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Hedgehog Signaling during Gut Formation in the Freshwater Leech, *Helobdella austinensis*

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Gut formation · Hedgehog signaling · Leech · Lophotrochozoans · NKL genes

Abstract

Introduction: The hedgehog signaling pathway plays a crucial role in inducing segment polarity through cell-cell interactions in various metazoans, including arthropods and annelids. However, its involvement in organogenesis and segmentation among lophotrochozoans remains inconsistent. This study aimed to explore the role of the hedgehog gene during gut development in the freshwater leech, *Helobdella austinensis*. ***Methods:*** Developmental RT-PCR and in situ hybridization were performed to examine the expressions of hedgehog genes. In addition, embryos were treated with cyclopamine (a hedgehog signaling antagonist) and purmorphamine (a *Smo* agonist) to examine the potential interactions between *Helobdella* orthologs to hedgehog and two NKL genes: *Hau-NK2* and *Hau-NK4*. ***Results:*** We examined the expressions of four core pathway members – Hedgehog (*Hh*), Patched (*Ptc*), Smoothened (*Smo*), and the downstream transcription factor *Gli* – spatiotemporally during the embryonic stages of *H. austinensis*. All four genes were expressed in the developing gut and proboscis during organogenesis but not during the segmentation stage. Additionally, the treatment of embryos with cyclopamine and purmorphamine revealed that NK genes are regulated by hedgehog signaling. Furthermore, NK2 and NK4 were expressed in the developing gut rather than in a segmental stripe pattern. ***Conclusion:*** This study confirms that the hedgehog signaling pathway is associated with gut development in the freshwater leech, *H. austinensis*. The expression patterns of hedgehog pathway genes and their interaction with NK genes suggest a role of hedgehog signaling in regulating gut development rather than segmentation in the freshwater leeches.

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Introduction

Studying cell-cell signaling pathways during embryogenesis helps unravel the complexity of biological processes. One such pathway, called the hedgehog pathway, is evolutionarily conserved [1–3], and its disruption can lead to disease and birth defects [4]. The hedgehog gene (*hh*) was first identified in *Drosophila melanogaster*, in which it has a role in generating segmental boundaries along the anteroposterior axis by interacting with wingless (*wg*) [5]. The studies of the hedgehog gene have been largely conducted in diverse animal taxa, and there is at least one hedgehog gene across bilaterians. However, three hedgehog genes have been identified in amniotes: Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*), and Sonic hedgehog (*Shh*); and four to five (*Dhh*, *Ihha*, *Ihhb*, *Shha*, and *Shhb*) in teleosts [6–8].

Hedgehog signaling plays a prevailing role in embryonic body segmentation in arthropods [9–12], tissue regeneration in planarians [13, 14], and neural development in vertebrates [15]. However, the gaps between the phyla regarding how the conserved signaling pathway differently affects them are not clear so far. The hedgehog gene and its cascade were involved in segmental formation during embryogenesis in *Platynereis* (Phylum: Annelida) [16]. Intriguingly, the hedgehog signaling pathway is involved in gut development but not in segmentation in the leech, *Helobdella robusta* [17]. This enigmatic variety even within the same phylum raises the question of how the function of the hedgehog signaling pathway has diversified during evolution, emphasizing the need to characterize the expression of its components in more species within the bilateria to get a clearer picture. Numerous studies have used hedgehog antagonists and agonists to examine the significance of hedgehog signaling during organogenesis [17–21]. For instance, the inhibition of hedgehog signaling using a steroidal alkaloid (cyclopamine) resulted in malformed embryos with the failure of proboscis invagination in leeches [17, 21], and the purine derivative (purmorphamine) activates the hedgehog pathway by targeting the pathway component "*Smo*" [20]. In vertebrates, there are genes from the NK2 family that are activated by Sonic hedgehog signaling [22], and one of the NK2-class genes (*Lox10*) is expressed in the crop of *Helobdella* [23]. In *Platynereis*, the *NK4/tinman* ortholog is involved in segmental patterning similar to hedgehog, but interestingly, it was not affected by cyclopamine treatment [16]. This remarkable variety of hedgehog and its cascade indicates that hedgehog signaling might have evolved differently in annelids. The present study examines whether hedgehog and its target genes, including NK2 and NK4, are involved during gut formation in the glossiphoniid leech, *H. austinensis*, using a range of experimental methods.

The glossiphoniid leech, *Helobdella*, is one of the valuable lophotrochozoan models resulting from laboratory-breeding systems with observations on its feeding behavior [24], mapping of cell lineage and developmental stages [25], available genome assembly [26], and functional study approaches [17, 27]. All these advantages have made this model widely used for evolutionary developmental biology (evo-devo), behavioral science, and physiology [28, 29]. *Helobdella* is a carnivore that feeds on a wide range of prey, exhibiting a unique feeding organ called the "proboscis," and well-organized muscle layers [18, 25]. The development of this feeding organ, along with overall gut formation, occurs from embryonic stages 9–11 [17, 19]. Herein, we characterize the hedgehog mRNA (*Hau-hh*) expression in *H. austinensis*, following up on a previous study to corroborate spatial evidence performed by Kang and collaborators [17] in 2003. Furthermore, we also identify its cascades for the first time, providing the spatiotemporal expression patterns of hedgehog ligands during gut organogenesis in *H. austinensis*. These include *Patched* (*Hau-ptc*), *Smoothened* (*Hau-smo*), *Cubitus interruptus/Gli* (*Hau-gli*), and two homeobox genes (*Hau-NK2* and *Hau-NK4*), assumed to be downstream targets of hedgehog signaling. We also demonstrate the correlation between hedgehog signaling and its downstream targets by treating *Helobdella* embryos with drugs, cyclopamine and purmorphamine.

Methods

Animals

Adult specimens were bred in the Laboratory of Cellular and Development Biology (Department of Biology, Chungbuk National University, Republic of Korea). *Helobdella* (leech) embryos were bred in petri dishes with lids containing *Helobdella triserialis* saline medium (4.8 mM NaCl, 1.2 mM KCl, 2 mM $MgCl_2$, 8 mM $CaCl_2$, and 1 mM maleic acid) according to the protocol for handling leech embryos [27–30]. The medium was changed once a day, and the specimens were scrubbed manually to get rid of any residual waste and kept in a biological oxygen demand incubator at 22°C. Their diet consisted of bloodworms purchased online (Hyangsan Co., Ltd, Daegu, South Korea) and bred in bowls in artificial freshwater in a biological oxygen demand incubator at 22°C.

Gene Cloning and Probe Synthesis

Specific primers for the genes of interest were designed (online suppl. Table S1; for all online suppl. material, see https://doi.org/10.1159/000543782), and genes were amplified from organogenesis stages (9–11). RNA extraction and cDNA synthesis from the embryos of *H. austinensis* were performed according to the manufacturer's instructions as described previously [29, 30]. Similarly, developmental RT-PCR was performed using the TaKaRa Ex Taq® kit (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions and under the following cycling conditions: predenaturation at 94°C for 5 min, denaturation at 94°C for 30 s, variable annealing temperature for 30 s, variable extension time at 72°C, and post-extension at 72°C for 5 min. These amplified fragments were cloned into a pGEM-T vector (Promega, Madison, WI, USA). RNA probes labeled with digoxigenin were constructed using the MEGAscript kit (Ambion, Austin, TX, USA) and DIG RNA Labeling Mix (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Drug Treatment and Developmental Semiquantitative RT-PCR

At stage 7, *H. austinensis* embryos were incubated in *H. triserialis* saline medium (4.8 mM NaCl, 1.2 mM KCl, 2 mM $MgCl_2$, 8 mM $CaCl_2$, and 1 mM maleic acid) including drugs: cyclopamine (Sigma-Aldrich, Saint Louis, MO, USA), a hedgehog signaling antagonist, and purmorphamine (Sigma-Aldrich, Saint Louis, MO, USA), a hedgehog signaling agonist [20, 21]. Total cyclopamine and purmorphamine concentrations were 10 mM and 5 mM in *H. triserialis* media, respectively. The PCRs were performed under the following cycling conditions: predenaturation at 94°C for 5 min, followed by 30–40 cycles of denaturation at 94°C for 30 s, elongation at 72°C for each sequence length-related times, and a final elongation step at 72°C for 5 min. A 10 µL aliquot of each PCR was removed after 25 cycles, while the remaining material underwent 5 additional cycles of amplification. The 18S rRNA primer was used as a standard [17]. The experiment was repeated a minimum of 5 times.

Immunostaining and Whole-Mount in situ Hybridization

Immunostaining was carried out as described previously [30]. Primary antibodies were directed against alpha-tubulin (T-7451; Sigma-Aldrich), and the secondary antibodies were goat anti-mouse IgG H&L Alexa Fluor 488 (ab150113; Abcam). Similarly, chemical and fluorescence in situ hybridizations were performed as described previously [21]. The details of riboprobes are described in online supplementary Table S1. The stained embryos were imaged with Leica DM6 B with a Leica DFC450 C camera (Leica, Wetzlar, Germany) and an LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany). All experiments were performed a minimum of 5 times.

Results

Phylogeny and Expressions of Hedgehog Orthologs in H. austinensis

The hedgehog precursor protein (HH) consists of two fragments: an amino-terminal (HH-N) and a carboxy-terminal (HH-C) polypeptide, each bearing a highly conserved domain known as "Hedge" and "Hog," respectively (Fig. 1a, b). Autoproteolytic cleavage of these fragments occurs, a process facilitated by the "Hint" region in the Hog domain (Fig. 1b). However, the presence of the "Hint" region within the Hog domain varies among lophotrochozoans and ecdysozoans (online suppl. Fig. S1). Specifically, the Hint domain is absent in helminths and leech annelids but is present in polychaetes and mollusks. In contrast, the Hint domain is absent in ecdysozoans and deuterostomes (online suppl. Fig. S1). To analyze the detailed expression patterns of *Hau-hh* in the leech, embryonic stages 10 and 11 were chosen as gut development in the leech initiates at embryonic stage 9 and completes by stage 11 [17]. At stage 10, *Hau-hh* expression was detected along the proboscis and midgut (Fig. 1c). The expression pattern intensified at stage 11, appearing along the proboscis, esophagus, reproductive organs, crop, intestine, and rectum (Fig. 1c). Additionally, developmental RT-PCR was conducted to examine the expression patterns of the core hedgehog pathway genes: *Hau-hh*, *Hau-ptc*, *Hau-smo*, and *Hau-gli* during embryonic stages 9–11. The expression patterns of *Hau-hh* and *Hau-ptc* were notably strong across stages 9–11 (Fig. 1e). In contrast, *Hau-gli* exhibited faint expression during stages 9–11 (Fig. 1e). Similarly, the expression pattern of *Hau-smo* was relatively weaker at stage 9 compared to stages 10 and 11 (Fig. 1e). Moreover, mRNA expression levels were also examined, and the results were consistent with RT-PCR (Fig. 1e-1). Furthermore, to assess the effectiveness of chemical treatment, immunostaining against alpha-tubulin was conducted. This revealed normal staining of the ventral nerve cord in both the control group (0.1% DMSO) and the experimental group (10 µM cyclopamine) (Fig. 1f).

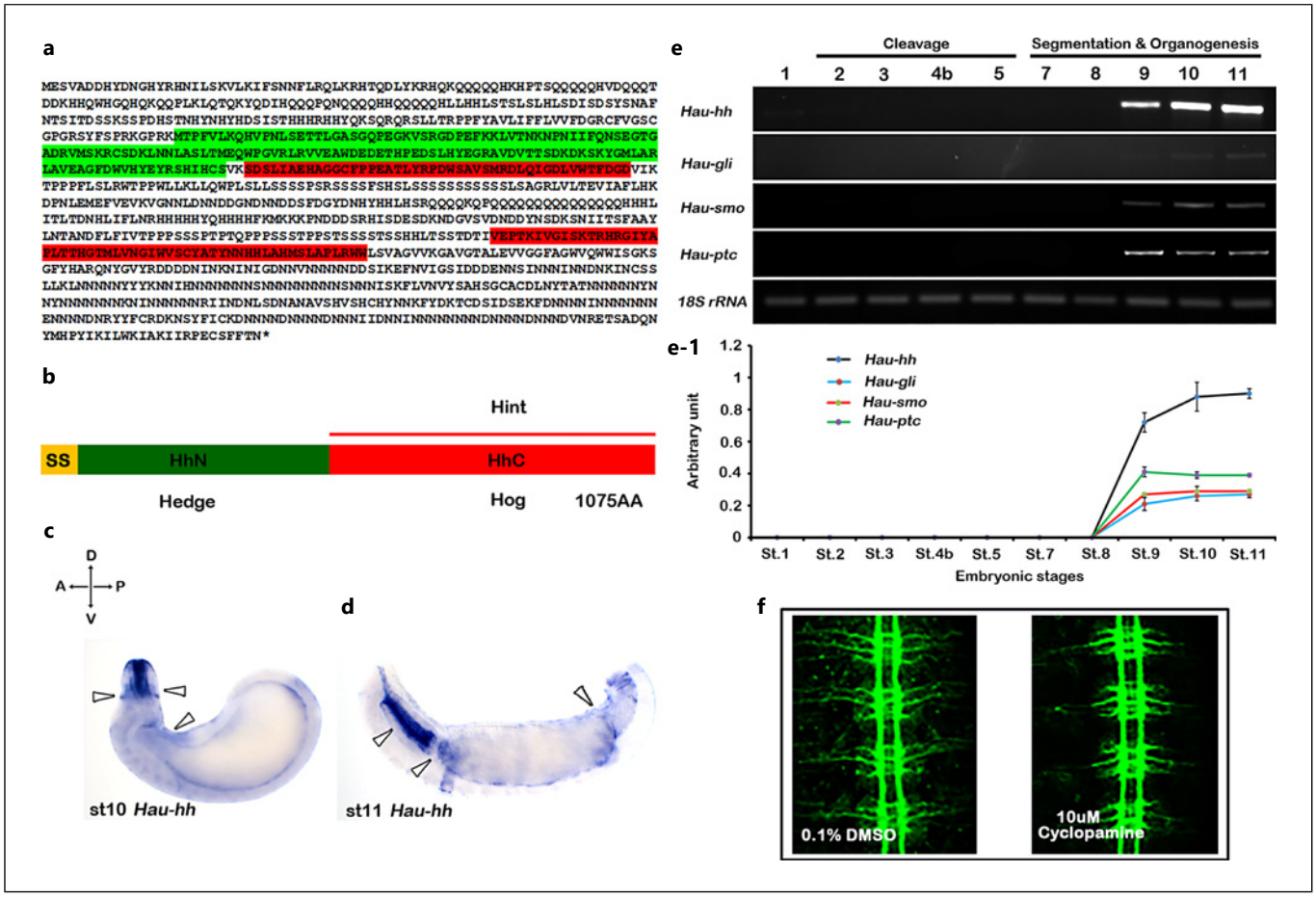


Fig. 1. Hedgehog ortholog in *H. austinensis*. **a** Amino acid sequence of the hedgehog ortholog in *H. austinensis*. Hedge and hog domains are highlighted in green and red color, respectively. **b** Schematic diagram showing SS, hedge, and hog domains with 1,075 AA sequences. **c**, **d** Whole-mount in situ hybridization showing the expression pattern of *Hau-hh* at embryonic stages 10 and 11 (lateral view). White arrowheads indicate the expression of *Hau-hh*. **e** RT-PCR showing the expression of hedgehog signaling molecules in *Helobdella* at embryonic stages 9–11. 18S ribosomal RNA used as a control. **e-1** mRNA expression levels of *Hau-hh*, *Hau-gli*, *Hau-smo*, and *Hau-ptc* during embryonic stages. **f** Immunostaining against alpha-tubulin showing the ventral nerve cord when embryos are incubated with 0.1% DMSO and 10 μM cyclopamine. A, anterior; P, posterior; D, dorsal; V, ventral; SS, signal sequence.

Embryonic Expression Patterns of Hedgehog Signaling Pathway Genes

The expression patterns of genes related to the hedgehog signaling pathway were examined via in situ hybridization (Fig. 2a–h). At early stage 9, the expressions of *Hau-hh*, *Hau-ptc*, *Hau-smo*, and *Hau-gli* were observed along the oral precursor stomodeum at the anterior part of the embryo (Fig. 2a–d). In addition, the expression patterns of *Hau-hh*, *Hau-ptc*, and *Hau-gli* were also observed along the germinal plate (Fig. 2a, b, d). By mid-stage 10, *Hau-hh* demonstrated robust expression throughout the innermost layer of the developing proboscis, which had undergone eversion [19], and formed a ring around the developing stomodeum (Fig. 2e, e-1, e-2). Additionally, *Hau-hh* showed weak expression along the edge of the syncytial yolk cell (SYC) at the embryo's posterior end (Fig. 2e). Conversely, *Hau-ptc* exhibited broad expression in the outer layer of the everted proboscis and faint expression in the inner layer, partially overlapping with *Hau-hh* (Fig. 2f, f-1, f-2). The faint expression of *Hau-ptc* was also observed along the ventral border of the SYC (Fig. 2f). Likewise, *Hau-smo* displayed strong expression along the outer layer of the proboscis, overlapping with the upper half of *Hau-ptc* expression by mid-stage 10 (Fig. 2g, g-1, g-2). Additionally, the weak expression of *Hau-smo* was detected in

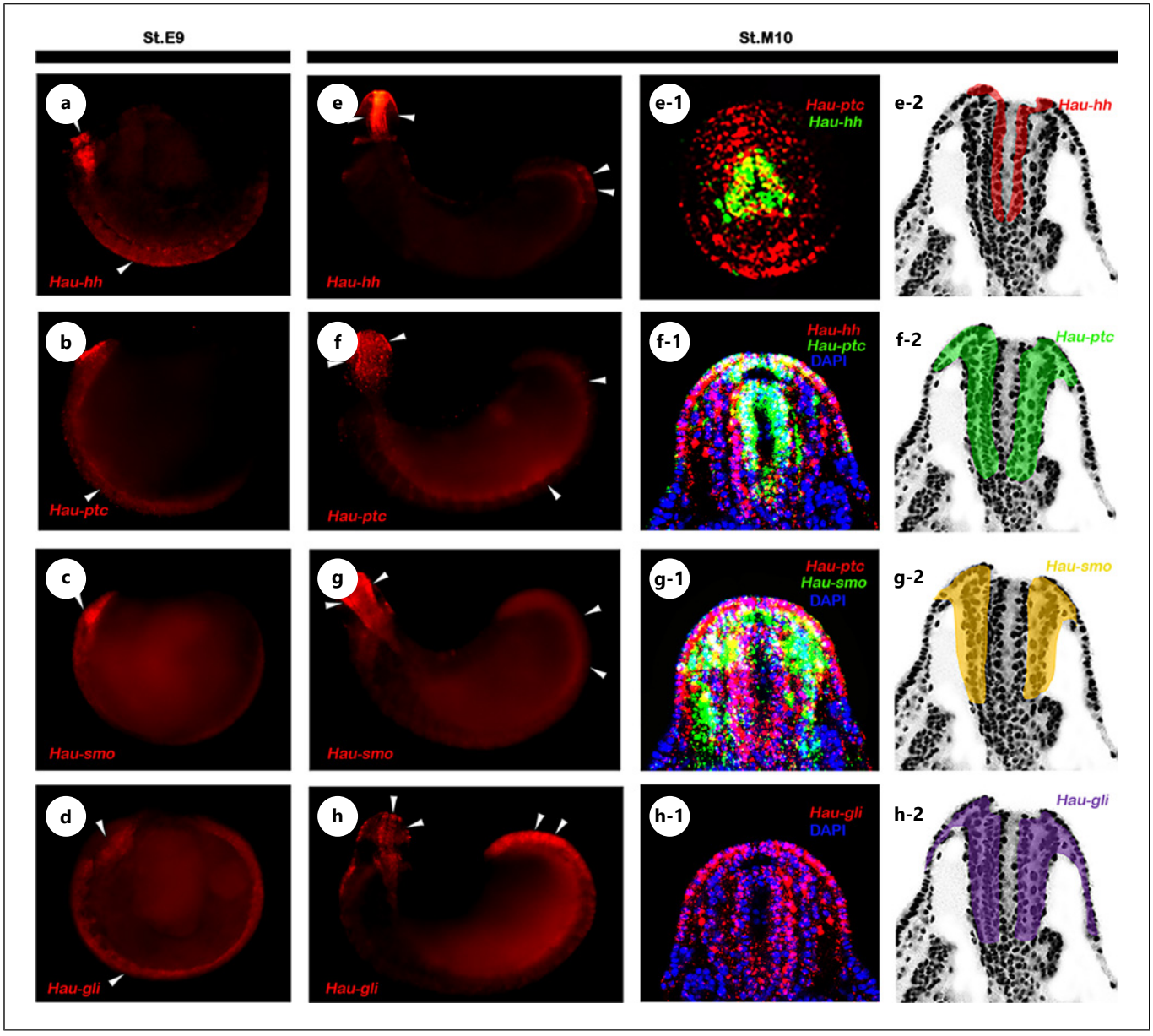


Fig. 2. Expression patterns of the hedgehog signaling pathway genes during organogenesis. Lateral view, anterior to the left and dorsal to the top unless indicated otherwise. **a–h** Expression patterns obtained through whole-mount fluorescence in situ hybridization at early stage 9 and middle stage 10. Expression of *Hau-hh* at early stage 9 (**a**) and mid-stage 10 (**e**). **e-1** Transverse section of midportion of the proboscis at mid-stage 10. Cells stained in green and red indicate *Hau-hh* and *Hau-ptc* expressions, respectively. Yellow color indicates the overlapping expression. **e-2** Schematic diagram showing positive *Hau-hh* cells in the proboscis. Expression of *Hau-ptc* at early stage 9 (**b**) and mid-stage 10 (**f**). **f-1** Close-up view of proboscis at mid-stage 10. Cells stained in green and red indicate *Hau-ptc* and *Hau-hh* expressions, respectively. Yellow color indicates the overlapping expression. Nuclear staining with DAPI shown in blue. **f-2** Schematic diagram showing positive *Hau-ptc* cells in the proboscis. Expression of *Hau-smo* at early stage 9 (**c**) and mid-stage 10 (**g**). **g-1** Close-up view of proboscis at mid-stage 10. Cells stained in green and red indicate *Hau-smo* and *Hau-ptc* expressions, respectively. Yellow color indicates the overlapping expression. Nuclear staining with DAPI shown in blue. **g-2** Schematic diagram showing positive *Hau-smo* cells in the proboscis. Expression of *Hau-gli* at early stage 9 (**d**) and mid-stage 10 (**h**). **h-1** Close-up view of proboscis at mid-stage 10. Cells stained in red indicate *Hau-gli*. Nuclear staining with DAPI shown in blue. **h-2** Schematic diagram showing positive *Hau-gli* cells in the proboscis. **a–h** White arrowheads indicate expressions of *Hau-hh*, *Hau-ptc*, *Hau-smo*, and *Hau-gli*.

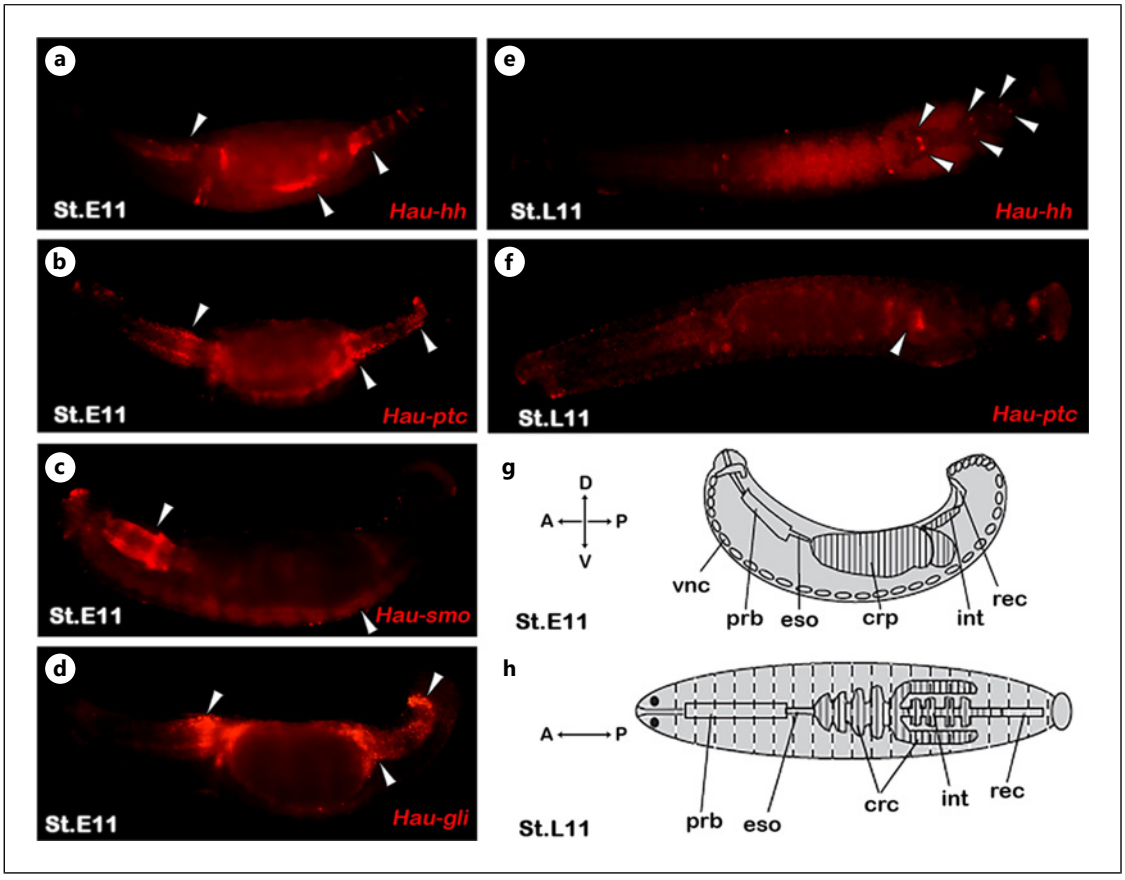


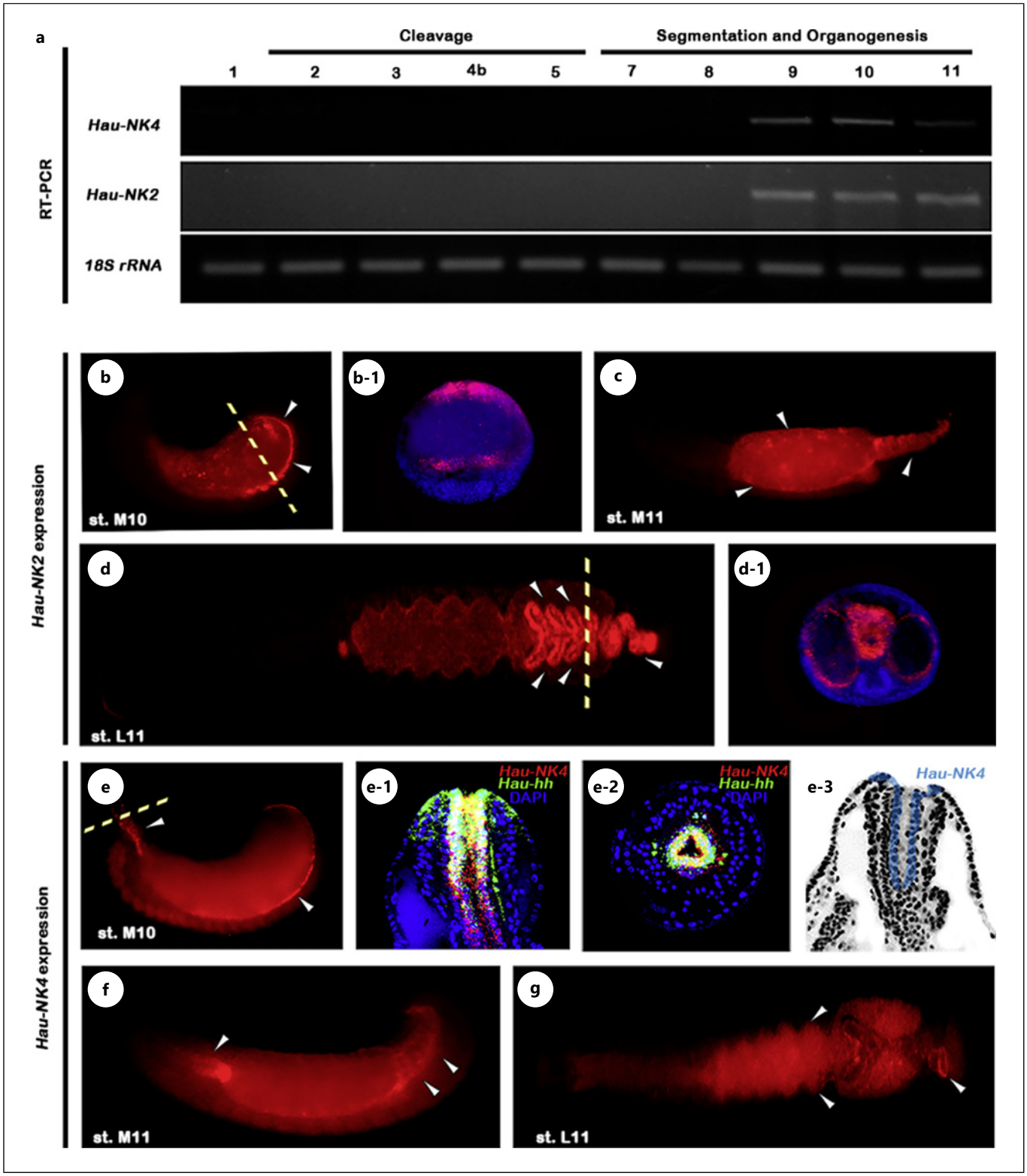
Fig. 3. Expression patterns of the hedgehog signaling pathway genes at embryonic stage 11. Lateral view, anterior to the left and dorsal to the top unless indicated otherwise. **a–d** Expressions of *Hau-hh*, *Hau-ptc*, *Hau-smo*, and *Hau-gli* at early stage 11. **e**, **f** Expressions of *Hau-hh* and *Hau-ptc* at late stage 11. Expression of *Hau-hh* as three sets of paired dots along the intestine at late stage 11 (arrowheads, **e**). **g**, **h** Schematic diagram depicting the internal morphology of the *Helobdella* embryo at early and late stage 11, retrieved from Kang et al. [17]. **a–f** White arrowheads indicate expressions of *Hau-hh*, *Hau-ptc*, *Hau-smo*, and *Hau-gli*. vnc, ventral nerve cord; prb, proboscis; eso, esophagus; crp, crop; int, intestine; rec, rectum; D, dorsal; V, ventral; A, anterior; P, posterior.

the esophagus and at the posterior border of the SYC (Fig. 2g). In contrast, the strong expression of *Hau-gli* was observed along the posterior region of the germinal plate, with faint expression in both the inner and outer layers of the proboscis by mid-stage 10 (Fig. 2h, h-1, h-2).

At early stage 11, when the proboscis retracts within the stomodeum, the expression of *Hau-hh* was observed diffusely in the retracted proboscis, in reproductive organs, as patches in the anterior, middle, and posterior regions of the crop, and as serial stripes in the intestine (Fig. 3a, g). Similarly, the expression of *Hau-ptc* was noted along the outer layer of the proboscis, crop, intestine, and rectum (Fig. 3b, g). The intense expression of *Hau-smo* was observed along the proboscis, with weak expression along the ventral nerve cord and in the region between the crop and the intestine (Fig. 3c, g). Conversely, the expression of *Hau-gli* at early stage 11 was notably strong in the esophagus, anterior and posterior ends of the crop, and intestine and rectum (Fig. 3d, g). By late stage 11, *Hau-hh* was expressed as three sets of paired dots along the intestine (Fig. 3e, h), whereas *Hau-ptc* appeared as a stripe at the base of the intestine and rectum (Fig. 3f, h).

Embryonic Expression Patterns of Homeobox NK2 *and* NK4

Similar to the aforementioned hedgehog pathway genes, the *Helobdella* homeobox genes NK2 (*Hau-NK2*) and NK4 (*Hau-NK4*) were expressed exclusively during organogenesis (Fig. 4a). The mRNA expression levels were also examined, which showed consistent results with the RT-PCR (online suppl. Fig. S2). At mid-stage 10, *Hau-NK2* exhibited expression on the ventral side of the SYC from the middle to the posterior end, corresponding to the presumptive midgut (Fig. 4b, b-1). By mid-stage 11,



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Hau-NK2 expression expanded throughout the crop, intestine, and rectum (Fig. 4c). Subsequently, at late stage 11, the intense expression of *Hau-NK2* was observed along the intestine and rectum, with transcripts detected on the borders of the crop caeca (Fig. 4d, d-1). Conversely, the homeobox gene *Hau-NK4* was expressed along the inner layer of the everted proboscis at mid-stage 10, substantially overlapping with *Hau-hh* in the middle upper half of the developing organ (Fig. 4e, e-1, e-2, e-3). Additionally, *Hau-NK4* transcripts were detected in the ventral edge of the SYC, similar to *Hau-NK2* expression (Fig. 4e). At early stage 11, *Hau-NK4* exhibited strong expression in the esophagus and the anteriormost region of the midgut and in the edges of the intestine and rectum (Fig. 4f). Meanwhile, at late stage 11, *Hau-NK4* was expressed as a stripe at the base of the intestine and formed a ring around the rectum (Fig. 4g).

Effects of Cyclopamine and Purmorphamine on the Expression of Hau-NK2 *and* Hau-NK4

To compare the effects of cyclopamine and purmorphamine on the expression of the homeobox genes *NK2* and *NK4* concerning Hedgehog expression, we treated *Helobdella* embryos with cyclopamine and purmorphamine, as previously described [17] (Fig. 5a–e). Subsequently, we conducted in situ hybridization to assess the impact of cyclopamine and purmorphamine on the expression of *Hh* genes. Treatment with cyclopamine resulted in inhibited proboscis development and malformed embryonic bodies (Fig. 5a, a-1). In these instances, *Hau-hh* expression appeared strong along the truncated oral opening and the ventral nerve cord (Fig. 5a, a-1). Conversely, treatment with purmorphamine led to a wider everted proboscis, and the expression of *Hau-hh* was notably strong only in the upper half of the inner layer of the proboscis (Fig. 5a, a-2). Nevertheless, although *Hau-hh* expression persisted as a ring surrounding the stomodeum, its pattern in the ventral edge of the prospective midgut became less distinct (Fig. 5a).

Under normal conditions, *Hau-NK2* exhibited strong expression in the midgut and hindgut (Fig. 5b). However, treatment with cyclopamine drastically decreased its expression in the midgut and rectum (Fig. 5b-1). Conversely, treatment with purmorphamine intensified its expression in the intestine, which was also detected in the esophagus (Fig. 5b, b-2). Normally, *Hau-NK4* was expressed in the innermost layer of the everted proboscis, esophagus, anterior half of the ventral nerve cord, and posterior half of the ventral border of the SYC (Fig. 5c). Treatment with cyclopamine resulted in the inhibition of *Hau-NK4* expression in the proboscis but increased expression along the ventral border of the SYC (Fig. 5c, c-1). Meanwhile, treatment with purmorphamine increased the expression of *Hau-NK4* in the intestine (Fig. 5c, c-2). Additionally, to validate further, transcript levels of *Hau-NK2* and *Hau-NK4* were examined after treatment with cyclopamine and purmorphamine (Fig. 5d, e). Interestingly, the transcript levels for *Hau-hh* decreased slightly after treatment with cyclopamine, whereas there was a more than 50% decrease in the transcript levels of *Hau-NK2* and *Hau-NK4* (Fig. 5d). On the other hand, transcript levels of *Hau-hh* increased slightly, and those of *Hau-NK2* and *Hau-NK4* increased by over 50% after treatment with purmorphamine (Fig. 5e).

Discussion

The present study demonstrates the role of hedgehog signaling molecules during gut formation in annelids using *H. austinensis* as an animal model. Our results showed that the expression of the hedgehog ortholog in *H. austinensis* resembles that of *H. robusta* in the gastrointestinal tract [17], indicating conserved gene expression across the genus *Helobdella* and other closely related glossiphoniid leeches. The spatiotemporal expression patterns of *Hau-ptc*, *Hau-smo*, *and Hau-gli* reveal the involvement of the hedgehog pathway in the mesodermal and endodermal cells, contributing to gut formation in leeches (Fig. 2, 3). Although the expression of *Hau-hh* was detected in differentiating neural cells, none of the pathway members were expressed in the

Fig. 4. a Expression patterns of *Hau-NK2* and *Hau-NK4*. Semi-quantitative RT-PCR showing expressions of *Hau-NK4* and *Hau-NK2*. 18S ribosomal RNA used as a control. **b–g** Fluorescence in situ hybridization showing expression patterns of *Hau-NK2* and *Hau-NK4* at embryonic stages 10 and 11. **b**, **b-1** Sagittal section (yellow dotted line in **b**) showing the *Hau-NK2* expression (red) at mid-stage 10. **c** *Hau-NK2* expression in mid-stage 11. **d** Ventral view of late stage 11. **d**, **d-1** Transverse section of late stage 11 (yellow dotted line in **d**) showing the *Hau-NK2* expression (red). **e** Expression patterns of *Hau-NK4* at mid-stage 10. **e-1** Close-up view of the anterior region of the proboscis showing the expression of *Hau-NK4* and *Hau-hh* in red and green color, respectively. Yellow dots indicate the overlapping expression. Nuclear staining with DAPI shown in blue. **e-2** Transverse section through the mid-region of the proboscis. **e-3** Schematic diagram showing positive *Hau-NK4* cells in the proboscis. **f**, **g** Expression patterns of *Hau-NK4* at mid- and late stage 11. **b–g** White arrowheads indicate expressions of *Hau-NK2* and *Hau-NK4*.

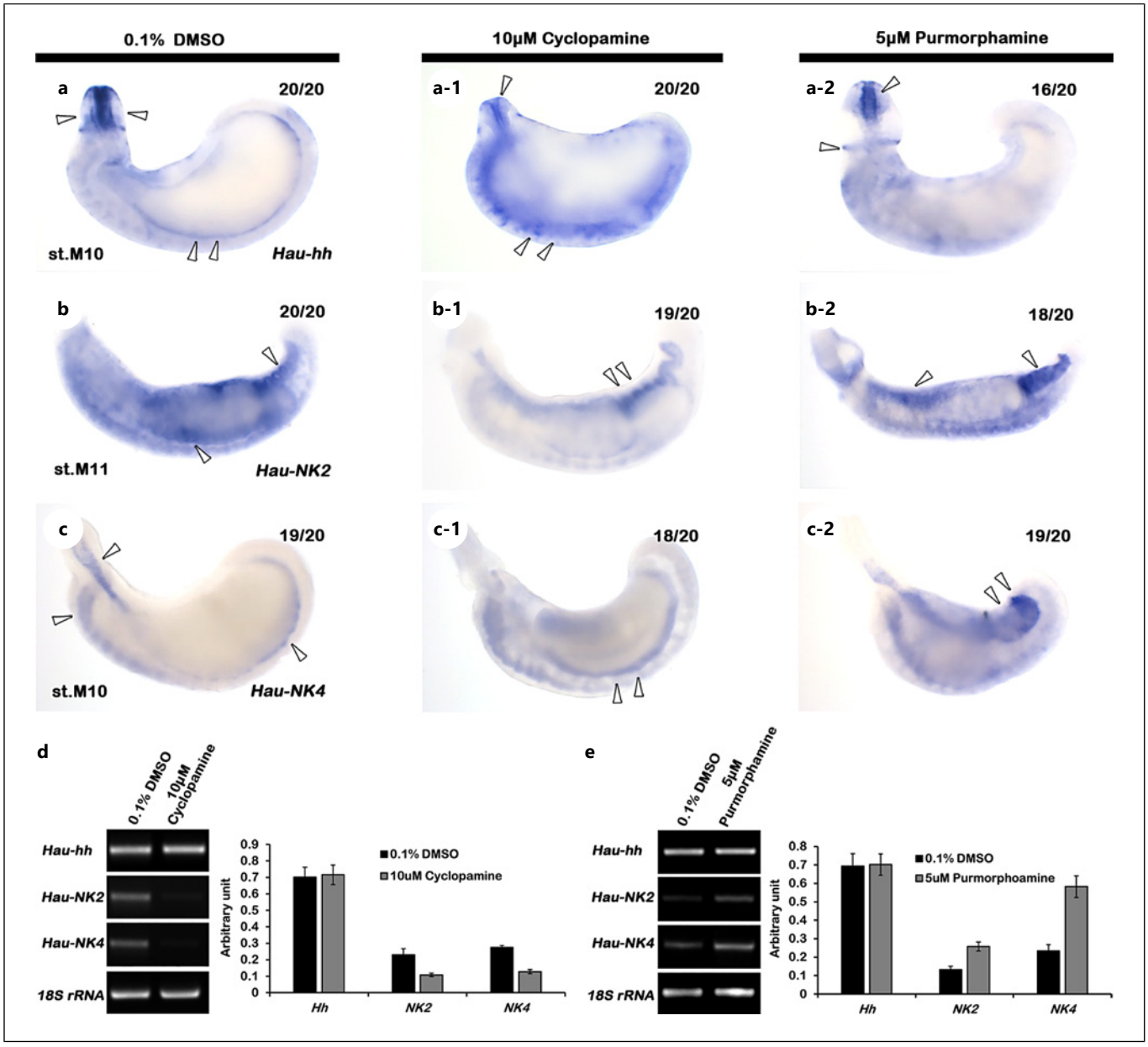

Fig. 5. Effects of cyclopamine and purmorphamine in hedgehog signaling. **a**, **a-1**, **a-2**, **b**, **b-1**, **b-2**, **c**, **c-1**, **c-2** Chemical in situ hybridization showing expressions of *Hh*, *NK2*, and *NK4* in *Helobdella* after treatment with cyclopamine and purmorphamine. White arrowheads indicate expressions of *Hau-hh*, *Hau-NK2*, and *Hau-NK4*. **d** RT-PCR and mRNA expression levels of *Hau-hh*, *Hau-NK2*, and *Hau-NK4* before and after treatment with 10 μM cyclopamine. **e** RT-PCR and mRNA levels of *Hau-hh*, *Hau-NK2*, and *Hau-NK4* before and after treatment with 5 μM purmorphamine. **d**, **e** 18S rRNA used as an internal control. The numbers in the upper right corner of each figure indicate the total number of embryos used in the experiment (denominator) and the number of embryos displaying the same expression patterns (numerator).

segmental tissue due to the lack of expression of *Hau-hh* during the segmentation stage (Fig. 1, 2).

Previous studies have already discovered the developmental role of the hedgehog signaling pathway during gut development in echinoderms, fish, amphibians, birds, and mammals [31–36]. The expression in the gastrointestinal tract of deuterostomes would suggest the evolutionary significance of hedgehog signaling in gut formation in metazoans. Interestingly, the involvement of hedgehog signaling in the determination of intestinal

stem cell fate in *Drosophila* development [37], and its expression in the gut of some cnidarians, lophotrochozoans, and ecdysozoans [17, 38–40] would provide clues of conserved hedgehog signaling during gut formation in protostomes as well. Furthermore, abnormal hedgehog signaling has been shown to result in malformed gut formation in both protostomes and deuterostomes [17, 33, 41], consistent with our findings, in which the loss and gain of hedgehog signaling using the hedgehog antagonist (cyclopamine) and agonist (purmorphamine) showed altered proboscis development and altered expressions of hedgehog in the gut of glossiphoniid leech (Fig. 5). Although the function of the hedgehog signaling pathway seems diversified even within the phylum Annelida [16], it can be inferred that the developmental role of hedgehog signaling molecules in gut formation suggests conserved signaling across metazoans.

In our study, the expressions of both *Hau-NK4* and *Hau-NK2* were detected during organogenesis, mainly in the gut tissue, similar to *Hau-hh* expression (Fig. 4). NK homeobox genes are considered important regulators for performing fundamental developmental processes in metazoans [42]. NK genes, as homeodomain transcription regulators, play a role in mesodermal and neural development in bilaterians [43]. Studies on the nereid annelid (*Platynereis*) highlighted the contribution of NK homeobox genes during segmentation [16, 44]. However, our results showed altered expressions of *Hau-NK2* and *Hau-NK4* along the gut after treatment with cyclopamine and purmorphamine (Fig. 5). This led us to provide indirect evidence of the involvement of *Hau-hh* in gut formation rather than segmentation as in previous reports [17–19, 21]. Additionally, the embryonic expression of segment polarity genes including hedgehog in the onychophorans corresponds to organogenesis [45], providing further support for the scenario in which hedgehog was ancestrally involved in organogenesis. Hedgehog components in leeches showed features similar to Platyhelminthes and Arthropods, i.e., the absence of the Hint domain (online suppl. Fig. S1); however, the Hint was already present as a hedgehog component in the Cnidaria, *Nematostella vectensis* [40]. It seems that the presence of the Hint domain and the expression of hedgehog signaling molecules during organogenesis are inconsistent in metazoans. Among annelids, there is also variation in the expression of hedgehog signaling during organogenesis [16, 46]. To understand the evolution of the signaling pathway in detail, functional analyses of hedgehog signaling in more invertebrate animal models are needed.

Conclusions

In summary, the present study revealed the developmental role of hedgehog signaling during gut formation in the glossiphoniid leech, *H. austinensis*. Different from the characteristic role of hedgehog as a segment polarity gene, our study revealed the involvement of hedgehog signaling during organogenesis. Despite being expressed during segmentation in other lophotrochozoans, *Hau-hh* expression during gut formation suggests an independent evolution for segmentation within the bilateria.

Statement of Ethics

This study protocol was reviewed and approved by Chungbuk National University, Institutional Animal Care and Use Committee (Approval No. CBNUA-2141-23-02).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

B.I.M.-J. and Y.P.A.: original draft preparation, formal analysis, review, and editing. H.-J.K.: conceptualization, methodology, original draft preparation, and formal analysis. C.-J.L., G.-H.J., I.-H.P., H.P., and S.K.: formal analysis. S.C.P.: original draft preparation, review, and editing. S.-J.C.: conceptualization, original draft preparation, funding acquisition, review, and editing.

Data Availability Statement

All data generated for the present study are included in the manuscript. The gene sequences used in this study are deposited in GenBank. GenBank accession numbers are presented in online supplementary Table S1.

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