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CYR61 is a Novel Gene Associated With Temperature-Dependent Changes in Fish Metabolism as Revealed by cDNA Microarray Analysis on a Medaka *Oryzias latipes* Cell Line

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Abstract A microarray comprising 3,514 cDNAs was constructed from a medaka EST library to elucidate the transcriptional responses associated with temperature shift from 25 to 15°C in a medaka cell line. Microarray analysis revealed that the mRNA levels of 313 clones were significantly different in at least one combination of different incubation periods up to 7 days at a given incubation temperature or between 25 and 15°C at a given incubation period ($P < 0.05$). These genes are known to be associated with various biological processes including morphogenesis, cell proliferation and response to stress. A number of genes encoding proteins which localize in extracellular areas were apparently up-regulated at 15°C, whereas those localizing in intracellular areas were down-regulated at this temperature. In addition, while a number of genes represented long-term expression changes, only a few responded to short-term inductions. A typical example was *CYR61*, a multifunctional matricellular signaling modulator, the mRNA levels of which increased after temperature shift from 25 to 15°C in 3 h, and then decreased rapidly to near the original level within 12 h. Another series of analyses by quantitative reverse transcription-PCR revealed that the mRNA levels of *CYR61* at 5°C were significantly higher even at 24 h after temperature shift compared to those of the cells successively maintained at 25°C. These analyses suggest that remodeling and reorganizing of extracellular structure of cells are important to offset the low temperature effect and *CYR61* is considered to be a novel gene associated with temperature response in poikilotherms. J. Cell. Biochem. 104: 1297–1310, 2008.

Key words: cell line; CYR61; medaka; *Oryzias latipes*; temperature adaptation; transcriptome

Environmental temperature markedly influences the physiology and behavior of poikilotherms, including fish. Seasonal temperature changes take place over weeks or months, and physiological reorganization to compensate for such changes is often referred to as an acclimation response [Hazel and Prosser, 1974]. Eurythermal temperate fish such as goldfish *Carassius auratus* (Linnaeus) and carp *Cyprinus carpio* (Linnaeus) are able to thrive in environments that expose them to a large variation in diurnal and seasonal temperatures. However, the mechanisms involved in alteration of gene expression which enable eurytherms to compensate for such thermal changes are poorly understood.

Culture cells from fish grow in temperature ranges where the corresponding fish inhabit. For example, RTG-2 cells derived from rainbow trout *Oncorhynchus mykiss* (Walbaum) gonad are not able to survive over 28°C [Mosser et al., 1986], whereas goldfish culture cells isolated

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from caudal fin grow at 37°C [Shima et al., 1980; Kondo and Watabe, 2004]. Medaka *Oryzias latipes* (Temminck and Schlegel) is a eurythermal species like goldfish and carp. It has several geographically and genetically distinct populations. Recently, we investigated temperature acclimation response in various medaka cell lines derived from different populations, and found that 15°C provided permissive growth in all cell lines from the Northern Japanese and East Korean populations, but not in the Southern Japanese population and medaka-related species *Oryzias celebensis* (Weber), which inhabits a tropical zone [Hirayama et al., 2006]. Reverse transcription-PCR (RT-PCR) for 102 temperature-responsive genes, previously reported in other species, revealed that the accumulated mRNA levels of the heat shock protein 47 (HSP47) gene was lower at 25°C than at 33°C, and vice versa for 12 genes including nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA, also called IκBα) and Rab35 (also called Rab-1c) genes, which are the inhibitor of a transcription factor NF-κB and the small Ras-like GTPase protein, respectively, in OLHNI-1 cell line established from the Northern Japanese population. Furthermore, quantitative RT-PCR (qRT-PCR) demonstrated that the accumulated mRNA levels of NFKBIA and Rab35 in OLHNI-1 and OLSOK-e7 cell lines from the East Korean population were increased when the culture temperature was shifted from 33 to 15°C, but not in OLHdrR-e3 cell line from the Southern Japanese population. These results suggested that immune responses and intracellular transports are associated with temperature adaptation in medaka.

Large scale expressed sequence tag (EST) and genome projects targeting at medaka have been carried out [Wittbrodt et al., 2002; Kimura et al., 2004; Kasahara et al., 2007]. The EST analysis is useful not only for the total genome sequencing, but also for a comprehensive work on gene expression as revealed for human [Miller et al., 1999], mouse [Marra et al., 1999] and *Fundulus heteroclitus* (Linnaeus) [Paschall et al., 2004]. More than 100,000 EST sequences of medaka are available, which comprise about 23,000 clusters generated from cDNA libraries of various adult tissues, embryos and cultured fibroblast-like cells [Kimura et al., 2004; Mitani et al., 2004].

Thus, it seems interesting to employ microarray using the above-mentioned comprehensive EST database to investigate the gene expression pattern of medaka in association with temperature changes. Microarray facilitates genome-wide examination of gene transcripts expression [Schena et al., 1995] and has also been adopted recently for fish [Oleksiak et al., 2002; Gracey et al., 2004; Buckley et al., 2006]. In this study, we examined temperature-dependent expression changes of EST sequences using medaka culture cells, which was one of the simplest materials to investigate the temperature adaptation mechanisms of fish, by a cDNA microarray technique.

MATERIALS AND METHODS

Cell Lines

OLHNI-e1 cell line from an embryo of HNI medaka inbred strain, and Odate-e1 and Kino-e2 cell lines from the embryos of Odate and Kinosaki wild subpopulations, respectively, from the Northern Japanese population were established as described in Hirayama et al. [2006]. Cells were cultured at 25°C with L-15 medium (Irvine Scientific, Santa Ana, CA) buffered with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and supplemented with 20% (v/v) fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan) and 50 μg/ml streptomycin. OLHNI-e1 cell line showed a fibroblast-like morphology, whereas Odate-e1 and Kino-e2 cell lines did epithelial cell-like morphologies (data not shown).

Fabrication of Medaka cDNA Microarray

The 3,514 clones without redundant sequences were selected by in silico screening on medaka OLb, OLc, OLd, OLe, and OLf EST libraries constructed in *Escherichia coli* using the OLestall0309asm Assemble Library of medaka EST database in the Medaka Genome Database, MBase (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html; Supplementary Table SI). cDNA inserts were amplified by PCR using 1 μl of the bacterial suspension in 100 μl of a standard PCR reaction mixture and primers specific to each library. PCR reaction mixture was prepared according to the supplier's protocol. Thermal cycling conditions consisted of the initial step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and polymerization at 72°C for

1 min, with the final extension step at 72°C for 5 min. The PCR products were purified using Montage^{TM} PCR_{96} cleanup kit (Millipore, Bedford, MA) and dissolved in 25% dimethyl sulfoxide (DMSO). PCR products were printed robotically onto high density amine coated slides for DMSO solution (Matsunami, Osaka, Japan) using an Affimetrix 417^{TM} Arrayer (Affimetrix, Santa Clara, CA). Medaka elongation factor 1 alpha (EF-1α) cDNA (OLe06.11c in the medaka EST database), Lucidea^{TM} Universal ScoreCard^{TM} (GE Healthcare UK Ltd, Buckinghamshire, England) and 25% DMSO solution without DNA were also printed as the internal, external and negative controls, respectively. A total of 7,680 spots were laid on one slide.

RNA Extraction

OLHNI-e1 cell line was cultured at 25°C to the confluent, and then transferred to 15°C, or successively maintained at 25°C. The medium of plates was changed to a fresh one on days 1 and 4 after the temperature shift. At 0, 1, 3, and 12 h and on days 1, 3, and 7 after the temperature shift (Fig. 1A), cells each in three dishes incubated for a given incubation period were lysed with ISOGEN (Nippon Gene, Toyama, Japan) and total RNAs were extracted from the cell lysates following the manufacturer's instruction. The quality of total RNAs was verified by agarose-gel electrophoresis using denaturing conditions with reference to 28S and 18S rRNA bands [Skrypina et al., 2003; Dumur et al., 2004]. Isolated total RNAs from three dishes were then pooled and subjected to the following analyses. Two independent series of experiments were carried out to examine the reproducibility, the first series (sample No.1 group) for cDNA microarray analysis and the second one (sample No. 2 group) for qRT-PCR analysis.

In another experiments OLHNI-e1, Odate-e1 and Kino-e2 cell lines were cultured at 25°C, and then transferred to 5 and 15°C, or successively maintained at 25°C, and subjected to qRT-PCR. Cell cultures and extraction of total RNAs were performed as described above, except for incubation periods of 1, 3, 6, 12, and 24 h (Fig. 1B).

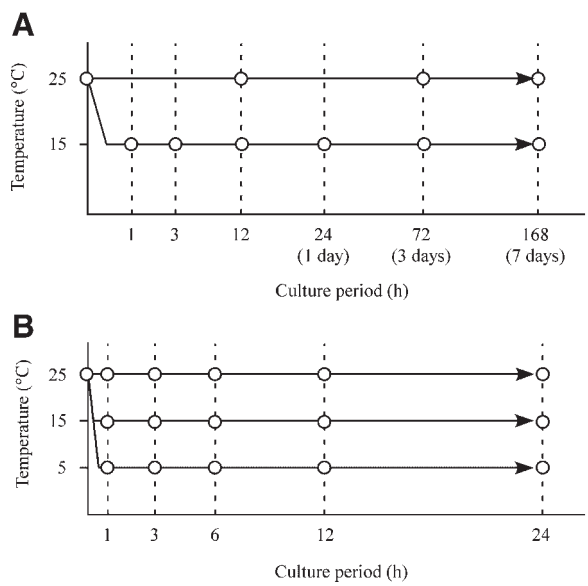

Fig. 1. Schematic diagram showing the time course of temperature shift and sampling regime for cDNA microarray experiments and quantitative RT-PCR experiments for CYR61. The culture temperature for medaka OLHNI-e1 cell line was shifted from 25 to 15°C within 1 h or successively maintained at 25°C for 7 days (**A**), whereas that for OLHNI-e1, Odate-e1 and Kino-e2 cell lines was shifted from 25 to 5°C and 15°C within 1 h or successively maintained at 25°C (**B**) for 24 h. Cells were collected at certain time intervals as indicated by vertical dashed lines.

Preparation of Probes and Hybridization in cDNA Microarray

First-strand cDNA probes labeled with cyanine 3 (Cy3) and Cy5 were generated from 15 μg of total RNAs from each sample using CyScribe^{TM} cDNA Post Labeling Kit (GE Healthcare UK Ltd.) following the manufacturer's instructions. The resulting labeled cDNA probes were purified using a CyScribe^{TM} GFX^{TM} Purification Kit (GE Healthcare UK Ltd.) according to the manufacturer's protocol, and concentrated using a vacuum centrifuge dryer. The fluorescence labeling reaction efficiency of each cDNA probe prepared was confirmed by measuring absorbance at 260, 550, and 650 nm. Hybridization reaction for labeled cDNA probes was performed at 65°C for 16 h on the microarray slides in a moist chamber with one sample containing Cy3-labeled cDNAs and the other containing Cy5-labeled cDNAs using a manufacturer's hybridization buffer (GE Healthcare UK Ltd.) containing 50% formamide. After hybridization, the slides were washed at room temperature three times in 2× standard saline citrate (SSC; 33.3 mM NaCl and 33.3 mM sodium citrate) containing 0.2% sodium dodecyl sulfate (SDS) for 3 min and three times in 0.2× SSC containing 0.2% SDS for 3 min followed by

washing at 60°C for 10 min twice in 0.2× SSC containing 0.2% SDS. The slides were subsequently washed at room temperature twice in 0.2× SSC containing 0.2% SDS for 3 min, three times in 0.2× SSC to remove SDS, and then twice in absolute ethanol for dehydration. Finally, the slides were dried by centrifugation at 300*g* for 3 min.

Labeled cDNAs bound to the microarray slides were quantified by fluorescence with a laser confocal scanner ScanArray® 4000 (Perkin-Elmer, Boston, MA) at 100% laser power with around 60% and 80% photomultiplier tube gains for low and high power settings, respectively. The fluorescence images were analyzed using the Packard QuantArray™ software (Perkin-Elmer). The resulting fluorescence intensities for each spot on the array slides were quantified by an algorithm for adaptive circle segmentation of an image, and the median of signal intensities of each pixel was subtracted from the median of the background intensities. The fluorescence signals less than twice the values of artificial negative control spots and those from low-quality areas of the array were regarded as artifacts and excluded from further analyses [Rahnenfuhrer, 2005]. The Cy5/Cy3 fluorescence ratios were $\log_2$ transformed, and a normalization factor was applied so that the median fluorescence ratio of the well-measured spots on each array was 0. Generally, the Cy5/Cy3 fluorescence ratios for a set of two spots embedded for one cDNA were averaged. However, in some cases, the ratio of one spot was considered when the duplicated spot failed to produce a significant value.

Experimental Design for cDNA Microarray Analysis

The loop design was employed in the present microarray experiments [Kerr and Churchill, 2001; Kerr, 2003]. This approach is based on a single hybridization combination between any given two samples either containing Cy3-labeled, or Cy5-labeled cDNAs (Fig. 2). Cultured cells incubated for various periods at different temperatures were subjected to total RNA preparation where one aliquot was labeled with Cy3 and the other with Cy5. The Cy3-labeled cDNAs from one sample were mixed with the Cy5-labeled cDNAs from the other and the two cDNA populations were hybridized on the slides.

Gene Annotation

Microarray experiments were performed based on the minimal information about a microarray experiment (MIAME) guideline (http://www.mged.org/workgroups/miame/miame.html).

Multiple data sets obtained as above were subjected to deduction of an accurate indication of the proportional changes in gene expression at different incubation periods following the temperature shift. The data from the array slides where signals were properly obtained were statistically analyzed by one-way analysis of variance (ANOVA) and Mann–Whitney test. When the genes displaying significant signals in

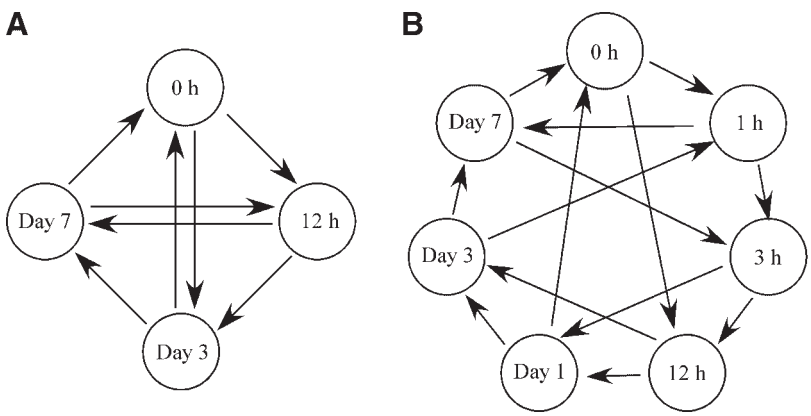

Fig. 2. Loop design used in microarray studies for a total of four samples from medaka culture cells incubated at 25°C (**A**), and seven samples from those at 15°C (**B**). Each arrow represents a microarray and connects the two individual samples hybridized to it. On each microarray, arrows indicated labeling (Cy3 or Cy5): one sample is labeled with Cy3 fluorescent dye (base of the arrow) and the other with Cy5 dye (arrow head).

all slides had no annotation data in the medaka EST database, further annotation work was operated using the medaka UT-genome (http://medaka.utgenome.org/) and Ensembl genome browsers (http://www.ensembl.org/index.html). The corresponding sequences of cDNA fragments were further annotated using GENSCAN program (http://genes.mit.edu/genscan.html) [Burge and Karlin, 1997], by submitting them to the NCBI Basic Local Alignment Search Tool (BLAST) server. The annotation of genes were considered significant when these showed the E values of less than 1.0×10^{-10} with model organisms including human, rat, mouse, zebrafish *Danio rerio* (Hamilton) and torafugu *Takifugu rubripes* (Temminck and Schlegel) homologues.

Gene Ontology Classification

Gene ontology (GO) classifications for "biological process" [Gene Ontology Consortium, 2000, 2004] were assigned for the clones when these were annotated to certain organisms and had significant GO information (Supplementary Table SII). The gene expression data in each GO group were clustered according to similarity in expression pattern using Avadis™ software (Strand Life Sciences, Bangalore, India). Pearson non-centered, complete linkage hierarchical clustering was used to integrate the data. To determine ontological categories significantly associated with temperature response, it was examined for each GO term using Avadis™ software whether an observed frequency distribution in a set of genes which changed their expression levels after temperature shift significantly differs from a theoretical distribution obtained from a set of genes which fulfilled the data criterion described above.

Quantitative Reverse Transcription-PCR Analysis

Total RNAs were extracted from medaka cell lines cultured under various conditions as described above and subjected to genomic DNA degradation and RNA purification using RNase-free DNase set and RNeasy® MinElute® Cleanup kit (Qiagen, Tokyo, Japan), respectively, according to the manufacturer's procedures. The synthesis of first strand cDNA was performed using a ReverTra Ace-α-® system (TOYOBO, Tokyo, Japan) following the manufacturers' instructions. qRT-PCR using SYBR® *Premix Ex Taq*™ (TaKaRa, Otsu, Japan) was performed with a Smart Cycler II system (Cepheid, Sunnyvale, CA) following the manufacturer's instructions using the same RNA set used for cDNA microarray experiments (sample No. 1) and another RNA set prepared separately (sample No. 2) as templates. Relative mRNA levels of target genes were calculated by normalizing the values relative to those of *EF-1α*. Primer pairs for amplification of the genes encoding high mobility group box 1 (HMGB1), ribonucleotide reductase M2 polypeptide (RRM2), decorin (DCN), cysteine-rich angiogenic inducer 61 (CYR61) and EF-1α are presented in Supplementary Table SIII.

Specificity of primers for genes to be amplified was examined using melting curves following the manufacturer's instructions. Further analysis for *CYR61* by qRT-PCR was performed using total RNAs as templates from OLHNI-e1, Odate-e1 and Kino-e2 cell lines cultured at 5, 15, and 25°C for 1, 3, 6, 12, and 24 h. Student's *t*-test was employed for statistical comparison among the mRNA levels at 5, 15, and 25°C.

RESULTS

Characterization of the Medaka cDNA Microarray

A total of 7,680 spots containing a duplicated set for sequences of 3,514 clones as well as internal, external, and negative controls were prepared on the array. The 3,514 clones selected by in silico screening on medaka EST libraries consisted of 158 duplicated, 12 triplicated and two quadruplicated sequences encoding the same gene. Such redundancy was derived from the sequences in different regions of single genes as identified by BLAST search, reducing the potential coverage of the array (Supplementary Table SI). However, this redundancy was useful in evaluating the reproducibility of the results obtained. In general, spots containing the same gene but different sequences showed very low variation in the fluorescence intensity with no significant differences for cells cultured under the same experimental conditions (data not shown).

Changes in Gene Expression in Response to Temperature Alteration

Gene expression levels were determined using loop design for the microarray analysis

(see Fig. 2) by comparing the amounts of the transcripts present in one experimental sample set to those in the other experimental one.

This study selected the genes expressing fluorescence signals at least twice those for the artificial negative controls and not flagged in the computational analysis as artifacts [Rahnenfuhrer, 2005] in all slides. Of 3,514 medaka genes, 925 genes fulfilled this criterion (Supplementary Table SII). The remaining 2,589 genes were not considered because they were considered as artifacts under the above criterion, although some of them showed changes in their mRNA levels at detectable levels for cells cultured for some given periods. The accumulated mRNA levels of *EF-1α*, which was spotted on the cDNA microarray as an internal control, were not significantly changed throughout the experiment (see Supplementary Table SII).

When the accumulated mRNA levels of 925 genes were compared between OLHNI-e1 cells successively cultured at 25°C and those at 15°C after temperature shift, 41, 121 and 114 genes showed significantly different levels at 12 h, on days 3 and 7, respectively ($P < 0.05$). Furthermore, 260 genes were also significantly different in at least one combination among different incubation periods during 7 days after temperature shift to 15°C ($P < 0.05$). The mRNA levels of a total of 313 clones were significantly different in at least one combination among different incubation periods up to 7 days at a given incubation temperature or between 25 and 15°C at a given incubation period in medaka cells (Supplementary Table SIV).

Biological Processes Related to Temperature Adaptation in Terms of Gene Ontology Classification

The genes with expression profiles that changed significantly in response to temperature shift were separated into various groups according to the GO classification for "biological process" [Gene Ontology Consortium, 2004]. Details of the genes of each cluster are shown in Supplementary Table SV. It must be noted, however, that since a particular gene can be implicated in numerous cellular processes, many genes are overlapped in different clusters.

As shown in Figure 3, we detected strong inductions for two genes in the early period (<12 h) after temperature shift to 15°C; *CYR61* belonging to clusters 5 (cell signaling), 6 (cell proliferation and growth), 8 (cell–cell or cell–matrix adhesion) and 15 (cell development), and pyrroline-5-carboxylate reductase-like gene (*PYCRL*) belonging to cluster 16 (cellular metabolism). The induction of a number of other genes were also detected in the late period (≥12 h) after temperature shift such as collagen type II alpha 1 gene (*COL2A1*) belonging to clusters 3 (protein synthesis), 7 (cellular structure and reorganization), 8, 14 (transport), and 15; *HMGB1* belonging to clusters 5, 7, 9 (transcriptional regulation), 12 (nucleic acid metabolism), 13 (apoptosis and cell death), 15 and 16; *NFKBIA* belonging to clusters 5, 6, 13, 14, and 15; *Rab35* belonging to clusters 5, 9, and 14; alpha-2-Heremans-Schmid-glycoprotein gene (*AHSG*) belonging to clusters 5, 14, and 15; prosaposin gene (*PSAP*) belonging to clusters 7, 11 (lipid and fatty acid metabolism), and 14; thioredoxin interacting protein gene (*TXNIP*) belonging to clusters 5 and 6; and transgelin gene (*TAGLN*) belonging to clusters 7 and 15.

On the other hand, majority of the genes were down-regulated at 1 h after temperature shift to 15°C and then recovered at 3 h, and the expression patterns of those genes at 1 h were similar to those at 12 h in 25°C-cultured cells.

Further analysis of the microarray data revealed that certain ontological terms have a number of related genes which significantly changed their mRNA levels, depending on incubation time periods and temperatures (Table I). The ontological genes related to development, morphogenesis and response to stimulus tended to be up-regulated at 15°C, whereas those related to cell proliferation and RNA localization were found to be down-regulated at this temperature. A number of genes encoding proteins which localize in extracellular areas was also apparently up-regulated at 15°C, whereas those localizing in intracellular areas were down-regulated at this temperature. It is, however, worth noting that about two third of the genes on the array (612 out of 925) did not significantly alter their expression levels after temperature shift. Besides, the genes encoding structural constituents of ribosome were not significantly changed at any points in the present study.

Verification of the Microarray Data

To examine the reliability of cDNA microarray analysis, qRT-PCR was performed for

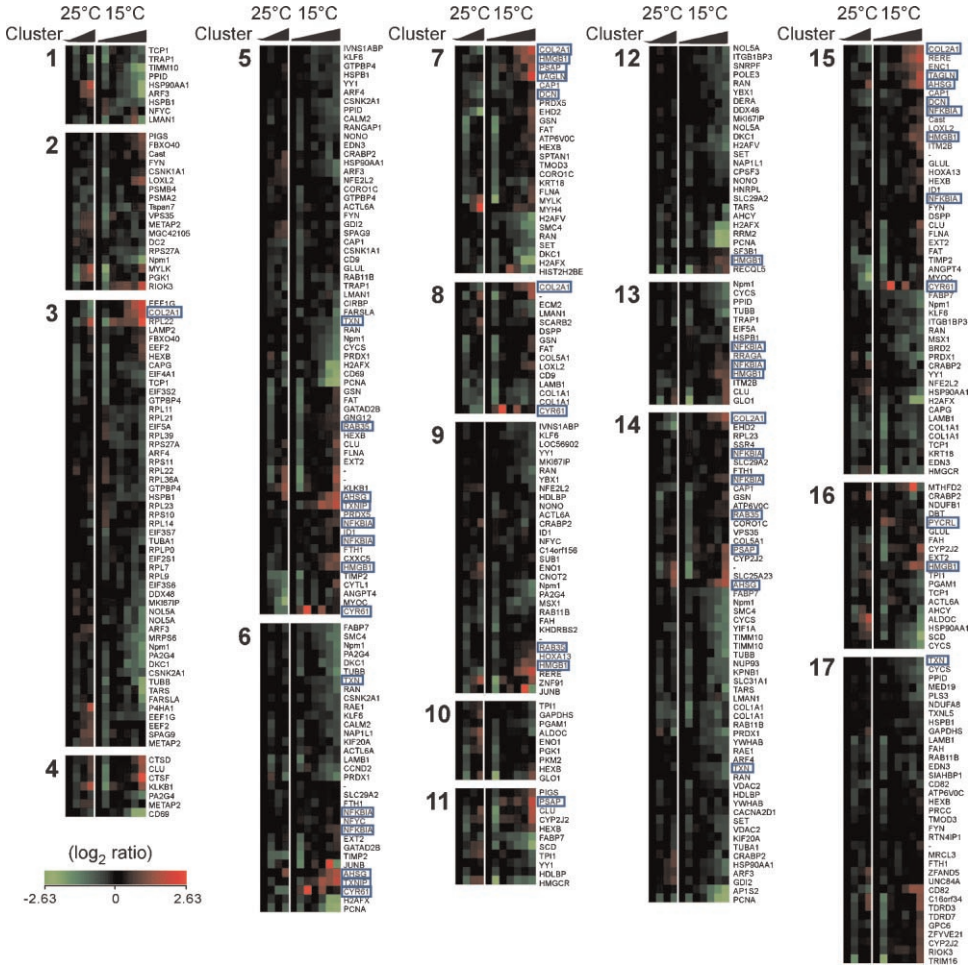

Fig. 3. Gene expression patterns in response to temperature shift in a medaka OLHNI-e1 cell line. Each row represents a single cDNA clone and each column represents an incubation period of culture cells for 0, 12 h, day 3 and 7 at 25°C and for 0, 1, 3, 12, 24 h, day 3 and 7 at 15°C. Genes depicted are those that were significantly different in at least one comparison as described in the text. The 313 genes that significantly changed in response to temperature shift were clustered by cellular processes according to their GO classification: clusters 1, protein rescue and folding; 2, protein modification and catabolism; 3, protein synthesis; 4 proteolysis; 5, cell signaling; 6, cell proliferation and growth; 7, cellular structure and reorganization; 8, cell–cell or cell–matrix adhesion; 9, transcriptional regulation; 10, carbohydrate metabolism; 11, lipid and fatty acid metabolism; 12, nucleic acid metabolism; 13, apoptosis and cell death; 14, transport; 15, cell development; 16, cellular metabolism; 17, other functions. The genes of interest (see text) are boxed. Details of the genes of each cluster are shown in Supplementary Table SV. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

four representative, arbitrarily selected genes following the previous experiment: *CYR61* for early period up-regulation, *HMGB1* for late period up-regulation, *RRM2* for down-regulation and *DCN* for genes showing weak changes. The results are shown in Figure 4. As expected, *CYR61* and *HMGB1* were strongly up-regulated in the early and late period, respectively, whereas *RRM2* was strongly down-regulated in the late period, and *DCN* belonging to clusters 7 and 15 represented weak changes. As shown in Figure 4, their gene expression patterns were in good agreement with those obtained by cDNA microarray analysis ($r = 0.934$), and this relationship was confirmed using total RNAs from two independent experiments ($r = 0.848$).

The Changes in Expression Patterns of CYR61 Associated With Exposure to Low Temperature

Further analysis by qRT-PCR for three medaka cell lines, OLHNI-e1, Odate-e1 and Kino-e2, revealed that the mRNA levels of *CYR61* at 25°C were not significantly changed. However, the mRNA levels of this gene from cells cultured at 15°C became fivefold higher than those at 25°C within 1 h ($P < 0.05$; Fig. 5). The differences in the accumulated mRNA

TABLE I. Gene Ontology Terms in Which the Number of Candidate Genes was Significantly Abundant

Compared time points between 15 and 25°C	Expression group [higher (Δ), lower (▼) or either (—) at 15°C than 25°C]	GO term: Principles[a]	GO term: Specific terms	Actual numbers (number of genes in the term/total number of genes): For the genes of expression group	Actual numbers (number of genes in the term/total number of genes): For all genes	*P*-value*
A, genes whose expression levels were significantly changed in association with temperature shift						
12 h	—	M	Electron transporter activity	5/41	21/925	0.00161
	—	M	Metal ion transporter activity	3/41	8/925	0.00388
	Δ	M	Oxidereductase activity acting on NADH, NADPH	2/17	8/925	0.00835
	Δ	M	Electron transporter activity	4/17	21/925	0.00039
	Δ	M	NADH dehydrogenase activity	2/17	21/925	0.00633
	Δ	M	Metal ion transporter activity	2/17	8/925	0.00835
	▼	M	Ion binding	6/24	59/925	0.00285
	▼	M	Metal ion binding	6/24	59/925	0.00285
Day 3	—	C	Nucleus	25/121	118/925	0.0058
	—	C	Chromosome	6/121	12/925	0.00211
	—	C	Membrane-endomembrane system	6/121	13/925	0.0035
	—	B	Development	74/121	489/925	0.00112
	—	B	Morphogenesis	13/121	44/925	0.00253
	—	B	Cell proliferation	7/121	15/925	0.00145
	—	B	Cell organization and biogenesis	15/121	62/925	0.0098
	—	B	Response to stimulus	17/121	58/925	0.00058
	—	B	Response to external stimulus	12/121	37/925	0.00153
	—	B	Protein localization	12/121	44/925	0.00767
	Δ	B	Development	11/48	61/925	0.00014
	Δ	B	Morphogenesis	9/48	44/925	0.00023
	Δ	B	Organogenesis	7/48	31/925	0.00066
	Δ	B	Organ development	7/48	33/925	0.00099
	Δ	B	Organismal physiological process	9/48	45/925	0.00027
	Δ	B	Neurophysiological process	4/48	17/925	0.00923
	Δ	B	Response to stimulus	11/48	58/925	0.00008
	Δ	B	Response to external stimulus	9/48	37/925	0.00005
	Δ	B	Maintenance of localization	2/48	3/925	0.00766
	▼	C	Nucleus	17/73	118/925	0.00692
	▼	C	Chromosome	5/73	12/925	0.00137
	▼	C	Organella membrane	8/73	36/925	0.0051
	▼	C	Mitochondrial inner membrane protein insertion complex	2/73	2/925	0.00615
	▼	B	Cell cycle	5/73	17/925	0.0078
	▼	B	Cell organization and biogenesis	12/73	62/925	0.00207
	▼	B	RNA localization	2/73	2/925	0.00615
	▼	B	Protein localization	9/73	44/925	0.00535
	▼	B	Regulation of hydrolase activity	2/73	2/925	0.00615
Day 7	—	M	Protein binding	23/114	108/925	0.00347
	—	M	Transcription factor binding	5/114	10/925	0.00395
	—	M	Enzyme activator activity	4/114	6/925	0.0027
	—	C	Cellular_component	69/114	450/925	0.00447
	—	C	Extracellular region	10/114	36/925	0.0088
	—	C	Extracellular matrix (sensu Metazoa)	6/114	15/925	0.00603
	—	B	Morphogenesis	12/114	44/925	0.00464
	—	B	Organogenesis	9/114	31/925	0.00915
	—	B	Organ development	10/114	33/925	0.00427
	—	B	Cell organization and biogenesis	17/114	62/925	0.00066
	—	B	Response to stimulus	14/114	58/925	0.00751
	—	B	Regulation of enzyme activity	3/114	4/925	0.00665
	Δ	M	Transcription factor binding	3/44	10/925	0.00954
	Δ	M	Enzyme regulator activity	4/44	18/925	0.00837
	Δ	C	Extracellular region	8/44	36/925	0.00015
	Δ	C	Extracellular matrix (sensu Metazoa)	4/44	15/925	0.00415
	Δ	C	Extracellular space	3/44	7/925	0.00308
	Δ	B	Development	9/44	61/925	0.00147
	Δ	B	Morphogenesis	8/44	44/925	0.00067
	Δ	B	Organogenesis	6/44	31/925	0.00246
	Δ	B	Organ development	6/44	33/925	0.00344
	▼	M	Molecular function	49/70	499/925	0.00332
	▼	M	Binding	35/70	328/925	0.00668
	▼	C	Cellular component	44/70	450/925	0.00923

TABLE I. (*Continued*)

Compared time points between 15 and 25°C	Expression group [higher (Δ), lower (▼) or either (—) at 15°C than 25°C]	GO term: Principles[a]	GO term: Specific terms	Actual numbers (number of genes in the term/total number of genes): For the genes of expression group	Actual numbers (number of genes in the term/total number of genes): For all genes	*P*-value*
	▼	C	Intracellular	39/70	372/925	0.00468
	▼	B	Chromosome	4/70	12/925	0.00937
	▼	B	Membrane-bound organella	27/70	227/925	0.00475
	▼	B	Intracellular membrane-bound organelle	14/70	123/925	0.00475
	▼	B	Biological process	47/70	489/925	0.00852
	▼	B	Physiological process	45/70	459/925	0.00736
	▼	B	Cellular physiological process	43/70	438/925	0.00987
	▼	B	Cell organization and biogenesis	12/70	62/925	0.0014
	▼	B	RNA localization	2/70	2/925	0.00565
	▼	B	Cellular process	47/70	358/925	0.00151
	▼	B	Regulation of hydrolase activity	2/70	2/925	0.00565
At least one time point	—	M	Molecular function	164/260	499/925	0.00031
	—	M	Binding	118/260	328/925	0.00006
	—	M	Nucleic acid binding	55/260	141/925	0.00155
	—	M	DNA binding	24/260	53/925	0.0048
	—	M	Protein binding	42/260	108/925	0.00653
	—	C	Cellular component	149/260	450/925	0.00063
	—	C	Cell	136/260	420/925	0.00523
	—	C	Intracellular	123/260	372/925	0.00384
	—	C	Nucleus	51/260	118/925	0.00012
	—	C	Chromosome	10/260	12/925	0.0001
	—	B	Biological process	160/260	489/925	0.00059
	—	B	Biological process unknown	6/260	8/925	0.00772
	—	B	Development	28/260	61/925	0.00164
	—	B	Morphogenesis	23/260	44/925	0.00045
	—	B	Organogenesis	16/260	31/925	0.00417
	—	B	Organ development	17/260	33/925	0.00323
	—	B	Physiological process	146/260	459/925	0.00791
	—	B	Biopolymer metabolism	30/260	73/925	0.00884
	—	B	Cell proliferation	11/260	15/925	0.00032
	—	B	Cell organization and biogenesis	30/260	62/925	0.00036
	—	B	Response to stimulus	28/260	58/925	0.0006
	—	B	Response to stress	18/260	38/925	0.00775
	—	B	Response to external stimulus	20/260	37/925	0.00062
	—	B	Response to biotic stimulus	15/260	30/925	0.00809
B, genes whose expression levels were not significantly changed						
	—	M	Structural constituent of ribosome	49/612	64/925	0.04315
	—	M	Carrier activity	18/612	21/925	0.04012
	—	C	Ribosome	49/612	64/925	0.04315

[a]M, molecular function; C, cellular component; B, biological process.
*P values were calculated with Avadis™ software (Strand Life Sciences) and the GO terms which represent less than the threshold of P-value (<0.01 for A and <0.05 for B) were shown.

levels between cells cultured at 15 and 25°C became insignificant after 12 h. Surprisingly, the accumulated mRNA levels at 5°C, unlike those at 15°C, were significantly higher than the levels at 25°C and more than 10-fold higher than those at 25°C even after 24 h.

DISCUSSION

We compared the gene expression profiles of the OLHNI-e1 medaka cell line at certain time intervals after temperature shift from 25 to 15°C or successively maintained at 25°C using a cDNA microarray comprising about 3,500 cDNA clones. It is assumed that the effects of incubation temperature on gene expression in fish culture cells provide useful information to disclose the molecular mechanisms involved in temperature adaptation of fish. It was reported that fish culture cells grow in the temperature range where fish inhabit [Bols et al., 1992; Kondo and Watabe, 2004; Hirayama et al.,

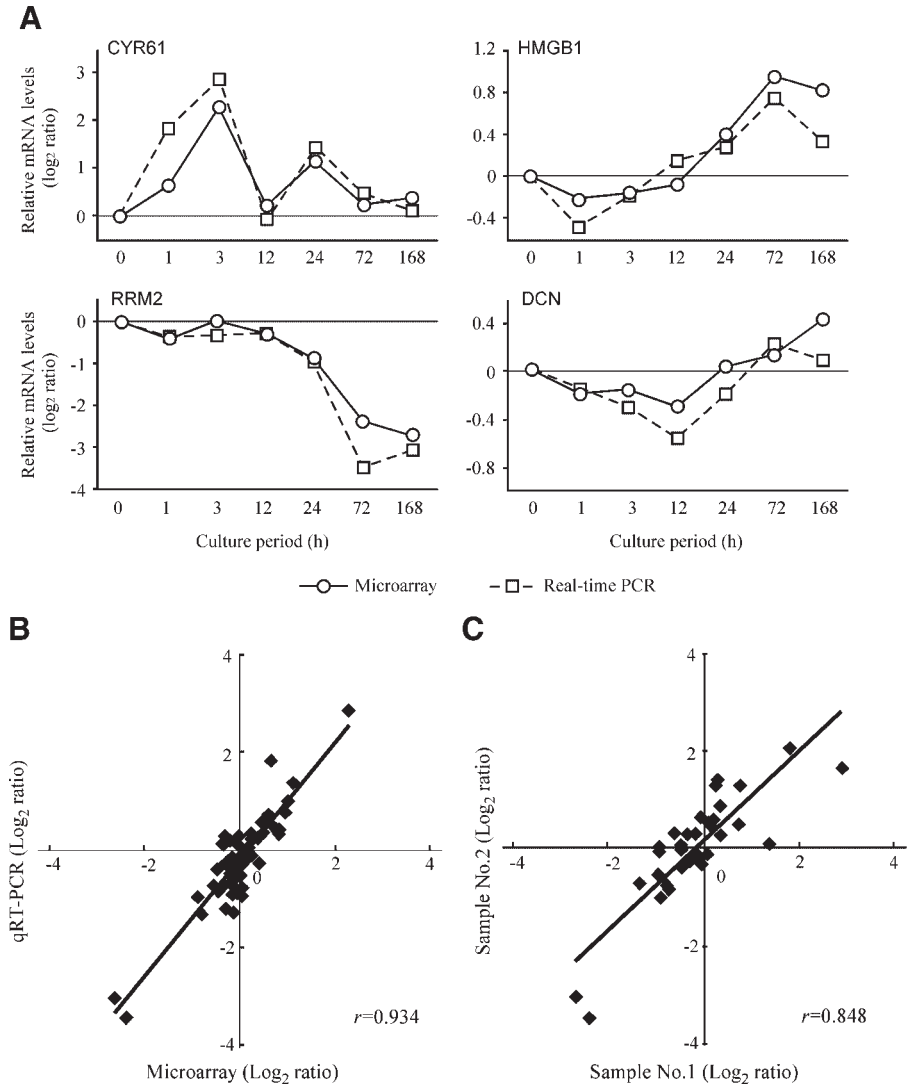

Fig. 4. Correlation of technical replicates for mRNA levels in scatter plots between gene expression data derived from microarray and quantitative reverse transcription-PCR (qRT-PCR; **A**,**B**) and that of biological replicates between samples No. 1 and No. 2 obtained by qRT-PCR (**C**) in medaka culture cells. The data obtained by microarray and qRT-PCR were normalized with global median normalization and the mRNA levels of *EF-1α*, respectively. qRT-PCR was performed for four genes encoding cysteine-rich angiogenic inducer 61 (CYR61), high mobility group B 1 (HMGB1), ribonucleotide reductase M2 polypeptide (RRM2), and decorin (DCN) using total RNAs as the templates for culture cells collected at various time intervals during incubation, as in the case of cDNA microarray analysis (1, 3, 12, day 1, 3, and 7 at 15°C; 12 h, day 3 and 7 at 25°C). In panel (A), the expression patterns of the four genes at 15°C are shown. The Pearson correlation coefficients for both technical (r = 0.934) and biological replicates (r = 0.848) are shown.

2006], clearly suggesting that poikilothermic culture cells are good models for investigating temperature adaptation of poikilothermic organisms.

In this study, since the medaka cell lines were cultured to the confluent before temperature shift, the expression levels of genes related to "cell proliferation and growth" (cluster 6 in Fig. 3) were not largely changed at 25°C. At 15°C, however, many genes belonging to this cluster were down-regulated or up-regulated; in particular, *TXNIP* and *AHSG* were strongly up-regulated in the late period. TXNIP inhibits the activity of thioredoxin (TXN), which is known to promote cell cycle progression [Powis and Montfort, 2001; Sheth et al., 2006], whereas AHSG blocks the activity of transforming growth factor-β (TGF-β) [Demetriou et al., 1996]. The expression levels of *TXN*, which belongs to the clusters 5, 6, 14, and 17, were decreased following the decrease of incubation temperature (see Fig. 3). These responses would

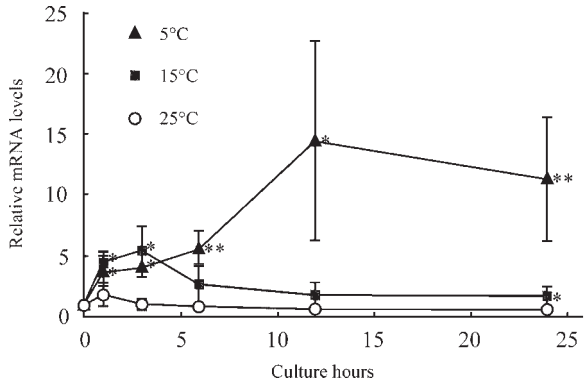

Fig. 5. Gene expression patterns of cysteine-rich angiogenic inducer 61 gene (CYR61) in medaka cell lines cultured at 5, 15, and 25°C. OLHNI-e1, Odate-e1 and Kino-e2 cell lines were cultured at 25°C to confluent, and then transferred to 5 and 15°C within 1 h or successively maintained at 25°C (see Fig. 1). Relative mRNA levels were determined using the levels of *EF-1α* as the standard and *y*-axis represents the ratio of the accumulated mRNA levels to those of 0 h. Student's *t*-test was employed for statistical comparison. An asterisk represents significantly different mRNA levels compared with those at 25°C of each period ($P < 0.05$). Double asterisks represent significantly different mRNA levels at 5°C compared with those at both 15 and 25°C of each period ($P < 0.05$). Data are given as means ± SD.

lead to the strong inactivation of cell proliferation and growth. On the other hand, the expression patterns of the genes belonging to the two clusters, "cellular structure and reorganization" (cluster 7) and "cell development" (cluster 15) were largely altered at 15°C. Thus, cells might compensate for lower temperature by repressing the cell proliferation and growth in a positive manner and by changing the cellular structures and organizations.

The eukaryotic cell has two competing pathways for managing proteins abnormally denatured due to stress: the molecular chaperone pathway with heat shock proteins (HSPs) performing the signal role in the eventual rescue and refolding of damaged polypeptides, and the ubiquitin–proteasome pathway, which results in the degradation of the abnormally folded protein [Ciechanover, 1998; Buckley et al., 2006]. The expression levels of the genes belonging to the "protein rescue and folding" (cluster 1) and "protein synthesis" (cluster 3) were overall down-regulated at 15°C, except only a few genes (Fig. 3). However, changes in expression of those belonging to the "proteolysis" (cluster 4) tended to be similar at both temperature regimes, although there were some differences. These expression profiles suggest that production of new proteins and misfolded proteins are decreased at 15°C and that a few misfolded proteins at low temperature might be processed mainly by protein degradation, but not rescued by molecular chaperones. These results are in contrast to those reported for heat shock experiment with goby (*Gillichthys mirabilis*) where genes belonging to clusters 1 and 4 were up-regulated [Buckley et al., 2006], probably because increased intracellular digestion and folding of macromolecules are needed during heat shock. In a similar way, the genes related to metabolisms of carbohydrate (cluster 10) and nucleic acids (cluster 12) were overall repressed at 15°C (see Fig. 3), whereas those belonging to "lipid and fatty acid metabolism" (cluster 11) showed considerable variation in their expression patterns after temperature shift to 15°C. It should be noted, however, that the expression levels of stearoyl-CoA desaturase gene (*SCD*) at 15°C, which is considered as a key gene involved in membrane adaptation by incorporating the first unsaturation bond into saturated fatty acids, were considerably lower than those at 25°C. This is quite unusual as *SCD* shows cold-responsive expression in many organisms [Nishida and Murata, 1996; Tiku et al., 1996; Murray et al., 2007]. Such unexpected expression pattern of *SCD* in medaka cell line might be due to the complexity of the control of desaturase expression [Tiku et al., 1996] and the different manners of adaptation capability among organisms under cold stress [Hsieh and Kuo, 2005].

Genes localizing in the extracellular region, including *COL2A1*, *AHSG*, *PSAP*, and *DCN*, increased their expression levels at 15°C compared with those at 25°C (see Table I). These four genes encodes the functional proteins as follows: COL2A1 is a type II collagen, which is abundant in chondrocytes [Eyre, 1980; Kosher et al., 1986]; AHSG is an anti-proliferative protein as described above; PSAP is a precursor of sphingolipid activator proteins (saposin A–D) involved in hydrolysis of sphingolipids [Kishimoto et al., 1992; Koochekpour et al., 2007] and DCN is a small leucine-rich proteoglycan, which plays roles in collagen fibrillogenesis, growth factor modulation and direct regulation of cellular growth [Reed and Iozzo, 2003]. Thus, cold-induced increased expression of these genes further suggests that medaka cells respond to cold stress by changing the cellular structures and organizations. On the other hand, the mRNA levels of RRM2, which induces

NF-κB-dependent matrix metalloproteinase 9 (MMP9) activation, decreased at 15°C (see Figs. 3 and 4). Low levels of RRM2 could keep MMP-9 inactivated, which belongs to a family of zinc binding proteolytic enzymes that are responsible for degradation of the components of the extracellular matrix, a tissue which is also a biological regulator of cell growth and differentiation [He, 1996; Duxbury and Whang, 2007]. Interestingly, MMP-9 is also repressed by TAGLN [Nair et al., 2006], the gene expression level of which was increased at 15°C (see Fig. 3). These results strongly suggest that remodeling and reorganizing of extracellular structure of cells are important to offset the low temperature effect through cell–cell interaction, cell–cell signaling, cell–cell, or cell–matrix adhesion and the extracellular structure itself.

In the present study, other interesting expression patterns of a certain genes were found. As shown previously, we detected the induction of the genes encoding NFKBIA and Rab35, which function to inhibit a transcription factor NF-κB [Baeuerle and Henkel, 1994] and to control an endocytic recycling pathway as a regulator of intracellular transport [Zerial and McBride, 2001; Kouranti et al., 2006], respectively, after temperature shift to 15°C (see Fig. 3) [Hirayama et al., 2006]. These results suggest that the NF-κB cascade, which plays a pivotal role in mediating not only inflammatory responses to a variety of stimulations, but also cell adhesion, cell growth and apoptosis [Baeuerle and Henkel, 1994; Ghosh et al., 1998; May and Ghosh, 1998; Chen et al., 2001], was activated, and that facilitating intracellular trafficking would be prerequisite at low temperature as many genes belonging to the GO cluster "transport" showed altered expressions at low temperature (see Fig. 3). We also detected strong induction of a high-mobility group protein, HMGB1, that modulates transcription through the alteration of chromosome conformation [Bustin, 1999; Thomas and Travers, 2001], suggesting that chromosomal DNA secondary structure might also be disturbed at low temperature. The expression of HMGB1 has also been shown to fluctuate with cycling temperatures in killifish [Podrabsky and Somero, 2004] and had been detected in cold acclimated carp [Gracey et al., 2004], providing further evidence that this gene is involved in temperature responses.

While a number of genes were found to be up-regulated in the late period of temperature shift, only two genes, *PYCRL* and *CYR61*, were found to be up-regulated in the early period. PYCRL has a similar structure to pyrroline-5-carboxylate (P5C) reductase, which is important for proline synthesis [Misener et al., 2001]. In species as divergent as flies, beetles and crickets, cold acclimation and cold tolerance are correlated with significant increases in proline levels [Shimada and Riihimaa, 1990; Bonnot et al., 1998; Ramløv, 1999]. Although the relationship between cold tolerance and proline levels in vertebrate has never been argued to our knowledge, it is expected that a higher proline level possibly produced by induced PYCRL might provide a certain positive effect to cold adaptation in fish. On the other hand, CYR61 belongs to the family of CYR61/connective tissue growth factor (CTGF)/Nov (CCN) proteins, which are structurally related to secreted matricellular proteins and function in adhesion, migration, proliferation and extracellular matrix synthesis [Brigstock, 2003]. In this regard, CYR61 seems to be an important candidate gene since it belongs to an important family of matricellular regulatory factors involved in internal and external cell signaling [Perbal, 2004]. It has been reported that CYR61 are immediately induced in cultured cells in response to physical and chemical stimuli, and that their expression at the very beginning of the stimuli is a prerequisite to establishment of a new cellular regime which is to be maintained thereafter [Brigstock, 2003; Perbal, 2004].

In the present study, transcripts of CYR61 were immediately increased during temperature shift from 25°C to 15, or 5°C followed by constitutive culture at respective temperatures for 1 week or 1 day and interestingly, high mRNA levels of *CYR61* at 5°C were maintained even at 24 h after temperature shift. These results suggest that medaka cells require CYR61 for their metabolic processes at low temperature and thus we think CYR61 as a promising biomarker for temperature adaptation of medaka. Although CYR61 has been well characterized for homeotherms, its functions in relation to temperature adaptation in poikilotherms remain to be understood.

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