Identification of WSB1 gene as an important regulator in the development of zebrafish embryo during midblastula transition

Wenjian Lv1,2*, Yunbin Zhang1,3*, Zhili Wu1, Lin Chu1, S. S. Koide4, Yuguang Chen2, Yuanchang Yan1*, and Yiping Li1*

1 Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Shanghai 200031, China
2 School of Life Sciences, Shanghai University, Shanghai 200444, China
3 Graduate School of the Chinese Academy of Sciences, Beijing 100864, China
4 Center for Biomedical Research, Population Council, New York, NY 10021, USA

Received: March 25, 2008        Accepted: April 24, 2008

Identification of WSB1 gene as an important regulator in the development of zebrafish embryo during midblastula transition

To uncover novel genes potentially involved in embryo development, especially at the midblastula transition (MBT) phase in the developing embryo of zebrafish, Affymetrix zebrafish GeneChip microarray analysis was carried out on the expression of 14,900 gene transcripts. The results of the analysis showed that 360 genes were clearly up-regulated and 119 genes were markedly down-regulated. Many of these genes were involved in transcription factor activity, nucleic acid binding, and cell growth. The present study showed that significant changes in transcript abundance occurred during the MBT phase. The expression of eight of these 479 genes was identified by reverse transcription-polymerase chain reaction analysis, confirming the microarray results. The WSB1 gene, found to be down-regulated by the microarray and reverse transcription-polymerase chain reaction analyses, was selected for further study. Sequence analysis of the WSB1 gene showed that it encodes a protein with 75% identity to the corresponding active human orthologs. In addition, WSB1 gene expression was detected at a higher level at 2 h post fertilization and at a lower level at 4 h post fertilization, consistent with the chip results. Overexpression of the WSB1 gene can result in the formation of abnormalities in embryos, as determined by fluorescence-activated cell sorting. The present study showed unequivocally that the occurrence of WSB1 expression is an important event during the MBT phase in the development of zebrafish embryos.

Keywords: zebrafish; microarray; WSB1; embryo development; midblastula transition

Zebrafish is an excellent vertebrate model to study embryogenesis and to carry out gene analysis because of the large and transparent embryos and rapid development. The animals attain sexual maturity within 3 months of age. Studying zebrafish using the genetic method of mutagenesis led to the discovery of many important developmental genes; however, many genes that play critical roles during the earliest stages of development remain unidentified because their mutations lead to lethal phenotypes.

The midblastula transition (MBT) phase represents a major event in embryo development and influences differentiation and growth and later biological processes such as segmentation and gastrulation, whereas some highly expressed genes remain quiescent [1]. Maternal gene products regulate early development of the embryo during the period when the newly formed embryo is transcriptionally inactive. During the maternal-zygotic transition of the embryos, transcription is initiated and many maternal RNAs are degraded [2]. In zebrafish embryos, the MBT phase occurs at the 512 cell stage, within a narrow 2.5 h window. Prior to the MBT phase, embryo development is mainly regulated by maternal mRNAs and proteins and manifested by a very low level of transcription [3]. During the MBT phase in Xenopus laevis and zebrafish, the cell cycle lengthens and cell divisions lose
their synchrony, while cell motility occurs and zygotic transcription is initiated [4–6]. Although quiescence of transcription is not so prominent in the mouse, there is also a significant delay in zygotic gene activation in this species [7]. However, the molecular pathways involved in triggering the onset of MBT should be investigated. The activation of the zygotic genome preceding the early events might be the key that opens the path for the transition of cells from pluripotency to undergo a sequence of committed embryonic developments such as cellular differentiation and the activation of early developmental genes [8].

The application of microarray technology to study various aspects of zebrafish physiology has increased extensively in recent years [9] and this methodology has emerged as a key tool for understanding developmental processes as well as basic physiology. In the present study, the differentially expressed genes in zebrafish at the MBT phase were determined using the GeneChip zebrafish genome array (Affymetrix, Santa Clara, USA). The purpose of the present study was to determine the genes whose expression level is up-regulated or down-regulated at the MBT phase in zebrafish to identify and investigate novel genes and their pathways in controlling zygotic gene activation and, subsequently, the early developmental processes. *WSB1* acts as an E3 ubiquitin ligase for the production of the thyroid hormone activating type 2 iodothyronine deiodinase (D2). In the developing tibial growth plate, hedgehog-stimulated D2 ubiquitination through Elongin BC-Cul5-Rbx1 induced the production of the parathyroid hormone-related peptide, thereby regulating chondrocyte differentiation [10].

In the present report, evidence for the occurrence of the expression of the *WSB1* gene during the MBT phase in developing zebrafish embryo is described.

**Materials and Methods**

**Zebrafish strain and embryo collection**

Zebrafish (AB strain) was raised and maintained according to standard laboratory conditions [11]. Embryos were allowed to develop and staged at 28.5 ºC, according to Kimmel *et al* [12].

**RNA isolation and reference RNA for microarray analysis**

Total RNAs were extracted from all embryos using Trizol reagent (Invitrogen, Carlsbad, USA). RNA quality was evaluated by gel electrophoresis, and the concentration was measured by ultraviolet spectrophotometer. RNAs were isolated from embryos at 2.5 hpf and 4 hpf stages and formulated into equivalent concentrations. As the initial step, sufficient amounts of the RNAs required for the entire project were prepared.

**Analysis of microarray data and evaluation**

The zebrafish microarray comprising 14,900 zebrafish oligonucleotides was designed and synthesized by Affymetrix. Two time points, namely 2.5 and 4 hpf, were examined in duplicate [13,14]. Total RNAs were biotin labeled, using the GeneChip one-cycle target labeling (Affymetrix) method and hybridized to the Affymetrix zebrafish genome array [15].

**Semiquantitative RT-PCR**

Total RNAs were isolated from zebrafish embryos at varying stages (2 hpf and 4 hpf) of development and from various organs of adult zebrafish. For RT-PCR analysis, first-strand cDNA was generated using oligo-dT primers and Superscript III reverse transcriptase (Invitrogen). The PCR annealing temperature and primers are shown in Table 1. To compare the levels of each mRNA, concentrations of cDNA were normalized to yield PCR products that were approximately equivalent to those of β-actin. All mRNA levels were quantified in the exponential phase of amplification, determined by analyzing PCR products synthesized with different numbers of cycles, using a fixed amount of cDNA.

**Isolation of zebrafish cDNA and RT-PCR analysis for *WSB1***

To obtain partial fragments, PCR was carried out using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). The PCR conditions were as follows: 94 ºC for 3 min, 33 cycles of 94 ºC for 15 s, 55 ºC for 30 s, and 68 ºC for 90 s, and finally 68 ºC for 10 min, using the primers as shown in Table 1. The full-length zebrafish *WSB1* cDNA was sequenced and found to be composed of 3452 bp. It spans an open reading frame (ORF) between nucleotides 283 and 1554 that encodes a protein of 423 amino acids (GenBank accession No. NM199633).

To establish the structural features of the zebrafish WSB1 protein, its amino acid sequence was compared with those of other WSB1 proteins using ClustalW version 1.82 online (http://www.ebi.ac.uk/clustalw2/index.html).

**Cloning of full-length *WSB1* cDNA**

A *WSB1* cDNA fragment with the complete coding region was amplified by RT-PCR (*WSB1*-F, 5'-GGCGAAATTCG-ATGGCAAGCTTCCCAGATTG-3’ and *WSB1*-R, 5'-GC-
GTCTAGATATGGGCTTTGAAGGTAAGAA-3'). The underlined \textit{EcoRI} and \textit{XbaI} cleavage sites were introduced. Total RNAs were extracted from 2 hpf zebrafish embryos and reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). The primers were used for PCR to obtain a fragment (1402 bp) of the gene. The PCR fragment was separated by gel electrophoresis (1% agarose) and purified. The fragment was then cut with \textit{EcoRI} and \textit{XbaI} and ligated into the pCS2+-\textit{myc} vector.

Capped RNA transcription and mRNA injection

\textit{WSB1} ORF was amplified as described above. The resulting fragments were subcloned into pCS2+-\textit{myc}, linearized and transcribed using the Ambion message machine kit (Ambion, Austin, USA). mRNA used in the injection experiments was synthesized as described previously [16]. The 6\textit{myc} tag upstream of \textit{WSB1} was used to determine the translation efficiency of the process. The 0.3–0.8 ng of \textit{WSB1} mRNA was injected into the yolk of one-cell or two-cell stage embryos. In addition, 0.3 ng \textit{GFP} mRNA without the \textit{myc} tag was injected as the lineage tracer as well as the negative control. Phenol red (0.05%; \textit{W/V}) was co-injected as a non-toxic visual marker.

Western blot analysis

Six hours after injection with green fluorescent protein (\textit{GFP}) and \textit{WSB1} mRNA, zebrafish embryos were processed for Western blot analysis. Each group had approximately 70 embryos. Experiments were carried out as follows: embryos were rinsed in cold saline, then pooled and homogenized (1:2; \textit{W/V}) with a Potter homogenizer in Tris-HCl buffered saline (0.1 M, pH 7.5) containing 1 \textmu M leupeptin, 10 \textmu M pepstatin, and 2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 15,000 \textit{g} for 30 min at 4 ºC, and the resulting pellet was dissolved in 10 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 100 mM dithiothreitol, and 10% glycerol at 4 ºC. Samples were heated to 95 ºC for 5 min and cooled on ice. The pellets were thawed and analyzed by electrophoresis in 15% discontinuous sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and blocked by immersion for 2 h in phosphate-buffered saline containing 5% dry milk, and 0.1% Tween-20. The membranes were then incubated at 4 ºC overnight with an antibody of \textit{myc} tag (9B11 diluted 1:1000; Cell Signaling Technology, Beverly, USA)/\beta-actin (diluted

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Annealing temperature (ºC)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC053206</td>
<td>GACTCGCTTTGCCCACAGAAT (F) CTCACGATAACCTTTAGAAGCCCT (R)</td>
<td>55</td>
<td>395</td>
</tr>
<tr>
<td>BC051620</td>
<td>ACTGTGCTTGGCCGCTCATTAC (F) ATCCCTGAGGTCTTCTTTGCT (R)</td>
<td>53</td>
<td>339</td>
</tr>
<tr>
<td>AI584468</td>
<td>TGGAGATTAACCAACTGCTT (F) GAAACCTTTGAACACCTGTA (R)</td>
<td>52</td>
<td>271</td>
</tr>
<tr>
<td>AI883911</td>
<td>GCACAGTAGAAGAGCGTGATA (F) TGTAATAGGGCTAAGTGGTG (R)</td>
<td>51</td>
<td>412</td>
</tr>
<tr>
<td>AW059114</td>
<td>TGCAAGAGCTACCTAGGTT (F) CTAGGAGGACATTACCTTCT (R)</td>
<td>53</td>
<td>334</td>
</tr>
<tr>
<td>AW128368</td>
<td>TAATAAGAGGAAAACCTACGCG (F) GGACATTAAAACCTTACCGTG (R)</td>
<td>53</td>
<td>340</td>
</tr>
<tr>
<td>BM402106</td>
<td>AACAAAGGTAACAACGCAGAG (F) TGACCCGAGGTATAAGCAG (R)</td>
<td>55</td>
<td>495</td>
</tr>
<tr>
<td>NM199633</td>
<td>CGGATCTCAGGCTTTT (F) CATCTTGAGGCCACACG (R)</td>
<td>58</td>
<td>702</td>
</tr>
<tr>
<td>\beta-actin</td>
<td>CAACGCTACGTTATG (F) TGCCAGGTATCATTGG (R)</td>
<td>55</td>
<td>892</td>
</tr>
</tbody>
</table>

1:5000; Sigma, St. Louis, USA), followed by incubation with peroxidase-conjugated secondary antibody (diluted 1:5000; KangChen, Shanghai, China), and detected by the SuperSignal West Pico Chemiluminescent system (Pierce, Rockford, USA).

**Embryo dissociation and fluorescence-activated cell sorting (FACS)**

Cell collection and the FACS technique and analysis used were carried out as previously reported [17]. In brief, cells comprising the embryo tissues were dispersed in 1 ml DMEM supplemented with 10% FBS, and the cell suspension was sieved through a 4 µm filter. Cells were collected, washed several times, and resuspended in phosphate-buffered saline, followed by the addition of propidium iodide to a final concentration of 1 µg/ml, and 2 µg DNase-free RNase. The suspension was incubated in the dark for 30 min. FACS analysis was carried out to obtain forward- and side-scatter characteristics. The propidium iodide exclusion test was carried out using a FACS vantage flow cytometer (FACSCalibur; BD Biosciences, San Jose, USA) and the cell cycle phase was analyzed using FlowJo (Tree Star, Ashland, USA) and FCS3.0 software.

**Results**

**Microarray analysis and gene categorization**

An enriched custom microarray, containing 14,900 gene transcripts prepared from several cDNA libraries, was used in this study. We chose two time points, 2.5 and 4 hpf, and each time point was repeated once again. Expression data from four independent microarray analyses were collected (Fig. 1). Chip results are depicted as a scatter diagram. From the entire dataset, reproducible expression data were summarized and the results from the microarray are listed in Table 2. Log base 2 transformed expression ratios were normalized to account for background fluorescence, mean intensity, and deposition order biases [18]. In general terms, the microarray analysis might not provide strictly quantitative data, as was suggested previously [15]. To derive the reproducible dataset, transcripts whose induction was repeatedly observed were selected. Next, a functional assignment of the respective transcripts was made. As many expressed sequence tag (EST) sequences occurred in the 5'- or 3'-untranslated region (UTR) and were non-conserved during evolution, longer sequence information was obtained by searching for overlapping EST sequences. Based on these longer or full-length amino acid sequences, a search for protein

<table>
<thead>
<tr>
<th>Transcription</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14,900</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>360</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>119</td>
</tr>
<tr>
<td>Others</td>
<td>14,421</td>
</tr>
</tbody>
</table>

**Table 2 Number of up-regulated and down-regulated genes from an enriched custom zebrafish microarray**

![Fig. 1 Scatter diagram of zebrafish gene expression profiles](scatter_diagram.png) The x axis shows signals emitted by the control group. The y axis shows signals from the test group. Red dots represent detection of signals from both control and test groups. Blue dots represent detection of signals from one of the groups. Yellow dots denote absence of signals from both groups.
homology and assignment of their possible functions was undertaken. The resultant proteins were classified according to the Gene Ontology Molecular Function, mainly based on DNA binding (eg, AL727131, CB353911), transcription factor activity (NM_131698.1), receptor activity (NM_131137.1), nucleic acid binding (AL724403, BI475158), zinc ion binding (AF207751.1, AA605796), and G protein-coupled receptor activity (BC050172.1).

**Confirmation of differential expression of zygotic genes**

The genes selected from the microarray analysis to test the validity of the chip results are listed in Table 3. The relative abundance of β-actin mRNA was similar at both stages and was used as the internal control. The abundance values of all seven up-regulated genes (AW059114, BC053206, BC051620, AI584468, AI883911, AW128368, and BM402106) were in accordance with those of the chip results (Fig. 2), as well as the single down-regulated gene (WSB1; NM199633). Fig. 2 shows the results of semiquantitative RT-PCR at 2 and 4 hpf and β-actin is used as the control.

### Table 3 Selected genes from zebrafish microarray analysis results and their corresponding changes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC051620</td>
<td>+6.3</td>
</tr>
<tr>
<td>BC053206</td>
<td>+5.4</td>
</tr>
<tr>
<td>AI584468</td>
<td>+4.9</td>
</tr>
<tr>
<td>AI883911</td>
<td>+5.8</td>
</tr>
<tr>
<td>AW059114</td>
<td>+6.0</td>
</tr>
<tr>
<td>AW128368</td>
<td>+5.7</td>
</tr>
<tr>
<td>BM402106</td>
<td>+5.4</td>
</tr>
<tr>
<td>NM199633</td>
<td>−1.2</td>
</tr>
</tbody>
</table>

**Identification and characterization of zebrafish WSB1**

The 3442 bp full-length cDNA of zebrafish WSB1 was comprised of a 283 bp 5'-UTR, a 1272 bp ORF encoding a deduced 423 amino acid polypeptide, and a 1887 bp 3'-UTR. The alignment of the Danio (zebrafish) WSB1 amino acid sequences against those of Homo (human), Mus (mouse), Rattus (rat), Xenopus, and Takifugu revealed a high degree of interspecies sequence conservation [Fig. 3(A)]. In particular, the SOCS-box-containing WD-40 protein was identical to the amino acid content among all species. A database search for sequence identity to the deduced amino acid sequence of WSB1 revealed significant homology with a number of different WD proteins. In the phylogenetic analysis, WSB1 protein had the highest homology to that of the Takifugu gene among the vertebrates [Fig. 3(B)].

**Expression pattern of WSB1**

As shown in Fig. 4(A), WSB1 was expressed at various stages, reaching a high level of expression at 2 hpf that subsequently decreased to a low level at 4 hpf, paralleling the chip results. The expression gradually decreased until the 24 hpf stage, except for a slight increase at 16 hpf [Fig. 4(A)]. The degree of expression of WSB1 in adult zebrafish tissues is shown in Fig. 4(B). Of the nine tissues studied, the highest level was found in the intestine and was practically undetectable in the fin.

**Effect of WSB1 overexpression on early embryo development**

To determine the function of WSB1 during zebrafish embryonic development, WSB1 was overexpressed by microinjecting its mRNA into early embryos at the 1-cell or 2-cell stage together with GFP. Western blot analysis was carried out to detect the expression of WSB1 (myc...
WSB1, an important regulator of embryo development in zebrafish

Fig. 3 Comparative sequences of WSB1 proteins from six species
(A) Alignment of Homo sapiens (AAF17193), Mus musculus (CAI25778), Rattus norvegicus (NP_001036026), Xenopus tropicalis (NP_989387), Danio rerio (NM199633), and Takifugu rubripes (AAD32247) WSB1 proteins. The shaded residues are either identical (black) or similar (gray) in at least two sequences. The amino acid sequences of Homo, Mus, Rattus, Xenopus, Danio, and Takifugu WSB1 were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein).

(B) Phylogenetic analysis of WSB1-related proteins. Comparison of amino acid alignment of WSB1 sequences among Homo, Mus, Rattus, Xenopus, Danio, and Takifugu were used to construct a phylogenetic parsimony tree with MEGA software.
**WSB1**, an important regulator of embryo development in zebrafish

A significant finding was the occurrence of numerous abnormally developed embryos among the **WSB1** overexpressed group. In contrast to the control group at 24 hpf, the development of nearly two-thirds of the treated embryos was markedly arrested (Figs. 6 and 7). In the control and untreated groups, the development of the embryos was comparable ([Figs. 6(A) and 7(A)]), whereas **WSB1** overexpression induced morphological abnormalities at gastrulation and segmentation stages. mRNA treatment produced defects in the extension movements during the early embryonic developmental stages that led to the shortening of the body axis at 30 hpf (Fig. 6) and at day 3 (Fig. 7).

The effectiveness of **WSB1** on inducing abnormal embryos was dose-dependent. When the amount of **WSB1** mRNA injected was 1 ng/embryo, approximately 70% of the embryos developed abnormal extension movements at 24 hpf (Table 4). The same dose of GFP (1 ng/embryo) was used as a control. The major effect of the treatment was a delay in the rate of embryonic growth. Approximately 30% of embryos injected with **WSB1** mRNA were arrested at the stage earlier than the 14-somite ([Fig. 8](#fig8)), designated as anomaly 1; approximately 50% of the treated embryos were slightly retarded ([Fig. 8(C)]), designated as anomaly 2.

**Fig. 4** Reverse transcription-polymerase chain reaction analysis of **WSB1** expression in developing zebrafish embryos and in adult tissues  
(A) Electrophoretic pattern of **WSB1** mRNA expressed at various stages of embryo development. (B) Electrophoretic pattern of **WSB1** mRNA in nine tissues of adult zebrafish. Note high content in the intestine and practically no content in the fin. hpf, hour post fertilization; M, DNA marker.

**Fig. 5** The expression level of the microinjection  
(A) Electrophoretic pattern of overexpressed WSB1 proteins, stained with anti-myc antibody. 1, green fluorescent protein (GFP)-injected group; 2, GFP/WSB1 co-injected group. (B) GFP expression examined by fluorescence microscopy of the GFP/WSB1 co-injected group at 6 h post fertilization. Scale bar=500 µm.

**Fig. 6** Morphological changes induced with RNAs encoding **WSB1** and green fluorescent protein (GFP) as control injected into zebrafish embryos at the 1-cell or 2-cell stages  
The embryos were cultured to 30 h post fertilization (hpf). Abnormalities of body shape and tail occurred in **WSB1**-treated embryos. Scale bar=250 µm.
WSB1, an important regulator of embryo development in zebrafish

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Death</th>
<th>Anomaly 1</th>
<th>Anomaly 2</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSB1 (%)</td>
<td>29 (16.8)</td>
<td>27 (15.6)</td>
<td>91 (52.6)</td>
<td>26 (15.0)</td>
</tr>
<tr>
<td>GFP (%)</td>
<td>15 (16.3)</td>
<td>0 (0.0)</td>
<td>6 (6.5)</td>
<td>71 (77.2)</td>
</tr>
<tr>
<td>Untreated (%)</td>
<td>4 (4.0)</td>
<td>0 (0.0)</td>
<td>3 (3.0)</td>
<td>93 (93.0)</td>
</tr>
</tbody>
</table>

Observations were made 24 h post-treatment. Anomaly 1, a delay in development to a stage earlier than the 14-somite; anomaly 2, a slight developmental retardation.

We suspected the retarded development in embryos overexpressing WSB1 might be due to abnormalities in the cell cycle, so FACS experiments were carried out to examine the cell cycle of embryos.

FACS was carried out twice (Fig. 9). Compared to GFP-treated control cells, the percentage of S phase cells decreased, whereas that of the M phase to G2 phase increased ($P<0.05$). The results suggest that overexpression of WSB1 protein could cause an arrest of embryonic cells in the G1 phase to S phase, as well as prevent cells from passing through the M phase ($P<0.01$) (Table 5). These findings suggest that WSB1 might participate in cell cycle regulation, although the mechanism is unclear.

Discussion

Although it is well accepted that the activation of the zygotic genome and the widespread onset of transcription are essential for the successful completion of the MBT stage, the specific gene products driving the process have not been completely identified [8]. The present Affymetrix GeneChip zebrafish microarray results show that approxi-
WSB1, an important regulator of embryo development in zebrafish

Table 5 Cell cycle analysis of zebrafish embryos treated with green fluorescent protein (GFP) or WSB1 mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Total</th>
<th>% M1 (cell death)</th>
<th>% M2 (G0−G1)</th>
<th>% M3 (S)</th>
<th>% M4 (G2−M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>100</td>
<td>5.7±3.3</td>
<td>59.2±6.2</td>
<td>21.4±2.9</td>
<td>12.1±0.3</td>
</tr>
<tr>
<td>WSB1</td>
<td>100</td>
<td>2.3±1.2</td>
<td>63.8±8.9</td>
<td>11.2±3.8*</td>
<td>21.7±3.8*</td>
</tr>
</tbody>
</table>

Values are percentages of cells in the various cell cycle phases, determined using fluorescence-activated cell sorting analysis. M1, cell death or apoptosis; M2, cells in G0−G1 phase; M3, cells in S phase; M4, cells in G2−M phase. *P<0.05 versus GFP-treated controls (n=3; 50,000 cells are shown).

Fig. 9 Fluorescence-activated cell sorting analysis of DNA synthesis and content of zebrafish embryo cells, determined by propidium iodide staining. (A) Green fluorescent protein (GFP)-injected embryos. (B) WSB1-treated embryos. M1, cell death or apoptosis; M2, cells in G0−G1 phase; M3, cells in S phase; M4, cells in G2−M phase. *P<0.05 versus GFP-treated controls (n=3; 50,000 cells are shown).

Currently 360 genes are up-regulated, exceeding the log base 2 transformed expression ratios. Several genes were selected for validation studies, including the five EST sequences (AI584468, AI883911, AW059114, AW128368, and BM402106), a mitochondrial carrier glutamate (BC053206), and a Ras oncogene family member (BC051620). All of these factors were highly expressed on completion of MBT. Likewise, a down-regulated gene, WSB1 (NM199633), was selected from the chip results. As predicted from the gene expression profiling, the semiquantitative RT-PCR results showed that these genes are expressed in accordance to the microarray findings. The reliability of the microarray analysis has been confirmed. Similarly, two genes, Biklf and Vegal, up-
regulated by 2.9 folds and 5.9 folds, respectively, in the present chip results, were also reported to be expressed after MBT [19,20]. However, nanor, which was reported to be expressed initially at MBT, was not found in our chip results or in others [21].

Microarray analysis offers an opportunity to simultaneously evaluate the expression of a series of genes in a single study [22]. The statistically derived results of the Gene Ontology Molecular Function deduced from the microarray results suggest that the functions of the up-regulated genes during MBT include DNA binding, transcription factor activity, nucleic acid binding, zinc ion binding, GTP binding, receptor activity, and G-protein coupled receptor activity. It is known that MBT in zebrafish embryos is characterized by the lengthening of the cell cycle, loss of cell synchrony, activation of transcription, and appearance of cell motility. It is not surprising to find that the regulatory factors of transcription and DNA-dependent protein genes are included in the up-regulated group [6]. The Gene Ontology Molecular Function of the down-regulated subgroup includes the structural constituent of ribosome transferase activity, DNA binding, and transcription factor activity. One of the defining events that occur during MBT is the activation of the zygotic genome manifested by the widespread onset of transcription. Several of the identified genes encode transcription factors [8]. Microarray results, including the up-regulated and down-regulated subgroups and the functional analysis findings, show that the expression level of transcription-related factors changed significantly during zygotic transcription. Based on these findings we theorized that alterations in the activities of expressed genes are the causes or facilitators of embryonic development.

WSB1, a gene included in the down-regulated subgroup, is rarely considered to be involved in embryonic development. However, a novel chick WD-protein, cSWiP-1, is expressed in somatic mesoderm and in developing limb buds as well as in other embryonic structures where hedgehog signaling has been proved to play a role [23]. WSB1 acts as an E3 ubiquitin ligase for thyroid hormone activating D2. In the case of the growth plate of developing chicks, WSB1-mediated D2 ubiquitination increases parathyroid hormone-related peptide production, thereby promoting chondrocyte proliferation. WD-40, a facilitator of WSB1, recognizes a novel 18 amino acid loop in D2 that confers metabolic instability, whereas the SOCS-box domain mediates its interaction with a ubiquitinating catalytic core complex, modeled as Elongin BC-Cul5-Rbx1 [10]. cSWiP-1 expression in somites integrates two signals originating from structures adjacent to the segmental mesoderm, a positive signal from the notochord, mediated by sonic hedgehog (Shh), and a negative signal from the intermediate and/or lateral mesoderm (possibly BMP4) [23]. WSB1 is induced by the signaling pathway. It is noteworthy that WSB1 is co-expressed with D2 in brain areas influenced by Shh [24]. This factor acts as a mitogen on precursor cells, regulating the fate of neural stem cells [25] in areas such as the spinal cord and hippocampus [25,26] as well as acting as an E3 ubiquitin ligase for the thyroid hormone activating D2.

WSB1 from zebrafish was cloned from the cleavage stage 2 hpf cDNA library. Protein sequencing revealed a putative peptide that belongs to the large family of WD-proteins. The members of this family all contain at least four WD repeats, a motif of approximately 40 amino acids, terminating with tryptophan and asparagines [23]. WD-40 repeats were originally recognized in the β-subunit of G proteins and have since been detected in a wide variety of cytoplasm proteins, many of which are involved in signal transduction [27].

The expression of the WSB1 gene declines gradually during the initial 24 h of embryo development. Micro-injection of WSB1 mRNA induces abnormalities in embryos that are readily observed within 24 hpf and the occurrence of embryo arrest at the 14-somite stage or earlier. At 30 hpf and 3 dpf, the WSB1-treated embryos showed distorted notochord and/or axial asymmetry. Former research showed that the notochord emits signals that could influence the production of cSWiP-1 in the medial portion of the somites during normal chick development [23].

It is well known that the commitment of cells to undergo chromosome replication occurs during the G1 phase of the cell cycle. Should the conditions allow passage of the cells through the “commitment point”, after a lag period, they will proceed into the S phase. Various parameters could influence the ability of a cell to make this decision, based on an assessment of whether the cell mass is sufficient to support the occurrence of a divisional cycle, perhaps some cell circle reference genes. The present cell cycle study shows that the percentage of S phase cells decreases, indicating that the cells are arrested from G1 to S phase. At the same time, the percentage of cells moving from G2 to M phase increases, indicating that the cells are prevented from proceeding to the G1 phase. The FACs results show that overexpressed WSB1 induces abnormalities in the developing embryos that can be attributed to the double-restrictive influence on the cell cycle. Cell cycle regulatory factors are considered to be
important controllers of embryo development [28].

In conclusion, the expression of the WSB1 gene is an important event during the MBT phase in the development of zebrafish embryos. Overexpressed WSB1 can cause embryo abnormalities and cell cycle arrest.

Acknowledgement

The authors thank Mr Yihong Wang for his kind assistance in caring for the fish.

References

2 Schier AF. The maternal-zygotic transition: death and birth of important controllers of embryo development [28].
3 Yang J, Tan C, Darken RS, Wilson PA, Klein PS.
9 Douglas SE. Microarray studies of gene expression in fish. OMICS 2006, 10: 474–489