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Thyroid hormone-upregulated hedgehog interacting protein is involved in larval-to-adult intestinal remodeling by regulating sonic hedgehog signaling pathway in *Xenopus laevis*

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Abstract

Sonic hedgehog (Shh) was previously shown to be involved in the larval-to-adult remodeling of the *Xenopus laevis* intestine. While Shh is transcriptionally regulated by thyroid hormone (TH), the post-transcriptional regulation of Shh signaling during intestinal remodeling is largely unknown. In the present study, we focused on a role of the pan-hedgehog inhibitor, hedgehog interacting protein (Hip), in the spatio-temporal regulation of Shh signaling. Using real-time RT-PCR and in situ hybridization, we show that Hip expression is transiently upregulated during both natural and TH-induced metamorphosis and that Hip mRNA is localized in the connective tissue adjacent to the adult epithelial primordia expressing Shh. Interestingly, the expression of bone morphogenetic protein (BMP)-4, a Shh target gene, is hardly detectable where Hip is strongly expressed. Finally, we demonstrate that Hip binds to the N-terminal fragment of processed Shh in vivo, suggesting that Hip suppresses Shh signaling through sequestering Shh.

Keywords

sonic hedgehog (Shh); hedgehog interacting protein (Hip); *Xenopus laevis*; metamorphosis; intestinal remodeling

INTRODUCTION

During amphibian metamorphosis, some adult organs such as limbs undergo de novo development and larva-specific organs such as tail are resorbed completely. The vast majority of the tadpole organs undergo extensive remodeling from the larval to adult form (Kikuyama et al., 1993; Tata, 1993; Shi, 1999). All changes during metamorphosis are triggered by thyroid hormone (TH) and thus can be easily manipulated by simply blocking the synthesis of endogenous TH or can be precociously induced by adding exogenous TH to the rearing water of premetamorphic tadpoles (Dodd and Dodd, 1976; Shi, 1999).

To investigate the molecular mechanisms of organ remodeling during amphibian metamorphosis, we have used the *Xenopus laevis* intestine as a model. The tadpole intestine is a simple tubular organ consisting of a single layer of primary (larval) epithelial cells surrounded by thin layers of muscles with the intervening connective tissue (Marshall and Dixon, 1978; Kordylewski, 1983). The connective tissue remains very thin except for a single fold, the typhlosole, throughout pre- (up to stage 54) and prometamorphosis (stage 54-58). During metamorphic climax (stage 58-65), the larval epithelium undergoes apoptosis (Ishizuya-Oka and Ueda, 1996). At the same time, primordia of the secondary (adult) epithelial cells, whose origin remains to be determined, appear as islets between the larval epithelium and the connective tissue, proliferate and differentiate into the adult intestinal epithelium with the progress of intestinal fold-formation (Hourdry and Dauca, 1977; McAvoy and Dixon, 1977). The adult epithelium after metamorphosis establishes a cell renewal system along the trough-crest axis of the intestinal folds (Shi and Ishizuya-Oka, 2001) similar to the mammalian crypt-villus axis (Cheng and Bjerknes, 1985; Madara and Trier, 1994).

Since the exogenous TH can induce the larval-to-adult epithelial cell replacement both in vivo and in vitro (Ishizuya-Oka and Shimozaawa, 1991), an important approach has been to analyze TH response genes to study the molecular basis of intestinal remodeling. Among a number of TH response genes isolated from the *X. laevis* intestine (Shi and Brown, 1993), we have identified sonic hedgehog (Shh) as a direct TH response gene, whose expression in the intestine is highly upregulated during metamorphic climax (Stolow and Shi, 1995). Shh is generally known to act as an important signaling molecule involved in the spatial patterning of various organs including the gut (Roberts et al., 1995; Sukegawa et al., 2000). By immunohistochemistry using anti-Shh antibody, we have previously shown that the epithelial-specific expression of Shh coincides well with active proliferation of the adult epithelial primordia during metamorphosis (Ishizuya-Oka et al., 2001b). More importantly, we have demonstrated that Shh induces the connective tissue-specific expression of bone morphogenetic protein (BMP)-4, which in turn promotes the adult epithelial differentiation during the intestinal remodeling (Ishizuya-Oka et al., 2006). Such a pivotal role of Shh in the larval-to-adult remodeling of intestinal epithelium prompted us to investigate how the action of Shh is controlled at both the mRNA and protein levels by TH.

Hedgehog interacting protein (Hip), a putative transmembrane glycoprotein, was originally isolated from the mouse limb bud cDNA library (Chuang and McMahon, 1999). Hip has been shown to directly interact with all mammalian hedgehog (Hh) proteins in vitro and to attenuate Hh activity upon targeted misexpression in transgenic mice (Chuang and McMahon, 1999; Treier et al., 2001). In the mouse small intestine overexpressing Hip under control of the villin promoter led to flattened epithelium with significant interference of villus formation and epithelial remodeling (Madison et al., 2005). *X. laevis* homologue of mouse Hip has been isolated, and its overexpression during embryonic development results in an increase of retinal structures and larger olfactory placodes by interfering with Hh as well as Wnt-8 and eFgf/Fgf-8 signaling pathways (Cornesse et al., 2005). Thus, we hypothesize that Hip regulates the Shh signaling during intestinal metamorphosis.

To investigate this possibility, we have analyzed the expression of Hip in the *X. laevis* intestine during both natural and TH-induced metamorphosis by real-time RT-PCR and in situ hybridization (ISH). We show here that spatial and temporal correlation of Hip expression with that of Shh and BMP-4 during the intestinal remodeling suggest a role for Hip in the attenuation of Shh signaling by the sequestration of N-terminal fragment of Shh to affect the spatial development and/or proliferation of adult epithelial stem cells during amphibian metamorphosis.

RESULTS

Upregulation of Hip during natural and TH-induced metamorphosis

To determine the temporal regulation of Hip expression during the intestinal remodeling, we first carried out real-time RT-PCR and compared the expression profile of Hip with that of Shh and BMP-4, one of target genes of Shh (Perrimon, 1995; Ingham, 1998), during natural metamorphosis (Fig. 1). As previously reported (Stolow and Shi, 1995), Shh mRNA was expressed at a very low level during premetamorphosis, upregulated at the onset of metamorphic climax (stage 58, about 10 fold), and reached a peak level at stage 62 (about 35 fold), when adult epithelial cells are actively proliferating (Ishizuya-Oka and Ueda, 1996; Fig. 1a). Hip expression was also upregulated during prometamorphosis and reached a maximal level at stage 61 (about 6 fold, Fig. 1c). Thereafter, the expression of both genes decreased toward the end of metamorphosis, and was at low levels 2 months after metamorphosis. Similarly, the expression of BMP-4 was transiently upregulated at the onset of metamorphic climax as previously reported (Ishizuya-Oka et al., 2001a) and reached its peak at stage 62 (about 7 fold, Fig. 1e). Then, the expression level decreased as metamorphosis proceeds. While all three genes were upregulated similarly during intestinal metamorphosis, it is of interest that the peak of Hip expression precedes that of Shh and BMP-4 expression (compare Fig. 1c to 1a or 1e), suggesting that Hip may inhibit Shh function during early metamorphic climax (up to stage 61).

As TH can induce precocious intestinal remodeling, if Hip plays a role in regulating Shh function, the temporal expression profiles should be reproduced during TH-induced metamorphosis. Thus, we analyzed the expression of those genes in the intestine of premetamorphic tadpoles at stage 54 treated with T3 for 1 to 5 days, which is known to induce intestinal remodeling (Shi and Hayes, 1994; Ishizuya-Oka et al., 1997). Shh expression was found to be highly upregulated after 1 day of T3 treatment (Fig. 1b), in agreement with the fact that Shh is a direct TH response gene (Stolow and Shi, 1995), and continued to rise even after 5 days of treatment. In contrast, the expression of BMP-4, which is known as a late TH response gene (Amano and Yoshizato, 1998; Ishizuya-Oka et al., 2001a), was upregulated only after 4 days of T3 treatment (Fig. 1f). On the other hand, Hip expression was slightly upregulated by T3 after 1 day of the treatment and was markedly increased after 2 or 3 days (Fig. 1d). Hip expression reached the highest level around 3 days of T3 treatment, while Shh and BMP-4 expression continued to increase significantly after 3 days. These expression profiles are similar those during natural metamorphosis. These results suggest that Hip is not only a late, probably, indirect TH response gene but also plays a role to delay the induction of BMP4 by Shh because of its earlier upregulation than that of BMP-4.

Distinct spatial localization of Hip, Shh and BMP-4 during intestinal metamorphosis

We next investigated the spatiotemporal expression of Shh and Hip mRNAs in the *X. laevis* intestine during natural metamorphosis by ISH. In agreement with the RT-PCR analysis shown in Fig. 1, the levels of both Shh and Hip mRNAs were low, if any, in the intestine during premetamorphosis (Fig. 2a and b), and then became high at metamorphic climax (Fig. 2c, d, e, k and l). Similar to the expression of Shh protein as previously reported (Ishizuya-Oka et al., 2001b), Shh mRNA was epithelium-specific throughout metamorphosis (Fig. 2c, e, g, k, m and n) and reached its highest level in the adult epithelial primordia (Fig. 2n), which could be conventionally identified by methyl green-pyronin Y staining (Fig. 2o) (Ishizuya-Oka and Ueda, 1996). At the end of metamorphosis, Shh was expressed in the adult epithelial cells at the trough region of the well-developed intestinal folds and in the entire epithelium of developing short intestinal folds (Fig. 2g and m). On the other hand, the hybridization signals for Hip mRNA were localized in the connective tissue at stage 61 (Fig. 2d and l). Then, the signals became weaker at stage 62 (Fig. 2f) and decreased to the background level toward stage

66 (Fig. 2h). Interestingly, the cells expressing Hip mRNA in the connective tissue were localized adjacent but not right beneath the primordia of adult epithelium which had the highest levels of Shh expression. In addition, the Hip expressing cells were located close to the muscle layer instead of the epithelium (compare Fig. 2k and l).

Similarly, when premetamorphic tadpoles at stage 54 were treated with 10 nM T3 for 3 days and the expression of Shh and Hip mRNAs was analyzed by ISH (Fig. 2p and q), the cells expressing Hip (Fig. 2q) were again localized in the connective tissue adjacent but not right beneath the epithelial cells expressing Shh (Fig. 2p) and were close to the muscles. The overall patterns of Shh and Hip expression induced by TH were essentially identical to those during natural metamorphosis (Fig. 2p, q and data not shown).

The ISH results above suggest that Hip might restrict Shh signaling spatially during the intestinal remodeling. To investigate this possibility, we compared the localization of Hip and BMP-4 mRNAs in the intestine by ISH on serial sections. Both Hip and BMP-4 are expressed in the connective tissue. Interestingly, at stage 61 (Fig 3a to d), the hybridization signals for BMP-4 mRNA were hardly detectable where Hip was strongly expressed (compare Fig. 3c and d). Conversely, at stage 62, when Shh was highly expressed throughout the proliferating adult epithelium (Fig. 2e), BMP-4 was strongly expressed throughout the connective tissue (Fig. 3f) while the expression of Hip was low (Fig. 3e). Thus, Hip may attenuate Shh signaling to spatially restrict the induction of Shh-target gene BMP-4 in the connective tissue in a stage-dependent manner.

Hip binds Shh in vivo

Hip has been shown to bind to Shh in vitro. Our findings above suggest that Hip binds to Shh in vivo to restrict spatial reach of Shh signal. Shh is known to be processed into N- and C-terminal fragments (namely N-Shh and C-Shh, respectively), which can be easily distinguished by their molecular weights (Bumcrot et al., 1995; Ishizuya-Oka et al., 2001b). N-Shh is responsible for Shh signaling and thus is expected to bind to Hip in vivo if Hip plays a role in attenuating Shh signaling. To investigate this possible interaction in vivo, we microinjected into fertilized eggs with mRNAs encoding a FLAG-tagged Hip without the transmembrane domain (to facilitate immunoprecipitation) and HA-tagged Shh (to determine which processed fragment of Shh binds to Hip, HA tag was inserted into two regions of Shh, yielding N-Shh-HA and C-Shh-HA after auto-processing) (Fig 4a). Embryo extracts were prepared for immunoprecipitation (IP) with anti-FLAG M2 agarose beads followed by Western blot analysis. Western blot analysis with the anti-FLAG antibody on the pre-IP extract confirmed the expression of FLAG-tagged Hip in embryos injected with the Hip mRNA with or without Shh mRNA (Fig. 4b, top left, arrow). This exogenous Hip was successfully immunoprecipitated by anti-FLAG M2 agarose beads and detected as a doublet (Fig. 4b, top right) as previously reported (Coulombe et al., 2004). Western blot analysis with the anti-HA antibody showed that overexpression of HA-tagged Shh produced two expected major bands corresponding to N-Shh (blank arrowhead) and C-Shh (black arrowhead) based on their molecular weights (Fig. 4b, bottom left). Although 2 minor bands were also detected, their origins were unknown and they might be Shh fragments that were improperly processed or impaired in the posttranslational modifications due to tag insertion (Fig 4b, bottom left). Immunoprecipitation of the overexpressed Hip with anti-FLAG M2 agarose from the extracts of embryos injected with mRNAs for both FLAG-tagged Hip and HA-tagged Shh led to the coprecipitation of N-Shh, but not of C-Shh (Fig. 4b, bottom right). Neither N-Shh nor C-Shh was co-immunoprecipitated when FLAG-tagged Hip and HA-tagged Shh were expressed individually (Fig. 4b, bottom right). These results demonstrate that Hip binds to N-Shh in vivo, in agreement with in vitro binding of Hip to N-Shh (Chuang and McMahon, 1999) and support

the model that Hip inhibits Shh signaling pathway by sequestering the signaling unit (N-Shh) of Shh in vivo.

DISCUSSION

There is a growing body of evidence that Shh plays a critical role in the gastrointestinal development. Shh is expressed in almost all of the gut endodermal cells during the formation of the gut tube. It has been shown by using mice with a targeted deletion that Shh is essential for foregut development (Litingtung et al., 1998). Mice lacking Shh exhibit morphological and histological abnormalities in the intestine in late gestation, although another member of Hh family, Indian hedgehog, is also involved in the organogenesis (Ramalho-Santos et al., 2000). In the *X. laevis* intestine, it has been shown that the constitutive Hh signaling by a mutated Hh receptor Smoothed results in failure of midgut epithelial cytodifferentiation, lengthening, and coiling (Zhang et al., 2001), indicating that downregulation of Hh signaling is required for the intestinal organogenesis during embryogenesis. In addition, Shh was identified as one of the early, direct intestinal target genes of TH, the causative agent of metamorphosis, implicating a role of Shh in the development of the adult intestine (Stolow and Shi, 1995). Indeed, our earlier work has provided strong evidence to support such a function for Shh (Ishizuya-Oka et al., 2006).

The organogenesis of the adult frog intestine during metamorphosis involves first the degeneration of the larval epithelium through apoptosis and then the de novo development of the adult epithelium in a process that requires extensive interactions among different tissue types, particularly between the epithelium and connective tissue. It is unclear how Shh participates in these processes and if/how Shh signal is regulated posttranslationally to facilitate spatiotemporal development of the adult epithelium. Our study here has provided evidence that Hip is involved in the spatiotemporal regulation of Shh signaling pathway in the development of the frog intestine from the larval form in *X. laevis*.

Our expression analyses have shown that Hip is transiently upregulated in the intestine during metamorphosis, suggesting that Hip is a TH response gene. Indeed, the expression of Hip can be induced by TH administration to premetamorphic tadpoles. This induction appears to be a slow, indirect effect of TH, in contrast to the direct induction of Shh by TH, but similar to the induction of BMP-4, a target gene of Shh in the intestine (Ishizuya-Oka et al., 2006). It has been demonstrated that Hip is a transcriptional target of Shh signaling during early development in mouse (Chuang and McMahon, 1999) and *X. laevis* (Cornesse et al., 2005). In *X. laevis* intestine, the initial induction of Shh by TH takes place prior to the upregulation of Hip by TH as Shh is a direct target gene of TH (Fig. 1). Thus, it is possible that Shh may play a role in the upregulation of Hip during early metamorphic climax. On the other hand, it is interesting to note that the peak of Hip expression precedes that of Shh during intestinal metamorphosis in *X. laevis* and Hip is downregulated at stage 62 when Shh expression is the highest. Thus, other factors yet to be determined are likely involved in the regulation of Hip expression in the remodeling intestine.

More importantly, the peak level of Hip expression precedes that of both Shh and BMP-4, a Shh target gene. This suggests that during early stage of intestinal remodeling, while Shh expression is induced as a direct response to TH, its function is attenuated/regulated by Hip, which leads to the delay/inhibition of the induction of BMP-4 expression by Shh. After stage 61, with the downregulation of Hip and increase in Shh, both Shh and BMP-4 expression peaks at stage 62. Similarly, during TH treatment, while Shh expression is induced immediately, Hip expression reaches highest levels before that of BMP-4, consistently with a role in attenuating/inhibiting Shh signaling. This inhibitory function of Hip is further supported by the spatial localization of Hip, since BMP-4 expression is hardly detectable where high levels of Hip

expression are present. The fibroblasts expressing Hip are close to the muscle layers of the intestine, suggesting that at this stage Hip serves to limit spatially the extent of Shh signaling from the proliferating adult epithelial islets. Although we do not yet have direct evidence to show the function of Hip to suppress BMP-4 expression, studies in other animals such as mice have demonstrated such a role for Hip (Chuang and McMahon, 1999; Chuang et al., 2003; Madison et al., 2005). In addition, we have demonstrated for the first time that in vivo Hip binds to N-Shh, which is responsible for Shh signaling, but not to C-Shh, which is important for the auto-processing of Shh to generate N-Shh. Aside from Shh, Hip may also affect other members of *X. laevis* hedgehog family similar to that reported for mice (Chuang and McMahon, 1999). Currently, it is unknown whether other hedgehogs are expressed or regulated by TH in *X. laevis*. Clearly, it will be important to study the molecular mechanisms of the inhibitory action of Hip in the future.

In summary, we have demonstrated a tight spatiotemporal correlation of Hip expression with Shh and a Shh target, BMP-4, during intestinal remodeling. This together with the ability of Hip to bind to the signaling unit of Shh, the N-Shh, suggests the following working model. During metamorphosis of intestine, the adult epithelial stem cells appear as islets from yet unknown origin and they express high levels of Shh. Shh then induces the underlying connective tissue to express BMP-4 (Ishizuya-Oka et al., 2001a), which in turn signals back to the proliferating epithelial cells. At this stage, Hip is also upregulated in the connective tissue cells adjacent to the proliferating adult epithelial cells. This upregulation of Hip expression thus serves a role to limit the extent of Shh signaling to the proliferating adult epithelium and immediate underlying fibroblasts. Once the proliferating adult epithelial cells replaces all the larval epithelial cells, high levels of Shh expression throughout the epithelium coupled with the down-regulation of Hip leads to strong expression of BMP-4 in throughout the connective tissue. The BMP-4 then promotes the differentiation of the adult epithelial cells to form the adult epithelium (Fig. 5) as we previously demonstrated (Ishizuya-Oka et al., 2006). Such a model implies an important role of Shh in stem cell development and proliferation while a role of Hip to spatially regulate this process. To test the validity of this model, future studies should be directed at clarifying functions of Hip in the adult epithelial development and interactions of Hip with other members of Shh signaling by using methodologies for functional analyses such as transgenesis (Kroll and Amaya, 1996; Pan et al., 2006; Sinzelle et al., 2006) organ culture (Ishizuya-Oka et al., 2000), and somatic gene transfer (de Luze et al., 1993; Nakajima and Yaoita, 2003).

EXPERIMENTAL PROCEDURES

Animal and treatment

Xenopus laevis tadpoles and froglets were obtained and maintained as previously described (Hasebe et al., 2007b). The developmental stages of tadpoles were according to Nieuwkoop and Faber (1994). Tadpoles at stage 54 were treated with 10 nM TH (3,5,3'-triiodothyronine or T3) for 1 to 5 days. At least 3 tadpoles were analyzed for each stage or day of TH-treatment. Animal rearing and treatment were done according to the guidelines set by Nippon Medical School animal use and care committee.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Total RNA from the small intestine of wild type and T3-treated animals was extracted by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) followed by DNase treatment with DNA-free (Ambion, Austin, TX, USA) to remove any DNA contamination. The integrity of RNA was checked based on 18S and 28S ribosomal RNAs by electrophoresis. Total RNA was mixed with RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan), and then quantitative real-time RT-PCR was performed by using ABI PRISM 7700 Sequence Detector

(Applied Biosystems, Foster City, CA, USA) according to the manufacture's instructions. For Shh detection, primers 5'- AGCGACTTCCTCATGTTTCATC -3' and 5'- GCCTTCAAGGTCATGGTCTTG -3' were used. For Hip detection, primers 5'- GGACACATACTGGGGTTTGG -3' and 5'- ATTAGCGGACGTTTGCATTC -3' were used. For BMP-4 detection, primers 5'- GGAGAATCTACCAAGCACAG -3' and 5'- GCAGCTATGGGTTTCATAAC -3' were used. As a loading control, ribosomal protein L8 (rpL8) (Shi and Liang, 1994) was amplified with primers 5'- CCACGTCAAACACAGAAAGG -3' and 5'- TGCCACAGTACACAAACTGTC -3'. The level of specific mRNA was quantified at the point where the thermal cycler detected the upstroke of the exponential phase of PCR accumulation, and normalized to the level of rpL8 mRNA for each sample. Samples were analyzed in triplicate for 3 times. The specific amplification was confirmed by the dissociation curve analysis and gel electrophoresis.

In situ hybridization (ISH)

A cDNA library was constructed as previously described (Hasebe et al., 2006). A cDNA encoding *X. laevis* Shh coding region was obtained by PCR using *Pfu*Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA) with primers 5'- AATATTACCGGTGCCGCCACCATGCTGGTTGCGACTCAATC -3' and 5'- AATATTGAATTTCGCTAGCTCAACTGGATTTCGTTGCCATGCC -3'. The PCR product was digested with *Age* I and *Nhe* I, inserted into T7Ts expression vector (Hasebe et al., 2007a) and sequenced (T7Ts_Shhs). A partial cDNA encoding Shh was obtained by PCR with primers 5'- AATATTTCTAGATACTTTTGTGGCCCAGACC -3' and 5'- TATATTAAGCTTGGGTGCAGGGAGTTACTGTC -3' using T7Ts_Shhs as a template. The PCR product was digested with *Xba* I and *Hind* III, inserted into pBSII-KS⁻ plasmid vector and sequenced (pBSII-KS⁻_Shh-probe).

X. laevis Hip cDNA (accession no.: BC046952) cloned into pCMV-SPORT6 (IMAGE: 5571858) was purchased from Open Biosystems (Huntsville, AL, USA). The coding region of this cDNA is not totally identical to that reported by Cornesse et al (2005), since the nucleotides encoding 19 amino acids at the C-terminal end corresponding to the transmembrane domain are missing. A *Bgl* II - *Hind* III fragment of Hip cDNA was inserted into pBSII-KS⁺ predigested with *Bam* HI and *Hind* III (pBSII-KS⁺_Hip-probe).

A partial cDNA encoding *X. laevis* BMP-4 was amplified with primers 5'- AATATTGAATTCAAGTCGCGGCCGACATTCAG -3' and 5'- TATATTAAGCTTTACCCTCGTGTCCAGCAGCC -3', digested with *Eco* RI and *Hind* III, inserted into pBSII-KS⁻ plasmid vector and sequenced (pBSII-KS⁻_BMP4-probe).

The plasmids were linearized to synthesize sense and antisense probes either with T3 or T7 RNA polymerase by using digoxigenin (DIG) RNA Labeling Mix (Roche Applied Science, Indianapolis, IN, USA). Intestinal fragments were isolated from the anterior part of the small intestine just after the bile duct junction in tadpoles at indicated stages as well as T3-treated tadpoles and fixed in MEMFA followed by cryosectioning. Tissue sections were prepared at 7 µm and subjected to ISH. ISH was performed by using sense or antisense probes of Shh, BMP-4 and Hip as previously described (Hasebe et al., 2006). Photographs were taken by using a digital CCD color camera (DP70, Olympus, Tokyo, Japan) attached to an optical microscope (BX51, Olympus).

Plasmid DNA constructs for microinjection

Hip—The transmembrane domain (TMD) was totally removed (Chuang and McMahon, 1999; Madison et al., 2005) and 3xFLAG tag was placed to the C-terminus of HipΔTMD by PCR. Briefly, using pCMV-SPORT6_Hip as the template, HipΔTMD-3xFLAG was amplified

with Hip-forward primer (5'-AATATTACCGGTGCCGCCACCATGAACAAGTTCCTGTTGGTGCAG -3') and HipΔTMD-3xFLAG-reverse primer (5'-ATTGCTAGCTCACTTGTCATCGTCATCCTTGCTAGTCGATGTCATGATCTTTATAA TCACCGTCATGGTCTTTGTAGTCCCTGGCCACTCTCCGGACG -3'). The resulting cDNA was digested with *Age* I and *Nhe* I, inserted into T7Ts expression vector and sequenced (T7Ts_HipΔTMD-3xFLAG).

Shh—HA tag was placed to the N-terminal region between Cys²⁵ and Gly²⁶ and the C-terminal end by PCR. At first, HA tag was inserted between Cys²⁵ and Gly²⁶ by two-step PCR to generate N-Shh-HA. Using T7Ts_Shhs as the template, the upstream fragment of N-Shh-HA was amplified with Shh-forward primer (5'-AATATTACCGGTGCCGCCACCATGCTGGTTGCGACTCAATC -3') and N-Shh-HA-reverse primer (5'-AGCGTAATCTGGAACATCGTATGGGTAACATGCCAGCCCAGGGGGGGTGC -3'). The downstream fragment of N-Shh-HA was amplified with N-Shh-HA-forward primer (5'-TACCCATACGATGTTCCAGATTACGCTGGACCTGGCCGAGGCATTGGCAAGAGG -3') and Shh-reverse primer (5'-AATATTGAATTCGCTAGCTCAACTGGATTTCGTTGCCATGCC -3'). Both upstream and downstream fragments were mixed and amplified without any primer for 5 cycles followed by another 20 cycles after addition of Shh-forward and Shh-reverse primers to obtain the full length N-Shh-HA. The resulting cDNA was digested with *Age* I and *Eco* RI, inserted into T7Ts and sequenced (T7Ts_N-Shh-HA). Next, HA tag was fused to the C-terminus to generate C-Shh-HA. Using T7Ts_Shhs as the template, C-Shh-HA was amplified with Shh-forward primer and C-Shh-HA-reverse primer (5'-TGAATTCTCAAGCGTAATCTGGAACATCGTATGGGTAACACTGGATTTCGTTGCCATGCCAGTG -3'). The resulting cDNA was digested with *Age* I and *Eco* RI, inserted into T7Ts and sequenced (T7Ts_C-Shh-HA). T7Ts_N-Shh-HA was digested with *Age* I and *Nco* I to obtain a 1 kb fragment. This fragment was inserted into T7Ts_C-Shh-HA predigested with *Age* I and *Nco* I. Finally, the construct for two HA tags within one molecule of Shh was generated (T7Ts_Shhs-2HA).

Microinjection

The capped mRNA encoding HipΔTMD-3xFLAG and Shh-2HA were synthesized from the plasmid DNA constructs linearized with *Xma* I by using mMESSAGE mMACHINE (Ambion) according to the manufacture's instructions. Fertilized *X. laevis* eggs were prepared as previously described (Hasebe et al., 2007a). At stage 2 (Nieuwkoop and Faber, 1994), embryos were injected with 2 ng of HipΔTMD-3xFLAG mRNA and/or 1 ng of Shh-2HA mRNA into one blastomere (Hasebe et al., 2007a).

Immunoprecipitation and Western blot analysis

Embryos injected with the indicated mRNAs were subjected to protein extraction 1 day after injection. Briefly, 30 embryos were lysed by pipetting in 300 μl of IP buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaCl, 0.1% Igepal CA-630 and protease inhibitor cocktail (Roche Applied Science)). After centrifugation at 15000 × g for 15 min at 4°C, the supernatant was collected and subjected to immunoprecipitation (IP) assay, or mixed with 1/5 volume of 6x SDS-loading buffer containing 6% 2-mercapthoethanol (2ME) and subjected to Western blotting (1/6 embryo equivalent/lane).

150 μl of the supernatant obtained after centrifugation above was mixed with 250 μl of IP buffer and 15 μl slurry of anti-FLAG-M2 agarose beads (Sigma-Aldrich, St. Louis, MO, USA).

After the incubation overnight at 4°C, the beads were washed 3 times with IP buffer. Immunoprecipitates were eluted with 100 µg/ml 3x FLAG peptide (Sigma-Aldrich) in Tris-buffered saline (TBS), then mixed with 1/5 volume of 6x SDS-loading buffer-6% 2ME and subjected to Western blotting (4 embryo equivalent/lane).

The protein samples were electrophoresed on a 5-20% polyacrylamide gel (Anatech, Tokyo, Japan) followed by transferring onto PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was immediately washed with TBS containing 0.1% Tween-20 (TBST), blocked with 5% skim milk (Wako, Osaka, Japan) in TBST for 30 min, and incubated overnight at 4°C with the indicated primary antibody diluted in either TBST-0.5% milk or Can Get Signal Immunoreaction enhancer solution (Toyobo). The antibodies used were anti-FLAG M2 mAb (Sigma-Aldrich, 1/1000 dilution with TBST-0.5% milk), and anti-HA mAb (Cell Signaling Technology, Danvers, MA, USA, 1/1000 dilution with Can Get Signal). After washing 3 times with TBST, the membrane was incubated for 1 hour at room temperature with the secondary antibody against mouse IgG conjugated with peroxidase (GE Healthcare, Buckinghamshire, England). After washing 3 times with TBST, peroxidase activity was detected by using ECL Western Blotting Substrates (Pierce Biotechnology, Rockford, IL, USA) with an imaging film (Kodak BioMax XAR Film, Carestream Health, Rochester, NY, USA).

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REFERENCES

- Amano T, Yoshizato K. Isolation of genes involved in intestinal remodeling during anuran metamorphosis. *Wound Repair Regen* 1998;6:302–313. [PubMed: 9824549]
- Bumcrot DA, Takada R, McMahon AP. Proteolytic processing yields two secreted forms of sonic hedgehog. *Mol Cell Biol* 1995;15:2294–2303. [PubMed: 7891723]
- Cheng H, Bjerknes M. Whole population cell kinetics and postnatal development of the mouse intestinal epithelium. *Anat Rec* 1985;211:420–426. [PubMed: 3993991]
- Chuang PT, Kawcak T, McMahon AP. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev* 2003;17:342–347. [PubMed: 12569124]
- Chuang PT, McMahon AP. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* 1999;397:617–621. [PubMed: 10050855]
- Cornesse Y, Pieler T, Hollemann T. Olfactory and lens placode formation is controlled by the hedgehog-interacting protein (Xhip) in *Xenopus*. *Dev Biol* 2005;277:296–315. [PubMed: 15617676]
- Coulombe J, Traiffort E, Loulier K, Faure H, Ruat M. Hedgehog interacting protein in the mature brain: membrane-associated and soluble forms. *Mol Cell Neurosci* 2004;25:323–333. [PubMed: 15019948]
- de Luze A, Sachs L, Demeneix B. Thyroid hormone-dependent transcriptional regulation of exogenous genes transferred into *Xenopus* tadpole muscle in vivo. *Proc Natl Acad Sci U S A* 1993;90:7322–7326. [PubMed: 8346251]
- Dodd, MHI.; Dodd, JM. The biology of metamorphosis. In: Lofts, B., editor. *Physiology of Amphibia*. Academic Press; New York: 1976. p. 467-599.
- Hasebe T, Hartman R, Fu L, Amano T, Shi Y-B. Evidence for a cooperative role of gelatinase A and membrane type-1 matrix metalloproteinase during *Xenopus laevis* development. *Mech Dev* 2007a; 124:11–22. [PubMed: 17055228]

- Hasebe T, Hartman R, Matsuda H, Shi Y-B. Spatial and temporal expression profiles suggest the involvement of gelatinase A and membrane type 1 matrix metalloproteinase in amphibian metamorphosis. *Cell Tissue Res* 2006;324:105–116. [PubMed: 16418836]
- Hasebe T, Kajita M, Fujimoto K, Yaoita Y, Ishizuya-Oka A. Expression profiles of the duplicated matrix metalloproteinase-9 genes suggest their different roles in apoptosis of larval intestinal epithelial cells during *Xenopus laevis* metamorphosis. *Dev Dyn* 2007b;236:2338–2345. [PubMed: 17654707]
- Hourdry J, Dauca M. Cytological and cytochemical changes in the intestinal epithelium during anuran metamorphosis. *Int Rev Cytol Suppl* 1977;5:337–385.
- Ingham PW. Transducing Hedgehog: the story so far. *Embo J* 1998;17:3505–3511. [PubMed: 9649421]
- Ishizuya-Oka A, Hasebe T, Shimizu K, Suzuki K, Ueda S. Shh/BMP-4 signaling pathway is essential for intestinal epithelial development during *Xenopus* larval-to-adult remodeling. *Dev Dyn* 2006;235:3240–3249. [PubMed: 17016847]
- Ishizuya-Oka A, Li Q, Amano T, Damjanovski S, Ueda S, Shi Y-B. Requirement for matrix metalloproteinase stromelysin-3 in cell migration and apoptosis during tissue remodeling in *Xenopus laevis*. *J Cell Biol* 2000;150:1177–1188. [PubMed: 10974004]
- Ishizuya-Oka A, Shimozaawa A. Induction of metamorphosis by thyroid hormone in anuran small intestine cultured organotypically in vitro. *In Vitro Cell Dev Biol* 1991;27A:853–857. [PubMed: 1748625]
- Ishizuya-Oka A, Stelow MA, Ueda S, Shi Y-B. Temporal and spatial expression of an intestinal Na⁺/PO4³⁻ cotransporter correlates with epithelial transformation during thyroid hormone-dependent frog metamorphosis. *Dev Genet* 1997;20:53–66. [PubMed: 9094212]
- Ishizuya-Oka A, Ueda S. Apoptosis and cell proliferation in the *Xenopus* small intestine during metamorphosis. *Cell Tissue Res* 1996;286:467–476. [PubMed: 8929349]
- Ishizuya-Oka A, Ueda S, Amano T, Shimizu K, Suzuki K, Ueno N, Yoshizato K. Thyroid-hormone-dependent and fibroblast-specific expression of BMP-4 correlates with adult epithelial development during amphibian intestinal remodeling. *Cell Tissue Res* 2001a;303:187–195. [PubMed: 11291765]
- Ishizuya-Oka A, Ueda S, Inokuchi T, Amano T, Damjanovski S, Stelow M, Shi Y-B. Thyroid hormone-induced expression of sonic hedgehog correlates with adult epithelial development during remodeling of the *Xenopus* stomach and intestine. *Differentiation* 2001b;69:27–37. [PubMed: 11776392]
- Kikuyama S, Kawamura K, Tanaka S, Yamamoto K. Aspects of amphibian metamorphosis: hormonal control. *Int Rev Cytol* 1993;145:105–148. [PubMed: 8500980]
- Kordylewski L. Light and electron microscopic observations of the development of intestinal musculature in *Xenopus*. *Z Mikrosk Anat Forsch* 1983;97:719–734. [PubMed: 6649786]
- Kroll KL, Amaya E. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 1996;122:3173–3183. [PubMed: 8898230]
- Litingtung Y, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. *Nat Genet* 1998;20:58–61. [PubMed: 9731532]
- Madara, JL.; Trier, JS. Functional morphology of the mucosa of the small intestine. In: LR, J., editor. *Physiology of the Gastrointestinal Tract*. Raven Press Ltd.; New York: 1994. p. 1577–1622.
- Madison BB, Braunstein K, Kuizon E, Portman K, Qiao XT, Gumucio DL. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* 2005;132:279–289. [PubMed: 15590741]
- Marshall JA, Dixon KE. Cell specialization in the epithelium of the small intestine of feeding *Xenopus laevis* tadpoles. *J Anat* 1978;126:133–144. [PubMed: 649494]
- McAvoy JW, Dixon KE. Cell proliferation and renewal in the small intestinal epithelium of metamorphosing and adult *Xenopus laevis*. *J Exp Zool* 1977;202:129–138.
- Nakajima K, Yaoita Y. Dual mechanisms governing muscle cell death in tadpole tail during amphibian metamorphosis. *Dev Dyn* 2003;227:246–255. [PubMed: 12761852]
- Nieuwkoop, PD.; Faber, J. Normal table of *Xenopus laevis* (Daudin). Garland Publishing Inc.; New York: 1994.
- Pan FC, Chen Y, Loeber J, Henningfeld K, Pieler T. I-SceI meganuclease-mediated transgenesis in *Xenopus*. *Dev Dyn* 2006;235:247–252. [PubMed: 16258935]
- Perrimon N. Hedgehog and beyond. *Cell* 1995;80:517–520. [PubMed: 7867057]
- Ramallo-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 2000;127:2763–2772. [PubMed: 10821773]

- Roberts D, Johnson R, Burke A, Nelson C, Morgan B, Tabin C. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* 1995;121:3163–3174. [PubMed: 7588051]
- Shi, Y-B. *Amphibian Metamorphosis: From morphology to molecular biology*. John Wiley & Sons, Inc.; New York: 1999.
- Shi Y-B, Brown DD. The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. *J Biol Chem* 1993;268:20312–20317. [PubMed: 7690754]
- Shi Y-B, Hayes WP. Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev Biol* 1994;161:48–58. [PubMed: 8293885]
- Shi Y-B, Ishizuya-Oka A. Thyroid hormone regulation of apoptotic tissue remodeling: implications from molecular analysis of amphibian metamorphosis. *Prog Nucleic Acid Res Mol Biol* 2001;65:53–100. [PubMed: 11008485]
- Shi Y-B, Liang VC. Cloning and characterization of the ribosomal protein L8 gene from *Xenopus laevis*. *Biochim Biophys Acta* 1994;1217:227–228. [PubMed: 8110841]
- Sinzelle L, Vallin J, Coen L, Chesneau A, Du Pasquier D, Pollet N, Demeneix B, Mazabraud A. Generation of transgenic *Xenopus laevis* using the Sleeping Beauty transposon system. *Transgenic Res* 2006;15:751–760. [PubMed: 16957880]
- Stolow MA, Shi Y-B. *Xenopus* sonic hedgehog as a potential morphogen during embryogenesis and thyroid hormone-dependent metamorphosis. *Nucleic Acids Res* 1995;23:2555–2562. [PubMed: 7630736]
- Sukegawa A, Narita T, Kameda T, Saitoh K, Nohno T, Iba H, Yasugi S, Fukuda K. The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* 2000;127:1971–1980. [PubMed: 10751185]
- Tata JR. Gene expression during metamorphosis: an ideal model for post-embryonic development. *Bioessays* 1993;15:239–248. [PubMed: 8517853]
- Treier M, O'Connell S, Gleiberman A, Price J, Szeto DP, Burgess R, Chuang PT, McMahon AP, Rosenfeld MG. Hedgehog signaling is required for pituitary gland development. *Development* 2001;128:377–386. [PubMed: 11152636]
- Zhang J, Rosenthal A, de Sauvage FJ, Shivdasani RA. Downregulation of Hedgehog signaling is required for organogenesis of the small intestine in *Xenopus*. *Dev Biol* 2001;229:188–202. [PubMed: 11133163]

Figure 1.

Shh and Hip are upregulated prior to BMP-4 in *X. laevis* intestine during natural and TH-induced metamorphosis. Real-time RT-PCR was performed using total RNA prepared from the intestine of animals at indicated developmental stages (a, c and e) or stage 54 tadpoles after T3 treatment (b, d and f). Levels of Shh (a, b), Hip (c, d) and BMP-4 mRNAs (e, f) are shown relative to levels of ribosomal protein L8 (rpL8) mRNA, with the values at stage 54 or 0 day treatment set to 1. 2 mo: 2 months after metamorphosis.

Figure 2.

Spatiotemporal localization of Shh and Hip mRNAs in the *X. laevis* intestine during natural and TH-induced metamorphosis. Cross sections of the intestine at premetamorphic stage 54 (a, b), at metamorphic climax stages 61 (c, d, j, k, l, n and o) and 62 (e, f and i) and at the end of metamorphosis (stage 66) (g, h and m), and the intestine isolated from stage 54 tadpoles treated with 10 nM T3 for 3 days (p and q) were hybridized with antisense Shh (a, c, e, g, k, m, n and p) and Hip (b, d, f, h, l and q) probes. Some of the sections were stained by methyl green-pyronin Y (MGPY) to identify the adult primordia (dashed arrow in the panel o). To compare the localization of Shh with that of Hip and with the adult primordia, serial sections were used (panels c with d, e with f, k with l, n with o, and p with q). Dark blue deposits indicate the sites of probe binding. Light or dark brown pigments in panels e, f, g and h are melanin. Arrowheads indicate the cells expressing Shh, while solid arrows indicate those expressing Hip. k, l and m: Higher magnification images of a boxed area in panels c, d and g, respectively. Shh and Hip mRNAs are hardly detectable at stages 54 (a, b). Signals for Shh become stronger at stage 61 (c), reach the maximal level at stage 62 (e) and then decrease at stage 66 (g). Shh mRNA is expressed only in the epithelium (Ep) but not in other tissues throughout metamorphosis (k and m). Particularly, at the climax, the cells expressing Shh correlate well with the adult primordia stained strongly red with MGPY (n and o). At stage 66 (m), signals

for Shh are detectable in the trough region of the mature intestinal fold (right side) and the entire epithelium of the developing intestinal fold (left side). On the other hand, signals for Hip are detected at stage 61 (d) only in the connective tissue (CT) close to the muscle layer (M) but beneath Ep (l), which expresses Shh (k). At stage 62, signals for Hip decrease (f), and then become hardly detectable at stage 66 (h). Overall patterns of Shh (p) and Hip expression (q) induced by exogenous TH are essentially identical to those during natural metamorphosis. Sense Shh (i) and Hip probes (j) do not give any signal. L: lumen. Ty: typhlosole. Scale bars are 100 μm (a-j) and 20 μm (k-q).

Figure 3.

Comparison of Hip and BMP-4 localization. Cross sections of the intestine at stage 61 (a-d) and stage 62 (e, f) were hybridized with antisense Hip (a, c and e) and BMP-4 (b, d and f) probes. To compare the localization of Hip with that of BMP-4, serial sections were used. Dark blue deposits indicate the sites of probe binding. Light or dark brown pigments are melanin. c and d: Higher magnification of a boxed area in panels a and b, respectively. The signals for BMP-4 mRNA (d, dashed arrow) are hardly detectable where Hip is strongly expressed (c, solid arrow). BMP-4 is highly expressed at stage 62 (f) when the expression of Hip decreases (e) and that of Shh peaks (Fig. 2c). Ep: epithelium. CT: connective tissue. M: muscle layer. Scale bars are 100 μ m (a, b, e and f) and 20 μ m (c and d).

Figure 4.

In vivo interaction of Hip with Shh. (a) Diagrams of Shh and Hip used for the study. For detection, HA-tag was placed to 2 regions of Shh and 3xFLAG-tag was fused at C-terminus of Hip in which the transmembrane domain was deleted (Δ TMD). sig: signal peptide. (b) The mRNAs encoding FLAG-tagged Hip and HA-tagged Shh were injected either alone or together into one blastomere of stage 2 embryos. Proteins were extracted 1 day after injection and subjected to Western blotting (WB) using anti-FLAG (top) or anti-HA (bottom) antibodies before (Input, left) or after immunoprecipitation with anti-FLAG M2 antibody (FLAG-IP, right). The arrows indicate the position of FLAG-tagged Hip (detected as a doublet after FLAG-IP). Shh is auto-processed into two major fragments, N-Shh (blank arrowhead) and C-Shh (black arrowhead). These major fragments were determined by their molecular weights, while the origin of other two minor fragments is not clear. However, they might be also Shh fragments produced from inaccurate processing or posttranslational modification due to tag insertion. N-Shh, but not C-Shh was coprecipitated with Hip. The non-specific bands detected in all samples are labeled by stars.

Figure 5.

A working model showing the possible mechanisms of intestinal remodeling of *X. laevis* via Shh signaling pathway. During prometamorphosis, when Shh is first expressed in the larval epithelial cells (not detectable by ISH due to sensitivity but could be detected by immunohistochemistry, see Ishizuya-Oka et al., 2001b), the connective tissue is largely a very thin layer. At this stage, Hip expression, detectable by PCR but not by ISH, are right beneath the larval epithelial cells. During metamorphic climax, high levels of Shh in the adult epithelial primordia induce BMP-4 expression in the underlying fibroblasts. BMP-4 then signals back to the proliferating adult primordial cells to promote their differentiation. Hip is also upregulated in the fibroblasts adjacent to the adult primordia but further toward the muscle layers to limit the extent of Shh signaling (e.g. suppressing BMP-4 expression).