Dosage-dependent hedgehog signals integrated with Wnt/β-catenin signaling regulate external genitalia formation as an appendicular program

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Embryonic appendicular structures, such as the limb buds and the developing external genitalia, are suitable models with which to analyze the reciprocal interactions of growth factors in the regulation of outgrowth. Although several studies have evaluated the individual functions of different growth factors in appendicular growth, the coordinated function and integration of input from multiple signaling cascades is poorly understood. We demonstrate that a novel signaling cascade governs formation of the embryonic external genitalia [genital tubercle (GT)]. We show that the dosage of Shh signal is tightly associated with subsequent levels of Wnt/β-catenin activity and the extent of external genitalia outgrowth. In Shh-null mouse embryos, both expression of Wnt ligands and Wnt/β-catenin signaling activity are downregulated. β-catenin gain-of-function mutation rescues defective GT outgrowth and Fgf8 expression in Shh-null embryos. These data indicate that Wnt/β-catenin signaling in the distal urethral epithelium acts downstream of Shh signaling during GT outgrowth. The current data also suggest that Wnt/β-catenin regulates Fgf8 expression via Lef/Tcf binding sites in a 3′ conserved enhancer. Fgf8 induces phosphorylation of Erk1/2 and cell proliferation in the GT mesenchyme in vitro, yet Fgf4/8 compound-mutant phenotypes indicate dispensable functions of Fgf4/8 and the possibility of redundancy among multiple Fgfs in GT development. Our results provide new insights into the integration of growth factor signaling in the appendicular developmental programs that regulate external genitalia development.

KEY WORDS: External genitalia, Genetic cascade, Hedgehog, Fgf, β-catenin (Ctnnb1), Cloaca, Appendages, Mouse

INTRODUCTION

Embryonic development is controlled by a series of basic regulatory processes, including the regulation of protrusion and outgrowth. It has become clear that such developmental processes require coordinated reciprocal interactions between epithelium and the adjacent mesenchyme, frequently mediated through hedgehog, Wnt and fibroblast growth factor (Fgf) pathways. Perturbation of these pathways causes developmental abnormalities in a variety of tissues due, at least in part, to failed cross-talk. Despite the importance of this cross-talk and reciprocal interactions, our understanding of signaling pathway interactions is limited.

An embryonic bud structure (an appendage) is a representative organ that is suitable for analyzing reciprocal interactions between signaling pathways. Protruding embryonic buds are often composed of the distal epithelium accompanied by adjacent proliferating mesenchyme, which eventually gives rise to a bud structure. Significant progress has been achieved in understanding the molecular network that regulates limb development (Capdevila and Izpisua Belmonte, 2001; Chen et al., 2004; Johnson and Tabin, 1997; Kmita et al., 2005; Niswander, 2003; Yamaguchi et al., 1999; Yang et al., 2006; Zhu et al., 2008). Vertebrate limb development depends on the establishment and maintenance of the apical ectodermal ridge (AER), a specialized epithelium at the distal tip of the limb bud. Epithelial-mesenchymal interactions between the AER and its adjacent mesenchyme are essential for limb bud outgrowth. Fgf gene family members are expressed specifically in the AER. The cumulative evidence indicates that AER formation and maintenance and Fgf expression are tightly controlled by intricate interplay among several growth factors (Lewandoski et al., 2000; MacArthur et al., 1995; Mariani et al., 2008; Moon and Capecchi, 2000; Sun et al., 2000; Sun et al., 2002; Yu and Ornitz, 2008). Wnt/β-catenin signaling in the limb ectoderm regulates AER maintenance and Fgf8 expression (Barrow et al., 2003; Soshnikova et al., 2003). Bmp signaling functions upstream of Wnt/β-catenin signaling in this process, as indicated by a failure of AER formation in mice lacking ectodermal bone morphogenetic protein receptor 1A (Bmpr1a) (Ahn et al., 2001). sonic hedgehog (Shh) is expressed in posterior limb bud mesenchyme [zone of polarizing activity (ZPA)] (Riddle et al., 1993), patterns the anterior-posterior axis of the limb, and supports expression of Fgf genes in the AER (Benazet et al., 2009; Laufèr et al., 1994; Niswander et al., 1994; Riddle et al., 1993; Zuniga et al., 1999).

Another embryonic appendage, the genital tubercle (GT), is the common primordium of male and female external genitalia. GT outgrowth is the result of mesenchymal proliferation around the cloaca, accompanied by formation of the urethral plate epithelium at the ventral midline of the GT. The amniotic cavity and cloacal lumen are separated by two epithelial components: a superficial...
layer of ectodermal epithelium and a thick inner endodermal cell layer. A unique developmental property of the GT is the coordinated formation of endoderm-derived tissues during its outgrowth and patterning. The endodermal epithelium expresses Shh, which influences gene expression in the adjacent mesenchyme (Haraguchi et al., 2001; Perriton et al., 2002). Shh-deficient embryos exhibit GT agenesis with loss of Bmp4 and Fgf10 expression (Haraguchi et al., 2001; Perriton et al., 2002), suggesting that Shh functions high up in a signaling cascade governing GT development.

As appendages, limb buds and the GT exhibit similarities in their development; for example, they undergo prominent outgrowth as an embryonic bud structure before differentiation of tissue components (Cobb and Duboule, 2005; Dolle et al., 1991; Kondo et al., 1997; Yamada et al., 2003; Yamada et al., 2006). Given the accumulating evidence of the importance of Fgf signaling (and of Fgf8 in particular) from the AER, Fgf8 is a prime candidate for mediating signaling from the GT epithelium to promote GT mesenchymal proliferation and outgrowth. The GT epithelium, termed the distal urethral epithelium (DUE), is located at the distal tip of the endoderm-derived epithelium (Haraguchi et al., 2001; Haraguchi et al., 2000; Lin et al., 2008; Ogino et al., 2001). Surgical removal of the tip of the GT, including the DUE, results in failure of GT outgrowth (Haraguchi et al., 2000). Fgf8 has therefore been proposed as a candidate regulator of initial GT development, although its function in GT formation is unknown.

The prospective GT region of Shh-deficient embryos fails to express Fgf8 (Haraguchi et al., 2001; Perriton et al., 2002), suggesting that Fgf8 is genetically downstream of Shh signaling. Here we show that Wnt/β-catenin signaling plays a key role in GT formation by inducing multiple factors, including Fgf8. The temporal requirement of Shh function and the importance of hedgehog signaling dosage are shown by analysis of conditional Shh mutants and of a series of Gli mouse mutants. We demonstrate that Wnt/β-catenin pathway activation by Shh signaling is responsible for subsequent GT outgrowth, and postulate that Wnt/β-catenin signaling functions in the DUE to stimulate mitogenic factors for the adjacent mesenchyme. Altogether, these data suggest a similar, and yet divergent, participation of growth factor signaling pathways, including Shh, Wnt/β-catenin and Fgf, in the development of appendicular structure, the external genitalia.

**Materials and Methods**

**Mouse strains and embryos**

The mutant mice used were Shh (Chiang et al., 1996), Gli2 (Mo et al., 1997), Gli3loxP (Hui and Joyner, 1993), ShhCreERT2 (Harfe et al., 2004), Isl1Cre+ (Yang et al., 2006), Hoxa3-Cre (Macatee et al., 2003), β-cateninflx−/−(Ctnnb1loxflx−/−) (Harada et al., 1999), β-cateninlox−/−(Ctnnb1Ex3lox−/−) (Huelsken et al., 2001), Shhlox−/− (Dassule et al., 2000), Fgf8lox−/− (Dong et al., 2004), Fgf8lox−/− (Park et al., 2006), R26R (Soriano, 1999), TopGal (DasGupta and Fuchs, 1999) and BatGal (Nakaya et al., 2005). To increase the efficiency of production of homozygous null embryos and decrease the incidence of mosaic deletion, β-catenin-null alleles were generated by utilizing CAGGS-Cre mice (Araki et al., 1997), which express Cre recombinase in the germline. Noon on the day when a vaginal plug was detected was designated as E0.5. Embryos for each experiment were collected from at least three pregnant females. All procedures and protocols were approved by the Committee on Animal Research at Kumamoto University, Japan.

The tamoxifen (TM)-inducible Cre recombinase system removes the floxed sequence from the target genome (Feil et al., 1997), which express Cre recombinase in the germline. Noon on the day when a vaginal plug was detected was designated as E0.5. Embryos for each experiment were collected from at least three pregnant females. All procedures and protocols were approved by the Committee on Animal Research at Kumamoto University, Japan.

**Histology**

Hematoxylin and Eosin staining, X-Gal staining, immunohistochemistry and in situ hybridization for gene expression were performed by standard procedures as previously described (Haraguchi et al., 2007). Immunohistochemistry employed the following primary antibodies: C44, β-catenin, E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA) and phospho-Erk1/2 (Cell Signaling, Danvers, MA, USA). For in situ hybridization, the following riboprobe templates were used: Tfl1 (H. Clevers, University Medical Center Utrecht, The Netherlands); Pitx1 (Y. Chen, Tulane University, New Orleans, L.A., USA); Fgf3 (C. Dickson, Imperial Cancer Research Fund, London, UK); Fgfr4 (G. Martin, University of California, San Francisco, CA, USA); Wnt3, Wnt5a, Wnt7a, Wnt6b (S. Takada, National Institutes of Natural Sciences, Okazaki, Japan); and Fgf4, Fgf8, Fgf10, Bmp4, Gli1, Ptc1, Shh, Dlx5 (Haraguchi et al., 2007; Suzuki et al., 2008). The template of Asx2 was obtained by standard RTPCR procedures using primers 5′-CACCTCAGGACGCTCGA-CAGA-3′ and 5′-TACCCAGCTCCTGGAGACTGA-3′.

**Cell proliferation and death analyses**

Pregnant females were injected with 100 mg BrdU (Sigma) per kg body weight. One hour after injection, embryos were collected. For cell culture experiments, BrdU (1 μg/ml) was added to the medium for 30 minutes and BrdU incorporation detected with anti-BrdU antibody (Roche, Mannheim, Germany). TUNEL assay for the detection of apoptotic cells was performed with the In Situ Apoptosis Detection Kit (Takara, Ohsu, Japan).

**Organ culture**

Filter-supported organ cultures for murine GTs isolated from embryos of ICR strain (E11.5; CLEA, Tokyo, Japan) were as previously described (Haraguchi et al., 2000). The tissues were cultured for 24 hours after bead application and processed for histological analysis. Heparin acrylic beads were soaked overnight with recombinant mouse Fgf8 protein at 1.0 μg/ml in PBS (Haraguchi et al., 2000). Control beads were treated with PBS containing 0.1% BSA.

**Plasmid DNA and luciferase assay**

The conserved region 3′ of the Fgf8 locus was described previously (Beermann et al., 2006). Genomic sequences of the 5′ region and conserved region 3 (CR3) of Fgf8 loci, obtained from Ensemble (www.ensembl.org), were submitted for analysis by rVISTA (genome.lbl.gov/vista). DNA fragments of CR3 of the murine Fgf8 locus, obtained from a BAC clone (RPC123-98F2) by PCR, were inserted into the pGL4.24 vector (Promega, Madison, WI, USA). The mouse β-catenin expression vector was kindly provided by Dr S. Kume (Katakashi et al., 2000).

The HaCat cell line was maintained in DMEM supplemented with 10% FBS. Cells were transfected with expression and reporter plasmids using FuGENE HD (Roche) according to the manufacturer’s instruction. Twenty-four to thirty hours post-transfection, luciferase activity was measured by chemiluminescence employing the Dual-Luciferase Reporter Assay System (Promega). The values were normalized against Renilla luciferase activity. At least three independent experiments were performed. Statistical analysis was performed using Student’s t-test or Welch’s t-test followed by F-test (P<0.05 considered significant).

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP Assay Kit (Upstate, Lake Placid, NY, USA) was used. The distal GT region containing the DUE, and the distal tip of the hindlimb containing the AER, were dissected from embryos at E12.5 and E10.5, respectively. β-catenin (Santa Cruz) and acetyl-histone H3 (Upstate) antibodies (2 μg) were used. For mock control, rabbit or mouse immunoglobulin (Dako, G. Martin, University of California, San Francisco, CA, USA); Wnt3, Wnt5a, Wnt7a, Wnt6b (S. Takada, National Institutes of Natural Sciences, Okazaki, Japan); and Fgf4, Fgf8, Fgf10, Bmp4, Gli1, Ptc1, Shh, Dlx5 (Haraguchi et al., 2007; Suzuki et al., 2008). The template of Asx2 was obtained by standard RTPCR procedures using primers 5′-CACCTCAGGACGCTCGAC-GA-3′ and 5′-TACCCAGCTCCTGGAGACTGA-3′.
RESULTS
Progressive GT formation revealed by conditional Shh mutation

The murine GT begins to visibly protrude as a bud structure at E10.5-11.5. Shh is expressed in the endodermal cloacal epithelium at this time (Fig. 1A,B) and the hedgehog-responsive genes Gli1 and patched 1 (Ptch1) are expressed in the adjacent mesenchyme and ectoderm (Fig. 1C,D). Shh knockout (KO; Shh–/–) embryos exhibit a complete failure of GT outgrowth (Haraguchi et al., 2001). We analyzed the temporal dependence of GT development on Shh genetically by crossing ShhCreERT2 and Shhlox alleles, which permits inducible Cre function and Shh inactivation in the cloacal endodermal cells and in cells of urethral plate epithelium (UPE) upon TM administration (Harfe et al., 2004). In control embryos, a prominent GT protrusion was observed, the bladder developed normally, and the urethral and rectal orifices were completely separated (Fig. 1E). In ShhCreERT2/lox mutants, administration of TM at E9.75 caused severe GT hypoplasia, accompanied by a persistent cloaca; the urethral and rectal ends were shared and opened at a common orifice in the rudimentary GT (Fig. 1F). Expression of the distal marker genes Wnt5a and Dlx5 was not detected in the mutants, although expression of Pitx1 as a dorsal marker was still observed (see Fig. S1 in the supplementary material). TM administration at sequentially later stages elicited progressively milder GT phenotypes (Fig. 1G-I), such that administration of TM at E13.5 resulted in morphologically normal GTs.

Decreased expression of Wnt ligand genes in Shh mutant GT

Shh elicits mesenchymal and epithelial cellular responses and regulates the expression of several downstream genes during GT development (Haraguchi et al., 2001). However, the signaling pathways downstream of Shh that are required for GT development have not been identified. Because of the importance of Fgf/Shh interactions in limb AER and mesenchyme, and given that Wnt ligands can regulate Fgf8 expression and limb outgrowth (Barrow et al., 2003; Kawakami et al., 2001; Kengaku et al., 1998), we examined the expression of Wnt ligand genes in the GT. Several Wnt ligand genes were expressed in the GT (as assessed by RT-PCR, data not shown). Among them, Wnt3, Wnt4, Wnt7a and Wnt9b were downregulated in the GT of Shh KO mutant embryos (Fig. 2A-D), particularly in the GT ectoderm (Fig. 2E-H).

Regulation of Wnt/β-catenin activity by Shh signaling

To examine whether the decreased expression of ectodermal Wnt ligands results in decreased Wnt/β-catenin signaling in Shh KO mutants, we assayed β-galactosidase (β-gal) activity from the TopGAL allele (DasGupta and Fuchs, 1999). During normal GT development, weak β-gal activity is detected in the distal region of the urethral GT (Fig. 1F). Expression of the distal marker genes Wnt5a and Dlx5 was not detected in the mutants, although expression of Pitx1 as a dorsal marker was still observed (see Fig. S1 in the supplementary material). TM administration at sequentially later stages elicited progressively milder GT phenotypes (Fig. 1G-I), such that administration of TM at E13.5 resulted in morphologically normal GTs.

Fig. 1. Temporal requirements for hedgehog signaling during GT initiation and outgrowth. (A,B) Murine GT development and Shh expression at E10.5 (A) and E11.5 (B). Red regions indicate Shh-expressing endoderm-derived epithelia. The cloacal region includes the thick endodermal epithelium (in red, B). The dark-blue line indicates adjacent ectoderm-derived epithelium. Cloacal cavity is in light blue. (C,D) Gli1 (C) and patched 1 (D) expression in GT mesenchyme and ectoderm at E11.5. (E-H) Phenotypes after temporally staggered ablation of Shh. GT phenotypes of ShhCreERT2/lox embryos treated with TM at E9.75 (F), E11.5 (G), E12.5 (H) and E13.5 (I) versus the control GT (no TM administration, E). TM administration at E9.75 induced severe GT aplasia with a persistent cloaca (asterisk). Arrowheads indicate GTs or GT rudiments. b, bladder; r, rectum; u, urethra. Scale bars: 100 μm.

Fig. 2. Expression of Wnt ligands in control and Shh KO mouse embryos at E11.5. (A-H) Ectodermal expression (arrowheads) of Wnt3 (A,E), Wnt4 (B,F), Wnt7a (C,G) and Wnt9b (D,H) is decreased in Shh KO embryos. Scale bar: 100 μm.
expression (Fig. 3E). This β-gal activity in the DUE persisted throughout multiple stages of GT development (see Fig. S2 in the supplementary material). In Shh KO embryos, TopGAL activity was almost undetectable in the cloaca at E10.5 and E11.5 (Fig. 3F-H), and was accompanied by a loss of Fgf8 expression in the DUE (Fig. 3I). We obtained similar results with the Shh KO embryos. TopGAL activity (N) relative to control (M) and Gli3\textsuperscript{lox/lox} embryos (O). Shh\textsuperscript{−/−};Gli3\textsuperscript{lox/lox} double mutants exhibit weak, but detectable, TopGAL activity (Q, arrowhead) as compared with Shh KO embryos (P).

Requirement for β-catenin during GT development

Wnt/β-catenin signaling is required for caudal body formation; Wnt3a\textsuperscript{−/−}, Left1\textsuperscript{−/−};Tcf1\textsuperscript{−/−} and Tcf1\textsuperscript{−/−};Tcf4\textsuperscript{−/−} embryos exhibit severe caudal truncation with GT agenesis (Dunty et al., 2008; Galceran et al., 1999; Gregorieff et al., 2004; Takada et al., 1994). To focus on the role of β-catenin specifically during GT formation, β-catenin
To examine the possibility that /H11032 assays were performed for a region 3 by Potential regulation of signaling at this later stage.

In developmental processes in the prospective GT region before GT outgrowth. In primers that amplify a CR3 genomic fragment that includes these material). We performed a ChIP assay followed by PCR using signaling epithelium. Using rVISTA, we found that CR3 contains the similarities between the DUE and the AER as a transient distal enhancer that regulates reporter expression in the AER, and we considered whether it might also function as a DUE enhancer given conserved region 3 [CR3 (Beermann et al., 2006)] is a candidate an evolutionarily highly conserved putative enhancer.

By contrast, the temporally inducible ShhCreERT2 line is suitable for analyzing gene function during later stages of GT development, as Shh-dependent outgrowth proceeds. In ShhCreERT2;Ctnnb1lox/lox embryos, the levels of β-catenin protein in the GT region (including the DUE) were reduced at E11.5 and GT outgrowth failed (Fig. 4B-E). This raises the intriguing possibility that an early phase of β-catenin activity is necessary for cloacal Shh expression.

### Potential regulation of Fgf8 expression by β-catenin

To examine the possibility that Fgf8 expression is directly regulated by β-catenin, chromatin immunoprecipitation (ChIP) and reporter assays were performed for a region 3′ of the Fgf8 locus that contains an evolutionarily highly conserved putative Fgf8 enhancer. Conserved region 3 [CR3 (Beermann et al., 2006)] is a candidate enhancer that regulates reporter expression in the AER, and we considered whether it might also function as a DUE enhancer given the similarities between the DUE and the AER as a transient distal signaling epithelium. Using rVISTA, we found that CR3 contains several Lef/Tcf binding sites (see Fig. S5 in the supplementary material). We performed a ChIP assay followed by PCR using primers that amplify a CR3 genomic fragment that includes these binding sites. β-catenin-specific enrichment was observed in the extracts from the GT and limb bud (Fig. 4L). PCR amplification of a putative enhancer 5′ of Fgf8 (Hu et al., 2004), which is also highly conserved in vertebrates but lacks Lef/Tcf binding sites, yielded no enrichment (Fig. 4L). Both regions were enriched in chromatin immunoprecipitated with anti-acetylated histone H3, as a positive control. Furthermore, overexpression of wild-type or constitutively active β-catenin activated transcription of a CR3 enhancer/luciferase reporter in HaCat cells, whereas a dominant-negative form did not (Fig. 4M). Taken together, these results suggest that Wnt/β-catenin signaling participates in the regulation of Fgf8 expression via CR3.

**Fgf8 can induce phosphorylation of Erk1/2 and cell proliferation in GT mesenchyme, but Fgf4 and Fgf8 are dispensable for GT outgrowth**

In the limb, AER-derived Fgf induces phosphorylated (p) Erk1/2 (Mapk3/1 – Mouse Genome informatics) activity in the adjacent mesenchyme (Kawakami et al., 2003). Organ culture experiments with explanted GTs revealed that GT mesenchyme adjacent to Fgf8-soaked beads has a higher level of pErk1/2 than that with a contralaterally implanted control bead (Fig. 5A). GT mesenchymal cell proliferation was also consistently increased by Fgf8b treatment (Fig. 5B). To investigate Fgf functions during GT development in vivo, we conditionally inactivated Fgf8 in the GT with a Hoxa3-Cre line, which exhibits strong Cre activity throughout the caudal embryo, including the presumptive cloacal region, GT primordium and DUE (see Fig. S6A in the supplementary material) (Macatee et al., 2003). In situ hybridization using an Fgf8 antisense riboprobe specific for the floxed exon 5 (Moon and Capecchi, 2000) confirmed the absence of Fgf8 expression in the Hoxa3-Cre;Fgf8 mutants (see Fig. S6B,C in the supplementary material). Notably, we found that although Fgf4 expression was barely detected in the normal GT, ablation of Fgf8 resulted in increased Fgf4 expression in the DUE.
Constitutively active β-catenin rescues GT outgrowth in Shh mutants

Our current findings suggest that β-catenin functions downstream of Shh to support GT outgrowth; if so, overexpression of β-catenin should at least partially rescue GT agenesis in Shh KO mutants. We generated Shh^{CreERT2/−};Ctnnb1^{Ex3/−} mutant embryos, which express a constitutively active β-catenin (Ctnnb1^{Ex3}) in a Shh-null background. Indeed, TM treatment at E9.5 resulted in visible outgrowth of the GTs of these compound mutants at E11.5, in contrast to GT agenesis in the Shh^{CreERT2/−};Ctnnb1^{Ex3/−} (equivalent to the Shh-null) embryos (Fig. 6A-C). At E13.5, GT outgrowth and a preputial fold were present, although smaller than normal (Fig. 6F-H). Surviving mutant embryos at E18.5 displayed a bud structure composed of mesenchymal tissue without the urethra (Fig. 6I,J; data not shown). It is noteworthy that hedgehog signaling was not activated in the Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos, judging by Gli1 expression (Fig. 6D,E), whereas Fgf8 was expressed at high level in the Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos (Fig. 7A-C). The Fgf8 expression domain coincided with the location of β-catenin augmentation, as evident by expression of Axin2, a downstream target of the Wnt/β-catenin pathway (Fig. 7D-F).

Cd44, another AER marker (Sherman et al., 1998), is expressed in a similar manner to Fgf8 in the DUE (Fig. 7G). Consistent with rescued Fgf8 expression in Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos, Cd44 expression was also restored (Fig. 7H,I). Expression of Fgf10 and Bmp4 in the GT mesenchyme is regulated by Shh and, consequently, their expression is decreased in Shh KO embryos (see Fig. S8A,B,D,E in the supplementary material) (Haraguchi et al., 2001; Perriton et al., 2002). Fgf10 and Bmp4 expression was not rescued in the GT mesenchyme of Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos (Fig. 7A-C). The Fgf8 expression domain coincided with the location of β-catenin augmentation, as evident by expression of Axin2, a downstream target of the Wnt/β-catenin pathway (Fig. 7D-F).

In wild-type mouse embryos, pErk1/2 immunoreactivity is present mainly in the mesenchyme (Fig. 7J), and is decreased in Shh KO embryos (Fig. 7K). By contrast, the mesenchymal domain of pErk1/2 expression was markedly enhanced in the GT of Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos (Fig. 7L), consistent with increased Fgf8 signaling. Abundant cell proliferation was observed in control embryos (Fig. 7M), but in Shh KO embryos mesenchymal cell proliferation was specifically decreased (Fig. 7N). Cell death in the endodermal epithelium and mesenchyme was increased in the distal GT (Fig. 7P,Q). It has been reported that the normal DUE, as well as with other signaling epithelia, displays a scattered pattern of apoptosis concomitant with reduced cell proliferation (Fig. 7M,P) (Haraguchi et al., 2001; Jernvall et al., 1998). In Shh^{CreERT2/−};Ctnnb1^{Ex3/−} embryos, mesenchymal cell proliferation was largely rescued (Fig. 7O), but mesenchymal cell death remained elevated above that seen in controls (Fig. 7R).

**DISCUSSION**

Coordinated growth factor signaling is essential for regulating morphogenesis of appendicular structures such as the limbs and external genitalia. Although previous studies significantly advanced our knowledge of individual gene contributions, understanding how these genes interact and how multiple signaling inputs are integrated during organogenesis remains a major challenge. Here we report that hedgehog signaling is essential for external genitalia development in a dosage-dependent manner, as demonstrated by analyses of different Gli compound mutants. Different levels of hedgehog signaling may regulate the output of the Wnt/β-catenin activity to regulate outgrowth of the embryonic external genitalia. Shh KO embryos display a persistent cloaca and failed GT outgrowth (Haraguchi et al., 2001; Mo et al., 2001; Perriton et al., 2002). Remarkably, we found that overexpression of constitutively active β-catenin rescues GT protrusion in the absence of Shh and Fgf8 expression in the endoderm is restored as is mesenchymal proliferation. To our knowledge, this is the first demonstration of genetic rescue of embryonic appendicular agenesis.
Integrated Gli and Shh signaling for GT development; hedgehog functions as a trigger for GT initiation and promotes subsequent outgrowth

Several reports have demonstrated the contribution of multiple growth factors to GT development, including hedgehog, Wnt and Fgf signaling (Haraguchi et al., 2001; Haraguchi et al., 2000; Lin et al., 2008; Morgan et al., 2003; Perriton et al., 2002; Suzuki et al., 2003; Yamaguchi et al., 1999). Shh plays a crucial role in external genitalia development (Haraguchi et al., 2001; Perriton et al., 2002). Here, stage-specific Shh conditional mutant analysis reveals the temporal requirements for Shh function during GT development. Early loss of Shh function induces minimal GT outgrowth and the cloaca persists, but later gene ablation elicits milder GT phenotypes. This might recapitulate early, transient requirements for Shh for the rapid growth of digit primordia (and digit patterning) in the limb (Zhu et al., 2008), and epithelium-derived Shh function in regulating tooth organ size (Dassule et al., 2000). Several pathways have been postulated to regulate proximal-distal and dorsal-ventral axis formation of the GT (Suzuki et al., 2003; Suzuki et al., 2008; Yamada et al., 2003; Yamada et al., 2006). If Shh only functions as a growth-promoting factor during early GT development, axis marker gene expression should have been normal in the early Shh conditional mutant GTs. However, Dlx5 and Wnt5a expression was abnormal, suggesting that proximal-distal axis formation was altered by early loss of Shh function. However, it is necessary to consider the unique character of GT development, as its robust outgrowth from the beginning depends substantially on Shh. Namely, the proximal-distal patterning is tightly linked with such robust outgrowth promotion of the anlage. Hence, it is not simply possible to make a conclusion for the status of proximal-distal axis formation by the current data. By contrast, expression of Pitx1, as a candidate dorsal marker during GT development (our unpublished results) (Haraguchi et al., 2007), was unaffected by loss of Shh function. It has recently been suggested from lineage analysis that the dorsal side of the GT develops in association with more-anterior regions, including the peri-cloacal mesenchyme (PCM) and the lower body wall regions (Haraguchi et al., 2007). The sustained dorsal marker gene expression might reflect such a unique program of GT development and the nature as a dorsal structure is less affected by Shh.

Hedgehog signal transduction comprises a series of complex intracellular events. Shh signaling modulates the function of Gli transcription factors. In mammals, Gli2 is a major positive transcriptional effector of hedgehog signal transduction. In the absence of Shh, Gli2 is completely degraded, and Gli3 is converted into a transcriptional repressor, Gli3R (Ingham and McMahon, 2001). By titrating the relative amounts of these factors, we found that both the extent of GT outgrowth and the level of Wnt/β-catenin activity are responsive to the effective level of hedgehog signaling. In the wild-type situation, Shh activates Wnt/β-catenin signaling and GT outgrowth, but in Shh KO embryos the net effect of the absence of Gli2 activator and presence of Gli3R leads to the most severe phenotype: GT agenesis. The contribution of Gli3R to the Shh KO phenotype is indicated by the restoration of TopGAL activity and moderate GT outgrowth in Shh; Gli3 double mutants (Shh<sup>−/−</sup>; Gli3<sup>−/−</sup>). Shh activity during GT outgrowth is likely to prevent formation of Gli3R. In Gli2 KO embryos, Shh prevents formation of Gli3R, but loss of Gli2 activator causes a modest decrease in downstream Wnt/β-catenin activity and the phenotypic outcome is GT hypoplasia. Thus, normal GT development requires the Shh pathway as balanced by the activation of Gli2 and Gli3. Integrated Gli function has been thoroughly explored with regard to anterior-posterior patterning of the limb, in which a major role of Shh is to prevent formation of Gli3R (Nieuwenhuis and Hui, 2005). In the GT, Gli2 activation downstream of Shh is essential for outgrowth, and inhibition of Gli3R production appears less important.

Context-dependent cross-talk between Wnt/β-catenin and Shh signaling

Shh KO embryos exhibit failed GT outgrowth and loss of Fgf8 expression in the DUE (Haraguchi et al., 2001; Perriton et al., 2002). In addition to our finding that hedgehog signaling regulates the level of Wnt/β-catenin activity in a dosage-dependent manner, we found that Wnt/β-catenin in turn regulates Fgf8 expression. Surgical removal of the ectodermal epithelium results in GT truncation, indicating an inductive role of the ectodermal epithelium in GT development (Murakami and Mizuno, 1986). We found that the expression of several ectodermal Wnt ligand genes was reduced in Shh KO embryos. Indeed, Wnt/β-catenin activity in the cloacal membrane and later DUE was affected by the level of hedgehog signaling. Ectoderm-derived Wnt ligands are likely candidates for induction of Wnt/β-catenin activity in the distal GT, including the DUE. In the limb, ablation of Wnt3 from the limb ectoderm results in agenesis as the most severe outcome, suggesting a similarly essential role for ectodermal Wnt ligands in appendicular outgrowth (Barrow et al., 2003).

Fig. 6. Rescue of GT outgrowth in Shh-null mutants by constitutively active β-catenin. (A-J) Control GTs (A,F) and Shh KO mouse embryos with GT agenesis (B,G,I). Shh<sup>CreERT2−/−; Ctnnb1<sup>Flox<sup>−/−</sup></sup> embryos have GT protrusion at E11.5 and continued GT outgrowth with preputial fold at E13.5 (C,H). Further outgrowth is evident by E18.5 (J). Gli1 expression is undetectable in Shh<sup>CreERT2−/−; Ctnnb1<sup>Flox<sup>−/−</sup></sup> embryos at E11.5 (D,E). Arrowheads indicate GT or prospective GT regions.
Fig. 7. Regulation of Fgf8 expression by Shh and β-catenin. (A-I) Fgf8 (A,B) and Cd44 (G,H) expression (arrowheads) in control (A,G) versus Shh KO (B,H) DUE. Expression is recovered in ShhCreERT2−/−;Ctnnb1lox/lox embryos (arrows in C,I). Fgf8 and Cd44 expression overlap with regions of augmented β-catenin and Axin2 (D,F,I). (J-L) pErk1/2 staining in control (J) versus Shh KO (K) and ShhCreERT2−/−;Ctnnb1lox/lox (L) embryos. (M-R) BrdU labeling (M-O) and TUNEL assays (P-R). Shh KO embryos show significantly decreased cell proliferation (N). By contrast, expression of stabilized β-catenin in Shh KO mutants restores cell proliferation (O). Mesenchymal apoptosis was unchanged in ShhCreERT2−/−;Ctnnb1lox/lox embryos as compared with Shh KO embryos (Q,R). Dotted lines in the midline GT indicate the basal layer of the endodermal and ectodermal epithelia. (S) Proposed signaling cascade for GT outgrowth. Shh signaling is required for GT initiation and subsequent outgrowth. Shh is expressed moderately in the DUE (red) and strongly in urethral epithelium (orange). Wnt ligands are expressed in the distal GT ectoderm, and Wnt/β-catenin expression appears to be downstream of Shh signaling. This Wnt/β-catenin activity, and possibly urethral plate-derived Shh, induce production of mitogenic factors leading to GT outgrowth. Yellow, mesenchyme; blue line, ectoderm-derived epithelium. Scale bars: 100 μm.

Given the strong Wnt/β-catenin activity in the endoderm-derived DUE, we used temporally inducible ShhCreERT2 to analyze the effects of β-catenin loss-of-function in the endoderm during GT outgrowth. ShhCreERT2−/−;Ctnnb1lox/lox embryos treated with TM at E9.5 exhibited markedly reduced Fgf8 expression. However, Gli1 expression was normal at E11.5, indicating that Shh signaling was not affected by disrupting Wnt/β-catenin signaling at this later stage. Taken together with the results of our β-catenin gain-of-function studies (see below), Wnt/β-catenin signaling appears to be downstream of hedgehog signaling during GT outgrowth and upstream of Fgf8 expression.

Wnt/β-catenin signaling is required during early embryogenesis (E9.0) for the formation of the caudal embryo and Wnt3a+/−, Lef1+/−, Tcf1−/− and Tcf4−/− embryos exhibit severe caudal truncation of the embryonic axis and GT agenesis (Dunty et al., 2008; Galceran et al., 1999; Gregorieff et al., 2004; Takada et al., 1994). Owing to the wide range of Wnt/β-catenin functions, the epistatic relationships between hedgehog and Wnt/β-catenin signaling are likely to vary in different developmental contexts. To investigate such a possibility with regard to GT development, we conditionally ablated β-catenin with Isl1Cre, in which Cre is active in the prospective GT region at the stage required for caudal body formation (~E9.0). Isl1Cre;Ctnnb1lox/lox embryos exhibited a loss of Shh expression at this early stage, consistent with the proposal that Shh lies downstream of β-catenin in the endoderm (Lin et al., 2008). The importance of Wnt/β-catenin activity during formation of the caudal embryo suggests that Wnt/β-catenin activity might contribute to cloaca formation, a possibility that merits further study.

Possible regulation of Fgf8 expression by β-catenin and functional redundancy among Fgfs during GT outgrowth

The CR3 enhancer (located 24 kb 3’ of the Fgf8 coding region) contributes to Fgf8 expression in the AER (Beermann et al., 2006). Fgf8 expression is augmented in the limb by overexpression of stabilized β-catenin in the ectoderm (Soshnikova et al., 2003). We questioned whether similar regulatory actions of β-catenin on Fgf8 expression operate in the DUE via CR3. We found that β-catenin is bound to a region of CR3 in nuclear extracts from GTs and that this region confers a transcriptional response to exogenous β-catenin in luciferase assays. Fgf8 expression is decreased in both GT and limb bud in β-catenin loss-of-function mutants (this study) (Lin et al., 2008; Soshnikova et al., 2003). These results suggest β-catenin as a candidate upstream regulator of Fgf8 expression in the DUE. Regulation of Fgf8 by β-catenin might be a shared characteristic of the AER and DUE. Such regulation through another candidate enhancer in intron 3 of Fgf8 was recently reported for tooth development (Wang et al., 2009).
Although we found that Fgf4/8 are largely dispensable for GT development, it is likely that compensation by other Fgf ligands occurs, as has been described in the limb, tooth, inner ear and brain (Boulet et al., 2004; Mariani et al., 2008; Moon and Capacchi, 2000). In the current study, a specific Fgf can be (also) ectopically induced upon mutation of another Fgf. Our data suggest that a feedback mechanism operates to maintain the amount of Fgf signaling emanating from the DUE, and that this mechanism can induce expression of Fgf ligand-encoding genes that are otherwise often undetectable. We have made similar observations in the pharyngeal tissues of Fgf8 mutants (A.M., unpublished) (Moon et al., 2000). The redundancy of Fgf function can also be analyzed in Fgf receptor (Fgfr) conditional mutants. Our preliminary analyses of conditional Fgfr mutants reveal GT defects (our unpublished results), and Fgfr1 null mutants have caudal agenesis (Deng et al., 1994; Yamaguchi et al., 1994).

Dispensable Fgf8 function was also recently demonstrated (Seifert et al., 2009).

The development of an embryonic bud structure requires an expansion of mesenchymal tissue during outgrowth (Sun et al., 2002). We found that constitutively active β-catenin in the DUE induced Erk1/2 phosphorylation and cell proliferation in the adjacent GT mesenchyme. This suggests that Wnt/β-catenin stimulates the distal GT, including the DUE, to secrete mitogenic factors required for GT mesenchymal growth (see Fig. S7 in the supplementary material). However, the nature of these factors is still unclear (see discussion of Fgfs above). We have shown that Bmp7 from the DUE has some role in GT outgrowth, suggesting Bmp signaling as an essential growth regulator of the GT (Suzuki et al., 2008). Bmp4 is induced by Shh and our previous data also suggest roles for Bmp signaling in the DUE and distal ectoderm in GT outgrowth (Suzuki et al., 2003).

Our work reveals both conserved and divergent features of the developmental programs that trigger GT and limb bud formation and how they interact to control subsequent outgrowth. Initiation and outgrowth of the GT primordia also critically influence anorectal/urogenital organ development (Hynes and Fraher, 2004), as revealed by the fact that Shh KO mutants display not only GT agenesis, but also a persistent cloaca (Mo et al., 2001). GlI2 and some conditional Shh mutant embryos show a severe form of defects for UPE formation in the GT. Overall, an improved understanding of the molecular mechanisms of genital development might shed light on the mechanisms that underlie congenital abnormalities of multiple organ systems, in addition to those affecting the external genitalia and anorectal/urogenital organs.

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