—Review—

Thyroid Hormone-Activated Signaling Pathways are Essential for Development of Intestinal Stem Cells

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Intestinal homeostasis is maintained by strict regulation of stem cell function. In mammals, several signaling pathways, including the formation of stem cell niches, are involved in stem cell regulation. However, little is known of the molecular mechanisms involved in postembryonic maturation of the vertebrate intestine, that is, the acquisition of cell renewal systems, including stem cell development and niche formation. Using thyroid hormone (TH)-dependent intestinal remodeling during amphibian metamorphosis as a model to study these mechanisms, we found that several signaling pathways, including the SHH/BMP4, WNT, Notch, and Hippo pathways, are regulated by TH and involved in stem cell regulation. In this review, we highlight findings regarding the role of these signaling pathways and discuss potential future avenues of study. (J Nippon Med Sch 2023; 90: 246–252)

Key words: intestine, stem cells, thyroid hormone, signaling pathway, amphibian metamorphosis

Introduction

The gastrointestinal tract is an important organ that is responsible for food digestion and nutrient absorption throughout an animal's life, and its homeostasis is thought to be maintained by elaborate regulatory mechanisms. In the small intestine (hereafter "intestine") of adult mammals, epithelial cells are derived from stem cells (SCs) that express leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)[1], a typical intestinal SC (ISC) marker in the crypts of Lieberkühn, and are renewed every 3-5 days[2]. Tissue SCs are maintained in a local microenvironment, termed a niche, that regulates their proliferation and differentiation[3,4]. Various niche factors have been identified in the adult mammalian intestine, and their roles in intestinal homeostasis are being increasingly investigated[5,6]. However, little is known of the molecular mechanisms underlying maturation/remodeling of the intestine, that is, the acquisition of a cell renewal system that takes place during the perinatal period in mammals, including niche factors and their functions, as well as signaling pathways involved in niche formation. Elucidation of these mechanisms is of interest from the perspective of SC and developmental biology, and is a subject of considerable importance in regenerative medicine and cancer therapy[7]. Although ISCs derived from fetal epithelium appear when plasma thyroid hormone (TH) levels reach a peak during intestinal maturation in mammals[8,9], it is difficult to manipulate uterus-enclosed mammalian embryos that are affected by maternal factors. To address this limitation, larval-to-adult intestinal remodeling during amphibian metamorphosis controlled by TH serves as a valuable and appropriate model[10] because it shares characteristics with mammalian intestinal maturation[11], as described below. It is also noteworthy that *in vivo* experiments are easy to conduct using free-living amphibian larvae (tadpoles) and that intestinal remodeling can be experimentally induced by administering exogenous TH both *in vivo* and *in vitro*[12].

We used as a model animal the African clawed frog (*Xenopus laevis*), which undergoes metamorphosis from a herbivorous larva to a carnivorous adult[13]. To adapt to this dietary change, the gastrointestinal tract is extensively remodeled during metamorphosis in response to TH[14,15], analogous to intestinal maturation in mammals during a dietary change from milk to solid food[16]. The in-

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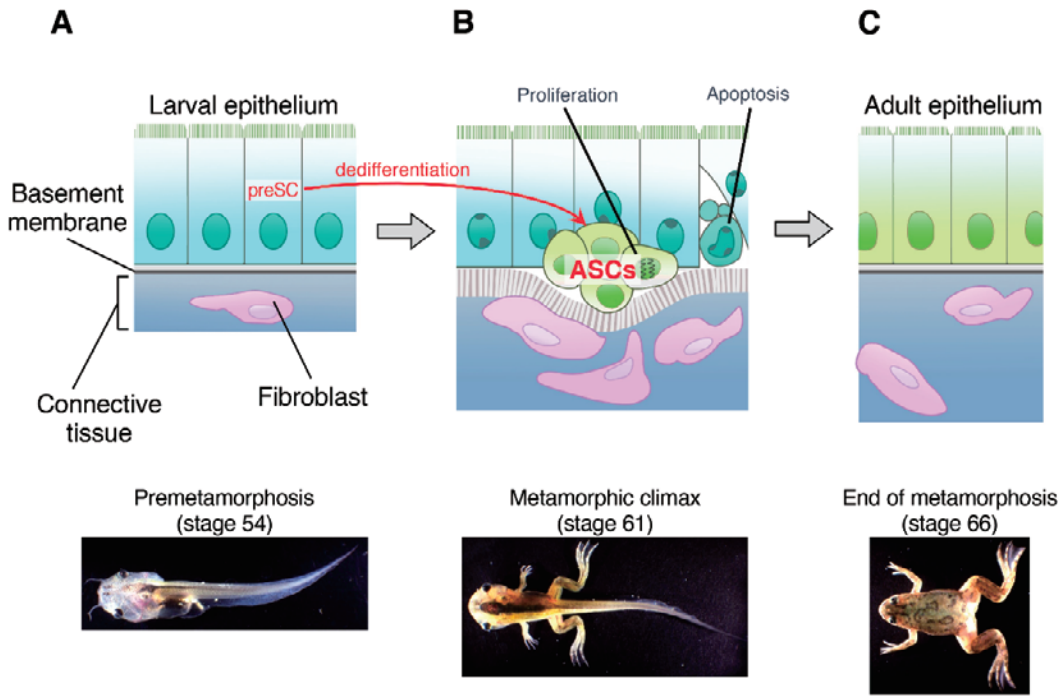

Fig. 1 Schematic illustration showing intestinal remodeling during metamorphosis of *X. laevis*. A. At premetamorphosis (stage 54), the larval intestine consists of larval epithelium surrounded by a thin basement membrane and connective tissue. The morphology of the intestine remains essentially unchanged until the end of prometamorphosis (stage 57), except for the size and length of the intestine. B. At early metamorphic climax (stage 60), some larval epithelial cells (precursors of stem cells: preSCs) dedifferentiate into adult stem cells (ASCs) that actively proliferate during metamorphic climax (stage 61-62). The remaining larval epithelial cells are removed by apoptosis. The photograph shows a tadpole at stage 61. C. Cells derived from ASCs differentiate to form adult epithelium, analogous to the mammalian intestine, by the end of metamorphosis (stage 66). These changes are controlled by TH.

testine of pre- and prometamorphic tadpoles (stage 54-57[17]), in which plasma TH levels are low, has a simple tubular structure. A single layer of larval epithelial cells lines the lumen of the intestine, which is surrounded by a thin basement membrane and thin connective tissue (**Fig. 1A**). When plasma TH levels begin to increase[18], the intestine exhibits metamorphic changes. At stage 59, larval epithelial cells begin to undergo apoptosis, and most of these cells are removed during the metamorphic climax[19]. At around stage 60 (early metamorphic climax), some larval epithelial cells (precursors of SCs, or preSCs) are induced by TH to dedifferentiate into adult epithelial SCs (ASCs)[20], which appear as small roundish islets between the connective tissue and larval epithelial cells (**Fig. 1B**). During the metamorphic climax, the basement membrane becomes thicker and more permeable, allowing connective tissue cells to readily make contact with ASCs. Initially composed of one or a few cells, the islets rapidly enlarge through active proliferation and invaginate into the connective tissue. They then differentiate into a single layer of adult epithelium, with the progression of intestinal fold formation by the end of metamorphosis at stage 66[13] (**Fig. 1C**). After completion of metamorphosis, the intestine acquires a cell-renewal system along the trough-crest axis of the intestinal fold, similar to the mammalian system along the crypt-villus axis[21,22].

TH binds to its nuclear receptor (TR), forming a heterodimer with the 9-cis retinoic acid receptor (RXR). In the presence of TH, the TR/RXR complex binds to DNA elements known as TH response elements (TREs) and activates the expression of direct TH-responsive genes[23–25]. The products of these genes affect downstream gene expression. Therefore, to clarify the molecular basis of ASC development during intestinal remodeling, it is important to examine TH response genes, regardless of the mechanism of response to TH. For this purpose, numerous TH response genes, including those encoding signaling molecules, have been identified by using several approaches[26–28]. To date, it has been shown that various signaling pathways are regulated by TH in the metamor-

phosing intestine, including sonic hedgehog (SHH)/bone morphogenetic protein 4 (BMP4), WNT, Notch, and Hippo. In this review, we summarize the roles of these signaling pathways in ASC development and discuss prospects for future investigations.

SHH/BMP4 Pathway

In the adult mammalian intestine, SHH is expressed more abundantly in the epithelial cells of the crypt base than in the villi and has been shown to be involved in cell proliferation and Paneth and goblet cell development[29,30]. In *X. laevis* intestine, SHH expression is directly upregulated by TH in ASCs during metamorphic climax[31]. Epithelial SHH travels to the connective tissue and is then received by cells expressing its receptor, PTC1[32]. These cells also express the SHH effector GLI1 transcription factor, which regulates expression of SHH target genes[33]. Among them, BMP4 expressed in connective tissue cells signals back to the epithelium and promotes differentiation into absorptive cells[34,35]. Although SHH enhances cell proliferation in both epithelial and connective tissues[36], BMP4 does not appear to affect epithelial cell proliferation but inhibits connective tissue cell proliferation[34].

We recently showed that SHH regulates the expression of the transcription factor FOXL1, the genomic DNA of which contains several GLI-binding sites[37]. In the adult mammalian intestine, FOXL1 is expressed in subepithelial telocytes, which are mesenchymal cells that are critical components of the SC niche[38]. In the *X. laevis* intestine, connective tissue cells just beneath the islets express FOXL1, and these cells are ultrastructurally similar to mammalian telocytes[37], suggesting that intestinal telocytes are evolutionarily conserved among terrestrial vertebrates.

Canonical and Non-Canonical WNT Pathways

The canonical WNT signaling pathway involving several WNT ligands[39] has important roles in promoting the proliferation of SCs in the adult mammalian intestine, and its hyperactivation often results in intestinal tumorigenesis[40]. It is unclear which WNT ligands are involved in SC regulation in the *X. laevis* intestine. However, we previously demonstrated that nuclear β-catenin, a hallmark of active canonical WNT signaling, is prominent in ASCs at metamorphic climax[41], indicating that canonical WNT signaling is activated by TH. In addition, we have experimentally shown that secreted frizzled-related protein 2 (SFRP2), which binds to WNT ligands and receptors and acts as a modulator[42], is essential for WNT signaling[41].

CD44 is a major target of WNT signaling and plays multiple roles, including cell adhesion[43,44]. It is also a primary receptor for hyaluronan (HA)[45] and is expressed in the crypt base of the mammalian intestine where ISCs reside[46]. In the *X. laevis* intestine, CD44 expression is transiently upregulated in ASCs and the connective tissue cells surrounding them during metamorphic climax[41,47]. We have shown that inhibition of HA synthesis results in failure to generate ASCs[47]. These results indicate that HA is an SC niche component that is essential for ASC formation.

Our investigation of the non-canonical WNT/planar cell polarity (PCP) pathway[48,49] focused on WNT5a/receptor tyrosine kinase-like orphan receptor 2 (ROR2) signaling, since both genes have been identified as TH response genes by microarray analysis[26]. Both WNT5a and ROR2 were transiently upregulated in *X. laevis* intestine during metamorphosis. In particular, ROR2 expression is scattered in the larval epithelium during pre- and prometamorphosis and then becomes specific to ASCs during metamorphic climax[50]. Therefore, we conclude that ROR2-expressing larval epithelial cells are preSCs destined to dedifferentiate into ASCs through the action of TH. Critically, we demonstrated that depletion of WNT5a leads to failure of ASC formation, indicating that WNT5 a/ROR2 signaling is indispensable for preSCs to dedifferentiate into ASCs[50].

Notch Pathway

In the adult mammalian intestine, Notch signaling regulates the renewal of ISCs and binary cell fate determination of absorptive and secretory cells that originate from a common ISC population[51–53]. Notch ligands delta-like ligand 1 (DLL1) and DLL4 are required for the maintenance of LGR5-expressing ISCs[53]. Inhibition of Notch signaling results in loss of the proliferative crypt compartment and conversion of progenitor cells into post-mitotic goblet cells[52,54]. In *X. laevis* intestine, NOTCH1 and DLL1 expression is transiently upregulated in ASCs in response to TH during metamorphosis. We have experimentally demonstrated that inhibition of Notch signaling modestly suppresses the TH-induced upregulation of LGR5, which suggests that this pathway has a role in ASC development. More importantly, Notch inhibition during intestinal remodeling leads to hyperplasia of secretory cells and reduction of absorptive cells, resembling that observed in the adult mammalian intestine[55]. These results suggest that Notch signaling plays a role in cell fate determina-

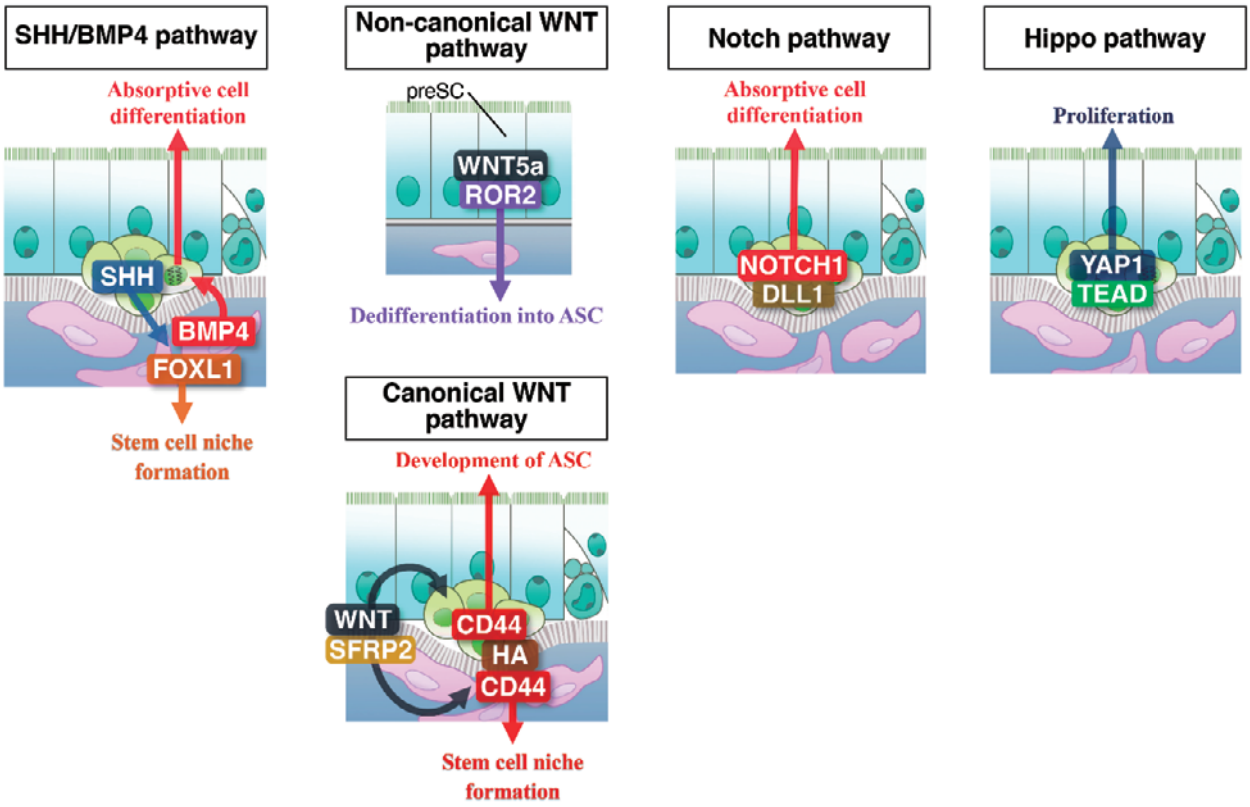

Fig. 2 Summary of signaling pathways involved in SC regulation. The SHH/BMP4, WNT, Notch, and Hippo pathways are shown.

tion during the initial formation of ASCs and is evolutionarily conserved.

Hippo Pathway

The Hippo signaling pathway plays a pivotal role in organ size control, regeneration, and tumor suppression[56,57]. In the adult mammalian intestine, this pathway has been reported to be crucial for maintaining normal tissue homeostasis through crosstalk with the signaling pathways described above[58]. The effectors of this pathway—the transcription cofactors YAP and TAZ—are regulated by the phosphorylation cascade of Hippo signaling. When Hippo signaling is activated, YAP/TAZ are phosphorylated and become inactive. In contrast, when Hippo signaling is inactive, non-phosphorylated YAP/TAZ translocate into the nucleus, where they bind to their key binding partner, the TEAD transcription factor, and promote target gene expression[59,60]. YAP is enriched in ISCs in the adult mammalian intestine[61]. Disruption of the Hippo signaling cascade results in disorganized villi and enlarged crypt structures[62,63]. In *X. laevis* intestine during metamorphosis, the expression of YAP1 is transiently upregulated in response to TH in ASCs and surrounding connective tissue cells, similar to that of CD44, suggesting that *YAP1* is a WNT target gene[64]. In addition, YAP1 is localized in the nuclei of some preSCs expressing ROR 2 at stage 59, which suggests the involvement of YAP1 in dedifferentiation of preSCs into ASCs[65]. Furthermore, we have experimentally shown that formation of the YAP-TEAD complex is required for ASC proliferation[65].

Conclusions and Future Directions

The role of signaling pathways in the acquisition of the cell-renewal system during TH-induced *X. laevis* intestinal metamorphosis is becoming clearer (**Fig. 2**). Because the mechanisms of action of each signaling pathway are, in many cases, common to vertebrates, findings from studies of amphibians could be applied to higher vertebrates, including humans[10].

The function of each pathway that we have elucidated is primarily based on blocking the pathway with an inhibitor. To analyze the function of each pathway more precisely, gene knockout and our recently developed simple and powerful transgenesis techniques[66] would be of great help. However, if pathways mutually interact, blocking one could lead to inhibition of another. To address this issue, combining an inhibitor of one signaling pathway with an agonist of another may be worthwhile.

Alternatively, it may be useful to treat transgenic tadpoles expressing a factor that inhibits or promotes one signaling pathway with an agonist or antagonist of another. Using the pharmacological and genetic approaches described above, we aimed to clarify the contribution and order of each pathway in ASC formation during TH-dependent intestinal remodeling. It is also important to assess the extent of similarity in the mechanisms of stem cell development and maintenance between frogs and mammals. We anticipate that future studies using amphibians will shed new light on the mechanisms underlying SC development in vertebrates.

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Conflict of Interest: The authors declare no potential conflicts of interest.

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