aptara	INS	ins12164	Dispatch: September 4, 2014	CE: AFL
	Journal	MSP No.	No. of pages: 10	PE: Rod

Insect Science (2014) 00, 1–10, DOI 10.1111/1744-7917.12164

ORIGINAL ARTICLE

Hedgehog signaling pathway regulated the target genes for adipogenesis in silkworm *Bombyx mori* L

Shuang Liang, Rui-Ting Chen, Deng-Pan Zhang, Hu-Hu Xin, Yan Lu, Mei-Xian Wang and Yun-Gen Miao

key Laboratory of Animal Virology of Ministry of Agriculture, College of Animal Sciences, Zhejiang University, Hangzhou, China

Abstract Hedgehog (Hh) signals regulate invertebrate and vertebrate development, yet the role of the pathway in adipose development remains poorly understood. In this report, we found that Hh pathway components are expressed in the fat body of silkworm larvae. Functional analysis of these components in a *Bm*N cell line model revealed that activation of the *Hh* gene stimulated transcription of Hh pathway components, but inhibited the expression of the adipose marker gene *AP2*. Conversely, specific RNA interference-mediated knockdown of *Hh* resulted in increased *AP2* expression. This further showed the regulation of Hh signal on the adipose marker gene. In silkworm larval models, enhanced adipocyte differentiation and an increase in adipocyte cell size were observed in silkworms that had been treated with a specific Hh signaling pathway antagonist, cyclopamine. The fat-body-specific Hh blockade tests were consistent with Hh signaling inhibiting silkworm adipogenesis. Our results indicate that the role of Hh signaling in inhibiting fat formation is conserved in vertebrates and invertebrates.

Key words adipocyte differentiation; adipogenesis; *Bombyx mori*; Hedgehog (Hh); signaling pathway; silkworm

Introduction

The intercellular signaling proteins in the hedgehog (Hh) family were isolated in the early 1990s. Since then the Hh signaling pathway has been recognized as a key regulatory component of many fundamental processes in embryonic development (Ingham & McMahon, 2001) with mutations in Hh and its downstream signaling molecules linked to numerous oncogenic and disease states (Nybakken & Perrimon, 2002). Hh signaling has also emerged as a potential mediator of adipocyte differentiation during post-natal development (Li *et al*., 2008). However, the specific role Hh plays is controversial. Injection of adult mice with a sonic Hh (Shh)-IgG (immunoglobulin G) fusion protein (a Hh agonist) caused a significant increase in fat mass and body weight without affecting food consumption and fluid intake (Martin *et al*., 2002). Consistent with this finding, suppression of Hh signaling in response to injected anti-Hh antibodies significantly reduced body weight gain in these animals (Buhman *et al*., 2004). These studies suggest that Hh signaling stimulates adipogenesis in adult mice. In contrast, Suh *et al*. (2006) reported that Hh blocks adipocyte differentiation in cultured mammalian adipocytic or multipotent mesenchymal cells. Still other reports indicated that genetic activation of Hh signaling inhibited fat formation in Drosophila and that Hh blocked white adipocyte differentiation in mice (Pospisilik *et al*., 2010; James *et al*., 2010). Further complicating our understanding of this pathway, Hh signaling has been shown to play different roles in adipogenesis in different species (Buhman *et al*., 2004; James *et al*., 2010; Zehentner *et al*., 2000). Activation of the Hh signaling pathway interfered with the early differentiation of pluripotent stem cells in mice, whereas in humans the Hh signaling pathway influenced the maturation of human fat

Correspondence: Yong-Gen Miao, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China. Tel: +86 131 86984135; fax: +86 571 88982312; email: miaoyg@zju.edu.cn

cells (Nybakken & Perrimon, 2002; Fontaine *et al*., 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 *Hh* and over-expression of *Hh* 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) *in vitro* on cultured adipose cells and *in vivo* on adipocyte differentiation in silkworm larvae.

Materials and methods

BmN cell and silkworm larvae

The *B. mori* cell line *Bm*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).

Cloning of the Hh pathway components from B. mori *by 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-*Taq* 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 *Xho*I and *Hin*dIII restriction enzymes and insert positive clones were sequenced.

Quantitative real-time PCR (qPCR) analysis of Hh signaling pathway components

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 *Actin 3* (A3) gene was used as a qPCR control gene (Teng *et al*., 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 *Actin 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 *et al*., 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.

Generation of transfected Hh over-expression cell lines

The *BmHh* was amplified by RT-PCR using the following primers: F, 5'-ggatccATGAACCAGTGGCC GGGAGT (*Bam*HI site is underlined) and R, 5'-gaattcTCGATATCTATACGATGCTG (*Eco*RI site is underlined). *BmHh* was inserted into the PXL-BACII-GFP-*Bm*U6–Neo^r vector via the same enzymes. The resulting transformation vector was designated as PXL-BACII-GFP-*Bm*U6-Hh-Neo^r. *Bm*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. *Bm*N cells were co-transfected with the transformation vector PXL-BACII-GFP-*Bm*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 G418 was achieved after 30 days when most of the non-transformed cells died. The live cells were cultured continuously to establish the transfected *Bm*N cell line.

Hh knockdown in BmN cells

BmHh double-stranded RNA (dsRNA) was synthesized and purified using a T7 RiboMAX^{TM} Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's instruction. Primers used to generate the RNA template were T7-Hh-F 5'-GGATCCTAATACGACTCACTATAGGATGAACCA GTGGCCGGGAGT and T7-Hh-R 5'-GGATCCTAATA CGACTCACTATAGGTCGATATCTATACGATGCTG. *Bm*N cells were seeded in a six-well tissue culture plate at a density of 5×10^5 cells per well and cultured in TC-100 medium with 10% FBS. Cells in each well were transfected with synthesized *BmHh* dsRNA. Cells were collected 3 days later for qPCR analysis of Hh signaling pathway genes.

In vitro *cyclopamine treatment*

Cyclopamine (Fig. S1 for molecular structure, Selleck Chemicals, Houston, TX, USA), a specific Hh signaling pathway antagonist, was dissolved in dimethyl sulfoxide (DMSO) at 92 mg/mL and then diluted 1 : 100 in cell culture medium. The dorsal fat bodies of 4th instar silkworm larvae were dissected and cultured *in vitro* in 12-well plates after digestion with trypsin (0.25% ethylenediaminetetraacetic acid [EDTA]-trypsin). Several hours later, after the adipose cells had become adherent, 10 μmol/L cyclopamine was added and morphological changes (i.e., growth, differentiation, death) were observed.

In vivo *cyclopamine treatment*

Fourth instar silkworm larvae were fed cyclopamine at a dose of 50 mg/kg/day for 10 days. Differentiation in the character of fat body adipose cells was determined by means of paraffin sectioning. Dissected fat body was fixed with 10% formalin for 24 h at room temperature. Paraffin embedding was done over a 16-h period consisting of: $2 \times$ 70% ethanol incubations at 1 h each, an 80% ethanol incubation for 1 h, a 95% ethanol incubation for 1 h, $3 \times$ 100% ethanol incubations for 1.5 h each, followed by three incubations of xylene or xylene substitute for 1.5 h each, and 2× paraffin wax (58–60°C) incubations at 2 h each. The paraffin blocks were trimmed, sectioned in 5 μm increments and observed using a Nikon Eclipse Ti-S Inverted Fluorescent Microscope.

Statistical analysis

All experiments were performed in three independent biological replicates and reactions of each sample were carried out in triplicate. Determinations were performed using one-way analysis of variance (ANOVA).

Results

Cloning of Hh pathway components from B. mori

To determine if the inhibitory role of Hh is conserved in invertebrates, we evaluated the expression of Hh pathway components in the fat body of 4th instar larvae silkworm. Using RT-PCR, we found that all of the Hh pathway components, Ptc, Smo, Ci, Fu and Cos2, as well as the adipose marker gene *AP2*, were expressed in silkworm fat tissues (Fig. 1A).

The full-length *B. mori Hh* gene product was amplified from 3rd instar larvae by RT-PCR (Fig. 1B) and was sequenced. The nucleotide sequence data for this transcript has been deposited in the GenBank/EMBL/DDBJ database under the accession no. KC494700. The

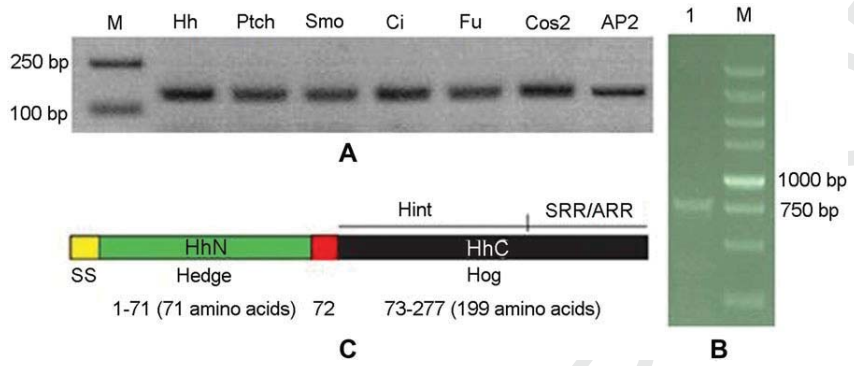

Fig. 1 Reverse transcription polymerase chain reaction (RT-PCR) analysis of Hedgehog (Hh) pathway components in silkworm fat body. A: RT-PCR amplification of fragments of the Hh pathway. B: RT-PCR amplification of the complete Hh coding sequence from silkworm fat body. The amplified product was cloned into pMD18-T vector and sequenced. *Bombyx mori Hh* 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: *Hh* 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).

Transcriptional expression profile of the Hh signaling pathway in different stages of early larvae

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 *Hh* and the transmembrane proteins *Smo* gene underwent a drastic decline, while the *Ptc* underwent a steady decline as the larval stages progressed (Fig. 2A). Highest expression for the transcription factor *Fu* appeared in the embryo and declined gradually throughout the early stages of larval development. Two

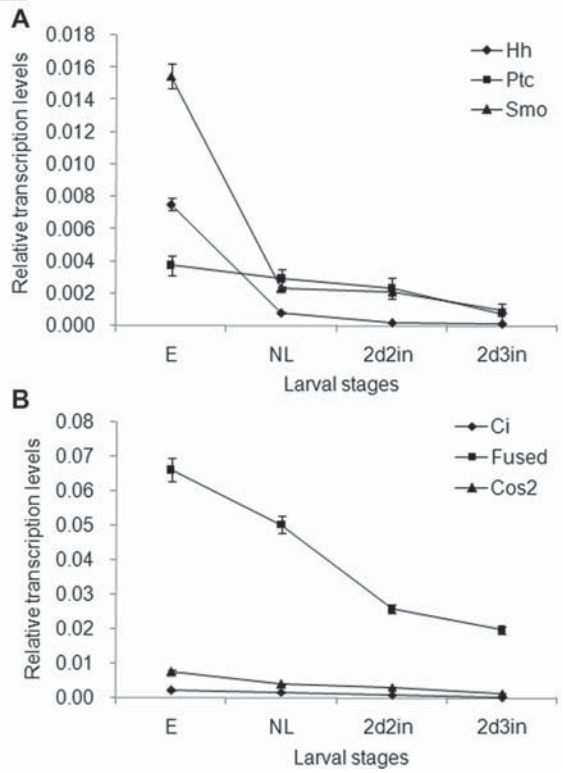

Fig. 2 Transcriptional levels of Hedgehog (Hh) pathway components at different larval stages. E, embryo; NL, newly hatched larvae; 2d2in, 2-day-old 2nd instar larvae; 2d3in, 2-day-old 3rd instar larvae. Graphs display the mean fold difference (triplicate biological replicates) ± SEM.

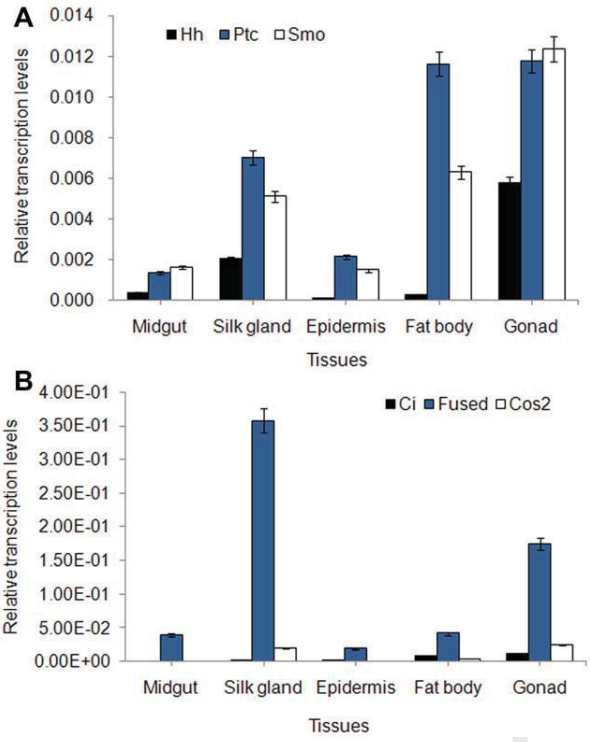

Fig. 3 Expression levels of Hedgehog (Hh) pathway components in different tissues from 3-day-old 5th instar *Bombyx mori* larvae. Tissues analyzed include midgut, silk glands, epidermis, fat body and gonad. Graphs display the mean fold difference (triplicate biological replicates) ± SEM.

other factors, *Ci* and *Cos2*, 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.

Transcriptional expression profile of the Hh signaling pathway in different tissues

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. mori* were likewise investigated by qPCR. The results presented in Figure 3A indicate expression of the transmembrane proteins *Ptc* and *Smo* in all tissues examined, with highest expression in particular silk glands, fat body and gonad. In contrast, *Hh* exhibited comparatively lower expression levels. Of the remaining genes, *Fu* was the more highly expressed transcript with Ci and *Cos2* exhibiting relatively low expression (Fig. 3B).

Expression level of Hh pathway components in the fat body of late-stage larvae

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.

Effects of over-expression or knockdown on transcription in Bm*N cells*

To investigate the regulatory role of *Hh* on various Hh pathway components (*Ptch*, *Smo*, *Ci*, *Fu* and *Cos2*) as well as the adipocyte marker gene *AP2*, we examined the effects of exogenous over-expression and suppression of *Hh* gene in cultured *Bm*N cells.

The expression vector PXL-BACII-GFP-*Bm*U6-*Hh*-Neo^r (Fig. S2) was used to generate a transfected *Bm*N cell line. After 2 months of G418 selection, a stable *Hh*-overexpression transfected *Bm*N cell line was obtained (Fig. S3). Expression levels of Hh pathway components in transfected *Bm*N cell in relation to non-transformed cells were examined by qPCR. Figure 5A shows that expression of *Hh* was much higher (about 50 times) in the transfected cells compared to that in the controls. The downstream genes *Smo*, *Ci* and *Fu* also showed higher transcription levels. Conversely, *Ptch* and *Cos2* had lower transcription levels. The *AP2* displayed a decreased transcription level in *Hh*-overexpression transfected *Bm*N cell line compared to controls.

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

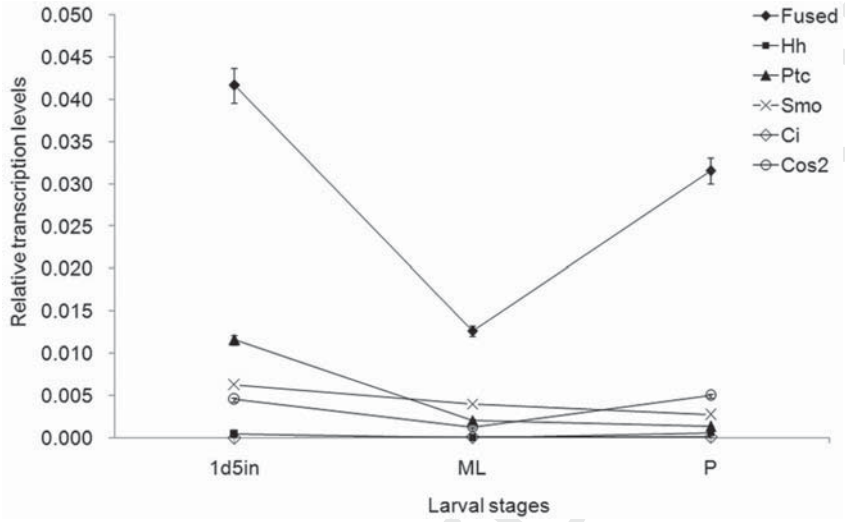

Fig. 4 Changes in the expression levels of the Hedgehog (Hh) signaling pathway components in fat body from 5th instar larvae through pupae. 1d5in, 1-day-old 5th instar fat body; ML, matured larva (just prior to spinning) fat body; P, pupal fat body. Graphs display the mean fold difference (triplicate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

Three days later, the cells were collected and the comparative expression levels of *Hh* and the related genes were determined. Figure 5B shows that the expression of *Hh* decreased about 44.8% compared with controls, confirming the knockdown efficacy of the *Hh* dsRNAs. Figure 5B also shows that the expression of the other Hh signaling pathway genes likewise decreased: *Smo*, *Ci* and *Fu*. Conversely, *Ptch* and *Cos2* increased. These results indicate that the target genes for adipogenesis were significantly regulated in the dsRNA-mediated *Bm*N cells. As expected, the transcription level of *AP2* underwent a notable increase after dsRNA-mediated depletion of *Hh*.

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 *Hh* transcription. *AP2* expression was significantly lower following *Hh* overexpression, but was higher when *Hh* 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.

Inhibiting Hh signaling increases silkworm adipogenesis

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 *in vitro* 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 *Hh* knockdown. That is, cyclopamine treatment decreased the transcriptional levels of *Hh*, *Smo*, *Ci* and *Fu*, but increased that of *Ptch*, *Cos2* and *AP2* (Fig. 6A). In contrast, fat-body-specific *Hh* inhibition stimulated fat formation in Drosophila (Pospisilik *et al*., 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).

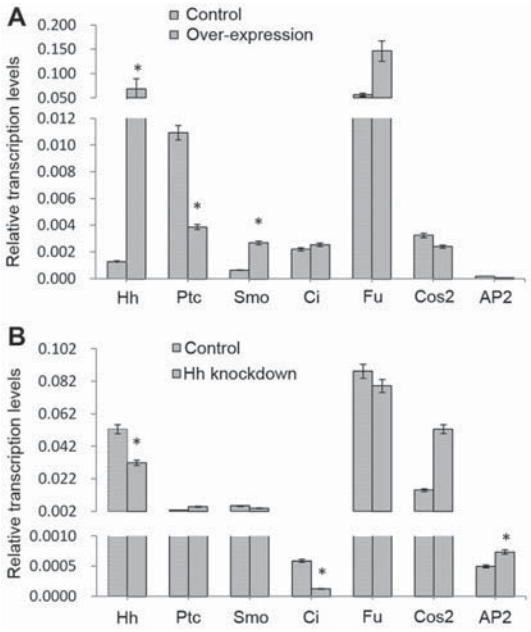

Fig. 5 Transcription levels of Hedgehog (Hh) pathway components and the *AP2* adipocyte marker gene after *Hh* overexpression and knockdown. A: quantitative polymerase chain reaction (qPCR) analyses of the expression levels of genes in normal *Bm*N cells and transfected cells. The blue bars represent expression levels in control *Bm*N cells, while the red bars represent expression levels in transfected cells. B: Expression of genes after addition of *BmHh* double-stranded RNA (dsRNA) (10 nmol/L) to *Bm*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 *et al*., 2007).

The Hh signaling pathway comprises several signaling molecules. The foremost level is the signal transfer activity of the secreted Hh glyocoprotein. The next level is the multiple-pass transmembrane proteins Ptch (Lie *et al.*, 2008) and Smo that interact at the cell surface, the interaction of which regulates Smo signaling. The third level is the high molecular weight protein complex that is formed by the kinesin-related microtubule motor protein Cos2, the serine/threonine kinase Fu, and the zinc finger transcription factor Ci (Nybakken & Perrimon, 2002; Dhawan & Gopinathan, 2003). Current models view Ptch and Smo as a preformed receptor complex that is activated by Hh binding (Denef *et al*., 2000), while Ptch functions in Hh binding, and Smo functions in transducing the signal.

In the absence of Hh stimulation, Smo is repressed by Ptc, the Fu/Ci/Cos2 complex is bound to microtubules and Ci is cleaved into a smaller N-terminal fragment called Ci75 which moves to the nucleus and represses Hh target genes (Nybakken & Perrimon, 2002). Binding of Hh to Ptc results in loss of Ptc activity and relieves the inhibitory effect that Ptc normally has on Smo. The subsequent activation of Smo transduces the Hh signal to the cytoplasm, ultimately leading to the activation of the Fu/Ci/Cos2 complex of transcription factors (Varjosalo & Taipale, 2007), which can then travel to the nucleus and function as a transcriptional activator of Hh target genes (Hao *et al*., 2011). The adipocyte fatty acid-binding protein AP2 (Chen & Tang, 2011) is a carrier protein for fatty acids that is primarily expressed in adipocytes, which is usually used as an adipose marker protein (Wang *et al*., 2011; Farmer, 2006). AP2 expression is increased in obesity (Furuhashi & Tuncman, 2007).

The development of mature adipocytes undergoes two distinct stages, the commitment of pluripotent stem cells to pre-adipocytes and the terminal differentiation of preadipocytes into mature adipocytes (Chen & Tang, 2011). In the present study, we compared the expression levels of Hh in different larval stages using RT-PCR and qPCR and revealed that high transcription levels were mainly observed in the embryo. This result implies that Hh signaling pathway mainly regulates the initial stage of adipocyte development in the embryo.

It has been shown that Hh signaling plays a significant role in osteoblastic and adipocyte differentiation *in vitro*. Previous studies have examined the role of Hh signaling in the balance of osteogenic and adipogenic differentiation in mouse adipose-derived stromal cells (ASCs) (James *et al*., 2010). Hh molecules have been shown to control the differentiation of different cell types, such as directing differentiation of mesenchymal precursor cells toward osteoblasts. The murine mesenchymal precursor cell line KS483 was used to examine the involvement of Hh signaling in the initiation of osteoblastic differentiation in the bone collar during endochondral bone formation (van

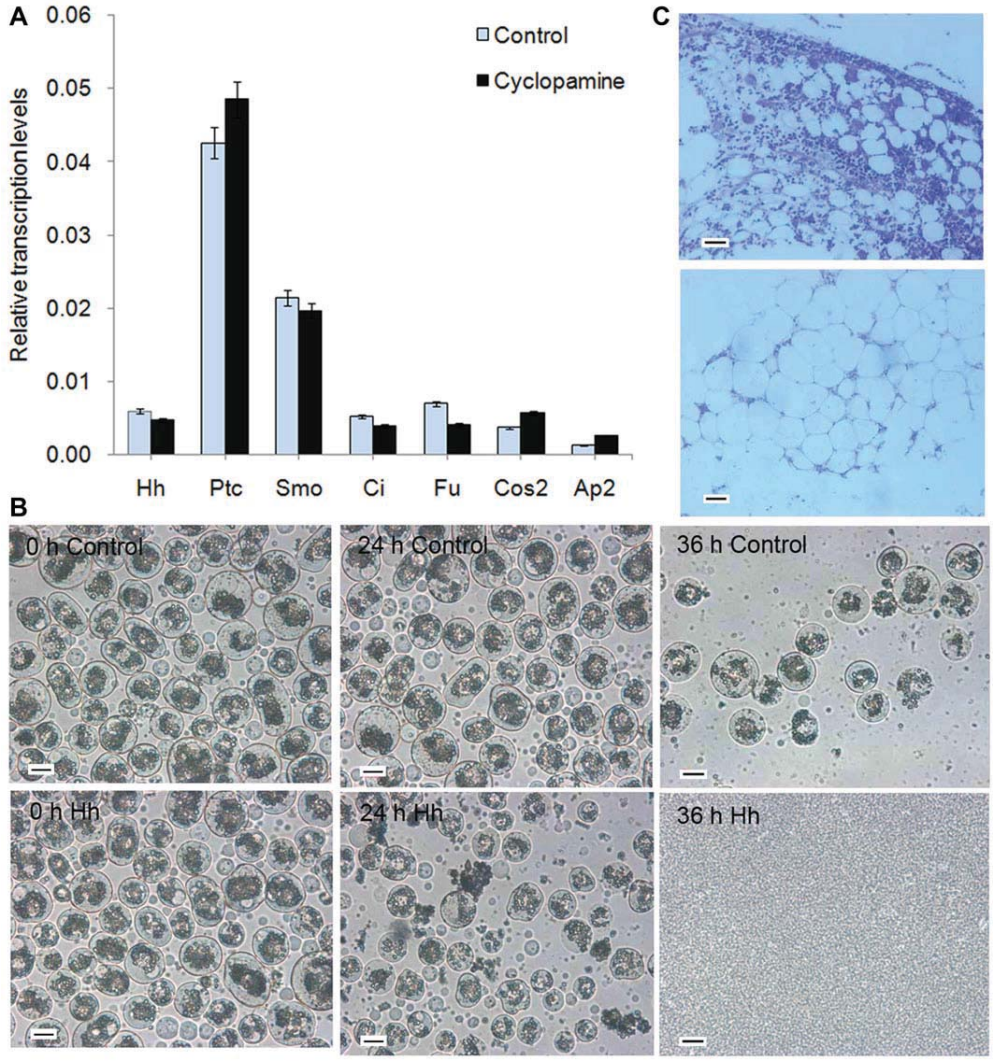

Fig. 6 Cyclopamine inhibits Hedgehog (Hh) pathway stimulates *Bombyx mori* fat formation. A: The expression levels of Hh pathway and adipocyte marker gene *AP2* after cyclopamine treatment in silkworm adipose cells. B: Adipose cells were incubated in TC100 media in the presence or absence (control) of 10 μmol/L cyclopamine, and lipid formation was assessed based upon morphology (B). C: Hematoxylin–eosin stained sections of adipose tissues from cyclopamine-treated (i.e., Hh inhibited) larvae and control larvae. Scale = 20 μm.

der *et al*., 2003). *In vitro*, Shh has been demonstrated to induce alkaline phosphatase (ALP), a marker of osteoblast differentiation, in the mouse mesenchymal cell line C3H10T1/2 (Zehentner *et al*., 2000; Spinella *et al*., 2001) and the osteoblast cell line MC3T3-E1 (Nakamura *et al*., 1997). A short treatment with N-Shh was sufficient to dramatically reduce the levels of the adipocytic-related transcription factors CCAAT-enhancer-binding protein α and peroxisome proliferator-activated receptor γ in both C3H10T1/2 and calvaria cell cultures (Spinella *et al*., 2001). Theoretically, the manipulation of Hh in *Bm*N cells presents an attractive avenue for analyzing this signaling pathway.

In the established transfected *Bm*N cell line, the *Hh* gene was inserted between the Neo^r gene and the green fluorescent protein (GFP) gene. *Hh* over-expression appears to have activated Hh pathway components as they showed higher transcription levels. Conversely, *Hh* over-expression decreased the expression levels of the adipogenesis marker gene *AP2*. This result revealed that

the Hh signal has a negative influence on the transcription level of *AP2*. Opposite results were obtained in the *Hh* 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 *et al*., 2002), was used to treat silkworm fat body *in vitro* and *in vivo*. 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 *in vivo* and *in vitro* support the notion that the Hh pathway inhibits adipocyte differentiation in *B. mori*.

Next to *Drosophila*, the silkworm represents an ideal model animal for basic research. The study of the silkworm Hh signaling pathway and its regulatory effects on adipogenesis are expected to have a signficant impact on our understanding of adipogenesis regulation in this species, which has both theoretical and practical importance for the sericulture industry. Further studies of this pathway in silkworms may also provide valuable insights into human obesity and/or diabetes and may help establish a molecular basis for screening various pharmaceuticals for care and prevention of both disease states.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (No. NSFC: 31372374/C1703) and the National Basic Research Program of China under grant No. 2012CB114601, the Key project of Zhejiang Government (No. 2011C14006), the Science and Technology Innovation Team of Zhejiang Province (No. 2010R50031) and Chinese Universities Scientific Fund. We thank Joe Hull for discussions and critical reading of the manuscript.

Disclosure

The authors have no conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

References

Buhman, K.K., Wang, L.C., Tang, Y.Z. and Swietlicki, E.A. (2004) Inhibition of Hedgehog signaling protects adult mice from diet-induced weight gain. *Journal of Nutrition*, 134, 2979–2984.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: Minimum information for publication of Quantitative Real-Time PCR experiments. *Clinical Chemistry*, 55, 611–622.

Cousin, W., Fontaine, C., Dani, C. and Peraldi, P. (2007) Hedgehog and adipogenesis: Fat and fiction. *Biochimie*, 89, 1447–1453.

Chen, J.K., Taipale, J., Cooper, M.K. and Beachy, P.A. (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to smoothened. *Genes & Development*, 16, 2743–2748.

Chen, J.W. and Tang, Q.Q. (2011) Hedgehog signaling pathway and adipocyte development. *Chinese Journal of Biochemistry and Molecular Biology*, 27(1), 6–10.

Denef, N., Neubuse, D., Perez, L. and Cohen, S.M. (2000) Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell*, 102, 521–531.

Dhawan, S. and Gopinathan, K.P. (2003) Expression pattern of Cubitus interruptus from the mulberry silkworm *Bombyx mori* in late developmental stages. *Development Genes and Evolution*, 213, 166–177.

Farmer, S.R. (2006) Transcriptional control of adipocyte formation. *Cell Metab*, 4, 263–273.

Fontaine, C., Cousin, W., Plaisant, M., Dani, C. and Peraldi, P. (2008) Hedgehog signaling alters adipocyte maturation of human mesenchymal stem cells. *Stem Cells*, 26, 1037–1046.

Furuhashi, M. and Tuncman, G. (2007) Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature*, 447, 959–965.

Hao, K., Xie, X.H. and Yang, Y.M. (2011) Advancement in researches of role of hedgehog signal pathway in pathogenesm of pancreatic cancer. *Chinic Journal of Hepatobiliary Surgery*, 17, 70–72.

Ingham, P.W. and McMahon, A.P. (2001) Hedgehog signaling in animal development: paradigms and principles. *Genes Development*, 15, 3059–3087.

James, A.W., Philipp, L., Levi, B., Carre, A.L., Xu, Y., Helms, J.A. and Longaker, M.T. (2010) Sonic Hedgehog Influences the Balance of Osteogenesis and Adipogenesis in Mouse Adipose-Derived Stromal Cells. *Tissue Engineeting Part A*, 16, 2605–2616.

Li, Z.L., Zhang, H., Denhard, L.A., Liu, L.S., Zhou, H.X. and Lan, Z.J. (2008) Reduced white fat mass in adult mice bearing a truncated Patched 1. *International Journal of Biology*, 4, 29–36.

Martin, P. L., Lane, J. and Pouliot, L. (2002) Increase in adipose and total body weight, but not in lean body mass, associated with subcutaneous administration of sonic hedgehog-Ig fusion protein to mice. *Drug Development Research*, 57, 107–114.

Nakamura, T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Pacifici, M., Kinto, N., Yamaguchi, A., Noji, S. and Kurisu, K. (1997) Induction of osteogenic differentiation by hedgehog proteins. *Biochemical and Biophysical Research Communications*, 237, 465–469.

Nybakken, K. and Perrimon, N. (2002) Hedgehog signal transduction: recent findings. *Current Opinion in Genetics & Development*, 12, 503–511.

Pospisilik, J.A., Schramek, D., Schnidar, H. and Shane, J.F. (2010) *Drosophila* genome-wide obesity screen reveals Hedgehog as a determinant of brown versus white adipose cell fate. *Cell*, 140, 148–160.

Spinella, J.S., Rawadi, G.., Kawai, S., Gallea, S., Faucheu, C. and Mollat, P. (2001) Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *Journal of Cell Science*, 114, 2085–2094.

Suh, J.M., Gao, X.H., McKay, J., McKay, R., Salo, Z. and Graff, J.M, (2006) Hedgehog signaling plays a conserved role in inhibiting fat formation. *Cell Metab*, 3, 25–34.

Teng, X., Zhang, Z., He, G., Yang, L. and Li, F. (2012) Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. *Journal of Insect Science*, 12, 1–17.

van der Horst, G., Farih-Sips, H., Löwik, C.W. and Karperien, M. (2003) Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells. *Bone*, 33, 899–910.

Varjosalo, M. and Taipale, J. (2007) Hedgehog signaling. *Journal of Cell Science*, 120, 3–6.

Wang, X.C., Shi, M.X., Li, H. and Wang, N. (2011) Hedgehog Signaling Pathway and Adipogenesis. *Chinese Journal of Cell Biology*, 33, 936–941.

Zehentner, B.K., Leser, U. and Burtscher, H. (2000) *BM*P-2 and sonic Hedgehog have contrary effects on Adipocyte-Like differentiation of C3H10T1/2 cells. *DNA and Cell Biology*, 19, 275–281.

Accepted June 12, 2014

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used for amplification of Hh signaling pathway components.

Fig. S1 Molecular structure of cyclopamine.

Fig. S2 Schematic representation of the *Hh*-expressing recombinant transformation plasmid. *BmHh* was amplified by RT-PCR and inserted to the vector PXL-BACII-GFP-*Bm*U6–Neo^r (preserved in our lab). The resulting transformation vector was designated as PXL-BACII-GFP-*Bm*U6-*Hh*-Neo^r.

Fig. 3S Stable over-expression of *Hh* in the transfected *Bm* cell line. a: Fluorescence of transfected *Bm*N cells observed under an inverted fluorescence microscope following excitation with ultra violet light; b, normal cells.