Hedgehog signaling regulates imaginal cell differentiation in a basally branching holometabolous insect

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ABSTRACT

The evolution of imaginal cells, or stem cell-like cells, contributed to the spectacular diversification of holometabolous insects, which undergo complete metamorphosis. The proliferation and differentiation of these imaginal cells is under the control of juvenile hormone (JH), but which patterning genes respond to JH is currently unknown. Here, the role of Hedgehog (Hh) signaling in the development of imaginal cells was investigated. RNA interference-mediated knockdown of the components of the Hh signaling pathway showed that Hh is required for the proliferation of polymorphic and imaginal cells in Tribolium castaneum. Hh was also necessary for the regeneration of larval appendages. In contrast, knockdown of Hh signaling antagonists, patched and costal 2 led to the overgrowth and precocious maturation of structures derived from imaginal cells and the occasional appearance of ectopic appendages from the head epidermis. In addition, JH suppressed the expression of hh both in vivo and in vitro. Our findings suggest that imaginal cells are created and maintained by modulating Hh signaling. Thus, Hh signaling may have played a critical role during the evolution of complete metamorphosis.

KEYWORDS

Imaginal cells; juvenile hormone (JH); metamorphosis; Hedgehog (Hh) signaling; Tribolium castaneum
INTRODUCTION

The origin of insect metamorphosis has been debated for centuries and remains unresolved. Various theories have been proposed over the last century yet there is little consensus about how metamorphosis may have evolved (Belles and Santos, 2014; Erezyilmaz, 2006; Konopova et al., 2011; Truman and Riddiford, 1999). Specifically, two alternative views have been proposed to explain the origin of larval morphology. One posits that the larva is equivalent to the nymphal stages of hemimetabolous insects, insects that undergo incomplete metamorphosis (Belles and Santos, 2014; Hinton, 1963; Konopova et al., 2011; Konopova and Zrzavy, 2005). The other proposes that the larva is equivalent to the hemimetabolous embryo stage and that heterochronic shift in the timing of adult tissue maturation led to the origin of larval morphology (Berlese, 1913; Truman and Riddiford, 1999). To resolve these issues, the developmental genetic underpinnings of adult tissue maturation need to be understood. A distinct feature of larval development is the formation of imaginal cells or the delay of complete differentiation of larval cells (Truman and Riddiford, 2007). How these cells gained the ability to delay maturation remains unknown, but this issue is critical to our understanding of the origin of insect metamorphosis. In particular, while we know how the endocrine system influences the timing of metamorphosis (Nijhout, 1998), how the metamorphic hormones interact with the developmental genetic regulation of patterning remains unknown.

Imaginal discs, which give rise to adult structures, have been extensively studied, but this mode of development is in fact highly derived; the more ancestral mode of development involves the formation of imaginal primordia that do not form discs (Truman and Riddiford, 1999; Svacha, 1992). These imaginal primordia differ from imaginal discs in that they do not detach from the larval epidermis and do not proliferate until metamorphosis is initiated (Truman and
Riddiford, 2002, 2007). These imaginal cells may be set aside in small clusters within the larval structures, or they may proliferate during metamorphosis from the larval epidermis, but they do not contribute to the larval structures (Tanaka and Truman, 2005; Truman and Riddiford, 2002; Fig. 1). In most insects, the wings and eyes develop from imaginal cells that proliferate and differentiate at the end of larval life. In some insects, such as Manduca sexta, imaginal cells are also found in legs (Tanaka and Truman, 2005). However, in the more ancestral state, holometabolous insects have simple larval legs and antennae whose cells are thought to create adult appendages (Truman and Riddiford, 2002). These cells are called polymorphic cells as they contribute to both the larval and adult appendages (Fig. 1). Alterations to patterning in the larval legs are carried over to the adult legs, indicating that there is developmental continuity between the larval and adult structures, unlike imaginal cells that can give rise to several different structures (Lee et al., 2013). The nature of these polymorphic cells remains unclear, however, and the cell fates of these polymorphic cells can be re-specified during metamorphosis (Angelini et al., 2009; Shippy et al., 2009), indicating that they retain some level of potency throughout their larval development. During metamorphosis, differentiation is completed and adult structures form. Thus, both imaginal cells and polymorphic cells represent undifferentiated or incompletely differentiated precursor cells. Insects, such as Megaloptera and Neuroptera as well as the hymenopteran suborder Symphyta all lack imaginal discs (Svacha 1992). Thus, imaginal cells and polymorphic cells represent the more ancestral mode of adult development, and studying the development and evolutionary origin of these cells holds the key to understanding the origin of metamorphosis (Truman and Riddiford, 2002). For the remainder of this paper, we will group them together under the broad term of precursor cells.
The sesquiterpenoid lipid hormone, juvenile hormone (JH), has been shown to act as a morphostatic hormone, which suppresses the morphogenetic growth of these precursor cells (Truman et al., 2006). JH is a key developmental hormone in insects, and it has been linked to the regulation of life history transition by acting as a status quo hormone (Riddiford, 1996; Truman et al., 2006). This action of JH is typically conferred through JH modifying the effects of the molting hormone ecdysteroids (Jindra et al., 2013). A much less understood action of JH is the morphostatic action of JH, which inhibits imaginal cell morphogenesis independent of the action of ecdysteroids (Truman et al., 2006; Truman and Riddiford, 2007). Understanding how JH inhibits the proliferation of polymorphic and imaginal cells has implications for understanding how these cells arose in the first place.

Here we wished to determine the mechanisms underlying the development of precursor cells using the red flour beetle Tribolium castaneum. In tenebrionid beetles, wings and compound eyes develop from imaginal cells at the end of the larval stage. In contrast, the simplified larval legs and antennae appear to be comprised of polymorphic cells (Huet and Lenoir-Rousseaux, 1976; Nagel, 1934; Truman and Riddiford, 2002). Thus, in this species, a more ancestral mode of development is used to pattern the adult appendages. In the present study, we wished to identify the mechanism that regulates the morphogenetic growth of precursor cells and determine how this mechanism interacts with JH. Specifically, we investigated the role of Hedgehog (Hh) signaling in appendage development because a preliminary RNA-seq analysis of late 6\textsuperscript{th} instar legs and prepupal legs revealed hh to be upregulated in prepupal legs.

Hh signaling pathway has been well characterized in Drosophila (Ingham et al., 2011). In the presence of the Hh ligand, the phosphorylation of transmembrane protein Smoothened (Smo)
allows the scaffold protein Costal 2 (Cos2) and the Suppressor of fused (Sufu) to be phosphorylated by Fused (Liu et al., 2007; Lum et al., 2003). Consequently, the transcription factor, Cubitus interruptus (Ci) separates from Sufu and Cos2, translocating into the nucleus where it activates the transcription of Hh target genes. In the absence of the Hh ligand, the Hh receptor Patched (Ptc) acts as an inhibitor of Smo (Beachy et al., 2010; Taipale et al., 2002). The inhibition of Smo results in Cos2 forming a signaling complex composed of multiple serine and threonine kinases. This complex recruits Ci where it is then phosphorylated and ultimately cleaved (Ingham et al., 2011; Sisson et al., 1997). The cleaved form of Ci then acts as a repressor transcription factor, preventing the transcription of Hh target genes (Ingham et al., 2011). Thus, Ptc and Cos2 both act as negative regulators of Hh signaling.

We examined the role of Hh signaling during larval development and metamorphosis in the flour beetle, Tribolium castaneum, by silencing the expression of hh and the Hh signaling antagonists ptc and cos2 through RNA interference. In Tribolium, knockdown of hh leads to severe deformation of embryonic limbs accompanied by disruption and loss of body segmentation (Farzana and Brown, 2008). However, we currently do not know what role it plays during postembryonic development. We also examined the effect of hh removal during larval limb regeneration. Limb regeneration in Tribolium follows three distinct stages, which are regulated by distinct processes: wound healing, blastema formation and re-patterning (Lee et al., 2013). We investigated whether Hh might play a role during any of these stages. Our study shows that Hh is necessary for proper limb regeneration. We also show that Hh signaling is required for postembryonic development and that it is repressed by JH to prevent precocious development.
MATERIALS AND METHODS

Beetle husbandry

Wildtype Tribolium castaneum strain GA1 was obtained from Dr. Richard Beeman (USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas). All beetles were raised on organic whole wheat flour containing 5% nutritional yeast and kept in an incubator at a constant temperature of 29ºC and 50% relative humidity. In our laboratory, most of the wildtype larvae undergo seven or eight instars.

RNA isolation

In order to amplify and clone cDNA from Tribolium larvae for eventual dsRNA synthesis and injection, RNA was isolated from Tribolium at random larval stages. Larval tissue was dissected in 1X-phosphate-buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, 0.0038 M NaH₂PO₄, 0.0162 M Na₂HPO₄; pH 7.4) to remove the fat body and gut. The remaining tissue was homogenized in TRIzol (Life Technologies) and treated with chloroform in order to isolate RNA. The RNA was sequentially treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer’s instructions.

cDNA synthesis and polymerase chain reaction

cDNA was synthesized from 1 μg of RNA using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The sequences for hh (GenBank
accession number NM_001114365), *ptc* (GenBank accession number NM_001134377) and *cos2* (GenBank accession number XM_968298) were obtained from GenBank and Beetlebase. Primers for these genes were designed (Table S1) and used to amplify cDNA through a polymerase chain reaction (PCR). PCR products were then analyzed by agarose gel electrophoresis. cDNA was extracted from the gel using the MinElute Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions.

**Cloning and dsRNA synthesis**

Isolated PCR product was cloned into the pCR-4 TOPO-TA vector (Life Technologies). Plasmids from the cloned vector were purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. After the verification of the inserts through sequencing, the plasmids were digested using Spe1 and Not1 restriction enzymes (NE Biolabs). Single-stranded RNA (ssRNA) was synthesized using the T3 and T7 MEGAscript Kits (Ambion) according to the manufacturer’s instructions. Equal amounts of complementary ssRNA were then used to create a 2 μg/μl solution of dsRNA in diethyl pyrocarbonate-treated water. dsRNA was annealed as described previously by Hughes and Kaufman (2000). The annealed product was analyzed by agarose gel electrophoresis and compared to ssRNA to confirm proper annealing.

**dsRNA injections**
Approximately 0.5 μg of either hh, cos2, or ptc dsRNA was injected into either day zero sixth or seventh instar larvae. For third or fourth instar, larvae were injected until the body expended. Larvae were anesthetized by placing the animals on ice. Injections were done using pulled-glass capillary tubes where the needle containing dsRNA was inserted between the first and second abdominal segments on the dorsal side of the larva. Control animals were injected with the same amount of bacterial ampicillin resistance (amp') dsRNA (plasmid obtained from Dr. Takashi Koyama, the Gulbenkian Institute of Science, Portugal). After injections, animals were separated into individual plastic containers with whole wheat flour and maintained under normal conditions. Phenotypes were observed every three to four days after injection and daily after the pre-pupal stage. To ensure that the Hh knockdown phenotypes were not due to off-target effects, another region of the gene was cloned and dsRNA was synthesized. Knockdown of hh using the two non-overlapping regions of the hh gene led to similar phenotypes, indicating that the phenotypes reported here were not due to off-target effects. Ptc and Cos2 both act as antagonists of Hh signaling, so they served as off-target controls for each other.

Leg ablations

Larval mid- and hind legs were ablated two days after injection to ensure that the RNAi-mediated knockdown was in full effect. Larvae anesthetized on ice were placed ventral side up and mid- and hind-legs were cut close to the base of the femur. All cuts were made with fine microscissors under a dissecting microscope. The forelegs and the contralateral mid- and hind-legs served as internal controls for regeneration analysis. Following leg ablations, larvae were kept in normal conditions, and observations on regenerating legs were recorded after each molt.
in the larval stage as well as after metamorphosis into the pupa. For antenna regeneration studies, larval antennae from the left side of the head were ablated using a razor blade. The contralateral antenna served as the internal control. Following antenna ablations, larvae were kept in normal conditions, and observations of regenerating antenna were recorded after each molt in the larval stage as well as after metamorphosis into the pupal stage. Animals were stored in a solution containing 15% glycerol and 70% ethanol at -20°C until imaged. Larval legs and antennae of both hh and amp control animals were then dissected and mounted in an 80% glycerol solution. Because larval tissues produce their own autofluorescence, mounts were examined using a Nikon Eclipse 80i fluorescence microscope. Images were taken using a QImaging camera (Diagnostic Instruments) and NIS Elements Imaging Software. All images were compiled using ImageJ.

BrdU staining of proliferating cells

In order to see the effect of hh knockdown on cell proliferation, BrdU cell proliferation staining assay was conducted on hh dsRNA-injected animals. Day zero prepupae were anesthetized and then dissected in 1X-PBS solution. The ventral section containing the larval limbs were dissected and incubated in a solution of 20 μg/μL of BrdU at room temperature for three hours. The dissected portion was fixed in a solution of 3.7% formaldehyde in 1-X PBS. Within an hour of formaldehyde fixation, the tissue was placed back in PBS solution where the leg tissues were dissected out and placed back in the formaldehyde solution. After an overnight fixation, the tissue was washed with a solution of PBS with 1% Triton-X 100 (PBS-TX). The tissue was then submerged in a solution of 2 N HCl in PBS-TX for one hour at 37°C. After treatment, the acid solution was removed and the tissue was rinsed in PBS-TX. Tissues were then blocked with 5%
NGS in PBS-TX for 30 min. at room temperature. Post-blocking, tissues were incubated in 1:200 anti-BrdU antibody in PBS-TX overnight at 4°C. Subsequently, the anti-BrdU antibody was removed and the tissue was rinsed again. The tissue was then incubated in a solution of 1:1000 Alexa Fluor 488 goat anti-mouse antibody in PBS-TX overnight at 4°C. The secondary antibody was removed and after several washes in PBS-TX, the tissue was mounted in either Vectashield or 100% glycerol. Mounted tissues were imaged using the Nikon Eclipse 80i fluorescence microscope, and images were taken using a QImaging camera (Diagnostic Instruments) and NIS Elements Imaging Software. Images were stacked using ImageJ (NIH Image).

TUNEL staining of apoptotic cells

To determine the pattern of cell death, prepupal legs were stained with a TUNEL cell apoptosis-staining assay. Early- and late-stage prepupae were dissected in 1X-PBS solution, and the thoracic segments were fixed for 30 minutes at room temperature in 3.7% formaldehyde. The prepupal mid- and hindlegs were then dissected out of the cuticle and fixed for an additional 30 min. The fixed legs were washed 3 times in PBS-TX, and then incubated in 20 µg/mL Proteinase K in PBS for 30 minutes at 37°C. The legs were then washed two times in PBS. Positive control legs were incubated in a 10 units/mL DNase solution for 30 minutes, after which positive control legs and experimental legs were treated with 125 µL of TdT solution (10% enzyme solution and 90% label solution) from the in situ cell death detection kit (Roche) as described by the manufacturer. Negative control legs were treated with 50 µL of 100% label solution. All tissues were covered immediately after treating to avoid light exposure. All legs were then incubated for 1 hr at 37°C in the dark. Finally, the legs were washed 5 times in PBS-TX and twice in PBS. The
legs were mounted in VectaShield and imaged using the Nikon Eclipse 80i fluorescence microscope. Images were taken with QImaging camera (Diagnostic Instruments) and NIS Elements Imaging Software. Images were stacked using ImageJ (NIH Image).

Methoprene treatment and tissue culture

To assess the expression of *hh* in the whole animal in the presence of JH, the JH analogue, 0.5 µl of methoprene (30 µg/µl in acetone) was ectopically applied to the dorsal side of day zero final instar larvae using a micropipette. For control larvae, 0.5 µl of acetone, the solvent used for methoprene treatments, was applied. Larvae were raised in flour as described above and whole bodies were harvested in TRIzol daily until day four. For *in vitro* assays of *hh* expression, ventral thoracic plates with six legs attached from day one eighth instar larvae were dissected and cultured in 1mL Grace’s insect tissue culture medium (Life Technologies/GIBCO) supplemented with 10% FCS and 10% microbial inhibitors (Life Technologies/GIBCO antibiotic-antimycotic).

Cultures were sealed in 95% oxygen and incubated at 29ºC. After 24 hrs, tissues were cultured with the addition of either no hormones or 1 µg/mL methoprene (Sigma-Aldrich) as described in Koyama et al (2008). After an additional 24 hours, tissues were collected and stored in TRIzol for qPCR analysis.

Quantitative PCR (qPCR)

Expression profile of *hh* and *ptc* in the presence and absence of juvenile hormone (JH) during metamorphosis was analyzed using qPCR. The treated animals were collected daily until day
four and their RNA was isolated as described above. RNA (1 µg for whole body or 0.5 µg for legs) was converted to cDNA as described above and mixed with SYBR Green Supermix (Bio-Rad) and qPCR primers of either hh, ptc, or Ribosomal protein 49 (rp49). Each sample underwent 50 cycles at 60°C. Three biological replicates of each treatment were made and analyzed to ensure the reproducibility of the data. Each biological sample was assayed in triplicate. For the whole body analysis, three animals were used per biological sample. For the tissue culture analysis, each biological replicate consisted of 6 leg plates for a total of approximately 36 legs.

Semi-quantitative RT-PCR and knockdown verification

Knockdown of hh, ptc and cos2 expression in dsRNA-injected animals was verified through semi-quantitative reverse transcription-PCR (RT-PCR). Day zero seventh instar larvae were injected with the respective dsRNA, and two or three pre-pupa were used for RNA isolation. amp′ dsRNA-injected prepupa were used as controls. After RNA isolation, 1 µg of RNA from each treatment group was converted into cDNA and semi-quantitative RT-PCR was done on these samples. rp49 was used as a control to ensure that equal amounts of cDNA were used for each reaction. Thirty-four or thirty-five cycles were used. Two replicate RT-PCRs were performed to ensure repeatability of the knockdown verification. Primers are given in Table S1.

RESULTS

Hh is required for adult limb patterning and compound eye development
To characterize how silencing hh expression affects normal development in Tribolium, hh dsRNA was injected into day zero sixth instar larvae. Of the larvae that survived past the first larval molt following injections (n=18), 30% (n=5) survived to the pupal stage, with the rest dying primarily during the larval molts or pre-pupal stage. The hh dsRNA-injected animals that survived to pupate died upon pupation. Hh knockdown pupae developed severe limb abnormalities. The wings were reduced to small outgrowths (Fig. 2G), and the pupal terminal appendages were rounded (Fig. 2I) and diminished in size compared to the control amp' dsRNA-injected pupae (Fig. 2A,C). The antennae of hh-knockdown animals were also considerably reduced in size compared to those of the control amp' dsRNA-injected animals (Fig. 2D,E,J,K). In addition, pupal legs were severely reduced in width, and some exhibited branching patterns that were not seen in normal pupal legs (Fig. 2F,L,M). The severely affected leg segments lacked the normal tarsal and tibial morphology, and instead were threadlike and often contorted (Fig. 2M). The femur also appeared mildly affected; its morphology was more bulbous than the normal femur (Fig. 2F,M).

To determine how leg development was impacted by hh knockdown, cell proliferation in prepupal legs was analyzed using BrdU staining (Fig. 3A). amp'-dsRNA injected early prepupae exhibited extensive cell proliferation throughout the legs, indicating that most of the larval leg transforms into the adult leg (n=4; Fig. 3A). This together with the observation that there is limited cell death (Fig. S1) indicate that larval legs are largely composed of polymorphic cells, as has been suggested previously (Huet and Lenoir-Rousseaux, 1976; Truman and Riddiford, 2002). In hh dsRNA-injected prepupal legs, cell proliferation was observed only in the proximal tibial region and the distal portion of the tarsus (n=9; Fig. 3B). These observations suggest that Hh is required for the proliferation of a large portion of the larval leg. However, the pupal legs...
still developed with more or less normal proximodistal pattern, indicating that either cell growth or cell proliferation in the two BrdU-positive regions contribute to the bulk of the proximodistal growth of the pupal leg.

Eye development during the pupal stage was also drastically impacted by the silencing of Hh expression. Compound eyes normally begin to develop in day zero pupae (Fig. 2B). However, hh knockdown animals lacked any signs of compound eye development during this stage (Fig. 2H). They still retained the larval stemmata, but the band of compound eyes normally seen in the amp dsRNA-injected day zero pupae were completely absent. Together, these results imply that Hh is required for proliferation of polymorphic limb patterning and imaginal cell derived compound eye development in Tribolium.

Knockdown of Hh expression prevents larval limb re-patterning

To investigate the role of Hh signaling during larval leg regeneration, we examined the effect of Hh knockdown during limb regeneration. hh dsRNA was injected into day zero sixth instar larvae, and the mid- and hind- legs, or the antenna, on one side of the animal were ablated two days later. In amp dsRNA-injected control animals, wound healing occurred and blastema-like structures formed after the first larval molt (Fig. 4A,5A). After the second larval molt, segments were reformed, and the legs and the antenna began to take on the normal morphology (Fig. 4B,5B).

In hh knockdown animals, all larvae showed complete wound healing of their ablated legs and antennae similar to amp dsRNA-injected control animals after one molt (Fig. 4C,5C). Along with wound healing, most of the hh dsRNA-injected larvae formed rounded blastema-like
structures at ablation sites. After the second molt, none of the hh knockdown larvae showed any signs of regeneration (Fig. 4D,5D). Rounded structures seen in the subsequent molt were the only apparent outgrowths. Similarly, larvae that developed into pupae after two molts did not show any signs of regenerated pupal legs (n=12; Fig. 4E,F). The rounded mass of cells apparent during the larval stage disappeared in the pupal stage and instead, sites of leg ablation were completely devoid of any limb structures as indicated by the flattened and smooth cuticle. In contrast, in the amp's dsRNA-injected pupae, legs were fully regenerated as long as the larva molted once before pupation. The hh-knockdown pupae also failed to regenerate their antennae (n=9; data not shown). Thus, Hh is required for the re-patterning of both the legs and antennae in Tribolium.

Knockdown of ptc and cos2 expression results in ectopic appendage outgrowths

Ptc and Cos2 act as antagonists of Hh signaling, and removing these results in the upregulation of Hh signaling (Ingham et al., 2011). In order to characterize the effect of overactivating Hh signaling in Tribolium development, ptc or cos2 dsRNA was injected into day zero sixth and seventh instar larvae. With the exception of a few larvae, 87% of all ptc dsRNA injected animals and 98% of all cos2 dsRNA injected animals showed a defective phenotype within the first two molts post injection (ptc: n= 53; cos2: n=51). Knockdown of either ptc or cos2 expression caused alterations to the larval limb and head morphology (Fig. 6). The comparable phenotypes observed suggest that both cos2 and ptc knockdowns disrupt the Hh signaling pathway in similar ways. Limb morphology was most commonly altered in these knockdown animals (ptc: n=36/53; cos2: n= 36/51). The legs were affected to varying degrees
but in all cases, outgrowths were apparent on the legs (Fig. 6M,N). These outgrowths were found along the femur, tibia, and tarsus and showed variability between individuals. Depending on the size and nature of the outgrowth, the knockdown led to legs with small bumps or dramatically thickened legs (ptc: n=19/53; cos2: n=17/51; Fig. 6M,N). In more severely affected legs, the leg segments disappeared (Fig. 6M). In addition, some legs had sclerotized teeth-like projections along the proximo-distal axis (Fig. 6N, arrow). Duplication of the claw was also seen in some animals (ptc: n=4/53; cos2: n=7/51; Fig. 6N). Normal Tribolium larvae only have a single claw on each leg; two clawed legs are only seen in the adult morphology.

Morphological changes were also seen in the larval head and body of ptc and cos2 knockdown larvae. Larval antennal morphology was severely modified by silencing either ptc or cos2 expression. In some animals, the normally smooth and straight segmented antennae became curved and thick (ptc: n=12/53; cos2: n=20/51; Fig. 6I). In more severely affected larvae, the antennae appeared flattened and spherical (ptc: n=1/53; cos2: n=2/51; Fig. 6G). In addition, ectopic bumps developed on the head of ptc and cos2 knockdown animals (ptc: n=4/53; cos2: n=7/51; Fig. 6F,I,J,K). In all ptc and cos2 knockdown animals with two outgrowths, the bumps were either associated with the antennae (Fig. 6F,I) or appeared as ectopic outgrowths from the head cuticle (Fig. 6J,K). Ectopic eyes were also occasionally seen (Fig. 6F; n=2/6 in cos knockdown larvae). In addition, in a few ptc and cos2 knockdown larvae, protrusions were observed in the anterior second and third thorax segments (Fig. 6C; n=3/6 in cos knockdown larvae). As these segments are where the wing buds emerge from, we suspect that these outgrowths may be premature wing buds. In addition, compound eye-like structures were observed in a few larvae (Fig. 6I; n=5/6 in cos knockdown larvae). In our laboratory, normal larvae undergo around seven larval molts before pupating. To determine whether similar
phenotypes might be obtained in earlier instars, third or fourth instar larvae were treated with 

\textit{cos2} dsRNA. In general, we saw more severe effects when knockdown was initiated at this 

earlier stage. Antenna and leg defects were observed in five out of seven, and three out of seven 

larvae, respectively, after one molt. One out of eight larvae showed development of compound 

eyes and wing-like projection after one molt; an additional two developed compound eyes and 

another developed wing-like outgrowths after a second molt. Thus, upregulation of Hh signaling 

causes precocious maturation of imaginal cell-derived structures.

In one larva, we saw ectopic appendages both near the antennae and in the more medial 

regions of the dorsal head after two molts (Fig. 6D). These structures developed ectopically as 

evidenced by the presence of normal mouthparts on the ventral side (Fig. 6H). Taken together, 

the ectopic structures on the head in \textit{cos2} or \textit{ptc} dsRNA-injected larvae varied considerably 

between animals, from those developing ectopic eyes to those developing ectopic limbs.

In \textit{ptc} knockdown animals, two animals survived through the pupal stage to the adult (n= 

2/18 pupae; Fig. 7). In one pupa, the wings were malformed, and several of the pupal legs 

appeared thicker (Fig. 7D). In another pupa, three ectopic head outgrowths were seen. The two 

more lateral growths had the appearance of compound eyes, while the center head bump 

resembled an appendage (Fig. 7E,F). The phenotypes seen in the pupa persisted to the adult stage 

where they developed into adult compound eyes and an antenna-like appendage (Fig. 7J-L). The 

antenna-like appendage grew out of a socket, indicating that this appendage had joints at the base 

(Fig. 7L, open arrowhead). Thus, in addition to causing precocious maturation of imaginal cell 

derived structures, \textit{ptc} and \textit{cos2} removal appears to cause duplication of head structures.
Confirmation of gene knockdown

In order to verify that the dsRNA-injections resulted in the corresponding gene knockdown, a semi-quantitative RT-PCR was performed. To verify knockdown of hh, seventh instar larvae were injected on day zero with either amp’ or hh dsRNA. cDNA from all animals was collected during the pre-pupal stage and used for knockdown verification. For verifying ptc, and cos2 knockdown of day zero seventh instar larvae were injected with amp’, ptc, or cos2 dsRNA (Fig. 8). RT-PCR showed that the expression of ptc and cos2 were knocked down in the ptc and cos2 dsRNA-injected larvae, respectively, relative to the amp’ dsRNA injected larvae.

hh expression is suppressed by JH

The phenotypes obtained from knockdown of ptc and cos2 suggested that Hh signaling might be suppressed during the larval stage and that it becomes reactivated during the prepupal stages. One potential regulator of Hh signaling is JH. In order to determine whether JH regulates the expression of hh, larvae were treated with the JH mimic methoprene. When methoprene was topically applied to day 0 seventh instar Tribolium larvae, they underwent a supernumerary molt (Table S2). In contrast, acetone-treated animals became prepupae on day five of the seventh instar (Table S2). Next, we used PCR analysis to determine the hh expression in the whole body of seventh instar larvae that were treated with either methoprene or acetone. While the hh expression in methoprene-treated or acetone-treated larvae did not differ significantly during the first three days (not shown), day 4 methoprene-treated larvae had a significantly lower level of hh expression than those treated with acetone (Student’s t-test; P<0.05; Fig. 9A). To determine whether JH can specifically inhibit the expression of hh in the legs in the absence of
ecdysteroids, we cultured the ventral thoracic plates with the six legs with either no hormones or methoprene. We found that in vitro cultured methoprene-treated legs had significantly lower hh expression relative to hormone-free cultures (Student t-test, p<0.0005; Fig. 9B). These results demonstrate that hh expression can be inhibited by JH.

DISCUSSION

One of the major gaps in our understanding of metamorphosis is the link between endocrine regulators and patterning. In insects, precursor cells have been shown to be under the control of a morphostatic action of JH (Truman et al., 2006), but the molecular mechanism underlying this process has not been understood (Truman and Riddiford, 2007). Our findings indicate that Hh is a key regulator of both imaginal and polymorphic precursor cell proliferation and that its activity is inhibited during the earlier larval stages. Based on our qPCR data, we think that JH inhibits the expression of hh during the larval stage to prevent proliferation of these precursor cells.

Knockdown of Hh expression disrupted the development of pre-existing appendages as well as adult specific structures in Tribolium. We observed that cell proliferation was dramatically diminished in the prepupal legs. This appears to be distinct from the role of Hh during embryogenesis as hh is not expressed at the tip of growing limbs (Peel et al., 2013). Hh-deficient embryos have been shown to have severely reduced limbs, although this was also accompanied by loss of segments (Farzana and Brown, 2008). Given absence of hh expression in wildtype limbs, the reduction of limbs in these hh-deficient embryos is likely caused by segmentation defects, not a direct effect of reduced hh expression in the limb tissue. Thus, hh
may play a distinct role during embryogenesis and metamorphosis. In addition, we have found that Hh plays a role during re-patterning during limb regeneration. Loss of hh led to the formation of a blastema but no re-growth. This is in contrast to crickets where removal of hh RNA leads to the regeneration of forked legs (Nakamura et al., 2008).

The knockdown effects observed here are similar to those of imaginal disc-derived structures observed in Drosophila. In Drosophila appendage imaginal discs, Hh is required for proper patterning and growth (Ingham, 1995; Ingham and Fietz, 1995), and localized upregulation of Ci in Drosophila results in increased cell proliferation in the wing imaginal disc (Duman-Scheel et al., 2002). In addition, Hh also plays a key role in the initiation of the morphogenetic furrow, which is required for the differentiation of eye development in Drosophila (Borod and Heberlein, 1998; Pappu et al., 2003). However, imaginal disc development in Drosophila and polymorphic and imaginal cell development in Tribolium differ in their timing of development. In beetles, the presence of JH inhibits morphogenesis of polymorphic and imaginal cells until the last larval instar (Truman and Riddiford, 1999). In contrast, Drosophila imaginal discs grow and develop throughout the larval stage, indicating that they have lost sensitivity to JH (Truman and Riddiford, 1999). Since knockdown of ptc and cos2, the antagonists of Hh signaling, led to outgrowths and precocious appearance of adult-specific structures in the larval stage, our study suggests that Hh signaling likely acts downstream of JH in Tribolium. This finding is corroborated by the finding that hh expression could be inhibited by JH in the legs. Since phylogenetic analyses show that imaginal disc-mode of development is a derived condition (Truman and Riddiford, 1999), our study suggests that the ancestral state of imaginal precursor cell development involved Hh signaling pathway that was sensitive to JH. The one structure that develops later in Drosophila is the ocellus, or simple eye, which also
depends on Hh signaling. Removal of Hh signaling leads to the loss of ocelli in a manner similar
to the loss of compound eyes in *Tribolium* (Aguilar-Hidalgo et al., 2013; Royet and Finkelstein,
1996). While these structures do not develop until the final instar, they are imaginal disc-
derivatives, so the extent to which they can be compared to *Tribolium* eye development is
unclear.

Our findings suggest that once JH declines at the end of the larval stage, Hh signaling
becomes reactivated, allowing the polymorphic cells to complete differentiation. In addition, our
results show that Hh overactivation leads to precocious maturation of imaginal cells. Thus,
suppression of Hh is necessary for proper maintenance of larval appendages, whereas activation
of Hh is required for appendage remodeling during metamorphosis. These observations are
consistent with Hh signaling being a target of the morphostatic action of JH (Truman et al.,
2006). It is particularly noteworthy that *hh* expression *in vitro* can be suppressed by JH in the
absence of 20E. This is consistent with the morphostatic action of JH which has been shown to
occur independently of 20E (Truman et al., 2006). Thus, we suggest that the absence of JH at the
onset of metamorphosis contributes to the Hh-dependent maturation of precursor cells.

The only larval tissues that overgrew when *ptc* or *cos2* was knocked down were those
tissues derived from polymorphic and imaginal cells. Interestingly, knockdown of *ptc* or *cos2*
also led to ectopic appendages and eyes on the dorsal head where appendages normally do not
develop. Since dsRNA was administered during the late larval stage, these structures had to have
developed from cells within the epidermis. This indicates that the cells within the larval
epidermis retain remarkable plasticity and that these cells can proliferate by altered expression of
Hh signaling. Perhaps it is not surprising that novel structures such as head horns of dung beetles
can evolve from the head epidermis. In fact, beetle horn precursor cells express limb patterning
genes (Moczek and Nagy, 2005; Moczek and Rose, 2009). It will therefore be interesting to determine the contribution of Hh signaling on the evolution of novel structures.

Finally, the origin of precursor cells remains an enigma that is the crux of the problem of the evolution of insect metamorphosis. Two major theories have been proposed, which remain contentious (Erezyilmaz, 2006; Konopova et al., 2011; Konopova and Zrzavy, 2005; Truman and Riddiford, 1999): the first proposes that the hemimetabolous pronymph stage seen in the embryo became the holometabolous larval stage through a heterochronic shift in the timing of tissue maturation (Berlese, 1913). In contrast, the second view holds that the hemimetabolous nymphs are equivalent to the holometabolous larva and that the final nymphal instar is equivalent to the holometabolous pupal stage (Hinton, 1963). Our study suggests that the shift in the timing of proliferation of Hh-dependent cells led to the evolution of larval morphology. Specifically, by delaying the complete differentiation of Hh-dependent cells, the complex adult morphologies do not develop until metamorphosis. Truman and Riddiford argued that holometaboly is a result of a heterochronic shift towards the embryonic state that was caused by a shift in the timing of embryonic JH secretion (Truman and Riddiford, 1999). A shift to an earlier secretion of JH would result in the retention of embryonic features upon hatching (Truman and Riddiford, 1999). Our study provides a potential mechanism by which JH would delay the maturation of Hh-dependent cells and create precursor cells. Alternatively, it is possible that Hh was recruited for appendage patterning in holometabolous insects. By recruiting Hh signaling, precursor cells could delay their maturation and form the distinct larval forms. In support of this hypothesis, preliminary studies in our lab show that legs can develop without dramatic patterning defects in hh-knockdown Oncopeltus fasciatus embryos (D. Jing, unpublished results).

We suggest that the recruitment of Hh signaling in adult tissue maturation allowed larvae
to retain tissues in an undifferentiated state. The Hh signaling pathway has been shown to maintain stem cell identity in many different types of stem cell niches (Lai et al., 2003; Martinez-Agosto et al., 2007; Ramalho-Santos et al., 2000; Zhang and Kalderon, 2001). Thus, the use of Hh signaling in patterning may have facilitated the evolution of metamorphosis by providing a mechanism for generating imaginal cells.

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FIGURE LEGENDS

Figure 1. Precursor cell types of various holometabolous insects. (A) Wing development in Tribolium, Manduca and Drosophila. Tribolium lack wing imaginal discs and instead form wings from imaginal cells during the final larval instar. Manduca and Drosophila develop wing imaginal discs during embryogenesis. (B) Leg development in Tribolium, Manduca and Drosophila. Tribolium legs appear to develop from polymorphic legs where most of the larval cells contribute to the adult legs. Much of the Manduca larval leg cells undergo apoptosis during the final instar. The adult leg derives largely from imaginal cells that begin proliferating during the last larval instar; a small portion of adult leg derives from polymorphic cells (Tanaka and Truman, 2005). Drosophila develop leg imaginal discs that develop during embryogenesis.

Figure 2. hh knockdown disrupts pupal appendage and eye phenotypes in Tribolium. (A-F) Day zero control pupa injected with 2 μg/μL amp' dsRNA at day zero sixth instar. (G-L) Day zero pupa injected with 2 μg/μL Hh dsRNA at day zero sixth instar. Images shown are the whole body (A, G), eyes (B, H), terminal appendages (C, I), dorsal head (D, J), ventral head (E, K), and midleg (F, L). (M) Strongly affected day two Hh knockdown pupal legs. The femurs in (F) and (M) are indicated by arrowheads.

Figure 3. hh knockdown causes reduced prepupal leg cell proliferation in Tribolium. (A) BrdU staining in prepupal leg injected with 2 μg/μL amp' dsRNA (A) or 2 μg/μL hh dsRNA (B) on day zero sixth instar larva. (B, right) Leg with colors inverted, and the localized regions
lacking cell proliferation highlighted in yellow to emphasize the contrast. Scale bars represent 0.5mm.

**Figure 4.** *hh* knockdown prevents larval leg regeneration in *Tribolium*. All animals were injected with 2 μg/μL *amp*’ (A, B) or *hh* dsRNA (C, D), and mid- and hind-legs were cut two days after. (A, C) Regenerating larval legs one molt after ablation (arrowheads). (B, D) Regenerating larval legs two molts after ablation (arrowheads). Middle panel shows the mid-leg and right panel shows the hind-leg. (E) Hindlegs of control pupa injected with 2 μg/μL *amp*’ dsRNA at day zero sixth instar and ablation performed two days later. (F) Hindlegs of strongly affected pupa injected with 2 μg/μL *hh* dsRNA at day zero sixth instar and ablation performed two days later. Right panels for E and F highlight the leg morphology. Ablated leg is indicated by an asterisk.

**Figure 5.** *Hh* knockdown prevents larval antennal regeneration. Larvae were treated with 2 μg/μL *amp*’ (A, B) and *hh* (C, D) dsRNA. The left panels show the head of the treated larvae, the middle panels show the unablated antennae and the right panels show the ablated antennae.

**Figure 6.** *ptc* and *cos2* knockdown leads to the limb overgrowth and formation of ectopic imaginal cell-derived structures on the head and body of *Tribolium* larvae. (A, E, I) Examples of ninth instar control larvae injected with 2 μg/μL *amp*’ dsRNA on day zero seventh instar. (B, F, I, N) Ninth instar larvae injected with 0.5 μg/μL *ptc* dsRNA on day zero seventh
instar. White arrows indicate antenna-associated bumps (F, J). Black arrows indicate the formation of the compound eye (F, J). White arrowhead indicates the modified antenna (J). (C, G, K, L) Ninth instar larvae injected with 2 μg/μL cos2 dsRNA on day zero seventh instar. (C) Dorsal view showing the wing-like outgrowths (green arrowhead). (G) Dorsal view with modified antenna (white arrowheads). (K, L) Lateral (K) and ventral views (L) showing the ectopic outgrowths (white arrows). (D, H) A sixth instar larva injected with 2 μg/μL cos2 dsRNA on day zero fourth instar. Ectopic mouthpart-like structures developed from the dorsal head (D, open arrowheads). The same animal also developed additional ectopic bumps (white arrow) near the antennae (white arrowheads). The structures are outlined with dotted white lines. These animals retained their normal mouthparts (open arrowheads) as seen from the ventral side (H). White arrowheads represent antennae. (M-O) Legs from ninth instar larvae injected with 2 μg/μL ampR dsRNA (M), 0.5 μg/μL ptc dsRNA (N) or 2 μg/μL cos2 dsRNA (O) on day zero seventh instar. Arrows indicate the duplicated claw and sclerotized teeth-like projections.

Figure 7. Knockdown of ptc leads to ectopic structures on the pupal and adult head of Tribolium. (A-C) Control pupa injected with 2 μg/μL ampR dsRNA on day zero seventh instar larva. (D-F) Pupa injected with 0.5 μg/μL ptc dsRNA on day zero seventh instar. (E, F) Ectopic structures on the head. (G-I) Adult injected with 2 μg/μL ampR dsRNA on day zero seventh instar larva. (J-L) Adult injected with 2 μg/μL ptc dsRNA on day zero seventh instar. Adult is the same animal as the pupa seen in (D-F). (L) ptc dsRNA-injected adult whose pupal cuticle was removed. The ectopic appendage on the head was removed. White arrowheads indicate the ectopic compound eyes (E, F, K, L). Arrows indicate the ectopic head appendage (E, J). Open arrowhead indicates the socket that the ectopic appendage grew out of.
Figure 8. Knockdown verification of dsRNA injected larvae. (A) Knockdown verification of hh. (B) Knockdown verification of cos2. (C) Knockdown verification of ptc. Day zero seventh instar larvae were injected with either 2 µg/µL amp<sup>r</sup>, hh or cos2 dsRNA, or 0.5 µg/µL of ptc dsRNA, and cDNA was synthesized from prepupae. PCR cycle number: amp<sup>r</sup>, cos2, and ptc = 35 cycles; hh and rp49 = 34 cycles.

Figure 9. Effect of JH on hh expression. (A) Whole body expression of hh in day 4 acetone- or methoprene-treated larvae. Error bars represent standard errors. Mean of three biological replicates are shown. Each biological replicate consisted of three animals. The difference is statistically significant (Student t-test, p<0.05). (B) Expression of hh after in vitro culture with no hormone and methoprene (1 µg/ml). Error bars represent standard errors. Mean of three biological replicates are shown. Each biological replicate consisted of 36 legs. The difference is statistically significant (Student t-test, p<0.0005).
Figure 1

A. Wing

Early larva

**Tribolium** (inferred)

Epidermis Imaginal cells

**Manduca/Drosophila**

Imaginal disc

Late larva

Pupa

B. Leg

Early larva

**Tribolium** (inferred)

Polymorphic cells

**Manduca**

Imaginal cells

**Drosophila**

Imaginal disc

Late larva

Pupa

Legend:

- Red: Imaginal cells and their fates
- Light green: Larval/polymorphic cells and their fates
- Blue: Imaginal disc and their fates
- Black: Cell death
Figure 3

A. amnRNAi

B. hhRNAi

C. amnRNAi

Scale bars indicate [unit of measurement].
Figure 4

1 molt after ablation

amp' RNAi

A

C

hh RNAi

B

D

2 molts after ablation

amp' RNAi

E

F

hh RNAi
**Figure 7**

**A**

**amps** RNAi

**B**

**ptc** RNAi

**C**

**amps** RNAi

**D**

**amps** RNAi

**E**

**ptc** RNAi

**F**

**amps** RNAi

**G**

**amps** RNAi

**H**

**ptc** RNAi

**I**

**amps** RNAi

**J**

**amps** RNAi

**K**

**ptc** RNAi

**L**

**amps** RNAi
Figure 8

amp' hh

hh

rp49

amp' cos2

cos2

rp49

amp' ptc

ptc

rp49