown in Fig 1. The GIT has been removed and treated as described above. In this photomicrograph, the GIT is oriented to best demonstrate Fgf10 expression in the rectum. The expression of Fgf10 is shown by dark staining. Note the expression is most specific for the mesenchymal cells surrounding the distal portion of the rectum. The rectum is found to have very intense and discreet expression in comparison with the proximal colon and adjacent small intestine, which have no Fgf10 expression at this stage. There is also notable expression in the cecum.

Phenotype of Fgf10−/− Mutant

Evaluation of Wt mice clearly shows the communication between the rectum and the anus at a time point between E12.5 and E13.5. Wt and Fgf10−/− mutants were evaluated at E13.5 by sagittal section as shown in Fig 2. The Wt mouse shown in Fig 2A is oriented with the pelvic structures at the lower portion of the panel. The anus, rectum, and bladder are centered in a higher magnification in (Fig 2B), showing the epithelium of the rectum in continuity with the anus, which is found between the tail and genital tubercle. The bladder is seen above the rectal structures. There is no communication of the urinary and gastrointestinal tracts. The Fgf10−/− mutant is shown in Fig 2C oriented similar to Wt. The bladder and anus are again identified. There is no rectal
epithelium present within the pelvis as shown under higher magnification in Fig 2D. The higher magnification confirms the absence of the epithelial layer and lumen in the expected location of the rectal structures.

Similar evaluation was conducted at a more mature stage (E17.5). Figure 3 shows the phenotype of the Fgf10−/− mutant compared with the Wt. These specimens were photographed as fresh samples. Note the Fgf10−/− mutant has no limbs or eyelids and is slightly smaller. Most importantly, the profile view of the perineum shows the genital tubercle is very close to the tail. The perineal body is profoundly deficient. This profile view will help illustrate the effects of Fgf10 invalidation on the perineal structures that will be shown in greater detail in Fig 4.

The effect of Fgf10 invalidation on the external anus and the continuity of the anorectal canal are shown in Fig 4. The tails were removed from the embryos from Fig 3 and photographed from a landscape view. E17.5 is a late stage of development and closely approximates the phenotype at the completion of gestation. The Wt embryo is shown in Fig 4A. Note the distance from the genital tubercle to the transected tail. The anus is a well-differentiated structure in this region. The darker cells superior to the anus are melanocytes, usually present in the perineal body. The Fgf10−/− mutant shows distinct phenotypic abnormalities (4B). The perineal body is almost completely absent and the anus is not identified. There is a small anal dimple. The melanocytes found in the Wt are greatly reduced in the mutant perineal body.

The anorectal structures from Fig 4A and B were embedded and sectioned as described earlier to more precisely define the anatomy of the anorectal structures. The Wt E17.5 sagittal section clearly shows mature continuity of the epithelium of the rectum and anus (Fig 4C). The bladder and urethra are immediately anterior to the rectum. There is no communication between the digestive and urinary tracts. The Fgf10−/− mutant fails to
Fig 3. Comparison of WT and Fgf10\(^{-/-}\) mutant embryos at E17.5. The mutant is slightly smaller than the WT. Note the absence of limbs (white arrow) and the absence of the eyelid (white arrowhead). In the Fgf10\(^{-/-}\) mutant, the distance from the genital tubercle to the tail (perineal body, shown in white box) is greatly decreased compared with WT. This area will be shown again in the landscape view of Fig 4.

Fig 4. Fgf10\(^{-/-}\) mutant embryos exhibit severe anorectal malformation at E17.5. A landscape view of the perineum of the wild type (WT) and Fgf10\(^{-/-}\) mutant at E17.5 is shown in (A, B). The tail has been removed to visualize the following structures. The WT male demonstrates the normal perineal structures, transected tail (tail), anus (a), perineal body (pb), and genital tubercle (gt) in (A). Note the distance from the tail to the genital tubercle. The dark skin near the anus is evidence of the presence of melanocytes (normally present in the perineal body). The equivalent view of the Fgf10\(^{-/-}\) mutant is shown in (B). The transected tail (tail), perineal body (pb), and genital tubercle (gt) are labeled. Note the distance from the genital tubercle to the anus is greatly reduced. The anus is not identified. The dark skin shown in the WT is present but to a much lesser degree. The perineal structures shown in (A, B) were processed, embedded, and sectioned as earlier described and shown in (C, D). A section of the WT perineal structures show the anus (a), rectum (r), seminal vesicle (sv), bladder (bl), and genital tubercle (gt) in (C). Note the epithelium of the rectum is in continuity with the epithelium of the anus in WT (C). The Fgf10\(^{-/-}\) mutant is shown in the same orientation with identical labeling (D). The obvious exception is the absence of rectum or rectal epithelium at E17.5. There is a small epithelial indentation that likely corresponds to the ectodermally derived structures of the anus (a). There is no evidence of a fistula between the digestive and urinary tracts.
show continuity of the rectal and anal epithelial structures (Fig 4D). On careful inspection, no rectal epithelium is present adjacent to the anus. There is no communication of the rectum with the structures of the pelvis. The sections shown in these photographs show a uniform phenotype with 100% penetrance.

**DISCUSSION**

The spectrum of anorectal malformations likely represents a common final pathway by which multiple errors of development occur. Anorectal development is a complex process, which requires multiple signaling molecules to act in concert. For anorectal continuity to develop, multiple tissue-specific events must take place. A greatly simplified process would require the colon and rectum to develop as a luminal structure deep in the pelvis. The urorectal septum, which divides the anorectal tract dorsally from the ventral urogenital tract, must develop along with the rectum, bladder, urethra, and external anus. The external cutaneous anus and muscular structures of the internal and external sphincters must also develop in the correct anatomic location. The anatomy of this region is responsible for the physiologic process of 3 complex organ systems (digestive, reproductive, and urinary). Our understanding of the developmental processes involved in this area is very basic. Fgf10 has proven to be one of many genes involved in this process.

We have shown that Fgf10 expression is present very early in the development of the anorectal structures, suggesting a regulatory role in the development of these structures. Figure 1 shows that Fgf10 expression is highly specific to the mesenchymal cells surrounding the distal rectal epithelium, the tissue that must migrate distally to allow anorectal continuity. Also of interest is the fact that the expression of Fgf10 is highly regional, indicating a specific regulatory role in these regions of the GIT. There is expression in the cecum and rectum, but no expression in the small intestine and proximal colon. In other tissues, Fgf10 has been shown to be responsible for both chemotaxis and proliferation. We have previously shown Fgf10 to cause an increase in the proliferative rate of the epithelium at the apex of the developing cecum (data not shown). The current results show that Fgf10 is expressed in the rectum at a critical time for normal anorectal development. These data invite further investigation of the mechanism of action and other key regulatory signaling molecules. Further studies will evaluate the control of Fgf10 expression and its relevance in the interaction between the ectodermal anus and endodermal rectum.

In the Wt mouse, anorectal continuity is established by E13.5. In Fig 2 we demonstrate the Fgf10−/− mutant fails to develop continuity between the rectum and the cutaneous anus. The rectal epithelium is not found in the pelvis on any of the sagittal sections, implying failure of distal development to meet the structures below in the pelvis. The cutaneous perineal structures at the site of anus in the Wt do not appear to be affected in the Fgf10−/− mutant. This normal cutaneous development would imply that the cutaneous anus develops independently of the Fgf10 pathway. These phenotypic findings are persistent to E17.5, which corresponds well to mature gestation. The anorectal malformation occurring in the Fgf10−/− mutant corresponds to what would be clinically classified as an imperforate anus without fistula.

We are now investigating the nature of this anomaly to determine if this anorectal malformation is a deficiency of rectal descent or a failure of migration of the urorectal septum. Both theories would appear plausible from these data. Of interest also is the development of the cutaneous anus, an ectodermal derivative. The interaction of the ectodermal and endodermal structures will likely prove to be a fruitful area of future investigation and provide greater detail about the precise mechanisms of development of anorectal continuity.

Fgf10 is responsible for regional and tissue-specific branching and budding morphogenesis. The deletion of both autosomal Fgf10 genes results in multiple developmental defects inherited in a classical autosomal recessive pattern. The Fgf10 mutant colony therefore is maintained in the heterozygous state. The pulmonary agenesis phenotype makes a complete germ line deletion of Fgf10 lethal at birth secondary to respiratory failure. The probability of a human surviving with a homozygous invalidation of Fgf10 is unlikely. A more likely hypothesis is point mutation leading to a hypomorphic phenotype in which the FGF10 protein is produced but has a decreased affinity for its receptor. The broad spectrum of phenotypic anomalies indicates Fgf10 is active in and critical for the organogenesis of multiple different systems. The expression of Fgf10 is likely modulated by tissue-specific regulatory mechanisms. Isolated anorectal malformation in the absence of other anomalies may occur not as a result of deletion of the Fgf10 gene itself, but rather a mutation of the regulatory elements controlling Fgf10 expression in the distal colon and rectum.

Anorectal malformations represent a complex challenge for both pediatric surgeons and investigators hoping to better understand this disease. Sonic Hedgehog (Shh) Signaling, Gli2, Gli3, and now Fgf10 mutations have been documented to cause a variety of anorectal malformations in genetically reproducible animal models. All-trans retinoic acid (ATRA), etretinate, and Adriamycin have all been shown to cause anorectal malformation in inducible teratogenic animal mod-
Clinically, several congenital syndromes (ie, Currarino, Towns-Brocks, Down’s, and VACTERL) have also been shown to develop anorectal malformations.14-17 Whereas the above-mentioned signaling molecules have been shown to be associated with development of anorectal malformations, their specific role in normal development, mechanism of action, and cause of pathogenesis are less well understood. The study of known signaling molecules active in anorectal development in our patients with anorectal malformation will lead to clinically applicable information.

We present here the identification of a uniform anorectal malformation with 100% penetrance in the Fgf10 mutant, providing a new animal model for the study of this pathology. Further investigation of the Fgf10 pathway and its effect on anorectal malformations may lead to a better understanding of this very complex process.

REFERENCES


Discussion

Bradley Rodgers: Tim, in all of these mice you saw no urinary tract abnormalities?

T.J. Fairbanks (response): Actually, we did see a genitourinary abnormality. We have not had a chance to fully study that because one of the interesting phenotypes of the Fgf10 mice is they do not breed so well, so we are concentrating our efforts on the anorectal malformations, but there is a consistent perineal hypospadias in the male mice.

C. Fekete (Paris, France): Did you look at the spine, and did you have any spine malformation associated with hindgut malformation?

T.J. Fairbanks (response): Yes, there is a spinal abnormality that has been previously published in the Fgf10 mice, which is well established. We are not specifically looking at that but it has been established. We did not see any presacral abnormalities with the exception of the anorectal malformation. The damage you saw on that slide is when we dissect the structures surrounding the pelvis to get a better histologic sample.