A spectrum of functional genes mobilized after *Trichinella spiralis* infection in skeletal muscle

Z. WU, I. NAGANO, T. BOONMARS *and* Y. TAKAHASHI*

Department of Parasitology, Gifu University Graduate School of Medicine, Yanagido 1-1, Gifu 501-1194, Japan

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SUMMARY

Trichinella spiralis infection causes the transformation of infected muscle cells, which leads to nurse cell formation. To search for the candidate genes responsible for nurse cell formation, cDNA microarray analysis of muscle tissues was performed before and after *Trichinella* infection. The Atlas mouse 1.2 cDNA expression microarray revealed the expression profiles of 1176 known genes. Out of these, 311 gene expressions were detected in normal and/or infected muscles. After the infection, 184 out of the 311 genes increased in expression by more than 3-fold. These included genes responsible for cell differentiation, proliferation, cell cycle and apoptosis. Thus this study suggested candidate genes for further investigation to dissect the molecular mechanisms of nurse cell formation.

Key words: *Trichinella spiralis*, microarray, gene expression profiling.

INTRODUCTION

Trichinella spiralis is an intracellular parasite of mammalian skeletal muscles. Infection by newborn larvae induces extensive changes of infected cells leading to prominent and unique cyst formation in infected muscle. The histopathological features have been well described by the previous authors (Despommier, 1975; Matsuo *et al*. 2000). Outside of the cyst wall, inflammatory cell accumulation and prominent angiogenesis occur (Baruch & Despommier, 1991; Despommier, 1993; Gabryel, Gustowska & Blotna-Filipiak, 1995; Liu *et al*. 1996; Polvere *et al*. 1997; Capo, Despommier & Polvere, 1998). Within the cyst wall, there are nurse cells that are not normal constituents of the host tissue. Of particular interest to *Trichinella* research is that the nurse cell is a transformed muscle cell. The question then arises ''How are terminally differentiated cells transformed?'' Based on this question, the processes of nurse cell formation have been intensively studied. The processes have been found to involve complex steps (see review by Despommier, 1998). The processes attempt to follow those seen during normal muscle cell repair, but are different in many respects. Infected muscle cells loose their integrity and transform to the nurse cell and undergo regeneration. Satellite cells proliferate but mis-differentiate to the nurse cell, not to the muscle cell (Matsuo *et al*. 2000; Wu *et al*. 2001; Boonmars *et al*. 2004*a*, *b*). These changes are thought to be triggered by parasite ES products (Despommier *et al*. 1990; Lee *et al*. 1991; Yao, Bohnet & Jasmer, 1998; Yao & Jasmer, 1998). In other words, muscle cells respond to the ES products in a different way from other causes of muscle cell damage, either overloading or drug-induced damage.

We have begun a series of molecular studies to determine the kinetics of certain genes that may play a key role in this unique transformation (Wu *et al*. 2001; Boonmars *et al*. 2004*a*, *b*). This cyst formation is, however, not simple because it involves numerous gene interactions. As such, the spectrum and kinetics of genes should be more extensively analysed. Recent genetic analysis techniques offer a powerful tool to match such a requirement. cDNA microarray assay is one of the methods that can examine the expression profile of thousands of genes simultaneously. In the present study, we adapted the Atlas mouse cDNA expression microarray (equipped with 1176 known genes) for analysing the gene expression in normal and *T. spiralis*-infected muscle tissues. The results provided fundamental knowledge for further understanding the mechanism of formation of the nurse cell and host–parasite relationship.

MATERIALS AND METHODS

Parasite and infection

Three nude mice ($n=3$) were orally infected with 600 larvae of *T. spiralis* (ISS413). The muscles of the hind limbs (containing about 1000 larvae per g of tissue) were collected at 23 days post-infection (p.i.) and subjected to RNA isolation (see next paragraph). Control samples were collected identically from uninfected nude mice ($n=3$).

* Corresponding author. Tel: +81 58 230 6365. Fax: +81 58 230 6368. E-mail: yu3@cc.gifu-u.ac.jp

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Table 1. Summary of microarray analysis

Gene classification	Total gene no.	Gene no. of signal detectable	Gene no. of expression increased
Basic transcription factors	171	37	22
Transcription activators and repressors	117	41	16
Cyclins, cell cycle-regulating kinases, CDK inhibitors	45	16	13
Cell adhesion receptors and proteins	59	13	8
Oncogenes and tumor suppressors	81	23	13
HSP and stress response	11	7	5
Death receptors, Bcl family proteins and apoptosis associated	58	16	14
Growth factor and chemokine receptors	21	7	5
Growth factors, cytokines and chemokines	87	31	21
Interleukin, interferon and their receptors	21	5	4
Hormones and hormone receptors	32	5	3
Intracellular transducers/effectors/modulators/adaptors/receptors	120	20	11
Proteases	19	7	5
Protease inhibitors	13	5	5
G-proteins and related proteins	15	4	3
Neuropeptides and neurotransmitter receptors	43	6	4
Xenobiotic metabolism and transporters	10	3	3
DNA polymerases, replication factors, topoisomerases, DNA damage repair proteins and ligases	43	10	6
Cytoskeleton and motility proteins	57	24	13
Cell surface antigens	25	2	1
Membrane channels and transporters, ligand-gated ion channels, symporters and antiporters	36	2	1
Functionally unclassified	25	2	1
Housekeeping genes	9	7	1
Others	58	8	3
Total	1176	311	184

RNA preparation

Total RNA was isolated from the muscle samples of the infected group and the normal mouse group using Trizol (GibcoBRL, Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, isolated total RNA was treated with 50 units/ml of DNase I (RQ1 RNase-free DNase, Promega Corporation, Madison, WI, USA) in the buffer (40 mM Tris-HCl, 10 mM $MgSO_4$ and 1 mM $CaCl_2$, pH 8·0) containing 400 units/ml ribonuclease inhibitor (Takara Bio INC. Shiga, Japan) at 37 °C for 30 min. The treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water.

cDNA microarray

The cDNA microarray assay was performed using Atlas™ mouse 1.2 Array (Clontech, Palo Alto, CA, USA), which contained 1176 different kinds of known genes dotted on 1 sheet of nylon membrane. The names and possible functions of the genes are listed in Table 1, according to the manufacturer's instructions.

The following pre-treatments (RNA purification, probe preparation, hybridization and signal visualization) were performed according to the manufacturer's user manual of Atlas™ mouse 1.2 Array (Clontech).

The probe was prepared using the Atlas™ Spotlight Labeling Kit (Clontech). In brief, 50 μg purified RNA was labelled by incubating with reaction buffer, labelling mixture and CDS (cDNA synthesis) primer mixture at 48 °C for 45 min, and then the labelled probe was purified by column chromatography.

Hybridization and signal detection were performed using the SpotLight™ Chemiluminescent Hybridization and Detection Kit (ClONTECH). In brief, the Atlas Array membrane was pre-hybridized in SpotHyb buffer containing 500 μg of sheared salmon testes DNA at 42 °C for 2 h, and hybridized with the probe at 42 °C overnight in a hybridization incubator. Then, the membrane was washed 3 times with 2X SSC (sodium citrate-sodium chloride) containing 1% SDS (sodium dodecyl sulfate) at 60 °C for 30 min, and twice with 0·1X SSC containing 0·1% SDS at 48 °C for 30 min. The membrane was incubated with block solution for 1 h and stabilized with streptavidin-HRP (horseradish peroxidase) conjugate for 15 min at room temperature. After washing with washing buffer, the membrane was incubated with substrate (a mixture of luminol/enhancer solution and stable peroxide

solution) for 5 min at room temperature, and finally exposed to Biomax MS film (Kodak, Rochester, NY, USA).

Microarray data analysis

Microarray experiments were performed 3 times with 6 independent samples from normal ($n=3$) and infected mice ($n=3$). The results were analysed densitometrically using NIH Image 1.95 software (developed at the U.S. National Institute of Health and available on their website at http://rsb.info.nih.gov/nih-image/). To compare the infected muscle and normal muscle, expression data from 3 microarrays were averaged and normalized as following. First, the average pixel intensities of the spot of each gene (A) in infected muscle and normal muscle were determined. The background signal (B) was determined by measuring the signals of negative controls spots on the array membranes. The corrected signal (C) was obtained from $C=A-B$. Next, the housekeeping gene G3PD was used to normalize the corrected signals. The normalized signals in infected muscle were expressed as C X (C of infected muscle G3PD signal/C of normal muscle G3PD signal). Then the ratio was calculated as the ratio of normalized signal of infected muscle to that of normal muscle. These results were expressed as increased or decreased folds. The fold inductions in Tables are averages of 3 independent experiments. In the present study, the ratio threshold was set at 3·0. Only those genes that were 3·0-fold or greater were considered to be differentially expressed.

Because many genes showed extremely low or undetectable signals in normal muscle, the ratio cannot be calculated accurately. Therefore, the expression levels of these genes were judged according to the size and density of each spot and rated as negative (−), or positive (1+ to 12+). Only that the normal muscle was negative and the infected muscle was 2+ or greater were considered to be differentially expressed.

RESULTS

Table 1 lists the gene classification made by the manufacturer and the summary of the present microarray results, including total gene numbers, the numbers of signal detectable genes and the numbers of the genes of expression which increased more than that 3-fold or over 2+. Fig 1 shows the example results of the microarray membrane. In Table 2, we listed the genes with signals that were detectable. Although the hybridization results were highly reproducible, there was some variation in density among the 3 independent analyses. Therefore the results are depicted by mean values.

Among the 1176 plotted genes, 311 showed a detectable signal in the normal and/or infected muscles,

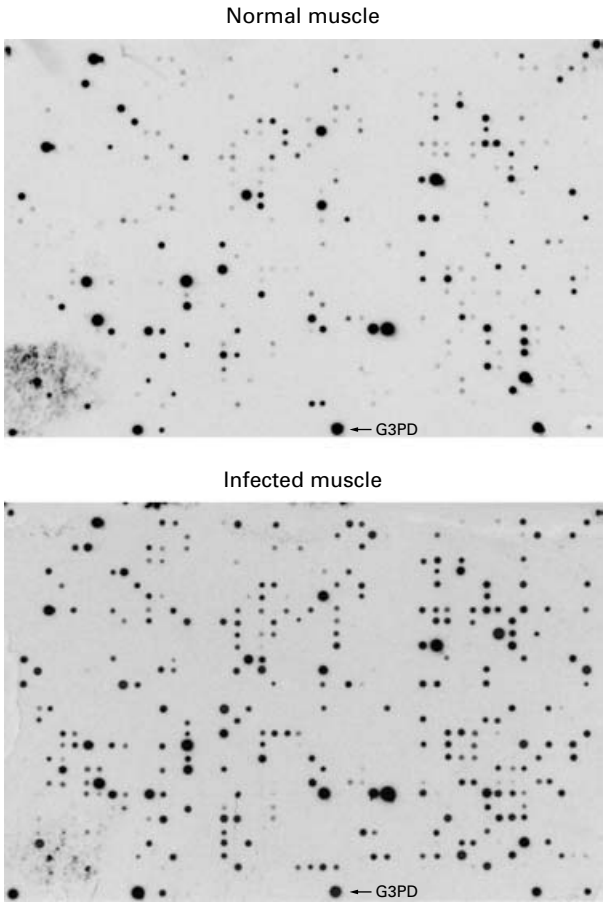

Fig. 1. The Atlas Mouse 1.2 Array images of cDNA hybridization. The upper panel and the lower panel represent gene expression in the hind limb muscles of normal mice and *Trichinells spiralis*-infected mice, respectively.

and the remaining 865 genes were undetectable in both normal and infected muscle. Out of the 311 detectable genes, 184 genes exhibited a 3-fold or greater increase in the expression, while the remaining 131 genes exhibited unchanged or less than a 3-fold increase.

Atlas 1.2 array contains 171 basic transcription factor genes, 37 of which produced detectable signals. Out of 37 genes, 22 showed more than a 3-fold increase expression in infected muscle. The highest increased expression was seen in the nuclear factor of activated T cells (NFAT1) (10+), which is a gene relative to cell differentiation and growth. The expressions of 7 homeobox genes were increased.

In 117 genes of transcription activators and repressors, 41 genes gave detectable signals. Out of the 41 genes, 16 genes showed more than a 3-fold or 2+ increase of expression, including 4 homeobox genes.

Out of 45 cyclins and cell cycle related protein, 16 genes gave detectable signals. Out of the 16 genes, 13 genes showed more than a 3-fold or 2+ up-regulatory expression. These genes included the cyclins that function mainly during G1 and G1-S phases, such as cyclin D2, cyclin D3, cyclin C, cyclin E1 and

Table 2. Differentially expressed genes between normal and *Trichinella spiralis*-infected muscles

GenBank Accession no.	Gene names	Expression level		Ratio
		Normal	Infected	
Basic transcription factors				
U02079	nuclear factor of NFAT1 isoform alpha	−	10+	
Y07609	max binding protein	−	6+	
Y11717	homeobox A3 (HOXA3)	−	6+	
D86603	BTB and CNC homology 1 (BACH1)	−	6+	
U45665	Cut-like protein 2 (CUTL2)	−	5+	
X59725	paired related homeobox 1 (PMX1)	−	4+	
U89487	LIM domain binding 3 (LDB3)	−	4+	
D43694	helix-loop-helix atonal homolog 1 (mATH1)	−	4+	
X63507	homeobox C10 (HOXC10)	−	4+	
AF001465	aristaless 4 homeobox protein (ALX4)	−	4+	
U57327	T-box 1 (TBX1)	−	3+	
X84814	sine oculis-related homeobox 5 homolog (SIX5)	−	3+	
L10075	DNA binding protein SMBP2	−	3+	
X86368	forkhead homolog 2 (FKH2)	−	3+	
U62523	msh-like homeobox 3 (MSX3)	−	3+	
X74937	hepatocyte nuclear factor 3 beta (HNF3B)	−	2+	
X99291	homeobox D13 (HOXD13)	−	2+	
U71208	eyes absent 2 homolog (EYA2)	1+	7+	9·7
X80338	sine oculis-related homeobox 2 homolog (SIX2)	1+	5+	6·7
U36340	Kruppel-like factor 3 (KLF3)	1+	5+	5·9
X56230	POU domain class 2 transcription factor 1	1+	4+	4·9
L13171	myocyte-specific enhancer factor 2C (MEF2C)	1+	4+	4·7
M98502	zinc finger protein 46	2+	4+	3·0
X74936	hepatocyte nuclear factor 3 afpha (HNF3A)	2+	4+	2·8
X74938	hepatocyte nuclear factor 3 afpha (HNF3G)	3+	3+	2·7
U47543	Ngfi-A binding protein 2 (NAB2)	3+	4+	2·1
S70632	homeobox protein 11 (HOX11)	5+	6+	2·0
L12140	amino-terminal enhancer of split (AES)	3+	4+	1·5
M30499	myogenic factor 6 (MYF6)	3+	4+	1·5
AF005772	paired homeobox 3 protein (PITX3)	6+	6+	1·3
X80339	sine oculis-related homeobox 1 homolog (SIX1)	2+	1+	1·2
S75837	developing brain homeobox 1 (DBX)	1+	1+	1·1
U65091	melanocyte-specific gene 1 (MSG1)	2+	2+	1·1
M97013	paired box gene 5	1+	1+	1·0
U81983	endothelial PAS domain protein 1	4+	4+	0·9
X92498	forkhead homolog 6 (FKH6)	2+	2+	0·9
U59322	homeobox A13 (HOXA13)	4+	3+	0·7
Transcription activators and repressors				
L12703	engrailed homeobox (EN19)	−	9+	
M20157	early growth response 1 (EGR1)	−	6+	
X14759	homeobox, msh-like 1	−	6+	
L12705	engrailed 2	−	6+	
U53228	ROR-related orphan receptor alpha	−	5+	
X90829	Lbx 1 transcription factor	−	5+	
U42554	single-minded 2 (SIM2)	−	5+	
X71327	MRE binding transcription factor 1	−	4+	
X59252	homeobox, msh-like 2	−	4+	
D00926	transcription elongation factor A3 (TCEA3)	−	4+	
AF015948	E2F transcription factor 3 (E2F3)	−	3+	
L21027	3-phosphoglycerate dehydrogenase (PGDH)	−	2+	
U41626	split hand/foot deleted gene 1 (SHFDG1)	−	2+	
L12147	early B-cell factor (EBF)	1+	6+	9·5
U36760	brain factor 1 (Hfhbf1)	1+	5+	6·5
J03168	interferon regulatory factor 2 (IFR2)	1+	4+	4·1
AF031814	orphan nuclear receptor PXR	1+	3+	3·8
J03770	homeobox D4	2+	4+	2·4
M98339	GATA binding protein 4 (GATA4)	1+	2+	2·2
U01036	erythroid transcription factor NF-E2	1+	2+	1·9
U19119	interferon inducible protein 1 (IFI1)	3+	4+	1·6
U59876	glial cells missing homolog 1 (mGCM1)	1+	1+	1·5
X63963	paired box gene 6	1+	1+	1·3
S74227	TEA domain family member 1	1+	1+	1·2
U51037	CCCTC-binding factor	1+	1+	1·2

Table 2. (*cont*.)

GenBank Accession no.	Gene names	Expression level		Ratio
		Normal	Infected	
M61909	relA proto-oncogene, p65	8+	8+	1·1
L03547	ikaros DNA binding protein	1+	1+	1·0
M31042	immediate early response 2	2+	2+	1·0
U29086	neurogenic differentiation 6	6+	6+	1·1
D49474	SRY-box containing gene 17	4+	4+	1·1
M58566	zinc finger protein 36	2+	2+	1·1
L39770	gastrulation brain homeobox 2 (GBX2)	1+	1+	1·1
X57621	nuclease sensitive element binding protein 1	3+	3+	1·1
U20532	nuclear factor erythroid 2-related factor 2	5+	5+	1·0
Z36885	ets domain protein elk4	1+	1+	1·0
L12721	delta-like 1 homolog (Drosophila)	1+	1+	1·0
D26046	AT motif binding factor 1	5+	5+	1·0
S53744	brain specific transcription factor NURR-1	2+	2+	0·9
U62522	trans-acting transcription factor 4	2+	2+	0·9
L35949	hepatocyte nuclear factor 3	7+	5+	0·7
U09419	nuclear receptor subfamily 1 group H member 2	3+	1+	0·5
Cyclins, cell cycle-regulating kinases, CDK inhibitors				
U43918	proliferation-associated 2G4 (p38-2G4)	−	7+	
X56135	prothymosin alpha (PTMA)	−	7+	
U62638	cyclin C (G1-specific)	−	6+	
U43844	cyclin D3	−	5+	
U05341	p55CDC	−	5+	
AF093416	NIMA-related expressed kinase 3 (NEK3)	−	4+	
M83749	cyclin D2	−	4+	
U20553	cyclin-dependent kinase inhibitor 1C (P57)	−	4+	
X66032	cyclin B2 (G2/M-specific)	−	3+	
M61177	mitogen activated protein kinase 3 (MAP kinase 3)	1+	5+	8·2
X75888	cyclin E1 (G1/S-specific)	1+	4+	7·6
L01640	cyclin-dependent kinase 4 (CDK4)	1+	4+	4·7
U10440	cyclin-dependent kinase inhibitor 1B (P27)	2+	4+	3·3
D78382	transducer of ErbB2·1 (TROB)	2+	3+	2·8
X84311	cyclin A1 (G2/mitotic-specific)	6+	7+	1·6
Z37110	cyclin G (G2/M-specific)	2+	2+	1·2
Cell adhesion receptors and proteins				
J04806	osteopontin (OP)	−	10+	
Y00769	integrin beta 1 (fibronectin receptor beta)	−	7+	
X52264	intercellular adhesion molecule 1 (ICAM1)	−	4+	
D13867	integrin alpha 3 (ITGA3, CD49C)	−	4+	
L06039	platelet/endothelial cell adhesion molecule (CD31)	1+	4+	5·4
M95633	integrin beta 7 (ITGB7)	2+	4+	3·7
X07640	integrin alpha M (ITGM)	2+	4+	3·3
X69902	integrin alpha 6 (ITGA6)	2+	4+	3·1
M31131	cadherin 2 (CDH2)	2+	3+	2·5
X97818	semaphorin G	3+	3+	1·4
X59990	catenin alpha 1	1+	1+	1·3
D14888	cadherin 4 (CDH4)	1+	1+	0·9
X53176	integrin alpha 4 (ITGA4, CD49D)	4+	4+	0·9
Oncogenes and tumor suppressors				
U14173	ski proto-oncogene	−	12+	
X81580	insulin-like growth factor binding protein 2 (IGFBP2)	−	6+	
M26391	retinoblastoma 1 (RB1, pp105)	−	4+	
X13664	neuroblastoma ras oncogene (N-ras)	−	3+	
Z50013	H-ras proto-oncogene	−	3+	
L10656	abl proto-oncogene	−	3+	
U58992	MAD homolog 1 (smad1)	1+	6+	9·7
X67812	ret proto-oncogene	2+	5+	5·1
X68193	nucleoside diphosphate kinase B (NDKB)	1+	3+	4·6
Z22819	ras-related protein RAB34	1+	3+	4·2
J04115	c-Jun proto-oncogene	1+	3+	3·7
X54924	neurofibromatosis 1	1+	3+	3·5
U18342	protein tyrosine kinase 3 (TYRO3)	1+	3+	3·4
M16449	myeloblastosis oncogene	3+	4+	2·3
U52945	tumor susceptibility gene 101	1+	2+	2·3

Table 2. (*cont.*)

GenBank Accession no.	Gene names	Expression level		Ratio
		Normal	Infected	
M64429	B-raf proto-oncogene	4+	5+	1·8
J05205	JunD1 proto-oncogene	1+	1+	1·1
X83974	transcription termination factor 1	4+	4+	1·1
X58876	transformed mouse 3T3 cell double minute 2	3+	3+	1·1
U65594	breast cancer 2	4+	4+	1·0
X68932	macrophage colony stimulating factor 1 receptor	6+	6+	1·0
X59421	Friend leukemia integration 1	1+	1+	1·0
Z32815	ets-domain protein elk3	1+	1+	1·0
Heat shock proteins				
U40930	osidative stress-induced protein (OSI)	−	5+	
M36830	heat shock protein, 86 kDa 1	−	3+	
D78645	78-kDa glucose-regulated protein (GRP78)	−	2+	
M36829	heat shock protein, 84 kDa 1	1+	5+	6·9
L40406	heat shock protein, 105 kDa	4+	6+	3·9
U03560	heat shock protein, 25 kDa	1+	2+	1·8
J05186	intestinal calcium binding protein (CAI)	3+	4+	1·4
Death receptors, Bcl family protein and apoptosis-associated proteins				
M59378	TNF receptor superfamily member 1b	−	12+	
D83698	activator of apoptosis harakiri (HRK)	−	5+	
L22472	Bcl2-associated X protein (BAX)	−	4+	
L28095	caspase 1	−	4+	
U57324	presenilin 2 (SP2)	−	4+	
S56660	retinoic acid receptor, beta	−	4+	
AA041883	cytoplasmic dynein light chain 1	−	4+	
M20658	interleukin 1 receptor	−	3+	
U83628	defender against cell death 1 (DAD1)	−	3+	
U10903	programmed cell death 2	1+	5+	7·2
U00182	insulin-like growth factor I receptor (IGFIR)	1+	4+	5·4
U05672	adenosine A2a receptor	1+	3+	3·5
U17162	Bcl2-associated athanogene 1 (BAG1)	1+	3+	3·3
M94335	akt proto-oncogene, protein kinase B (PKB)	2+	4+	3·3
U77714	survival motor neuron	1+	2+	2·1
U37522	TNF superfamily member 10	1+	1+	0·8
Growth factor and chemokine receptors				
L47240	erbB3 proto-oncogene	−	4+	
M34563	T-cell specific surface glycoprotein (CD28)	−	3+	
D16250	bone morphogenetic protein receptor 1A (BMPR1A)	−	3+	
M28998	fibroblast growth factor receptor 1	−	2+	
L47239	erbB2 proto-oncogene	3+	5+	4·0
M85078	colony stimulating factor 2 receptor	2+	3+	2·6
J04843	erythropoietin receptor	3+	3+	1·0
Growth factors, cytokines and chemokines				
X81583	insulin-like growth factor binding protein 5 (IGFBP5)	−	10+	
X16053	prothymosin beta 4 (PTMB4)	−	6+	
X70514	nodal	−	5+	
X81582	insulin-like growth factor binding protein 4 (IGFBP4)	−	4+	
X56848	bone morphogenetic protein 4 (BMP4)	−	4+	
L24755	bone morphogenetic protein 1 (BMP1)	−	4+	
X04480	insulin-like growth factor 1 (IGF I)	−	3+	
X83376	inhibin beta-B (INHBB)	−	4+	
S82648	bone morphogenetic protein 3B (BMP3B)	−	3+	
L33406	uromodulin	−	3+	
M95200	vascular endothelial growth factor A (VEGF)	−	3+	
Z68889	wingless-related MMTV integration site 8A	−	3+	
U12983	ephrin B1	−	3+	
M92415	fibroblast growth factor 6 (FGF6)	1+	6+	11·5
M11434	nerve growth factor alpha (NGFA)	1+	4+	6·1
AF020738	fibroblast growth factor 12 (FGF12)	1+	3+	5·1
X12531	small inducible cytokine A3 (SCYA3)	1+	3+	4·0
Z22703	fibroblast growth factor 7 (FGF7)	4+	6+	3·5
M16819	lymphotoxin A (LTA)	1+	3+	3·4
M97017	bone morphogenetic protein 8a (BMP8A)	2+	4+	3·3
M89799	wingless-related MMTV integration site 5B	2+	4+	3·2

Table 2. (*cont*.)

GenBank Accession no.	Gene names	Expression level		Ratio
		Normal	Infected	
X81584	insulin-like growth factor binding protein 6 (IGFBP6)	2+	4+	2·8
X57413	transforming growth factor beta 2	2+	4+	2·7
M14951	insulin-like growth factor 2 (IGF II)	7+	8+	2·6
U32330	endothelin 3	2+	3+	2·1
M95200	vascular endothelial growth factor A	1+	1+	1·1
U66203	fibroblast growth factor 11 (FGF11)	1+	1+	1·1
M13177	transforming growth factor beta 1	1+	1+	1·0
J00380	epidermal growth factor	1+	1+	1·0
M32502	wingless-related MMTV integration site 3	2+	2+	0·9
M14220	glucose phosphate isomerase 1 complex	5+	5+	0·8
Interleukin, interferon and their receptors				
D90205	interleukin 5 receptor alpha (IL5RA)	−	4+	
D17630	interleukin 8 receptor beta (IL8RB)	−	4+	
M29697	interleukin 7 receptor alpha (IL7RA)	−	3+	
X07962	interleukin 7	1+	5+	7·8
M29855	colony stimulating factor 2 receptor beta 2	6+	6+	0·9
Hormones and hormone receptors				
J05149	insulin receptor (INSR)	−	4+	
M33324	growth hormone receptor (GHR)	1+	4+	5·9
U14420	gamma-aminobutyric acid (GABA-A) receptor	1+	4+	5·3
U22516	angiogenin	3+	4+	2·1
AF028242	calcitonin gene-related peptide-receptor component	7+	6+	0·7
Intracellular transducers, effectors and modulators, adaptors and receptor-associated proteins				
U43187	mitogen activated protein kinase kinase kinase 3	−	6+	
X99063	zyxin (ZYX)	−	5+	
U25844	serine proteinase inhibitor 3 (SPI3)	−	3+	
M24086	retinal S-antigen	−	3+	
M20473	cAMP dependent protein kinase I beta	−	3+	
U29678	chemokine (C-C) receptor 1 (CCR1)	−	3+	
D84372	protein tyrosine phosphatase non-receptor type 11	−	3+	
X63615	calcium/calmodulin-dependent protein kinase II	−	2+	
U43319	frizzled homolog 6	2+	9+	15·3
U43205	frizzled homolog 3 (FZ3)	1+	4+	6·3
M34476	retinoic acid receptor, gamma	1+	3+	3·8
Z22532	syndecan 1 (SYND1)	2+	4+	3·4
U10871	mitogen activated protein kinase 14	1+	3+	2·9
X76850	MAP kinase-activated protein kinase 2	1+	3+	2·8
M84817	retinoid X receptor alpha	1+	2+	2·3
D32210	Notch gene homolog 2	2+	3+	2·0
U15159	LIM-domain containing, protein kinase	2+	2+	1·4
M83336	interleukin 6 signal transducer	3+	3+	1·3
M60778	integrin alpha L	2+	2+	1·1
L03529	coagulation factor II (thrombin) receptor	1+	1+	1·0
Proteases				
M14222	cathepsin B (CTSB)	−	6+	
X53337	cathepsin D (CTSD)	−	6+	
X06086	cathepsin L (CTSL)	−	4+	
X12822	granzyme C (GZMC)	1+	5+	7·2
AF030065	hepsin	2+	5+	4·8
M58588	kallikrein B, plasma 1	2+	2+	1·2
Protease inhibitors				
M33960	plasminogen antivator inhibitor 1 (PAI1)	−	10+	
X62622	tissue inhibitor of metalloproteinase 2 (TIMP2)	−	5+	
J05609	serine proteinase inhibitor J6	−	5+	
L19622	tissue inhibitor of metalloproteinase 3 (TIMP3)	−	4+	
X70296	serine protease inhibitor 4 (SPI4)	−	3+	
X59379	amyloid beta (A4) precursor protein	2+	3+	1·5
G-proteins				
L34290	guanine nucleotide binding protein beta 5	−	5+	
X95403	RAB2 member RAS oncogene family	−	4+	
X57277	RAS-related C3 botulinum substrate 1 (RAC1)	−	3+	

Table 2. (*cont.*)

GenBank Accession no.	Gene names	Expression level		Ratio
		Normal	Infected	
Neuropeptides and Neurotransmitter receptors				
U09421	opioid receptor kappa 3 (OPRM1)	−	3+	
AF019371	cholecystokinin B receptor	−	3+	
X15830	7B2 neuroendocrine protein	−	3+	
U14420	gamma-aminobutyric acid (GABA-A) receptor	1+	4+	5·3
M55181	preproenkephalin 1	5+	5+	1·2
U05342	ciliary neurotropic factor	4+	4+	0·9
Y00703	GNAS	6+	5+	0·8
Xenobiotic metabolism and transporters				
U13705	glutathione peroxidase 3 (GPX3)	−	3+	
M10021	cytochrome P450 1A1 (CYP1A1)	1+	6+	10·5
J04696	glutathione S-transferase mu 2 (GSTM2)	1+	3+	4·9
DNA polymerases, replication factors, topoisomerases, DNA damage repair proteins and ligases				
U59883	MLH1 DNA mismatch repair protein	−	5+	
X74351	xeroderma pigmentosum complementation group A	−	4+	
D26090	Cell division cycle 46 homolog (CDC46)	−	3+	
X92411	DNA damage repair & recombination 23B	−	3+	
Z32767	DNA damage repair & recombination 52	1+	4+	6·2
S71186	DNA excision repair protein ERCC3	2+	3+	2·9
X81464	translin	1+	2+	2·8
D86725	MCM2 DNA replication licensing factor	3+	4+	2·5
X96859	ubiquitin-conjugating enzyme E2B	2+	3+	1·7
X96618	recombination activating gene 1 gene activation	5+	4+	0·7
Cytoskeleton and motility proteins				
X51438	vimentin (VIM)	−	8+	
M87276	thrombospondin 1 (TSP1)	−	8+	
L20276	biglycan (BGN)	−	6+	
X82402	fibronectin 1(FN1)	−	6+	
X66405	procollagen, type VI, alpha 1	−	5+	
X72091	vitronectin (VTN)	−	5+	
D00472	cofilin 1, non-muscle	−	5+	
L27220	cytokeratin 4 (CK4)	−	4+	
L07803	thrombospondin 2 (TSP2)	−	4+	
X05211	laminin gamma 1 (LAMC1)	−	3+	
X61435	kinesin family member 5A	1+	4+	6·3
U92949	kinesin motor protein C2 (KIFC2)	1+	4+	4·9
X15475	peripherin (PRPH)	2+	5+	4·1
U42624	laminin, beta 2	3+	4+	2·6
U72520	enabled homolog (Drosophila)	3+	4+	2·2
Z22923	procollagen, type IX, alpha 2	3+	3+	1·2
U12147	laminin, alpha 2	1+	1+	1·0
X53929	decorin	2+	2+	1·0
D17577	kinesin heavy chain member 1B	1+	1+	1·0
X03491	keratin complex 2, basic, gene 4	3+	2+	0·7
U04443	myosin light chain, alkali, nonmuscle	6+	3+	0·6
M76601	myosin heavy chain, cardiac muscle, adult	5+	1+	0·4
M19436	myosin light chain, alkali, cardiac atria	6+	2+	0·3
Z22866	myomesin 1	3+	−	0·3
Cell surface antigens				
D13664	osteoblast specific factor 2 (OSF2)	−	6+	
X63099	gap junction protein beta 3 (GJB3)	3+	3+	1·1
Membrane channels and transporters, ligand-gated ion channels, symporters and antiporters				
U20107	synaptotagmin 8 (SYT8)	−	2+	
U70068	potassium channel subfamily Q member 1	2+	3+	2·1
Function unclassified				
U26967	cordon-bleu (COBL)	1+	6+	9·3
AF023458	protein phosphatase with EF hand 2	2+	5+	5·1
L28819	involucrin	2+	3+	1·8

cyclin B2. The cyclin dependent kinase 4 (CDK4), a protein that forms a complex with cyclin D family and regulates cell cycle progression during G1/S phase, showed an up-regulatory expression in infected muscle. While some cyclin-dependent kinase inhibitors (P57 and P27), which are mitotic inhibitors inducing cell cycle arrest in G1 and G2/M, showed an up-regulatory expression.

The expression of many heat shock protein genes was upregulated in infected muscle. Within 11 heat shock protein genes, 5 genes showed more than a 3-fold increase in expression, including oxidative stress-induced protein (OSI) and heat shock proteins (HSP84, HSP86 and HSP105).

Of 58 apoptosis-associated protein genes, expression of 14 genes was upregulated. The most prominently upregulated gene was tumour necrosis factor receptor 1 (TNF-R1), which showed a 12+ increase in expression, compared with it being undetectable in normal muscle. The expression of activator of apoptosis hara-kiri (HRK) and Bcl2-associated X protein (BAX) was increased.

The expressions of many growth factors were increased, for example, 5 insulin-like growth factors (IGF) (IGF I, IGF I receptor, IGF binding protein 5, IFG binding protein 4, and IGF binding protein 2), 3 fibroblast growth factors (FGF) (FGF6, FGF12, and FGF7), and 4 bone morphogenetic proteins (BMP) (BMP4, BMP1, BMP3, and BMP8).

Out of 19 protease and 13 protease inhibitor genes, the expression of 5 proteases and 5 protease inhibitors increased more than 3-fold or 2+, for example, the proteases cathepsin B, cathepsin D, cathepsin L, and protease inhibitor plasminogen activator inhibitor 1 (PAI1), tissue inhibitor of metalloproteinase 2 (TIMP2), TIMP3, and 2 serine proteinase inhibitors (SPI) (SPIJ6 and SPI4).

The expression of some genes was downregulated after infection. Such examples included homeobox A13, nuclear receptor 1H2, and some cytoskeleton and motility proteins (myosin heavy chain, myosin light chain, myomesin 1 and keratin complex 2).

DISCUSSION

In this study we revealed the expressions profile of as many as 1176 genes in muscle tissues before and after *Trichinella* infection. In total, 311 of the 1176 genes were detected by the present microarray analysis. The expression of 184 genes out of the 311 genes was increased after the infection. Although the 184 genes may have been expressed in the process of inflammatory reactions and/or angiogenesis seen near the cyst, at least some of them must have been directly involved in nurse cell formation.

An urgent need is the identification of such genes in these processes. Towards this goal, we paid careful attention to some important genes such as cell differentiation, apoptosis, cell cycle, homeobox and growth factors, because *Trichinella* infection causes permanent phenotypic (morphology, metabolism) changes of host muscle cell, including re-entry into the cell cycle, and arresting in G2/M of the cell cycle (Despommier, 1975; Jasmer, 1993).

Our unpublished preliminary data, however, showed that cDNA of *T. spiralis* muscle larvae hybridize the two genes on the Atlas 1.2 array (eyes absent 2 and nerve growth factor alpha). As such, these two genes should be excluded from further analysis.

After larvae enter the muscle cell, the cell exhibits prominent changes such as hypertrophy and enlarged nuclei, and satellite cells are activated (Despommier, 1975; Wu *et al*. 2001). These features are similar to those seen during muscle differentiation (Smith, Janney & Allen, 1994; Seale & Rudnicki, 2000; Hawke & Garry, 2001). In our study, as expected, cell differentiation genes were upregulated. Examples were given by myocyte-specific enhancer factor 2C (MEF2), Lbx 1 transcription factor and NFAT. MEF2 is involved in the activation of muscle-specific gene expression (Naya & Olson, 1999), and acts in concert with myogenic regulatory factors (MRFs) in muscle cell differentiation (Parker, Seale & Rudnicki, 2003). NFAT plays a role in regulating the expression of MRFs in satellite cells (Friday & Pavlath, 2001). Prominent angiogenesis seen around the cysts (Baruch & Despommier, 1991; Capo *et al*. 1998) is another candidate that may account for the increased expression of at least some cell differentiation genes.

After *T. spiralis* infection, the expression of 11 homeobox genes was upregulated, as shown in Table 3. Homeobox genes are regulatory genes encoding nuclear proteins that act as transcription factors, regulating aspects of morphogenesis and cell differentiation during normal embryonic development (see review by Abate-Shen, 2002) and muscle cell differentiation (see review by Olson & Rosenthal, 1994).

The expression of all 3 homeobox MSX family genes (MSX1, MSX2 and MSX3) was increased after infection. Interestingly, MSX genes inhibit the differentiation of many cell types. For example, MSX1 is known to regulate proliferation and prevent differentiation of cells (Song, Wang & Sassoon, 1992; Hu *et al*. 2001). This homeobox transcription factor down-regulates the expression of MRFs and inhibits differentiation (Woloshin *et al*. 1995; Bendall *et al*. 1999; Odelberg, Kollhoff & Keating, 2000). Moreover, MSX1 is known to trigger the de-differentiation of multinucleate myotubes into mononucleated cells which are then able to re-enter the cell cycle and re-differentiate into myogenic cells (Woloshin *et al*. 1995; Odelberg *et al*. 2000). Therefore the unusual phenomenon of *Trichinella*-infected muscle cells, mis-differentiation of satellite

Table 3. Expression of homeobox genes after *Trichinella spiralis* infection

GenBank Accession no.	Gene/Protein name	Expression level		Ratio
		Normal	Infected	
L12703	engrailed homeobox (EN19)	−	9+	
X14759	homeobox, msh-like 1	−	6+	
L12705	engrailed 2	−	6+	
X59725	paired related homeobox 1 (PMX1)	−	6+	
Y11717	homeobox A3 (HOXA3)	−	6+	
X59252	homeobox, msh-like 2	−	4+	
X63507	homeobox C10 (HOXC10)	−	4+	
AF001465	aristaless 4 homeobox protein (ALX4)	−	4+	
X84814	sine oculis-related homeobox 5 homolog (SIX5)	−	3+	
U62523	msh-like homeobox 3 (MSX3)	−	3+	
X99291	homeobox D13 (HOXD13)	−	2+	
X80338	sine oculis-related homeobox 2 homolog (SIX2)	1+	5+	6·7
J03770	homeobox D4	2+	4+	2·4
S70632	homeobox protein 11 (HOX11)	5+	6+	2·0
X80339	sine oculis-related homeobox 1 homolog (SIX1)	1+	1+	1·2
S75837	developing brain homeobox 1 (DBX)	1+	1+	1·1
L39770	gastrulation brain homeobox 2 (GBX2)	1+	1+	1·1
U59322	homeobox A13 (HOXA13)	4+	2+	0·7

Table 4. Expression of IGF and FGF genes after *Trichinella spiralis* infection

GenBank Accession no.	Gene/Protein name	Expression level		Ratio
		Normal	Infected	
IGF				
X81583	insulin-like growth factor binding protein 5 (IGFBP5)	−	10+	
X81580	insulin-like growth factor binding protein 2 (IGFBP2)	−	6+	
X81582	insulin-like growth factor binding protein 4 (IGFBP4)	−	4+	
X04480	insulin-like growth factor 1 (IGF I)	−	3+	
U00182	insulin-like growth factor I receptor (IGFIR)	1+	4+	5·4
X81584	insulin-like growth factor binding protein 6 (IGFBP6)	2+	4+	2·8
M14951	insulin-like growth factor 2 (IGF II)	7+	8+	2·6
FGF				
M28998	fibroblast growth factor receptor 1	−	2+	
M92415	fibroblast growth factor 6 (FGF6)	1+	6+	11·5
AF020738	fibroblast growth factor 12 (FGF12)	1+	3+	5·1
Z22703	fibroblast growth factor 7 (FGF7)	4+	7+	3·5
U66203	fibroblast growth factor 11 (FGF11)	1+	1+	1·1

cells, should be discussed in light of the increased expression of MSX1 genes.

Trichinella-infected muscle cells exit from G0 and re-enter the cell cycle (Jasmer, 1993). Therefore cell cycle regulation genes are of particular interest. Out of 45 cell cycle related genes, 13 genes were upregulated. For example, cyclin C, B2, D2 and D3 were increased in expression.

The mammalian cell cycle is regulated by a series of cell cycle regulatory molecules, including cyclin, cyclin-dependent kinase (CDK) and CDK inhibitor. Different cyclins bind specifically to different CDKs to form distinct complexes at specific phases of the cell cycle and thereby drive the cell from one stage of cycle to another. Upon stimulation, D-type cyclins assemble CDK4 and CDK6 to form complexes, which facilitate cells to exit from G0 phase and re-enter the cell cycle of G1 (Reed *et al*. 1994; Weinberg, 1996; Wuarain & Nurse, 1996). Our present data showed that genes of these proteins (cyclin D2, cyclin D3 and CDK4) were increased after infection, which supports the thesis that the infected cells re-enter the cell cycle.

Of particular interest is that the expression of retinoblastoma (Rb), p27 and p57 is increased. These proteins are known to play an important role in the growth arrest of differentiating cells, because they specifically inhibit CDKs, which leads to the withdrawal of cells from cycle and differentiation (Liu *et al*. 1996; Jacks & Weinberg, 1996; Pines 1997; Matushansky, Radparvar & Skoultchi, 2000). This implies that the increased expression of cycle

Table 5. Expression of BMP and associated genes after *Trichinella spiralis* infection

GenBank Accession no.	Gene/Protein name	Expression level		Ratio
		Normal	Infected	
U14173	ski proto-oncogene	−	12+	
X56848	bone morphogenetic protein 4 (BMP4)	−	10+	
L24755	bone morphogenetic protein 1 (BMP1)	−	6+	
D16250	bone morphogenetic protein receptor 1A (BMPR1A)	−	7+	
S82648	bone morphogenetic protein 3B (BMP3B)	−	6+	
AF003942	bone morphogenic protein receptor II (BMPR2)	−	6+	
U58992	MAD homolog 1 (smad1)	1+	5+	9·7
M97017	bone morphogenetic protein 8a (BMP8A)	2+	4+	3·3

inhibitor genes may be responsible for the arrest in G2/M of infected cells.

During the process of cystogenesis, apoptosis plays an important role in remodeling the host tissue (Boonmars *et al*. 2004*b*). The present microarray analysis revealed that out of 58 apoptosis related genes, expression of 14 genes was upregulated after the infection. The examples include tumour necrosis factor receptor I (TNFRI), Bcl2-associated X protein (BAX), activator of apoptosis hara-kiri (HRK) and protein kinase B (PKB). Our previous study showed that expressions of mitochondrial apoptosis related genes (BAX and PKB) were elevated in the *T. spiralis*-infected muscle cells during cyst formation (Boonmars *et al*. 2004*b*), which is reconfirmed by the present microarray results.

After *T. spiralis* infection, the expression of many growth factors was elevated. These upregulated growth factors are likely to be associated with satellite cell activation, re-entry to the cell cycle, muscle hypertrophy and regulation of differentiation and proliferation. Satellite cells are known to modulate their cell cycle state in response to growth factors (Hawke & Garry, 2001; Stamler & Meissner, 2001). In fact, many reports have indicated that growth factors (HGF, FGFs and IGFs) regulate the satellite cell activation (Doumit, Cook & Merkel, 1993; Coleman *et al*. 1995; Tatsumi *et al*. 2001). Of particular interest is FGF-6, which is muscle specific and upregulated during muscle regeneration (Floss, Arnold & Braun, 1997; Kastner *et al*. 2000).

The IGF family plays a crucial role in regulating cell proliferation and differentiation, and has mitogenic and anti-apoptosis effects on normal and transformed cells. IGF-I and IGF-II levels are upregulated in skeletal muscle undergoing regeneration (Levinovitz *et al*. 1992; Krishan & Dhoot, 1996). Both factors are able to alter MRFs expression and promote both the proliferation and differentiation of myoblasts (Coleman *et al*. 1995; Engert, Berglund & Rosenthal, 1996). IGF can induce skeletal muscle hypertrophy that resembles the pathological changes seen in *Trichinella*-infected muscle cells. The hypertrophic effects of IGF I are attributed to the activation of satellite cells. On the other hand, IGFBP-5 can inhibit both proliferation and differentiation. Therefore, the elevated expression of IGFs may be related to the muscle hypertrophy induced by the infection, and to regulation of proliferation and differentiation of infected cells during nurse cell formation.

BMPs are a large subgroup within the transforming growth factor-beta (TGF-beta) family. They are involved in growth and differentiation and are crucial regulators of early embryogenesis and subsequent organogenesis as well as tissue homeostasis in adults (Wozney, 2002). For example, BMP-4 inhibits myogenesis (Reshef, Maroto & Lassar, 1998). BMPs elicit their effects through activation of type I and type II kinase receptors, which initiate the signal transmission of Smad proteins to the nucleus. Activated Smads were transported into the nucleus and play a direct role in gene transcription, which is also regulated by other molecules such as Id, c-Ski. The expression of c-ski in infected muscle was highly upregulated (12+). This gene was identified as a transcriptional co-repressor of Smad2 and Smad3 in a TGF-beta dependent manner. High expression of c-ski may suppress TGF signal transcription, leading to withdrawal of infected muscle cells from differentiation.

All of the detectable BMP genes showed increased expression after the infection, as shown in Table 5. The upregulation of these genes is very likely due to many types of cell growth and differentiation during completion of cyst formation.

Thus, the present cDNA microarray analysis showed kinetics of the genes mobilized in response to *Trichinella* infection, and gave hints about key genes in muscle transformation. The usefulness of the present microarray results are now being confirmed by more quantitative analysis whereby the cyst components are separated by laser-capture microdissection and subjected to RT-PCR for gene expression. Such examples included tumour necrosis factor receptor 1, insulin-like growth factor, ski proto-oncogene, smad 1, cyclin D3 and osteopontin, which provided more direct and precise data about

functional involvement of examined genes (to be published elsewhere).

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