cells (Nybakken & Perrimon, 2002; Fontaine *e a* ., 2008). Thus, to better reveal the mechanisms that lie beneath this critical signaling pathway, alternative model systems and innovative experimental strategies should be considered.

The silkworm is an important economic insect that has become an ideal model animal for basic research. The silkworm fat body is an essential organ that functions in nutrition storage, detoxification, and the production of various biologically active metabolites. During maturation of the 5th instar silkworm larvae, both the silk gland and fat body are developing extremely fast. During this time, the regulation of adipocyte differentiation through activation or repression of the Hh signaling pathway may play a role in controlling the quality of both silk and eggs.

In this paper, we investigated the transcriptional profile of Hh signaling pathway components and target genes in adipocyte differentiation throughout the silkworm larval developmental period. The effects of RNA interference (RNAi)-mediated knockdown of *H* and over-expression of *H* on the Hh signaling pathway and the marker gene for adipogenesis were examined. We also examined the effects of cyclopamine (a specific Hh signaling pathway antagonist) on cultured adipose cells and on adipocyte differentiation in silkworm larvae.

Materials and methods

B N ce a d H a ae

The *B.* cell line *B* N (originated from the ovary) was maintained in our laboratory. Silkworm larvae (Strain name P50) were reared under standard conditions (25 ± 2°C, 65% ± 5% relative humidity).

C e H a a c e B. mori *b RT-PCR*

The Silkworm Genome Database (http://www.silkdb.org/silkdb/) was used to identify Hh pathway components: the secreted Hh protein itself, the transmembrane proteins Patched (Ptch) and Smoothened (Smo), the zinc finger transcription factor Cubitus interruptus (Ci), the kinesin-like protein Costal-2 (Cos2), and the protein kinase Fused (Fu). The adipocyte marker gene, AP2, was selected as a candidate gene for adipocyte differentiation. Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA) was used to design primers for reverse transcription polymerase chain reaction (RT-PCR) and real time PCR of these genes (Table S1).

Total RNA was isolated from the fat body of 4th instar larvae using RNAiso Plus (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). The concentration and quality of the isolated RNA was determined on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, NC, USA). First-strand complementary DNA (cDNA) synthesis was performed using 2 μg RNA as the template and the reverse transcriptase, PrimeScript™ RTase (TaKaRa) according to manufacturer's recommendations. The resulting cDNAs were used as templates for PCR expression profiling. PCR was carried out with Ex-*Ta* polymerase for 35 amplification cycles (95°C/30 sec, 52°C/40 sec and 72°C/1 min). PCR products were examined by electrophoresis on a 1% agarose gel stained with ethidium bromide. Amplified PCR products were recovered using an agarose gel DNA purification recovery kit (Sangon Biotech Co. Ltd., Shanghai, China) and cloned into the pMD18-T vector. Insertion was verified by digesting with *X* I and *H* dIII restriction enzymes and insert positive clones were sequenced.

Q a a e ea- e PCR (PCR) a a H a a , a c e

Total RNA extracted from embryos and 1st, 2nd and 3rd instar larvae using RNAiso Plus (TaKaRa) was used as a template in first-strand cDNA synthesis as before. The NCBI EST database (http://www.ncbi.nlm.nih.gov/) and silkDB (http://silkworm. genomics.org.cn/) were used to design specific qPCR primers for amplifying 150–200 bp fragments of the target genes (Table S1). The *Ac 3* (A3) gene was used as a qPCR control gene (Teng *e a* ., 2012).

qPCR was performed on an ABI7300 (Applied Biosystems, Foster City, CA, USA) using the fluorescence dye SYBR® Premix Ex Taq™ (TaKaRa) and a two-step amplification protocol consisting of 30 s at 95°C followed by target amplification via 40 cycles at 94°C for 5 s and 60°C for 31 s. After PCR, the absence of nonspecific amplimers was confirmed by automated melt curve analysis and agarose gel electrophoresis of the products. The transcript levels of the target fragment were normalized to *Ac 3* transcript levels in the same samples. All reactions were performed in triplicate. Gene expression was quantified using the comparative Ct (threshold cycle) method with Pfaffl method for accounting the variations in efficiencies (Bustin *e a* ., 2009). The transcriptional level of the Hh signaling pathway genes in different tissues (epidermis, gonad, fat body, midgut and silk glands) of 1st-day 5th instar larvae as well as gonads and fat body of matured larvae and pupae were similarly quantified.

Ge e a a ec ed H, e -e e ce e

The *B H,* was amplified by RT-PCR using the following primers: F, 5'-ggatccATGAACCAGTGGCC GGGAGT (*Ba* HI site is underlined) and R, 5'-gaattcTCGATATCTATACGATGCTG (*Ec* RI site is underlined). *B H,* was inserted into the PXL-BACII-GFP-*B* U6–Neo^r vector via the same enzymes. The resulting transformation vector was designated as PXL-BACII-GFP-*B* U6-Hh-Neo^r. *B* N cells were seeded onto 24-well plates at a density of 2.0×10^6 cells per well in TC-100 medium (AppliChem GmBH, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 27°C. *B* N cells were co-transfected with the transformation vector PXL-BACII-GFP-*B* U6-Hh-Neo^r and the transposase-producing helper vector DNA (1 : 1) using the transfection agent Lipofectin2000 (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Green fluorescence was observed 24 h post-transfection on an inverted fluorescence microscope. On the 2nd day, the medium was removed, cells were washed twice with phosphate-buffered saline (PBS) and new media containing neomycin G418 (800 μg/mL) was added. This media were replaced every 3–5 days. Clone selection with

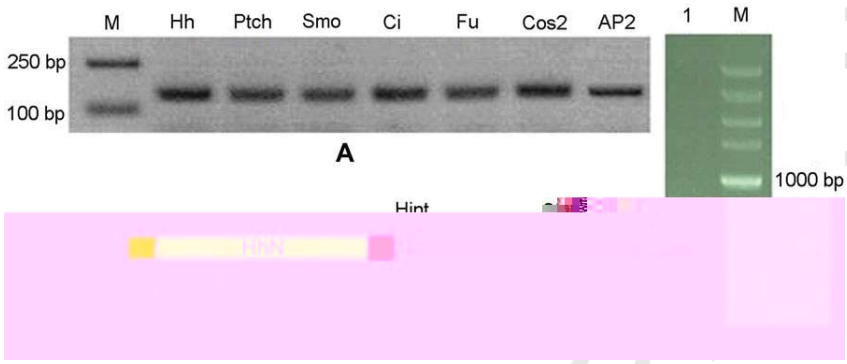

Fig. 1 Reverse transcription polymerase chain reaction (RT-PCR) analysis of Hedgehog (Hh) pathway components in silkworm fat body. A: RT-PCR amplification of fragments of the Hh pathway. B: RT-PCR amplification of the complete Hh coding sequence from silkworm fat body. The amplified product was cloned into pMD18-T vector and sequenced. *B b H,* complementary DNA containing an open reading frame of 834 bp and the deduced protein sequence containing 277 amino acid residues. The predicted molecular weight (MW) was 45 kDa. Lane M: DNA marker; lane 1: *H,* gene. C: The deduced Hh protein has two parts: the Hh amino-terminal signaling domain and a hint module. Domains were determined using the Pfam database (http://pfam.janelia.org/).

sequenced amplimer contained an open reading frame (ORF) of 834 bp and the deduced protein sequence consisted of 277 amino acid residues. The deduced Hh protein has two parts: the Hh amino-terminal signaling domain and a hint module (Fig. 1C). In Figure 1C, the signal peptide sequence for protein export (SS, yellow), the amino-terminal signaling domain (HhN, green), and the autocatalytic carboxy-terminal domain (HhC, black) are indicated. The Hog domain itself can be separated into two regions; the first two-thirds comprise a module termed Hint that shares similarity with self-splicing inteins, whereas the carboxy-terminal third binds cholesterol in Hh proteins and has been named the sterol-recognition region (SRR). In Hog proteins other than the Hh, this region is referred to as ARR (adduct recognition region).

T a c a e e e , e H, a a, a d e e a e ea ad ae

To provide insights into the potential functional period of the Hh pathway in silkworm, we extracted RNA from different stages of silkworm larvae and used qPCR to examine Hh pathway component expression. We found that in the early stage of development (embryo to 3rd instar) the transcriptional levels of *H,* and the transmembrane proteins *S* gene underwent a drastic decline, while the *P c* underwent a steady decline as the larval stages progressed (Fig. 2A). Highest expression for the transcription factor *F* appeared in the embryo and declined gradually throughout the early stages of larval development. Two

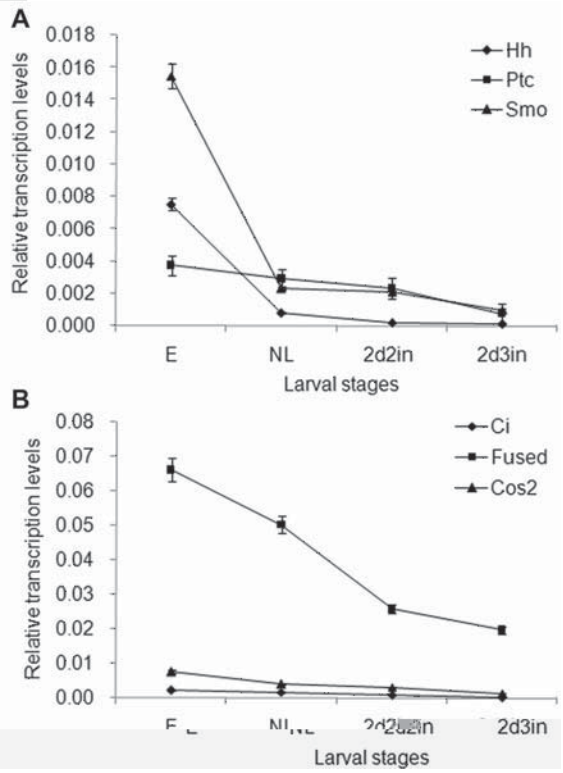

Fig. 2 Transcriptional levels of Hedgehog (Hh) pathway components at different larval stages. E, embryo; NL, newly hatched larvae; 2d2in, 2-day-old 2nd instar larvae; 2d3in, 2-day-old 3rd instar larvae. Graphs display the mean fold difference (triplicate biological replicates) ± SEM.

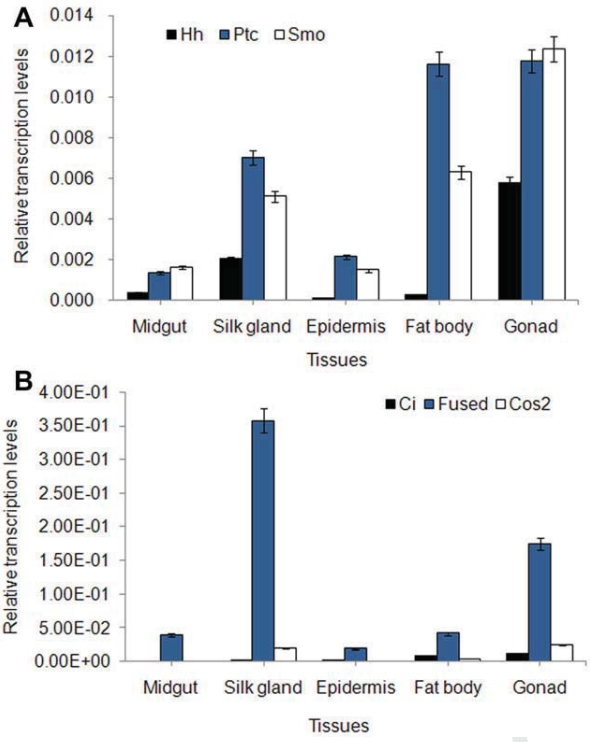

Fig. 3 Expression levels of Hedgehog (Hh) pathway components in different tissues from 3-day-old 5th instar *B b* larvae. Tissues analyzed include midgut, silk glands, epidermis, fat body and gonad. Graphs display the mean fold difference (triplicate biological replicates) ± SEM.

other factors, *C* and *C 2*, in contrast exhibited low expression levels during this same time period (Fig. 2B). These results indicate significant transcriptional activity of the Hh signal pathway in embryos and imply a significant role for these gene products during this developmental period.

T a c a e e e e H a a d e e e H H

The expression levels of Hh pathway components in different tissues (midgut, silk glands, epidermis, fat body and gonad) from 3-day-old 5th instar larvae of *B.* were likewise investigated by qPCR. The results presented in Figure 3A indicate expression of the transmembrane proteins *P c* and *S* in all tissues examined, with highest expression in particular silk glands, fat body and gonad. In contrast, *H* exhibited comparatively lower expression levels. Of the remaining genes, *F* was the more highly expressed transcript with Ci and *C 2* exhibiting relatively low expression (Fig. 3B).

Hed e a a a e H 5

E e e e H a a c e e a b d a e- a e a ae H H

Because 5th instar larvae undergo significant accumulation of adipose cells for pupal development and adult emergence (i.e., cocoon spinning), we next sought to examine the expression profile of the Hh components in the fat body of larvae from 5th instar through pupal development. Highest expression for Hh components was observed in 1-day-old 5th instar larvae but dramatically decreased in mature larvae before rising again at the pupal stage (Fig. 4). According to the growth mode of silkworm larvae, the 5th instar is a transition period for metamorphosis from larva to pupa. During the 5th instar, the growth and development rates of fat body are extremely fast, which are much faster than other growth instars. Then after a short stable period in mature larvae, the fat body will experience a rapid dissociation to scattered cells in blood before recombine to a new fat body. So, during the 5th instar and pupal stages, the fat body will organize a significant change in cell morphology and function. The data that indicate significant transcriptional activity of the Hh signal pathway in these two stages suggest the Hh pathway plays a role in fat body metabolism.

E ec e -e e H cHl a c Bm*N ce*

To investigate the regulatory role of *H* on various Hh pathway components (*P c* , *S* , *C* , *H* and *C 2*) as well as the adipocyte marker gene *AP2*, we examined the effects of exogenous over-expression and suppression of *H* gene in cultured *B* N cells.

The expression vector PXL-BACII-GFP-*B* U6-*H* -Neo^r (Fig. S2) was used to generate a transfected *B* N cell line. After 2 months of G418 selection, a stable *H* -overexpression transfected *B* N cell line was obtained (Fig. S3). Expression levels of Hh pathway components in transfected *B* N cell in relation to non-transformed cells were examined by qPCR. Figure 5A shows that expression of *H* was much higher (about 50 times) in the transfected cells compared to that in the controls. The downstream genes *S* , *C* and *F* also showed higher transcription levels. Conversely, *P c* and *C 2* had lower transcription levels. The *AP2* displayed a decreased transcription level in *H* -overexpression transfected *B* N cell line compared to controls.

In parallel experiments, we sought to examine the effects of *H* knockdown on the transcription of the aforementioned genes. *B H* dsRNA was synthesized to correspond to the full length of *H* and added to *B* N cells.

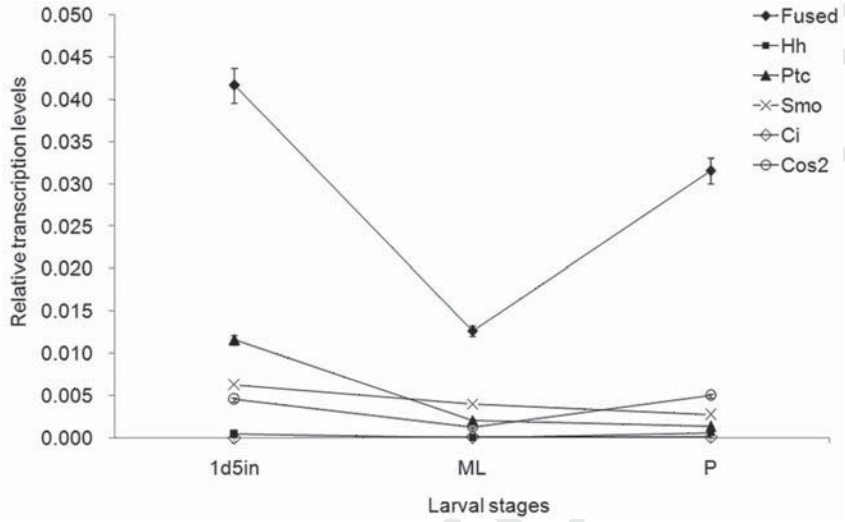

Fig. 4 Changes in the expression levels of the Hedgehog (Hh) signaling pathway components in fat body from 5th instar larvae through pupae. 1d5in, 1-day-old 5th instar fat body; ML, matured larva (just prior to spinning) fat body; P, pupal fat body. Graphs display the mean fold difference (triplicate biological replicates) ± SEM

Three days later, the cells were collected and the comparative expression levels of H_{l} and the related genes were determined. Figure 5B shows that the expression of H_{l} decreased about 44.8% compared with controls, confirming the knockdown efficacy of the H_{l} dsRNAs. Figure 5B also shows that the expression of the other Hh signaling pathway genes likewise decreased: *S* , *C* and *F* . Conversely, *P c*$_{l}$ and *C 2* increased. These results indicate that the target genes for adipogenesis were significantly regulated in the dsRNA-mediated *B* N cells. As expected, the transcription level of *AP2* underwent a notable increase after dsRNA-mediated depletion of H_{l} .

These data support the notion that Ptch and Cos2 are negative regulatory factors, while Smo, Ci and Fu are positive regulatory factors of the Hh signaling pathway. The data also show that expression of the adipocyte marker gene *AP2* changes in relation to H_{l} transcription. *AP2* expression was significantly lower following H_{l} overexpression, but was higher when H_{l} was knocked down. These data are consistent with the idea that the Hh pathway has a negative effect on *AP2*, which implies that this pathway may inhibit silkworm adipose differentiation.

I , b H, a c ea e H ad e e

To provide a baseline for observing adipose cell differentiation in the silkworm, the morphological characteristics of fat body cells were observed. After trypsin digestion (0.25% EDTA-trypsin), fat body cells were visualized as scattered round cells (adipose cells) that attach as a monolayer to culture plates within 12 h. These cells underwent growth and differentiation and exhibited lipid droplet accumulation. The lipid droplets initially accumulated as small clusters, but gradually increased in size as the adipose cells began to differentiate. After a short period, the adherent cells underwent a sharp increase in cell proliferation. In this process, the lipid droplets fused and encompassed the cells within 24 h as the cell membranes thinned, with the adipose cells eventually rupturing. Using these initial observations as a baseline, we next sought to examine the effect of cyclopamine (a Hh antagonist) on the Hh pathway and fat body differentiation.

Adipose cells that had been in primary culture for 12 h (marked as 0 h in Fig. 6B) were treated with 10 μmol/L cyclopamine and its effects on the transcriptional levels of Hh pathway components and *AP2* were determined. We found that the effects of cyclopamine on the Hh pathway were similar to H_{l} knockdown. That is, cyclopamine treatment decreased the transcriptional levels of H_{l} , *S* , *C* and *F* , but increased that of *P c*$_{l}$, *C 2* and *AP2* (Fig. 6A). In contrast, fat-body-specific H_{l} inhibition stimulated fat formation in Drosophila (Pospisilik *e a* ., 2010). We also found that cyclopamine stimulated the morphological changes associated with adipogenesis; that is, the adipose cells treated with cyclopamine accumulated much higher levels of lipid droplets, with most rupturing (Fig. 6B).

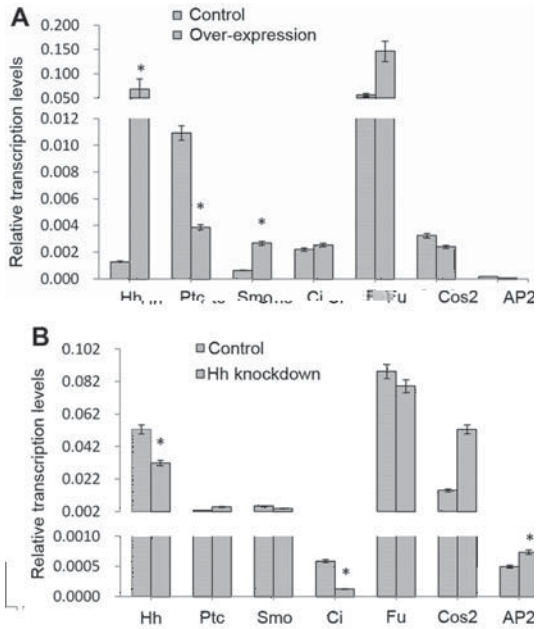

Fig. 5 Transcription levels of Hedgehog (Hh) pathway components and the *AP2* adipocyte marker gene after *Hh* over-expression and knockdown. A: quantitative polymerase chain reaction (qPCR) analyses of the expression levels of genes in normal *B* N cells and transfected cells. The blue bars represent expression levels in control *B* N cells, while the red bars represent expression levels in transfected cells. B: Expression of genes after addition of *B Hh* double-stranded RNA (dsRNA) (10 nmol/L) to *B* N cells. Cells were analyzed for expression levels via qPCR 3 days after addition of dsRNAs. Graphs display the mean fold difference (triplicate biological replicates) ± SEM. *$P < 0.05$.

To further examine this effect, we treated 4th instar larvae with cyclopamine, explanted the larval fat body, and examined the cellular morphology via paraffin sectioning. We found that the size of adipose cells were much bigger in *Hh*$^-$ silkworms (silkworms that were treated with cyclopamine) (Fig. 6C), which implied a higher degree of fat body differentiation.

Discussion

Studies in both vertebrates and invertebrates have identified proteins of the Hh family of secreted signaling molecules as key organizers of tissue patterning (Nybakken & Perrimon, 2002). Despite the importance of the Hh pathway, its role in adipogenesis is unclear (Cousin *e a* .,20ce7(o9s38J/F.121-1.1998TD[(f)e)-227.1(4h)-236.1(5ignaling)-250.1(5ithw)25.4(a)35.6(y)8550.4(com-)pris 2ecr5.6(gev5.7(y).3(re))

the Hh signal has a negative influence on the transcription level of *AP2*. Opposite results were obtained in the *H,* RNAi-mediated knockdown experiment, which showed that the inhibition of the Hh signal could increase AP2 expression. Thus, Hh and AP2 are inversely related. These data are consistent with the idea that Hh pathway components are expressed in the silkworm fat body and that Hh signals may block adipose differentiation.

To determine the potential role Hh signals play in silkworm adipogenesis, a Hh signaling pathway antagonist, cyclopamine, which has the ability to specifically block cellular responses to Hh signaling (Chen *e a*., 2002), was used to treat silkworm fat body and . Through a series of assays, we confirmed the suppressive effects of cyclopamine on Hh signals and the fat body molecular marker AP2, and found that blocking Hh signaling increased fat accumulation and lipid droplet size. Adipose cells from silkworm larvae treated with cyclopamine differentiated faster and secreted more lipid droplets when the Hh pathway was blocked than non-treated controls. Taken together, the specific suppression studies of the Hh signals and support the notion that the Hh pathway inhibits adipocyte differentiation in *B.* .

Next to *D* , *a*, the silkworm represents an ideal model animal for basic research. The study of the silkworm Hh signaling pathway Hh