Animal Model

Anorectal Malformations Caused by Defects in Sonic Hedgehog Signaling

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Anorectal malformations are a common clinical problem affecting the development of the distal hindgut in infants. The spectrum of anorectal malformations ranges from the mildly stenotic anus to imperforate anus with a fistula between the urinary and intestinal tracts to the most severe form, persistent cloaca. The etiology, embryology, and pathogenesis of anorectal malformations are poorly understood and controversial. Sonic hedgehog (Shh) is an endoderm-derived signaling molecule that induces mesodermal gene expression in the chick hindgut. However, the role of Shh signaling in mammalian hindgut development is unknown. Here, we show that mutant mice with various defects in the Shh signaling pathway exhibit a spectrum of distal hindgut defects mimicking human anorectal malformations. Shh null-mutant mice display persistent cloaca. Mutant mice lacking Gli2 or Gli3, two zinc finger transcription factors involved in Shh signaling, respectively, exhibit imperforate anus with recto-urethral fistula and anal stenosis. Furthermore, persistent cloaca is also observed in Gli2^{-/-}; Gli3^{+/-}, Gli2^{+/-};Gli3^{-/-}, and Gli2^{-/-};Gli3^{-/-} mice demonstrating a gene dose-dependent effect. Therefore, Shh signaling is essential for normal development of the distal hindgut in mice and mutations affecting Shh signaling produce a spectrum of anorectal malformations that may reveal new insights into their human disease equivalents. (*Am J Pathol* 2001, 159:765-774)

Anorectal malformations encompass a broad spectrum of congenital defects that frequently necessitates urgent surgical treatment in the newborn period, most often because of intestinal obstruction and sepsis. One of the most common anomalies, imperforate anus, has an incidence of 1 in 5000 and carries with it significant chronic morbidity, particularly with fecal incontinence. The most severe form of anorectal malformation, the cloacae, where distal intestinal and genitourinary tracts remain in a common channel, is much less common than imperforate anus (1 in 50,000) but has more significant serious long-term medical problems including gender assignment.¹⁻⁴ Despite its clinical relevance, however, the etiology and pathogenesis of anorectal malformations are still poorly understood.

Several congenital syndromes have been associated with malformations affecting the development of anus and rectum, including Currarino syndrome and Townes-Brocks syndrome.⁵⁻⁸ The Currarino syndrome (MIM no. 176450) is an autosomal-dominant genetic disorder characterized by the partial agenesis of sacral vertebrae, presacral teratoma, and anorectal malformations. Linkage and mutational analyses have identified the major locus for Currarino syndrome to be a homeobox gene, *HLXB9*, located on chromosome 7q36.⁵⁻⁷ However, the exact role of *HLXB9* in anorectal malformations is unclear because *Hlxb9* mutant mice do not display any anorectal, genitourinary, or sacral disturbances.^{9,10} Townes-Brocks syndrome (MIM no. 107480) is a rare dominant malformation syndrome, with a combination of anal, renal, limb, and ear anomalies, caused by mutations in the *SALL1* zinc-finger protein.⁸ The VACTERL syndrome, which represents a nonrandom association of vertebral, anorectal, cardiac, tracheoesophageal, renal, and limb anomalies

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includes another important cohort of patients with anorectal malformations.^{11,12} Among these anomalies, malformations involving foregut and hindgut development are highly associated.^{11,12} The genetic basis of the VACTERL syndrome is currently unknown.

Development of anus and rectum from the distal (or posterior) hindgut has been described in normal human fetuses at different stages.¹³ Similar studies on anorectal malformations in human fetuses, however, have not been performed because of the lack of available tissue. Several murine models are known to display various anorectal malformations but, most importantly, the naturally occurring *Sd* mouse (Danforth's short-tail mouse).^{14–17} These mice have an autosomal-dominant trait for imperforate anus and pass on this semi-dominant trait with high penetrance, where all of the homozygotes and most of the heterozygotes are affected. To date, the gene defective in *Sd* mice has yet to be identified. Domestic pigs have a naturally high incidence of anorectal malformation and can be bred to generate an animal model to study the anatomy and embryology of anorectal malformation.^{18–20} Like the *Sd* mice, however, the specific genetic mechanism for the anorectal malformation is not known. In both mice and rats, several teratogenic models were discovered in which anorectal malformation occur frequently using agents such as etretinate, retinoic acid, or adriamycin.^{21–23} They have accompanying abnormalities involving the skeletal and genitourinary systems. The specific gene(s) influenced by these teratogens is not known.

Recently, mutant mice involving Sonic hedgehog (Shh), a secreted signaling molecule that plays diverse roles in vertebrate development,^{24,25} were shown to display an imperforate anus phenotype,²⁶ in addition to other anomalies including complete agenesis of the vertebral column, heart-looping defects, tracheoesophageal fistula, and loss of distal limb structures.^{27–33} Shh is expressed throughout the rostral-caudal extent of the gut endoderm and has been implicated in the first phase of signaling from the endoderm to the mesoderm.^{34–37} A role for Shh signaling in foregut development has been reported.^{32,33,38,39} Shh null mice exhibit esophageal atresia/stenosis, tracheoesophageal fistula, and lung anomalies indicating that Shh signaling is required for the normal development of esophagus, trachea, and lung.^{32,33} Perturbation of Shh signaling in transgenic mice and in chick embryos also affects the development of the pancreas, another derivative of the foregut.^{38,39} However, the exact role of Shh in hindgut development is not known. Shh is specifically expressed in the endoderm of the developing hindgut and embryological studies in chick embryos reveal that overexpression of Shh can induce the expression of *Bmp4* and *Hoxd13* in hindgut mesoderm.³⁴ The initial observations of hindgut abnormalities in Shh null mice and in mice defective in Shh transcription factors, *Gli2* and *Gli3* prompted us to investigate the role of Shh signaling pathway in anorectal malformations in these mutant mice.

In this study, we performed a systematic analysis of the distal hindgut phenotypes in Shh null mice as well as in mutant mice that are deficient for *Gli2* and/or *Gli3*.^{29,40–42}

We report here the strongest genetic association thus far for a developmental pathway and anorectal malformations in mice. These mutant mice display a spectrum of anorectal malformations similar to that observed in humans. These observations establish the role of Shh signaling in the development of the distal hindgut and suggest that mutations affecting Shh signaling might be involved in anorectal malformations in humans.

Materials and Methods

Mice

Gli2 mutant mice carry a targeted deletion of the DNA-binding zinc-finger motifs of the gene.²⁴ *Gli3* mutant mice are spontaneous null mutants with a large 3' deletion of the gene.²⁵ *Gli2*^{+/-}, *Gli3*^{+/-}, and *Gli2*^{+/-};*Gli3*^{+/-} mice were maintained in a mixed 129 and CD1 background. Intercrosses of *Gli2*^{+/-};*Gli3*^{+/-} mice were used to generate *Gli2*^{-/-};*Gli3*^{+/-}, *Gli2*^{+/-};*Gli3*^{-/-} and *Gli2*^{-/-};*Gli3*^{-/-} embryos. Shh mutant mice carry a targeted deletion of most of the protein-coding region of the gene.²⁹ The generation of mutant embryos were performed as described previously.^{29,41,43} Genotype of mutant embryos was determined by polymerase chain reaction analysis of yolk sac DNA.

Dissection, Histology, and Paraffin Embedding

Midday of the day of the vaginal plug was considered as E0.5 in the timing of the embryo collection. Embryos were dissected out and fixed in 4% paraformaldehyde overnight at 4°C. They were dehydrated, processed, and embedded in paraffin wax before sectioning at 7 μ m. Slides were then dewaxed, rehydrated, and stained with hematoxylin and eosin.

Microdissection and Radiography

Embryos were aseptically dissected from uterine decidua and staged by external features at E14.5 and 18.5. Omnipaque radiographic dye was injected into the distal intestine of each embryo and standard anteroposterior radiographs were taken.

Scanning Electron Microscopy (SEM)

Dissected embryos were fixed in osmium tetroxide, dehydrated in ethanol, mounted with carbon paint, and examined on JEOL820 SEM as described.⁴⁴

Histology and *in Situ* Hybridization

Whole-mount *in situ* hybridization was performed according to the standard protocol.^{43,45,46} Section *in situ* was performed according to published procedures.^{41,47} The probes used were *Gli1*, *Gli2*, and *Gli3*.⁴⁸

Figure 1. Development of anus, rectum, and lower urinary tract in wild-type mouse embryos. The SEM of developing anal and urethral orifices in the perineal region of E10.5 to E14.5 embryos are shown [original magnification, $\times 40$] (a–d). **Boxed areas** indicate the perineum where future urethral and anal orifices open. **Arrowheads** and **arrows** represent urethral and anal orifices, respectively. No external features of anal and urethral development are observed on E10.5 (a) and E12.5 (b). Closely located anal and urethral openings are first visible at E14.5 (c). **d:** Higher magnification of c unveils morphologically distinct cell types of the uroepithelium and intestinal mucosa in the urethral and anal openings, respectively. Sagittal sections of E10.5 to E18.5 mouse embryos (e–h) reveal that bladder and urethra are not completely separated from anorectum by the urorectal septum (indicated by the **arrow** in e) until E14.5. Expression of *Gli1*, *Gli2*, and *Gli3* in the mesodermal component of the developing hindgut as revealed by RNA *in situ* hybridization on transverse sections of E14.5 mouse embryos (i–l). Abbreviations: a, aorta; b, bladder; cm, cloacal membrane; hg, hindgut; n, neural tube; nc, notochord; r, rectum; tg, tail groove; u, urethra; uc, urachus; ugs, urogenital sinus; urs, urorectal septum.

Results

Development of Anus, Rectum, and Lower Urinary Tract

In mice, the first external sign of developing anus, rectum, and lower urinary tract is the appearance of urethral and anal orifices in the tail groove of the perineum between the hindlimb buds and tail on E14.5 (boxed area in Figure 1; a–d). Between E12.5 and E13.5, the most distal hindgut and a more anteriorly located urethral compartment at the base of genital swelling, share a residual common cloacal cavity (marked as c in Figure 1e). The cavity is covered by a thin cloacal membrane, which degenerates at approximately E14.5 (Figure 1g). By SEM, the anal orifice can be easily identified by the

cobbled appearance of the villi in the anorectal mucosa and the urethral orifices by the ridged surface of the uroepithelium (Figure 1d; data not shown). The onset of the partition of the cloaca into the ventral urogenital sinus and dorsal anorectum is observed clearly by E10.5 (Figure 1e). However, a complete partition between the lower urinary and intestinal tracts does not occur until E14.5 when anus and urethra open independently into the perineum (Figure 1g). The appearance of male and female external genitalia in the perineum remains indistinguishable on E14.5. Between E14.5 and birth (E19.5 to E20.5), the distance between the anal and urethral orifices lengthens with the growth of genital tubercle and swellings (Figure 1, g and h) revealing more characteristic male (Figure 2a) and female (Figure 3a) external features at birth. The three *Shh*-responsive *Gli* transcription fac-

Figure 2. Cloacal malformation in *Shh*^{-/-} mice. SEM of E18.5 wild-type and *Shh*^{-/-} mice showing the normal female genitalia of E18.5 wild-type mice (**a**) as compared to the complex cloacal opening with poorly developed external genitalia in the perineum of E18.5 *Shh*^{-/-} mice (**b**) and a 22-week aborted human fetus (**c**). In **b**, the bulge in the cloaca was lined with uroepithelium as revealed by higher magnification (data not shown). In **c**, the boxed area indicates the partially covered cloacal opening. In E14.5 wild-type embryo (**d**), bladder and urethra are well separated from the anorectum. In E14.5 *Shh*^{-/-} embryos, distal intestinal tract and ureters are connected to a common cloacal cavity (**e**) similar to *en bloc* dissected specimen from the aborted human embryo (**f**), which demonstrates ureters and distal intestinal tract (metal probes in place) draining into the cloacal cavity. Abbreviations: b, bladder; c, cloaca; ft, fallopian tube; gt, genital tubercle; gs, genital swelling; hg, hindgut; k, kidney; r, rectum; u, urethra; ur, ureter.

tors (Gli1, Gli2, and Gli3) are all highly expressed in the visceral mesoderm of the developing hindgut suggesting that Shh signaling might play a critical role in hindgut development (Figure 1; i-l).

Shh Null Mice Fail to Develop an Anus, Rectum, and Lower Urinary Tract

To determine the role of Shh in hindgut development, we analyzed E14.5 (*n* = 5) and E18.5 (*n* = 7) *Shh*^{-/-} mutants by SEM, light microscopy, and histological examination. Strikingly, all *Shh*^{-/-} mutants displayed a persistent cloaca where the lower urinary tract and anorectum share a common outlet. E18.5 *Shh*^{-/-} mutants exhibited a single poorly developed perineal opening (Figure 2b) similar to those observed in human cloacal malformations in which the perineal area lacks any obvious external features of genitalia and is partially covered by amniotic membrane (see Figure 2c for example). The dissected autopsy specimen from the same patient clearly demon-

strates a persistent cloaca with distal intestine and ureters draining into a common cavity (Figure 2f). Histological analysis of E14.5 *Shh*^{-/-} mutants reveals a similar condition with a ventrally located hypoplastic bladder and an intestinal tract draining into a common cloacal channel, which is joined by shortened ureters (Figure 2e). SEM and histological examination revealed that the mucosal architecture of the distal intestine is colonic suggesting epithelial colonic differentiation occurs in the proximal hindgut of *Shh*^{-/-} mutants (data not shown). These observations indicate that Shh signaling is essential for the development of the distal hindgut, including the rectum and anus.

Imperforate Anus in Gli2 Mutant Mice

We examined E14.5 (*n* = 7) and E18.5 (*n* = 30) *Gli2*^{-/-} mice to determine the function of Gli2 in hindgut development. All *Gli2*^{-/-} mice displayed an imperforate anus

Figure 3. Imperforate anus in *Gli2*^{-/-} mice and anal stenosis in *Gli3*^{-/-} mice. The SEM of genital and anorectal regions of E18.5 wild-type (**a**), *Gli2*^{-/-} (**b**), and *Gli3*^{-/-} (**c**) mice. **Large** and **small arrowheads** indicate urethral and future vaginal openings, respectively, and **arrows** represent anal orifices (**a** and **c**). *Gli2*^{-/-} mice display a single urethral opening in the perineum (indicated by the **white arrowhead** in **b**), whereas *Gli3*^{-/-} mice exhibit anal stenosis (**c**). Sagittal sections reveal the absence of anus with a fistula (indicated by **arrow**) between distal intestinal tract and proximal urethra in *Gli2*^{-/-} mice (**e**), and a narrowed anus (indicated by **arrow**) with normal lower urinary tract and rectum in *Gli3*^{-/-} mice (**f**). Injection of radiographic contrast material into distal intestinal tract demonstrates a communication between the intestinal and urinary tracts in *Gli2*^{-/-} mice (**h**), whereas a simple outline of the anus and rectum is revealed in the wild-type animal (**g**). *En bloc* microdissection of the embryos reveals that the distal intestine is connected to the lower urinary tract in *Gli2*^{-/-} mice (**h**), and a narrowed anus with wedged meconium demonstrating anal stenosis in *Gli3*^{-/-} mice (**i**). The lower urinary and intestinal tracts are outlined by **white dots**. Abbreviations: a, anus; b, bladder; c, cloaca; gt, genital tubercle; r, rectum; u, urethra.

(absence of anus and lower rectum) with recto-urethral/recto-vaginal fistula and exhibited a single urethral opening in the perineum (Figure 3, b and e).⁴² The fistula is found between the distal intestine and bladder (Figure 3e) and can be clearly shown by the injection of a radiographic contrast dye into the distal intestine (Figure 3h). In wild-type mice, the radiographic contrast dye outlines

only the rectum and anus (Figure 3g). These data demonstrate that mice lacking *Gli2* exhibit a severe anorectal defect that is milder than the cloacal phenotype of *Shh* null mice. The distal hindgut anomalies found in *Gli2*^{-/-} mice are identical to the anorectal agenesis with recto-prostatic urethral/recto-vaginal fistula commonly seen in humans.

Figure 4. Embryology of anorectal malformation. Sagittal and transverse sections at stages E11.5 and E12.5 are displayed for wild-type (wt) (a–d), *Gli2*^{−/−} embryos (e–h), and *Shh*^{−/−} embryos (i–l). For wild-type and mutant embryos at E11.5, the alimentary and genitourinary systems enter into a cloaca (marked c). The presence of a dividing and descending septum was not seen on any section in wild-type embryos (a–d). Normal development of hindgut involves the gradual separation of dorsal hindgut from ventral urogenital tract in c, most of which takes place between E11.5 and E12.5 dpc (a–d). A similar transition is not seen in *Gli2*^{−/−} and *Shh*^{−/−} embryos, in which the dorsal hindgut remains fully adjoined to urogenital tract (e–l). Both mutant embryos also seem to have a blunted or missing dorsal aspect of their cloaca as compared to wild-type in a, e and i. Abbreviations: hg, hindgut; c, cloaca; nt, neural tube; ugt, urogenital tract.

Anal Stenosis in *Gli3* Mutant Mice

Gli3^{−/−} mice have a subtle distal hindgut phenotype (*n* = 29). Although their bladder, urethra, and rectum developed normally, the anus in all *Gli3*^{−/−} mice examined is significantly narrower by 30% as compared to that of wild-type mice (Figure 3; c, f, and i). In addition, in approximately one third of the *Gli3*^{−/−} mice, the anus appears ectopic with a location more ventral at the junction between the lower abdomen and tail (data not shown).

Embryology of Anorectal Malformation

The onset of normal development of ventral urogenital and dorsal anorectum from the proximal cloaca is first observed between E11.5 and E12.5. To determine whether anorectal malformation observed in *Shh* and *Gli2* mutants are because of failure in the development of a septum, which partitions the cloaca into ventral urogenital sinus and dorsal anorectum, we specifically examined the sagittal and transverse sections of E11.5 and E12.5 embryos from wild-type, *Gli2*, and *Shh* mutant embryos. These analyses reveal that the partitioning of cloaca into ventral urogenital and dorsal anorectal tracts does not occur by development of a septum as previously suggested.^{49,50} Presumptive urorectal septum on sagittal images is not corroborated by any anatomical equivalent on the transverse sections in wild-type embryos (*n* = 4) (Figure 4; a, b, e, and f). Furthermore, on transverse sections, there was no evidence of lateral ridge formation or fusion during normal development of anus and rectum in wild-type embryos (*n* = 4) (Figure 4b). These obser-

vations suggests that the development of dorsal anorectum and ventral urogenital tract from the cloaca is likely because of an anterior-to-posterior progression of regional specification and differentiation of these structures by endodermal-mesenchymal interaction.⁵¹ In the absence of either the *Shh* signaling or *Gli2*, the major transducer of *Shh* signaling, the most posterior end of the hindgut fails to differentiate into anorectum whereas epithelial differentiation of urogenital tract remains intact (*n* = 4) (Figure 4, e and i). It is notable that *Shh* and *Gli2* mutant mice have blunted or missing dorsal aspects of their most posterior aspect of cloaca (*n* = 4) (Figure 4, e and i).

Cloacal Malformations in *Gli2*;*Gli3* Mutant Mice

Previously, we have shown that *Gli2* and *Gli3* possess overlapping functions in foregut development; *Gli2*^{−/−}; *Gli3*^{+/−} mice exhibit tracheoesophageal fistula and severe lung hypoplasia, and *Gli2*^{−/−}; *Gli3*^{−/−} mice do not develop any esophagus, lung, and trachea.⁴³ To determine whether *Gli2* and *Gli3* possess overlapping functions in hindgut development, we examined the anorectal phenotype in *Gli2*;*Gli3* mutant mice. *Gli2*^{+/−}; *Gli3*^{+/−} mice are viable and do not display any anorectal malformations. *Gli2*^{−/−}; *Gli3*^{+/−} and *Gli2*^{+/−}; *Gli3*^{−/−} mice can survive until birth, whereas most *Gli2*^{−/−}; *Gli3*^{−/−} mice die at E10.5 and only few of them develop up to E14.5.

All E18.5 *Gli2*^{−/−}; *Gli3*^{+/−} (*n* = 8) and *Gli2*^{+/−}; *Gli3*^{−/−} mice (*n* = 8) displayed a complex and poorly developed genital swelling in the perineal area (Figure 5; a, b, c, and

Figure 5. Cloacal malformations in *Gli2*/*Gli3* mutant mice. Uroepithelium-covered cloacal openings are outlined by open squares. The SEM of genital and anorectal regions of E18.5 *Gli2*^{-/-}; *Gli3*^{+/-} (a and b) and *Gli2*^{+/-}; *Gli3*^{-/-} (c and d) mice. Both male (b and d) and female (a and c) mutant mice exhibit poorly developed external genitalia with the cloacal openings in perineum. All mutants demonstrate a confluence of lower intestinal and urinary tracts characteristic of the cloacal phenotype (f and g). E14.5 *Gli2*^{-/-}; *Gli3*^{-/-} mice (h) exhibit a very severe cloacal malformation similar to that observed in *Shb*^{-/-} mice (see Figure 2e). Abbreviations: b, bladder; cm, cloacal membrane; r, rectum; u, urethra; ur, ureter.

d). Although the external appearance of perineal area and external genitalia was variable depending on the sex and genotype, all double mutants exhibited a persistent cloacal malformation. The perineal openings in all *Gli2*^{-/-}; *Gli3*^{+/-} and *Gli2*^{+/-}; *Gli3*^{-/-} mice were partially or fully covered by a bulging cloacal membrane lined with uroepithelium (revealed at higher SEM magnifications; data not shown). Histological sections of E14.5 mutant embryos confirmed that all double mutants, including *Gli2*^{-/-}; *Gli3*^{-/-} mice (*n* = 2), develop a common outlet for urinary and intestinal tracts (Figure 5; f, g, and h). Persistent cloaca is observed when either one dose of *Gli3* is removed in a *Gli2*^{-/-} mutant background or one dose of *Gli2* is removed in a *Gli3*^{-/-} mutant background. The cloacal phenotype of *Gli2*^{-/-}; *Gli3*^{+/-} and *Gli2*^{+/-}; *Gli3*^{-/-} mice is less severe than that of *Shh* null mice, and *Gli2*^{-/-}; *Gli3*^{-/-} mice have a severe cloacal phenotype (Figure 5h), which resembles that of *Shh* null mice (Figure 2e). These observations illustrate that *Gli2* and *Gli3* possess both distinct and overlapping function in the development of distal hindgut.

Discussion

Our results establish that *Shh* signaling is essential for the development of the distal hindgut. Furthermore, this is the first demonstration of a specific genetic pathway capable of recapitulating the broad spectrum of anorectal malformations found in humans. We show that mutations in *Shh*, and its downstream mediators, *Gli2* and *Gli3*, result in the different forms of anorectal malformations (summarized

in Figure 6). Moreover, gene dosage of *Gli2* and *Gli3* was found to modulate the severity of malformation suggesting that a precise degree of *Shh* signaling is required for the normal development of rectum and anus.

The mutant phenotypes strongly suggest that defects in *Shh* signaling may be involved in the pathogenesis of human anorectal malformations. In further support of our hypothesis, some patients with Pallister-Hall syndrome (MIM no. 165240), which is caused by frameshift mutations in *GLI3*, also exhibit anorectal malformations, such as imperforate anus with recto-urethral fistula.⁵² The best known genetic form of human anorectal malformations, Currarino syndrome, has been attributed to the homeobox gene *HLXB9*, which is closely linked to *SHH* on chromosome 7q36. Although *SHH* was once a strong candidate gene for sacral agenesis, mutations could not be found in both the coding region and 5' flanking region of *SHH*.^{53,54} Instead, multiple mutations in the coding region of *HLXB9* were found in patients with sacral agenesis.⁵⁻⁷ The role of *HLXB9* in sacral agenesis is however controversial because mice lacking *Hlxb9* do not exhibit any defects in the development of sacral vertebrae and anorectal malformations.^{9,10} Our observations here raise an interesting possibility that sacral agenesis might be caused by mutations in the long-range regulatory elements of *SHH*, which reside in the coding region of *HLXB9*. It is noteworthy that the *SALL1* gene, which is mutated in Townes-Brocks syndrome, is also a putative target gene of *Shh* signaling.⁸ It will thus be of great interest to re-examine the role of *Shh* signaling in sacral agenesis by testing whether mutations found in the cod-

Figure 6. Summary of anorectal malformations observed in mutant mice with various defects in Shh signaling. Abbreviations: a, anus; b, bladder; r, rectum; s, reproductive tract; u, urethra.

ing region of *HLXB9* can affect the activity of the Shh gene in an experimental system like mice.

Biochemical and genetic studies have shown that the three mammalian Gli transcription factors (Gli1, Gli2, and Gli3) are involved in Shh signal transduction.^{39,40,43,45} Gli2 seems to be the major mediator of Shh signaling *in vivo*. In the developing foregut, Gli2 is required for the normal development of esophagus, trachea, and lung.²⁷ Gli2^{-/-} mice exhibit defects that are similar but milder than that observed in Shh null mice: stenosis of the esophagus and trachea, and lung lobulation defects.⁴³ Gli3 plays a minor role in foregut development; Gli3^{-/-} mice develop a normal trachea and esophagus, and only display subtle lung hypoplasia.⁵⁵ Analogous to the foregut, Gli2 is important for the development of dorsal anorectum from the distal hindgut, whereas Gli3 mutant mice display anal stenosis, a less severe phenotype, which is the most common type of anorectal malformation in humans.² In addition to their specific role in the development of distal hindgut, a gene dose-dependent effect observed in Gli2;Gli3 double mutant phenotypes suggest a conserved role of Shh signaling in both anterior and posterior ends of gut development.

Several theories on the embryogenesis of anorectal malformation have been presented in the past with considerable controversy. The first theory formulated by Tourneux⁴⁹ and Retterer⁵⁰ after examining embryos of sheep, rabbits, and pigs suggests that the crucial step in dividing the dorsal anorectum from the ventral urogenital tract is the formation of a septum. This remained the basis of our understanding until 1986 when more extensive anatomical and histological works refuted this theory because of the absence of morphological evidence to suggest septum formation either by the descent of a septum caudally or the formation and fusion of lateral ridges in the cloaca.¹⁸ More recently, studies of *Sd* mice noted that the most consistent finding in this animal model was the loss of the dorsal cloaca and the presence of a short cloacal membrane.^{16,56} In our anorectal mouse models, we found no evidence of active septation or lateral ridges

formation/fusion on transverse sections of our mutant embryos, and similar to the *Sd* mice, we also observed a lack of formation of a normal dorsal cloaca. Our data now introduces a developmental model that furthers our understanding of anorectal malformation beyond the previous anatomical-based theories. Our data strongly suggest that morphogenetic events in the hindgut depend on a precise degree of Shh signal as demonstrated by the graded phenotypes seen with different gene dosages of Gli2 or Gli3. Furthermore, Shh signaling is likely to determine the spatial and temporal organization of tissue in the hindgut and aberrations in signaling is responsible for abnormal morphogenesis of the hindgut.

Mutant mice involving Shh signaling demonstrates a whole spectrum of anorectal malformations similar to those in humans and introduces, for the first time, a genetic basis for anorectal malformation. We conclude that *Shh*, *Gli2*, and *Gli3* mutant mice are excellent animal models for studying the pathogenesis of anorectal malformations. The manipulation of the anatomical phenotype by molecular methods provides a powerful tool to rapidly expand our understanding of the morphogenesis of anorectal malformation.

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