Transcriptome Responses of Insect Fat Body Cells to Tissue Culture Environment

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Abstract

Tissue culture is performed to maintain isolated portions of multicellular organisms in an artificial milieu that is outside the individual organism and for considerable periods of time; cells derived from cultured explants are, in general, different from cells of the corresponding tissue in a living organism. The changes in cultured tissues that precede and often explain the subsequent cell proliferation of explant-derived cells have been partially studied, but little is known about the molecular and genomic basis of these changes. Comparative transcriptomics of intact and cultured (90 hours in MGM-450 insect medium) Bombyx mori tissues revealed that fewer genes represented a larger portion of the transcriptome of intact fat body tissues than of cultured fat body tissues. This analysis also indicated that expression of genes encoding sugar transporters and immune response proteins increased during culture and that expression of genes encoding lipoproteins and cuticle proteins decreased during culture. These results provide support for hypotheses that cultured tissues respond immunologically to surgery, adapt to the medium by accelerating sugar uptake, and terminate their identity as part of an intact organism by becoming independent of that organism.

Introduction

Tissue culture was devised as a method for studying the behavior of plant and animal cells in an environment that is free from systemic variations that might arise in vivo both during normal homeostasis and under the stress of an experiment. Today, this technique is essential for cell engineering. More than 500 insect cell culture lines have been established and continuously maintained [1]; these lines are used as research tools in virology, immunology, and physiology, and several cell lines are used commercially to produce recombinant proteins of biomedical significance. Since most insect cells have tolerance against changes in temperature [2], pH [3], and osmotic pressure [4], many of these cell lines are very useful as cell culture models of cellular phenomena. However, no general method has been developed to establish a cell line from an arbitrary tissue of an arbitrary insect species. Cell lines are established from primary culture of tissue when a population of proliferating cells derived from the primary tissue explant undergo immortalization [5]. In primary cultures of insect cells, it usually takes several months for active cell proliferation to start [6]. It is thought that during the early stage of primary culture, isolated explants activate immune responses to the culture conditions and then, ultimately, some subpopulation of cells adapts to the culture conditions. In mammalian tissue culture, the changes in the explant itself are the subject of considerable discussion [7]. Hadda (1912) noted that the cells derived from the explant are different from cells in the primary tissue. Champy (1913) proposed that two the phenomena, cell proliferation and cell dedifferentiation, are essential to establishment of tissue cultures. While many tissues in adult animals rarely show evidence of mitosis, active cell division does occur in culture, and this enhanced cell division is accompanied by dedifferentiation. However, the lack or loss of primary tissue characteristics in cultured cells is not primarily due to dedifferentiation, but rather to selective overgrowth of specific cells [8]. Dedifferentiation and selective cell survival and proliferation are clearly two important changes that take place as subpopulation of cells adapt to tissue culture. Recently it is widely known that there are many differences in cell behavior between cultured cells and their counterparts in vivo [9], but many of the changes that occur during this adaptation remain unclear. With the introduction of next-generation sequencing [10], genomic sequence data from many species became available, and profiling of whole transcriptomes became possible [11]. This type of profiling could provide new information on the reaction of insect cell as they respond to artificial culture conditions. The silkworm, Bombyx mori, is the first Lepidoptera for which a genome sequence has been openly released [12]. The B. mori genome is 368 Mb, and there are 14,625 predicted genes in the current version of the annotated sequence. Although annotation has just started and not all the genes are correctly predicted, this species was useful for transcriptome analysis. Fat body is principally responsible for intermediary metabolism and nutrient storage in insects. Here, the transcriptomes of intact fat body tissues and cultured fat body tissues were compared to evaluate the effects of culturing on genome-wide transcription in an insect tissue.
Results

Samples of total RNA were prepared from acutely dissected intact silkworm larval fat body and from fat body tissues that had been cultured for 90 hours in MGM-450 insect medium. Each sample was sequenced on a separate single lane of a flow cell. Sequencing resulted in 23-29 million 36-base-pair reads per lane (Table 1) passing Illumina’s quality filter; in all, 53 million reads and 7.9 GB of silkworm fat body transcript sequence data were generated. Mapping analysis was performed using CASAVA 1. 7. 0 [13]; 17 095 689 (58%) intact fat body and 14 257 793 (60%) cultured fat body high quality reads were mapped to GLEAN loci in the silkworm genome. Based on the KAIKObase [14] annotation, there are 14,623 predicted genes in the annotated silkworm genome; 9,850 genes of these were expressed in intact fat body, and 11,106 genes in cultured fat body. Expression of 11,451 genes was detected, and 9,505 of these genes were expressed in both samples. Reads per kilobase of exon model per million mapped reads [11] (RPKM) were calculated for all genes using CASAVA 1. 7. 0. In intact fat body, lipoprotein-encoding genes (BGIBMGA004399, BGIBMGA004394, BGIBMGA004396, BGIBMGA004457, BGIBMGA004397, BGIBMGA004395) occupied half of the transcriptome (Fig. 1A). In cultured fat body, these genes occupied about a quarter of the transcriptome (Fig. 1B). The expression of a gene encoding a heat shock protein-like protein (BGIBMGA005781) occupied a larger portion of the cultured fat body transcriptome than of the intact fat body transcriptome. Fewer genes represented a larger portion of the transcriptome of intact fat body than of cultured fat body (Fig. 1A and 1B). To compare heterogeneity of both transcriptomes, Shannon’s entropy [15] was calculated for all RPKMs. Shannon’s entropy was higher in the sample from cultured fat body (6.486697 shannon in intact fat body and 8.518913 shannon in cultured fat body) indicating that heterogeneity decreased during culture. The relative RNA production in the two tissue samples was estimated using edgeR [16] which is an R software package for statistical analysis [17], and based on this analysis, total RNA production in fat body was reduced by two-fifths during culture. We found 54 differentially expressed genes (false discovery rate (FDR) < 0.05) with DESeq [18] using read-count data (Fig. 2). During culture, expression of 11 genes increased (Table 2), and expression of 43 genes decreased (Table 3). The morphology of cultured fat body tissues were examined by stereoscopic microscope and phase contrast microscope (Fig. 3). There was no crucial difference.

Discussion

The transcriptome of lepidopteran fat body was analyzed using next-generation sequencing. Approximately 60% of reads obtained for Bombyx mori fat body transcripts were aligned with the genome of silkworm; this result was similar to the result from a previous study on silk glands (52.7% and 60.2%) [19]. Lipoprotein-encoding transcripts occupied approximately half of

| Sample               | Reads   | Mapped Reads | % Aligned |
|----------------------|---------|--------------|-----------|
| Intact fat body      | 29,532,006 | 17,095,689   | 57.89%    |
| Cultured fat body    | 23,938,758 | 14,257,793   | 59.56%    |

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the transcriptome in intact fat body; in contrast, these transcripts occupied a smaller portion of the transcriptome of fat body tissues that had been maintained in culture medium for 90 h. This result indicated that the cultured tissue had initiated the process of abrogating their tissue-specific function by becoming independent from the donor and their identity as a part of an individual, living organism. The independence of cultured fat body was also indicated by an increase in Shannon’s entropy of RPKMs. This finding indicated that gene expression in the fat body that resulted from inter-tissue communications between the constituent tissues of the insect donor had become dysregulated in the cultured fat body.

Expression of sugar-transporter genes (BGIBMGA004427, BGIBMGA004426) increased during culture of fat body, indicating that, under culture conditions, cells take up sugar actively. Expression of immunity-related genes (BGIBMGA004969, BGIBMGA012864, BGIBMGA013866) also increased during culture, indicating that the process of dissection from donor, the transfer into culture medium, and maintenance in culture medium are harmful for fat body tissue and could stimulate immune responses. In addition, expression of genes related to juvenile hormone synthesis (BGIBMGA002314, BGIBMGA001324) also increased during culture, and expression of cuticle protein genes (BGIBMGA000329 etc) decreased. Expression of genes related to hypoxia (BGIBMGA004893, BGIBMGA004894) also decreased, indicating that culture conditions were oxygen rich. Microsensor measurements of physically dissolved O2 in the hemolymph of living buzzer midge larvae indicate very low O2 concentrations (11 μmol/L, O2) [20]; this empirically determined concentration is lower than that in insect culture media [21]. Expression of troponin genes (BGIBMGA001030 etc.) and myosin genes (BGIBMGA014226 etc.) also decreased during culture, indicated that the fat body explants contained some myocytes.

The expression of genes relating to a particular function or processes may have changed in a coordinated fashion during culture. To assess whether such changes occurred, RPKMs were compared for 147 immunity-related genes [22], 54 apoptosis-related genes [23], and 10 juvenile hormone-related genes [24]. Of the 147 immunity-related genes, expression of 60 was increased more than 5-fold during culture; in two extreme examples, expression of the genes encoding gloverins and a gene encoding leboicin increased more than 1000-fold. Expression of most of the 54 apoptosis-related genes did not change during culture. Expression of all 10 genes related to juvenile hormone synthesis increased during culture, and expression 6 of these genes increased more than 5-fold. Cultured tissues were derived from living donor organisms, which have circulating hormones, and were transferred into culture medium lacking hormones. To avoid exposing cultured explants to abrupt changes in insect hormone levels, 5th instar larva were harvested 3 days after the 4th ecdysis; these larva have juvenile hormone and 20-hydroxyecdysone levels that are lower than those of other developmental stages. Hence, cultured fat body would not progress according to the original program of development.

Table 2. Differentially expressed *Bombyx mori* genes for which expression increased during culture of fat body.

| Gene ID        | Intact | Cultured | Log Ratio | FDR  | Seq. Description                  | eValue      | ACC          |
|---------------|--------|----------|-----------|------|-----------------------------------|-------------|--------------|
| BGIBMGA004427 | 0      | 865      | Inf       | 0.00777717 | sugar transporter-like protein   | 4.65E-07    | XP_001850640 |
| BGIBMGA004969 | 0      | 736      | Inf       | 0.010526917| protease inhibitor-like protein  | 2.03E-22    | ABG72724     |
| BGIBMGA004426 | 1      | 1431     | 9.36439731| 0.012002245| sugar transporter-like protein   | 4.91E-06    | XP_001659148 |
| BGIBMGA001007 | 1      | 925      | 8.734898908| 0.020018747| monocarboxylate transporter-like protein | 1.60E-86    | XP_001659154 |
| BGIBMGA012864 | 2      | 1907     | 8.778678481| 0.020177428| peptidoglycan recognition protein-like protein | 4.00E-16    | BAF03520.1   |
| BGIBMGA001347 | 1      | 832      | 8.582029071| 0.022363186| fibrinogen p25-like protein      | 1.06E-125   | BAB39500     |
| BGIBMGA002314 | 0      | 497      | Inf       | 0.027138743| farnesoid acid o-methyl transferase-like protein | 1.24E-26    | ABF18352     |
| BGIBMGA001324 | 0      | 490      | Inf       | 0.027857698| juvenile hormone binding protein e96h-0303 | 1.67E-136   | NP_001036949.1|
| BGIBMGA007462 | 3      | 4360     | 9.386739272| 0.039513702| sodium-dependent phosphate transporter-like protein | 0          | XP_001662636.1|
| BGIBMGA001358 | 3      | 1124     | 7.431053173| 0.04523946 | -NA-                             | -           | -            |
| BGIBMGA013866 | 4      | 3152     | 8.503641173| 0.046425762| gloverin-like protein            | 3.25E-18    | NP_001037884.1|

*“Intact” and “Cultured” columns indicate the number of read counts in intact fat body and cultured fat body of *Bombyx mori*. doi:10.1371/journal.pone.0034940.t002
fetuin, cytochrome c, inosine and tryptose phosphate broth. Six silkworm cell lines were established using MGM-450 insect medium [28,29] and a research about in vitro hemocyte differentiation of silkworm also performed using this medium [30].

This study also revealed that fewer genes occupied more of transcriptome in intact fat body than in cultured fat body. To understand characteristics of these differences, we compared the share of transcriptome of tissues from different species and of Drosophila cell lines as follows: silkworm fat body (our data), Table 3.

| Gene ID | Intact | Cultured | Log Ratio | FDR | Seq. Description | eValue | ACC |
|---------|--------|----------|-----------|-----|------------------|--------|-----|
| BGIBMGA001030 | 1093 | 2 | -10.21248833 | 0.002673821 | troponin i | 1.49E-31 | ACN86369.1 |
| BGIBMGA000329 | 3010 | 4 | -10.67959842 | 0.00388079 | larval cuticle protein LCP-20 precursor | 0.33E-99 | NP_001037490.1 |
| BGIBMGA002549 | 881 | 4 | -8.90408856 | 0.00388079 | cuticular protein RR-1 motif 5 | 2.84E-47 | NP_001166743.1 |
| BGIBMGA009184 | 902 | 5 | -8.81346675 | 0.00388079 | cathespin-like protein | 1.69E-58 | NP_001164088.1 |
| BGIBMGA014226 | 3156 | 3 | -11.15732964 | 0.00388079 | myosin heavy chain-like protein | 0 | EFN88457.1 |
| BGIBMGA010112 | 179 | 0 | -Inf | 0.004045802 | –NA– | - | - |
| BGIBMGA011714 | 499 | 4 | -8.08130652 | 0.004045802 | cuticular protein hypothetical 27 | 3.00E-25 | NP_001166750.1 |
| BGIBMGA008023 | 723 | 5 | -8.29434389 | 0.004190128 | –NA– | - | - |

Differentially expressed *Bombyx mori* genes for which expression decreased during culture of fat body.

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Table 3. Differentially expressed *Bombyx mori* genes for which expression decreased during culture of fat body.
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*Intact* and *Cultured* columns indicate the number of read counts in intact fat body and cultured fat body of *Bombyx mori*.

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cultured silkworm fat body (our data), Drosophila larval fat body [31], Drosophila larval midgut [31], a Drosophila cell line (SRR029023: modENCODE [32]), human liver [33], human kidney [33], and mouse muscle [11] (Fig. 4 and Fig. 5). The insect fat body had characteristic transcriptomes: fewer genes occupied more of the transcriptome in insect fat body when compared with the transcriptome in the Drosophila cell line and with those in the mammalian tissues. These comparisons may support the hypothesis that characteristic transcriptomes of insect tissues contribute to the broad diversity of insects in the process of evolution.

Materials and Methods

Establishment of the Primary Culture

The p50 strain of the silkworm, Bombyx mori, was reared on the fresh leaves from the mulberry, Morus bombycis. 5th instar larvae were harvested 3 days after the 4th eclosion. The larvae were surface-sterilized by submersion in 70 percent ethyl alcohol, and the fat body were pulled out, care being taken not to injure the digestive system. The fat bodies of 6 larvae were dissected and soaked in 0.75 ml of TRIzol LS (Invitrogen, CA, USA); then, the tissues were kept at −80°C until use. More than 100 chunks of fat body were immediately after dissection. (B) Fat body tissue 45 hours after culture. (C) Fat body tissue 90 hours after culture. (D–F) Phase contrast micrographs of silkworm fat body tissue during culture. (D) Fat body cells immediately after dissection. (E) Fat body cells 45 hours after culture. (F) Fat body cells 90 hours after culture.

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Figure 3. Stereoscopic micrographs and phase contrast micrographs of silkworm fat body tissue during culture. (A–C) Stereoscopic micrographs of silkworm fat body tissue during culture. Scale bars are 1 mm. (A) Fat body tissue immediately after dissection. (B) Fat body tissue 45 hours after culture. (C) Fat body tissue 90 hours after culture. (D–F) Phase contrast micrographs of silkworm fat body tissue during culture. (D) Fat body cells immediately after dissection. (E) Fat body cells 45 hours after culture. (F) Fat body cells 90 hours after culture.

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Figure 4. Comparison of transcriptomes using read-count data. A: Bombyx mori intact fat body, B: Bombyx mori cultured fat body, C: Drosophila melanogaster intact fat body, D: Drosophila melanogaster intact midgut, E: Drosophila melanogaster Kc167 cell line, F: Homo sapiens liver, G: Homo sapiens kidney.

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body tissue left intact as lobes (approx. 2 mm³) were excised from fat bodies dissected from 24 larvae. These tissue particles were incubated in cell culture dishes (ø = 35 mm; BD Biosciences, NJ, USA) with 2 ml of MGM-450 insect medium and with no gas change. The tissue particles were cultured without antibiotics for 90 hours at 25°C. We examined the morphology of cultured fat body cells by stereoscopic microscope (Leica EZ4D) and by phase contrast microscope (Leica DM IRB). The presence or absence of microbial infection of the culture was monitored by microscopic inspection. The culture was terminated by soaking the tissues in 0.75 ml of TRIzol LS (Invitrogen, CA, USA), and the tissue particles were kept at -80°C until use.

RNA Isolation

Total RNA was extracted following the manufacturer’s instructions using TRIzol LS (Invitrogen, CA, USA). Silkworm fat bodies soaked in TRIzol LS were homogenized. The homogenates were incubated for 5 minutes at 25°C. Chloroform (0.2 ml) was added to each sample, the homogenates were shaken vigorously for 15 seconds and then incubated at 25°C for 15 minutes. Samples were subjected to centrifugation at 12,000 × g for 15 minutes and then incubated at 25°C for 15 minutes. The supernatant was transferred to a new tube, and RNA was precipitated out of solution by the addition of isopropanol and then washed twice with 75% ethanol. The RNA pellets were dissolved in 40 µl of distilled water. More than 6.9 µg of total RNA was obtained from each sample. The A260/A280 ratio was between 1.98 and 2.03 for each RNA sample. The integrity of rRNA in each sample was checked by agarose gel electrophoresis [34].

Library Preparation and Sequencing: RNA-seq

All libraries were prepared using the mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA USA) according to the manufacturer’s instructions. In brief, magnetic beads containing poly-T molecules were used to purify mRNA from 10 µg of total RNA. Samples of purified mRNA were then chemically fragmented and reverse transcribed into cDNA. Finally, end repair and A-base tailing was performed before Illumina adapters were ligated to the cDNA fragments. After a gel-size fractionation step to extract fragments of approximately 200 bp, 30 ml of each purified samples was used to amplify cDNA over 15 cycles of PCR. Amplified material was validated and quantified using the Agilent 2100 bioanalyzer and the DNA 1000 Nano Chip Kit (Agilent, Technologies, Santa Clara, CA, USA). Each library was diluted to 10 nM, and 8 pM of each library was loaded onto cBot (Illumina) for cluster generation with cBot Single Read Cluster Generation Kit (Illumina). Sequencing reactions (36 cycles) were performed on a Genome Analyzer IIx (Illumina) using the 36 Cycle Sequencing Kit v4 (Illumina).

Data Analysis and Programs

Sequence read quality was controlled using FastQC program (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Short-read sequences were mapped to annotated silkworm genome sequence obtained from KAIKOBASE (http://sgp.dna.affrc.go.jp/) using the CASAVA mapping algorithm. A maximum of two mapping errors were allowed for each alignment. Multi-hit alignments were discarded. Alternatively, at most two internal N characters are permitted. Scaffolds and contigs data were organized with the locus information, which was integrated with a consensus gene set by merging GLEAN on the chromosomes. We defined the adaptor of the mRNA Sequencing Sample Preparation Kit (Illumina), mitochondrial DNA (32 sequences), ribosomal RNA

![Figure 5. Comparison of transcriptomes using RPKM or FPKM data. A: Bombyx mori intact fat body, B: Bombyx mori cultured fat body, C: Drosophila melanogaster intact fat body, D: Drosophila melanogaster intact midgut, E: Drosophila melanogaster Kc167 cell line, F: Mus musculus muscle. doi:10.1371/journal.pone.0034940.g005](http://www.plosone.org/attachments/201204/06/34940-Figure5.png)
obtained from Ensembl (http://uswest.ensembl.org/index.html) using Bowtie [36] (options: -n 2 -l 36 –best; http://bowtie-bio.sourceforge.net/index.shtml). To estimate fragments per kilo base of exon per million mapped fragments (FPKM), TopHat [37] (http://tophat.cbcb.umd.edu/) and Cufflinks [30] (http://cufflinks.cbcb.umd.edu/) were used.

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**Author Contributions**

Conceived and designed the experiments: NO KI. Performed the experiments: NO. Analyzed the data: NO. Contributed reagents/materials/analysis tools: NO TY KI. Wrote the paper: NO KI.

**References**

1. Lynn DE (2001) Novel techniques to establish new insect cell lines. In vitro cellular & developmental biology Animal 37: 319–321.
2. Eppler A (1980) Invertebrate systems in vitro. E. Kurstak KM, A Du¨bendorfer., editor: Elsevier/North-Holland Biomedical Press. pp 59–66.
3. Kurtti TJ, Traynor MA In; Rodriguez JG, editor. Insect and mite nutrition. North-Holland Publishing Co., Amsterdam. pp 387–395.
4. Kurtti TJ, Chaudhary SP, Brooks MA (1975) Influence of physical factors on the growth of insect cells in vitro. II. Sodium and potassium as osmotic pressure regulators of moth cell growth. In vitro 11: 274–283.
5. Grace TD (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 193: 788–789.
6. Grace TDC (1959) Prolonged survival and growth of insect ovarian tissue under in vitro conditions. Annals of the New York Academy of Sciences 77: 275–282.
7. Carleton HM (1923) Tissue culture: A critical summary. Journal of International Silkworm Genome Consortium (2008) The genome of a lepidopteran. Nature 195: 788–789.
8. Gordon Sato LZ, Mills SE (1960) Tissue culture populations and their relation to the tissue of origin. Proc Natl Acad Sci 46: 963–972.
9. Freshney RI (2005) Culture of animal cells: a manual of basic techniques. New Jersey: A John Wiley & Sons, Inc., Publication. 7 p.
10. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, et al. (2008) Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456: 53–59.
11. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and normalization of read numbers between samples and differential expression analysis was performed using DExSeq. The homology search and local alignments were determined using Blast2go [35]. Sequence data from *Drosophila melanogaster* fat body (SRR072380), *Drosophila melanogaster* midgut (SRR072381), and the *Drosophila melanogaster* Kc167 cell line (SRR029023) were obtained from DDBJ Sequence Read Archive [http://trace.ddbj.nig.ac.jp/dra/index.shtml]. Short-read sequences were mapped to the fly genome.
12. International Silkworm Genome Consortium (2008) Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456: 963–972.
13. Hosseini P, Tremblay A, Matthews BF, Alkharouf NW (2010) An efficient annotation and gene-expression derivation tool for Illumina Solexa datasets. BMC research notes 3: 183.
14. Shimomura M, Minami H, Suetusu Y, Ohyamagi H, Satoh C, et al. (2009) KAObase: an integrated silkworm genome database and data mining tool. BMC genomics 10: 486.
15. Shannon C (1948) A Mathematical Theory of Communication. Bell System Technical Journal 27: 379–423 and 623–656.
16. Robinson MD, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. Genome biology 11: R25.
17. R Development Core Team (2011) R: A Language and Environment for Statistical Computiong. Vienna, Austria. ISBN 3-900051-07-0. The R project website. Available: http://www.R-project.org. Accessed 2012 Mar 11.
18. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol. 11: R106.
19. Xiang H, Zhu J, Chen Q, Dai F, Li X, et al. (2010) Single base-resolution methylation of the silkworm reveals a sparse epigenomic map. Nature biotechnology 28: 516–520.
20. Steif P, Eller G (2006) The gut microenvironment of sediment-dwelling Chironomus plumosus larvae as characterised with O2, pH, and redox microsensors. Journal of comparative physiology B, Biochemical, systemical, and environmental physiology 176: 673–683.
21. Weiss NA, Orr T, Smith GC, Kalter SS, Vaughn JL, et al. (1982) Quantitative measurement of oxygen consumption in insect cell culture infected with polyhedrosis virus. Biotechnology and bioengineering 24: 1145–1154.
22. Tanaka H, Ishibashi J, Fujita K, Nakajima Y, Sagisaka A, et al. (2008) A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. Insect biochemistry and molecular biology 38: 1087–1110.
23. Zhang JY, Pan MH, Sun ZY, Huang SJ, Yu ZS, et al. (2010) The genomic underpinnings of apoptosis in the silkworm, *Bombyx mori*. BMC genomics 11: 577.
24. Shimoda T, Ioyama K (2003) Juvenile hormone acid methyltransferase: a key regulatory enzyme for insect metamorphosis. Proceedings of the National Academy of Sciences of the United States of America 100: 11986–11991.
25. Cherbas L, Willingham A, Zhang D, Yang L, Zou Y, et al. (2011) The transcriptional diversity of 25 Drosophila cell lines. Genome research 21: 301–314.
26. Mitsuhashi J, Inoue H (1988) Obtainment of a continuous cell line from the fat bodies of the mulberry tiger moth, *Spilosoma imparilis* (Lepidoptera: Arctiidae). Annals of the New York Academy of Sciences 77: 275–282.
27. Wyatt GR, Loughheed TG, Wyatt SS (1956) The chemistry of insect hemolymph; organic components of the hemolymph of the silkworm, *Bombyx mori*, and two other species. The Journal of general physiology 39: 853–866.
28. Imanishi S, Ohtsuki Y (1986) Characteristics of cell lines established from embryonic tissues of several races of the silkworm, *Bombyx mori* cultured in vitro. *J Sciric Sci Jpn* 57: 184–188.
29. Imanishi, Tomita S (1992) Suspension-type Cell Cloned Lines from Embryo Tissues of *Bombyx mori* JARQ 26: 196–202.
30. Yamashita M, Iwabuchi K (2001) *Bombyx mori* prohemocyte division and differentiation in individual microcultures. *J Sciric Sci Jpn 57*: 184–188.
31. Nordman J, Li S, Eng T, Macalpine D, Orr-Weaver TL (2011) Developmental control of the DNA replication and transcription programs. Genome research 21: 173–181.
32. Celniker SE, Dillon LA, Gerstein MB, Gunsalus KC, Henikoff S, et al. (2009) Unlocking the secrets of the genome. Nature 459: 927–930.
33. Marioni JC, Mason CE, Mane SM, Stephens M, Gilad Y (2008) RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome research 18: 1509–1517.
34. Jing T, Wang Z, Liu K, Qi F (2006) A modified one-step procedure for rapid RNA isolation from insect. Journal of forestry research 17: 129–131.
35. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676.
36. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology 10: R25.
37. Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25: 1105–1111.
38. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, et al. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology 28: 511–515.