의학박사학위논문

Exosomes Secreted by *Toxoplasma gondii*-infected L6 cells: Their Effects on Host Cell Proliferation and Cell Cycle Changes

톡소포자충에 감염된 L6 세포에서 분비된 엑소좀이 숙주 세포 주기 및 세포 증식에 미치는 영향

2016년 8월

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의학과 기생충학 전공

김 민 재

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이 논문을 의학박사 학위논문으로 제출함

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Exosomes Secreted by *Toxoplasma gondii*-infected L6 cells: Their Effects on Host Cell Proliferation and Cell Cycle Changes

By

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ABSTRACT

Exosomes Secreted by *Toxoplasma gondii*-infected L6 cells: Their Effects on Host Cell Proliferation and Cell Cycle Changes

Toxoplasma gondii infection induces alteration of the host cell cycle and cell proliferation. These changes are not only seen in directly invaded host cells but also in the neighboring cells. It is known that the soluble factor which mediates these changes is heat-labile and larger than 10 kDa. We tried to identify whether this alteration can be mediated by exosomes secreted by *T. gondii*-infected host cells.

L6 cells, a rat myoblast cell line, and RH strain of *T. gondii* were selected for this study. L6 cells were infected with or without *T. gondii*. The cellular growth patterns were identified by cell counting with trypan blue under confocal microscopy and cell cycle changes were investigated by flow cytometry. L6 cells infected with *T. gondii* showed decreased proliferation compared to uninfected L6 cells and revealed a tendency to stay at S or G2/M cell phase.

The exosomes were isolated from the L6 cells infected with or without *T. gondii* infection (RH exosome and L6 exosome, respectively) by sequential ultracentrifuge. The treatment of RH exosome into L6 cells resulted in the slight enhancement of S phase at 12 hour post treatment and the attenuation of cell proliferation at 36 hour post treatment. These changes were transient and disappeared at 48 hour after the exosome treatment. This transient result could be the result of the dilution of RH exosome by the exosomes secreted from the neighboring uninfected L6 cells.

To investigate whether miRNAs contained in the exosome can induce such changes, microarray analysis was performed on the exosomal miRNAs and cellular mRNAs of the exosome-treated cells. Significantly differently expressed miRNAs were identified and their target mRNAs were predicted based on sequence matching using web-based tools. These predicted mRNAs were compared with the actually differently expressed mRNAs. As a result, 1 miRNA-mRNA pair, rno-miR-216a-5p and Hmgb1 (high mobility group box 1), revealed as candidate for molecular mediator of changes on cell proliferation.

In conclusion, our study demonstrated that the exosomes originating from *T. gondii*-infected cells could change the host

cell proliferation and alter the host cell cycle. *Hmgb1* could be the possible responsible gene inducing such changes.

Keywords: *Toxoplasma gondii*, L6 cell, Exosome, miRNA, cell cycle, proliferation, *Hmgb1*, miR-216a-5p

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INTRODUCTION

Extracellular vesicles and exosome

In multicellular organisms, cell-to-cell communication is essential for many biological functions. It can be mediated through direct cell-to-cell contact or through transfer of active molecules. Recently another method of intercellular communication through the transfer of extracellular vesicle is intensively investigated [1]. The extracellular vesicles can not only directly stimulate other cells by receptor-mediated contact but also can transfer the genetic materials, proteins and lipids [2].

Several different types of extracellular vesicles have been described. Exosome is usually referred to those originating from intracellular multivesicular endosome (MVE) whereas microvesicle to those that are shed from the plasma membrane [2]. In contrast to the plasma membrane, the membranes of

exosomes contain abundant cholesterol, sphingomyelin, and hexosylceramides and low amount of phosphatidylcholine and phosphatidylethanolamine [1]. The tetraspanins, a family of transmembrane protein, such as CD53, CD63, CD81, CD82, CD53, are also known to be enriched on the exosomes [3]. In addition to these proteins and lipids, mRNAs were found to be contained in exosome and these mRNAs were shown to be translated into proteins in the targeted cells [4]. Small noncoding RNAs including miRNA are also known to be the cargo of the exosome. These miRNAs can interact with the mRNAs of the target cells resulting in the alteration of the protein expression [5].

The function of exosome was primarily considered as a mechanism to remove the useless molecules or to release transferrin receptors during reticulocyte maturation [6]. Later it was found that MHC complex is contained in the exosome and the exosome can present antigens to immune cells [7]. With the findings of exosome-associated mRNAs and miRNAs, the function of exosome in post-transcriptional modification is

extensively studied especially in the homeostasis and disease development [8,9].

Parasite and exosome

Recently extracellular helminth worms are found to secret exosomes into the surrounding environment. In trematode such as *Fasciola hepatica* and *Echinostoma caproni*, electron microscopy study revealed exosome-like vesicle on the tegumental surface. These exosomes were internalized by the host cells [10]. Another study revealed that *Schistosoma japonicum* adult worm secrets exosome-like vesicles and these vesicles could induce the host macrophage cell to polarize into M1 type [11].

In a study on the rodent intestinal nematode *Heligmosomoides polygyrus*, the parasite-secreted exosomes were internalized by the host cell. The study also showed the parasite-associated miRNA contained in the exosome were

internalized and could interact with the mRNA of host cell gene showing the inter-species RNA interference [12].

The protozoans are also found to release exosomes. The presence of exosome secreted from the promastigote of the *Leishmania donovani* was confirmed by the electron microscopy and proteomic analysis. These exosomes per se modulated the host immunologic response such as cytokine production and lymphocyte differentiation [13,14]. *Trypanosoma cruzi*, the etiologic agent of the human Chagas disease, also released the exosomes [15]. When mice were inoculated with the exosome secreted by the trypomastigotes stage of *T. cruzi* and subsequently infected with *T. cruzi*, they developed increased cardiac parasitism and intense inflammatory response [16]. The exosomes from the *Trichomonas vaginalis* also fused with host cells and modulated host cell cytokine production [17]. The exosome was also identified in the plasma of patients with uncomplicated *Plasmodium vivax* infection. The main sources of the exosome were platelets, erythrocytes and leukocytes. The amount of

exosome increased when patient was febrile at time of blood collection or the acute symptoms persisted longer [18].

The functions of exosome were extensively studied in the field of the immune modulation. In addition, the exosome also mediated the cell-to-cell communication of the parasite. The exosome released from the erythrocytes infected with *Plasmodium falciparum* induced other asexual parasites to differentiate into the sexual form, the gametocyte [19] . The exosome from the well-adherent *T. vaginalis* induced the poorly adherent *T. vaginalis* into the well-adherent type [17].

Toxoplasma gondii and exosome

Recently many aspects about the exosome of *T. gondii* are revealed. *T. gondii*-infected macrophages and human fibroblasts produced exosomes different from that of the uninfected cells [20,21]. The exosomes secreted by dendritic cells pulsed with *T. gondii*-derived antigen showed protective

effect against *T. gondii* infection [22,23]. The exosomes contained the major histocompatibility complex class I and II (MHC I and II), T-cell constimulatory molecules and antigen of *T. gondii*. This composition enabled the antigen presentation and induced a strong Th1-modulated *T. gondii* specific immune response [24]. In addition to the proteins in the exosomes, the role of miRNAs in the exosome is also investigated. The small RNA producing machinery of *T. gondii* was identified and was functionally and phylogenetically related to that of plants and fungi [25]. Bioinformatics approach suggested a possibility that *T. gondii* derived miRNA could directly regulate the host gene expressions [26].

Toxoplasma gondii and host cell cycle

T. gondii and host cell relationship is complex. As *T. gondii* is obligate intracellular organism, *T. gondii* utilizes the host cell apparatus for survival. It acquires nutrients from the host cells [27] and reorganizes host cell cytoskeleton for invasion and

parasitophorus vacuole formation [28]. *T. gondii* also uses host cell ion regulating system to adjust cellular environment to fit for parasite invasion and egress [29]. Many transcriptional changes occur when *T. gondii* invades the host cell [30,31]. Recent studies also identified miRNAs changes of the host cell infected with *T. gondii* [32]. Among the many changes associated with *T. gondii* infection, we want to focus on the host cell cycle dysregulation.

Many viruses are famous for their dysregulation of the infected host cell cycle. Different changes of cell cycle occur for each virus. Some viruses induce the quiescent host cell to enter the cell cycle [33]. Some other viruses arrest cells in the particular cell cycle phase [34]. Previous studies showed *T. gondii* infection also resulted in the host cell cycle dysregulation.

When human trophoblast and fibroblast cells were infected with *T. gondii*, *T. gondii* induced both the infected cells and the neighboring cell to stop proliferation. *T. gondii*-infected cells

transited from G0/G1 phase to S phase and finally arrested at G2/M phase. The conditioned culture media from the *T. gondii*-infected cells were tested whether soluble factors can cause the changes of the neighboring cells. However they did not resulted in any changes. Since *T. gondii* proliferated better in the G2-arrested cells than the G1-arrested cells, the host cell cycle changes were favorable for the parasite [35]. Another study using the human fibroblast confirmed that *T. gondii* invasion caused the quiescent cells to enter S phase. Not only infected cells but also the neighboring cells also showed similar changes. In contrast to the previous study, the conditioned culture media could reproduce the changes of cell cycle. The soluble factors in the conditioned culture media were heat-labile and larger than 10 kDa in size [36]. This study also confirms this cell cycle changes are beneficial for *T. gondii* because *T. gondii* is known to invade cells in the S phase most efficiently [37]. The cell cycle changes, promotion of S phase entry and arrest at G2/M phase, were once more verified in another study [38]. Various cell cycle regulators such as cyclin, cyclin-dependent kinases (Cdk), E2F transcription factor and

retinoblastoma (Rb) proteins were checked. Cell cycle regulators associated with G1-S transition were up-regulated.

Function of exosome as modulator of the host cell cycle

The previous studies showed that soluble factor can mediate the cell cycle changes induced by *T. gondii* infection. Many parasites and the infected cells secreted exosomes and these exosomes were associated with cell-cell communications. Thus we supposed that exosomes secreted from the *T. gondii*-infected cells would have effects on host cell cycle regulation and host cell proliferation. In addition, we tried to identify whether the miRNAs capable of regulating cell cycle or cell proliferation are really contained in the RH exosome and whether the target gene of the identified miRNA is really differently expressed in the exosome-treated cell.

MATERIALS AND METHODS

1. L6 cells and *T. gondii*

L6 rat myoblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene), 4 mM L-glutamine, 0.2 mM penicillin, 0.05 mM streptomycin (Welgene) at 37°C in humidified air containing 5% CO_2. The RH tachyzoites of *T. gondii* were maintained under in vitro condition using Vero cells. The tachyzoites were collected from the culture supernatant of infected Vero cells by serial centrifugation at 1,500 rpm for 5 min and 3,000 rpm for 10 min.

2. Changes of cell number and morphology after *T. gondii* infection

To investigate the cell proliferation patterns, L6 cells were inoculated in 6-well plates with or without *T. gondii* infection. The cell numbers in a well were counted after trypan blue staining at pre-determined time points with interval of 12 or 24 hours.

The phase-contrast images of *T. gondii*-infected cells were

acquired with confocal microscope (FV 1000, Olympus, Japan) after fixation with 4% paraformaldehyde at the pre-determined time points.

3. Isolation of exosome

Exosome-depleted culture media were prepared by ultracentrifugation of DMEM with 10% FBS at 100,000 *g* for 16 hour at 4°C followed by filtration through a 0.22-μm filter (Nalgene, Rochester, New York, USA). These media were used for isolation of exosomes. At first, 5×10^5 L6 cells were inoculated in 100 mm culture dish with exosome-depleted culture media. After 12 hour of incubation, the RH tachyzoites (multiplicity of infection; MOI = 20) were added. The culture media were exchanged with fresh exosome-depleted culture media 24 hr after *T. gondii* inoculation, thereby removing the non-invaded parasites. The exosomes of control group, i.e., L6 cells without *T gondii* infection, were also collected by the same protocol except that PBS was added instead of RH tachyzoites.

Exosomes were isolated from the culture supernatant by differential centrifugation, which is the most widely used method. Briefly, the L6 cell culture supernatants were

harvested and centrifuged at 300 *g* for 10 min at 4°C. The supernatant was serially transferred to a new tube and centrifuged at 2,000 *g* for 10 min at 4°C and at 10,000 *g* for 30 min at 4°C. The supernatant was then ultracentrifuged at 100,000 *g* for 70 min at 4°C with ultracentrifuge (Optima XE-100 Ultracentrifuge, Beckman Coulter, Miami, Florida, USA). The observed exosome pellets in each tube were collected together and ultracentrifuged once more at 100,000 *g* for 70 min at 4°C. The final pellet was re-suspended in 300 μl of PBS for RNA or protein analysis. The concentration of isolated exosome was determined by NanoDrop 2000 Spectrophotometer.

4. Changes of cell cycle

L6 cells were grown in 6-well plates. Initially, 2×10^5 L6 cells were inoculated in exosome-depleted culture media. After 12 hour, 1×10^6 RH tachyzoites (MOI 20) or exosomes from L6 cells with or without *T. gondii* infection were added at 100 μg/ml. The tachyzoites were allowed to invade the cells for 24 hr, and then non-invaded parasites were washed away. The culture media containing exosomes were maintained without media change. At pre-determined time points, the cells were

trypsinized and washed 3 times in 1 ml of PBS. After centrifugation at 1,200 rpm for 5 min, cell pellets were re-suspended in 0.3 ml PBS. The cells were fixed by incubation for 1 hr on ice with addition of 0.7 ml of 70% ethanol. The fixed cell suspensions were incubated in 37°C for 1 hr with RNase A. Finally, the cells were stained with propidium iodide (PI) and analyzed on a flow cytometry, FACS caliber (BD bioscience, USA) at 488 nm.

5. Microarray of miRNAs in the L6 and RH exosome

Exosomal RNA was extracted from L6 cell-derived exosomes using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined by NanoDrop 2000 Spectrophotometer. RNA quality for microarray was assessed by Agilent 2100 Bioanalyser (Agilent Technologies, Amstelveen, Netherlands).

The miRNA expression profiling was performed using miRCURY^{TM} LNA microRNA Array, 7th generation-has, mmu, and rno array (EXIQON, Vedbaek, Denmark). We used 250-1,000 ng of exosomal RNA for Cy3 dye labelling. Labeled samples were subsequently hybridized onto a microarray slide

using a hybridization chamber kit (Agilent Technologies, Santa Clara, California, USA) and hybridization gasket slide kit (Agilent Technologies). Hybridization was performed over 16 hr at 56°C followed by washing the microarray slide as recommended by the manufacturer. The microarray slides were then scanned with Agilent G2565CA Microarray Scanner System (Agilent Technologies). Scanned images were imported by Agilent Feature Extraction software version 10.7.3.1 (Agilent Technologies), and fluorescence intensities of each image were quantified using the modified Exiqon protocol and corresponding GAL files.

6. Confirmation of exosome uptake using endocytosis inhibitor, dynasore

To confirm the uptake of isolated exosomes by L6 cells, known endocytosis inhibitor, dynasore, was used. At first, exosomes from L6 cells were stained using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, St.Louis, MO, USA). 2×10^4 L6 cells were incubated in cover glass bottomed dishes with PKH26-labeled exosomes (red) under addition of 50 μM dynasore, 100 μM dynasore or 5% DMSO as negative control for 24 hours at 37°C in a 5% CO_2 incubator. The extracellular exsomes were washed

away with PBS and cells were fixed with 4% paraformaldehyde. L6 cells were stained with DAPI (blue) to stain the cellular nuclei. The cells were observed with fluorescent confocal microscopy, Leica TCS SP8 STED CW confocal system (Leica Microsystems, Mannheim, Germany) and Olympus CKX 41 (Olympus, Japan).

7. RNA purification from exosome-treated L6 cells

2×10^5 L6 cells were incubated in a 6 well plates. A 100μg/ml of L6 exosome or RH exosome was added in each well of culture plate. After 12 hours and 24 hours of incubation, cells of each group were harvested by trypsinization. The total RNA was extracted by miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined by NanoDrop 2000 Spectrophotometer.

8. Microarray of mRNA from exosome-treated L6 cells

For the quality control, RNA purity and integrity were evaluated by OD 260/280 ratio, and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

The Affymetrix Whole transcript Expression array process was executed according to the manufacturer's protocol (GeneChip

Whole Transcript PLUS reagent Kit). The cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal labeling kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Rat 2.0 ST Array at 45°C for 16 hour. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. The data were summarized and normalized with robust multi-average (RMA) method implemented in Affymetrix® Expression Console™ Software (EC). We exported the result with gene level RMA analysis and perfomed the differentially expressed gene (DEG) analysis. Statistical significance of the expression data was determined using fold change.

9. Analysis of relationship between altered miRNAs and

mRNAs

9.1 Significantly differently expressed miRNAs and mRNAs

Significantly differently expressed exosomal miRNAs and cellular mRNAs were identified from the results of microarray. Among the miRNAs identified by microarray, those showing signal intensity more than 9.0 (log2) were only included in the analysis. The differentially expressed miRNAs were determined as those showing greater than 4.0-fold change of signal intensity between 2 groups, miRNAs from L6 exosome and RH exosome. Among the mRNAs, differentially expressed mRNAs were identified as those with greater than 1.5-fold change of signal intensity between L6 cells treated with L6 exosome and RH exosome.

9.2 Identification of target mRNAs of the significantly differently expressed miRNAs via web-based tools

The target mRNAs of the significantly differently expressed miRNAs were identified from the sequence based match using miRNA target prediction web tool, miRDB (http://mirdb.org/miRDB). The mRNAs with target score larger than 70 were selected.

9.3 Comparison of the actually altered mRNAs with the expected target mRNAs

The predicted target mRNAs were compared with the actually altered mRNAs identified from the microarray. The mRNAs changed in opposite direction with the miRNAs were only identified.

9.4 Selection of mRNAs related with cell cycle or cell proliferation among the significantly differently expressed mRNAs

The cellular functions of the significantly differently expressed mRNAs were searched using web-based database, DAVID Bioinformatics Resources 6.7 (http://david.ncifcrf.gov) and Gene Ontology (http://geneontology.org). Gene ontology term 'cell cycle', 'mitotic cell cycle', 'regulation of mitotic cell cycle', 'cell cycle process', 'positive regulation of cell cycle', 'positive regulation of cell cycle process', 'cell proliferation', 'negative regulation of cell proliferation' and 'regulation of cell proliferation' were used for the search.

9.5 Identification of mRNAs possibly associated with miRNAs

The mRNAs selected by gene ontology research were compared with the predicted target mRNAs of the significantly differently expressed miRNAs. Also the mRNAs changed in opposite direction with the miRNAs were only selected.

10. Determination of cellular HMGB1 mRNA level

The cDNAs were synthesized from the total RNA derived from L6 and RH exosome treated L6 cells using Reverse Transcription Premix (Elpis, Daejeon, Korea). The levels of HMGB1 (High mobility group box 1) mRNA were determined by real-time PCR using SYBR green PCR master mix (Applied Biosystem, UK). GAPDH was used as housekeeping gene. The primers for HMGB1 were 5'-AGTTCAAGGACCCCAATG-3' (upstream) and 5'-TGCTCTTCTCAGCCTTGACCA-3' (downstream). Those of GAPDH were 5'-TGAACGGGAAGCTCACTGG-3' (upstream) and 5'-TCCACCACCCTGTTGCTGTA-3' (downstream) [39,40]. DNA was amplified for 40 cycles of denaturation for 15 seconds at 95°C and annealing for 60 seconds at 60°C. The specificity of reactions was determined by melting curve analysis.

11. Statistical Analysis

The independent t-test was used to compare the means between two groups. Statistical Package for the Social Sciences (SPSS) package version 18 was used for the statistical analysis.

RESULTS

1. *T. gondii* infection resulted in decrease of L6 cells proliferation and cell cycle arrest at G2/M and S phase

The L6 rat myoblast cell line was selected to study interaction between the host cell and *T. gondii*. L6 cells are known to differentiate into myocytes under confluent condition or with special differentiation medium [41]. Experiments were performed at early stages of cell culture and under normal growth medium to maintain proliferation of L6 cells. Cells were incubated in culture medium alone or with *T. gondii* RH tachyzoites. Non-invaded parasites were washed away after 24 hour of incubation. The cell numbers were counted, and the phase-contrast images were acquired at the pre-determined time points. The DNA contents were measured at 24 hr and 48 hr after the RH tachyzoite inoculation by flow-cytometry analysis of PI-stained cells.

T. gondii-infected L6 cells showed decreased proliferation compared to uninfected cells (Fig. 1). The difference in cell numbers between the 2 groups appeared early after *T. gondii* inoculation and became obvious at 48 hour post infection. After

72 hour post infection, the total cell numbers in infected cells began to decrease. The phase-contrast images showed continuous intracellular proliferation of *T. gondii* (Fig. 2). The cell lysis was obvious after 72 hour post infection, explaining the decrease of the cell counting. In *T. gondii*-infected cells, G2/M and S phase were increased, however, G0/G1 phase were decreased compared to uninfected cells. Between 24 hour and 48 hour post infection, the proportion of cells in the G2/M stage decreased in uninfected L6 cells from 20.8% to 15.4%. However, these changes did not occur in *T. gondii*-infected cells (Fig. 3).

2. Exosomes were internalized by L6 cells by endocytosis.

To confirm the exosome uptake by the L6 cells, exosomes were stained with red fluorescent dye, PKH, and added in the culture media of L6 cells. The confocal microscopic images after 24 hour shows internalized exosomes at the cytosol (Fig. 4). Dynasore is known endocytosis inhibitor. It is known to inhibit both clathrin-dependent and independent endocytosis [42]. When 50 μM or 100 μM of dynasore was added along

with the PKH stained exosome, the confocal microscopic images showed exosome uptake was highly reduced (Fig. 5).

3. Exosomes from *T. gondii*-infected cells showed attenuation of cell proliferation and slight enhancement of S phase and decrease of G2/M phase in L6 cells

To identify whether exosomes can mediate changes on cell cycle and cellular proliferation, cells were incubated with the exosomes isolated from L6 cells with or without *T. gondii*, respectively. Cells were trypsinized and analyzed by phase contrast microscopy for counting of cell number or by flow cytometry to analyze the distribution of cell cycle stages. All experiments were duplicated.

In L6 cells cultured with the exosomes derived from *T. gondii*-infected cells (considered as RH exosome), the host cells proliferated less than those grown in culture media alone and also than those grown with the exosomes derived from uninfected L6 cells (considered as L6 exosome). The cells treated with RH exosome reached only to 208.1 percent of the initial cell number, where those treated with L6 exosome to 367.5% at 36 hour post treatment. And L6 cells without

treatment of exosome proliferated to 376.9%. The difference was statistically significant with P=0.01 and P<0.001, respectively. At 48 hour post infection, these changes disappeared (Fig. 6).

By comparison, changes in the cell cycle were not as evident as the changes of the cell number. L6 cells cultured with the RH exosome showed increase in S stage (16.9% vs 15.8%, P=0.028), and decrease in G2/M stage (20.9% vs 25.0%, P=0.030) compared to those treated with L6 exosome at 12 hour post treatment. These changes also disappeared gradually (Fig. 7 and Fig. 8). At 48 hour post infection, both cells grown with L6 exosome and RH exosome revealed similar distribution patterns on the cell cycle stage. These patterns were also similar to that of the control group, L6 cells grown only in usual culture media.

4. Significantly differently expressed miRNAs in RH exosome compared to L6 exosome

The microarray analysis was performed to investigate the difference in the levels of miRNAs contained in RH exosome and L6 exosome. Among the total 701 miRNAs screened, 34

miRNAs revealed increased signal intensity and 30 miRNAs showed decreased signal intensity. The names of these significantly changed miRNAs are shown in Fig. 9. The target mRNAs of the significantly changed miRNAs were acquired by searching web-based database, mirDB (http://mirdb.org/miRDB). Genes with target score 70 and more were selected.

5. Significantly differently expressed mRNAs in L6 cells treated with RH exosome compared to L6 exosome

With microarray analysis, the levels of mRNAs were compared between L6 cells treated with RH exosome and L6 exosome 12 and 24 hour after exosome treatment. The mRNAs of 22259 genes were screened. Finally mRNAs of 308 genes were significantly differently expressed in L6 cells treated with RH exosome than L6 exosome 12 hour after exosome treatment. After 24 hour, 392 genes were significantly differently expressed.

6. Identification of mRNAs with possible relation with

significantly altered miRNAs

To identify the mRNAs possibly regulated by the significantly altered miRNAs, the result of microarray on mRNAs was compared with the predicted target mRNAs of the significantly altered miRNAs. The mRNAs whose level of expression changed in opposite to those of the miRNAs were only included. As a result, 10 and 15 possible mRNAs were identified at 12 hour and 24 hour time point respectively (Table 1).

The functions of these 25 mRNAs were searched using multiple web-based tools, DAVID Bioinformatics Resources 6.7 (http://david.ncifcrf.gov) and Gene Ontology (http://geneontology.org) [43]. When mRNAs whose functions were related with cell proliferation or cell cycle regulation were searched, only 1 mRNA, *HMGB1*, remained.

Conversely, from the significantly differently expressed mRNAs, those related with cell cycle or cell proliferation was selected using the same web-based tools. As a result, 14 and 13 mRNAs were selected at 12 hour and 24 hour time point respectively (Table 2). Among them, a total of 4 mRNAs were included in the predicted target genes of the significantly differentiated miRNAs. When the direction of changes were considered, also 1 mRNA, *Hmgb1*, remained as biologically

valid candidate gene.

7. Hmgb1 mRNA expression was increased at 12 hour and decreased at 24hour in RH exosome treated cells

As a result of microarrays and web-based analysis on miRNAs and mRNAs, rno-miR-216a-5p and *Hmgb1* gene revealed as possible candidate for mediator of cell proliferation and cell cycle changes. To confirm the temporal changes of the *Hmgb1* expression, real-time PCR was perform using cDNAs from L6 cells treated with RH and L6 exosome. At 12 hour after exosomes treatment, hmgb1 expression was more than three times increased in RH exosome treated cells than L6 exosome treated cells. This change was reversed at 24 hour after exosomes treatment (Fig. 10). Collectively, hmgb1 was more expressed in RH exosome treated L6 cells at 12 hour and its expression level declined at 24 hour. In contrast, *hmgb1* expression level increased in L6 exosome treated L6 cells.

8. Possible target genes of exosomal miRNAs related to the cell cycle

The microarray of the mRNAs could not identify any significantly changed mRNAs related with cell cycle regulation. The mRNAs involved in cell cycle regulation and also associated with the significantly changed miRNAs could be estimated by the searching target genes of the miRNAs. When analyzed by the web-based tools, the possible cell cycle regulating mRNAs include cell cycle related transcription factors such as *E2F* families and *DP1* and *DP2*, their regulators such as *p130* and *p107*, cyclin E1 (*CCNE1*) and cell cycle inhibitory kinase *WEE*. Genes involved in other signaling pathways, *Smad4*, *Stag1*, *Stag2* and *14-3-3*, are also suggested as a possible candidate (Fig. 11).

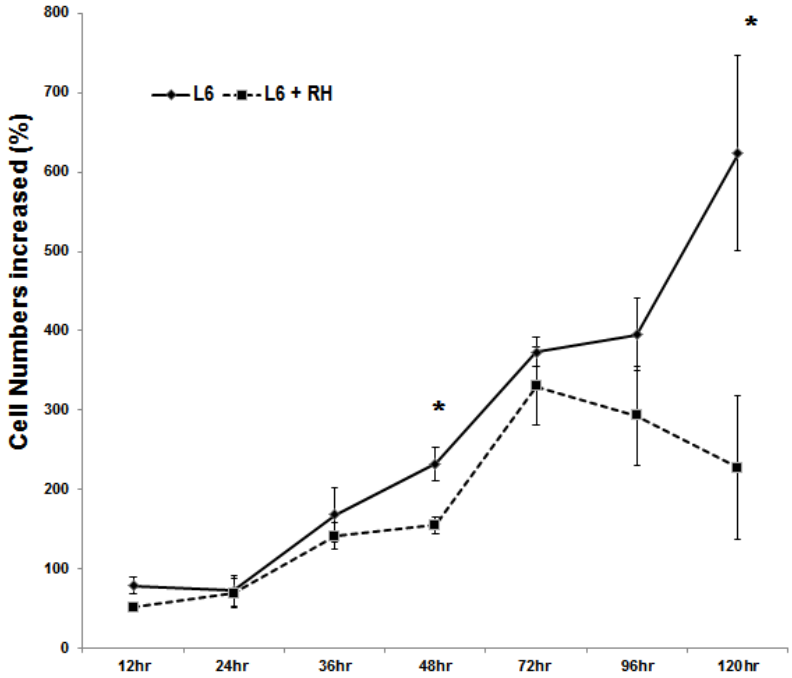

Figure 1. Cell growth patterns of uninfected and *T. gondii*-infected L6 cells. The cell numbers differed significantly at 48 hour and 120 hour post infection (* $P < 0.05$)

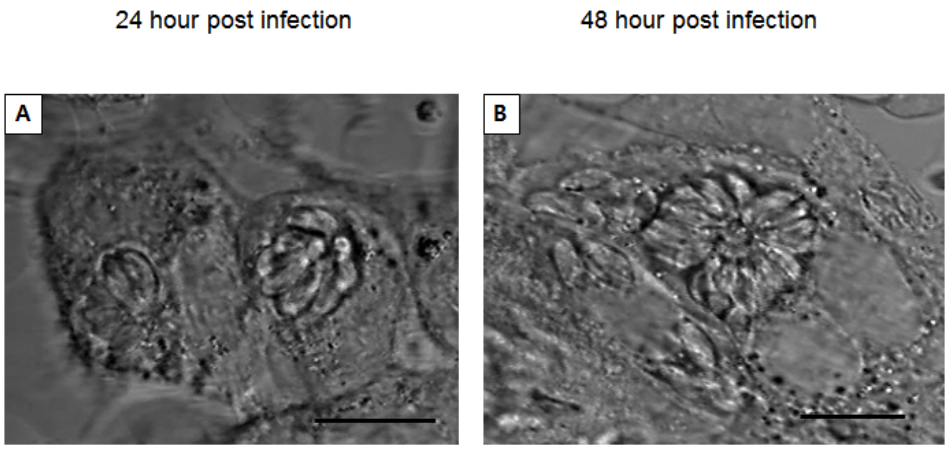

Figure 2. Phase-contrast images of *T. gondii*-infected cells at 24 hour and 48 hour post infection (MOI=20), respectively. The images show continuous proliferation of *T.gondii* in host cells. Bar = 10 μm.

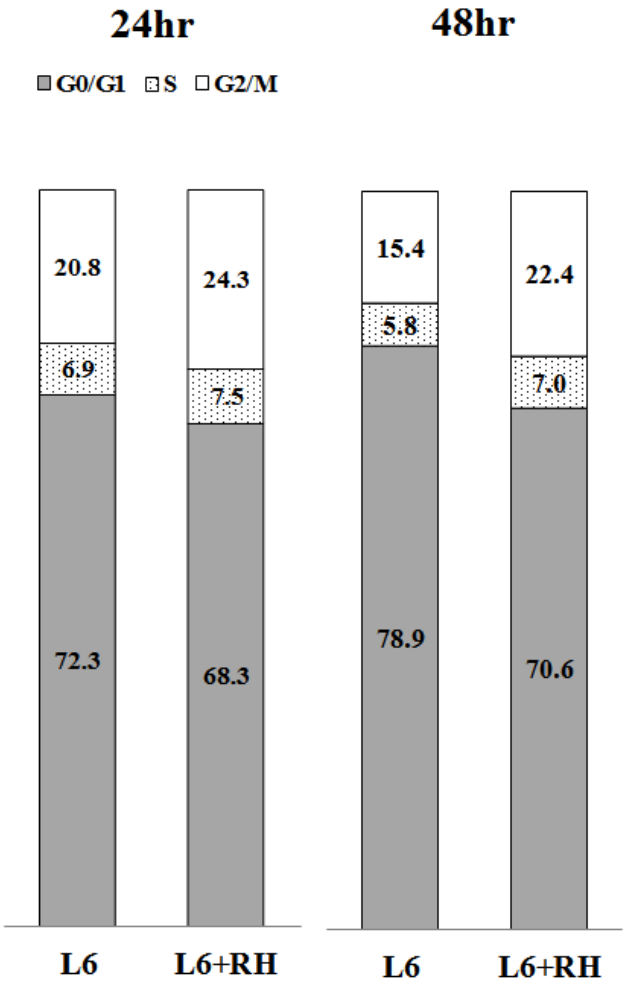

Figure 3. Cell cycle changes induced by *T. gondii* infection at 24 hour and 48 hour post infection.

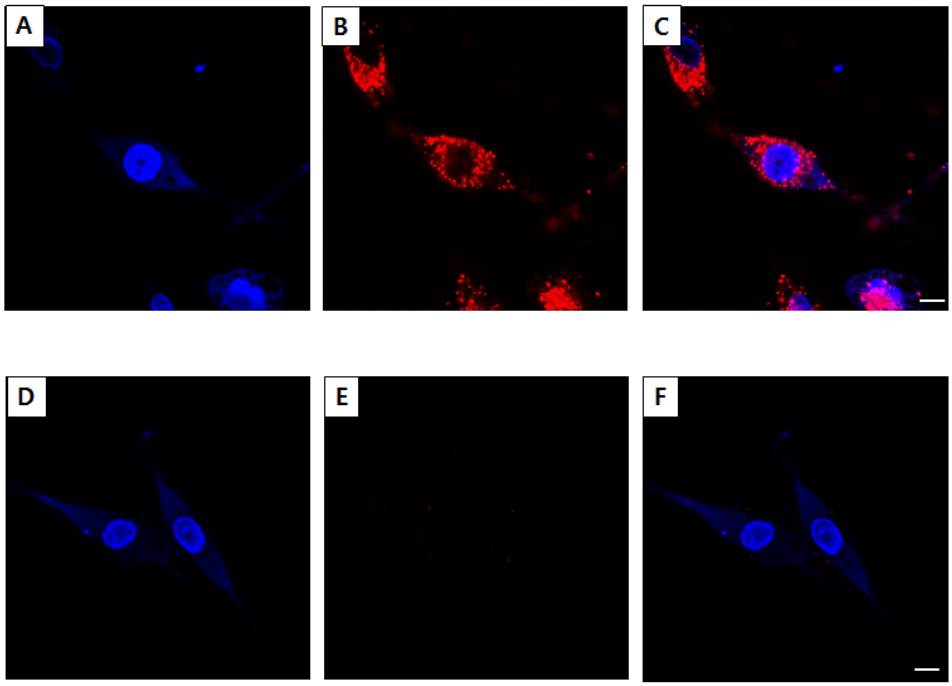

Figure 4. Confocal microscopic images of L6 cells 24 hour after exosome treatment with or without dynasore. Cellular nuclei is stained with DAPI and shown blue (A and D). PKH stain exosome is shown red and mainly observed in the cytoplasm (B). When the endocytosis is inhibited by dynasore, exosome is not observed in the cytoplasm (E). The merged images are shown in C and F. Bar = 10 μm.

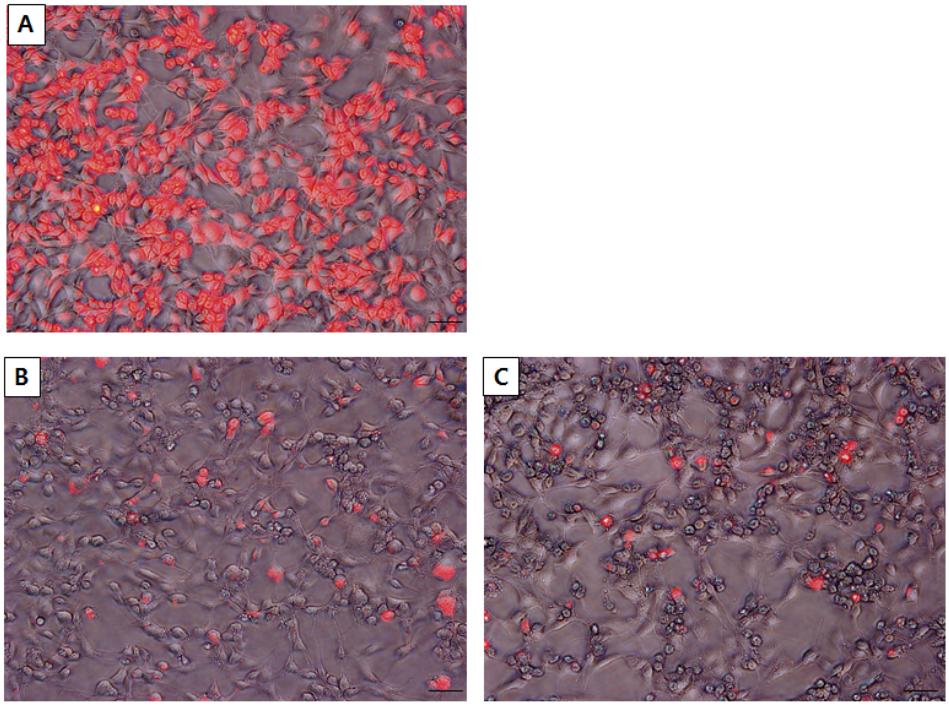

Figure 5. Confocal microscopic images showing exosome internalization was inhibited by dynasore. L6 cells were culture with PKH stained exosome in addition with 5% DMSO (A), 50 μM dynasore (B), and 100 μM dynasore (C). Bar = 50 μm.

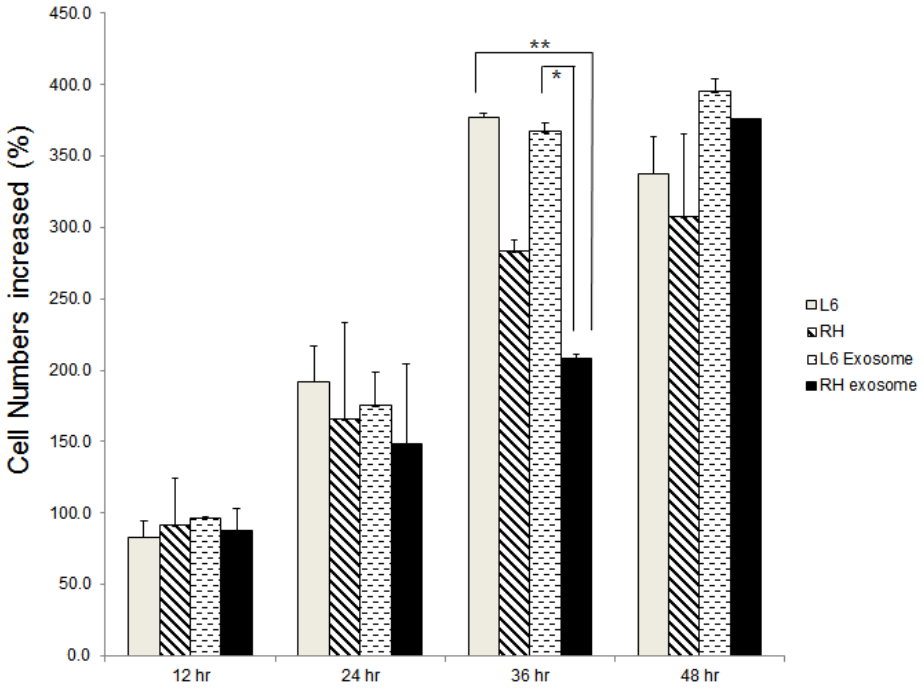

Figure 6. Effects of RH and L6 exosome on cell proliferation.

L6 cells were grown in usual culture condition, *T. gondii*-infected, treated with 100 μg/ml of exosomes from uninfected (L6 exosome) and *T. gondii*-infected (RH exosome) cells. The number of L6 cells treated with RH exosome was significantly different from that of the cells treated with L6 exosome (*P = 0.001) or that of the cells grown with usual culture media (**P < 0.001) at 36 hour by independent t-test.

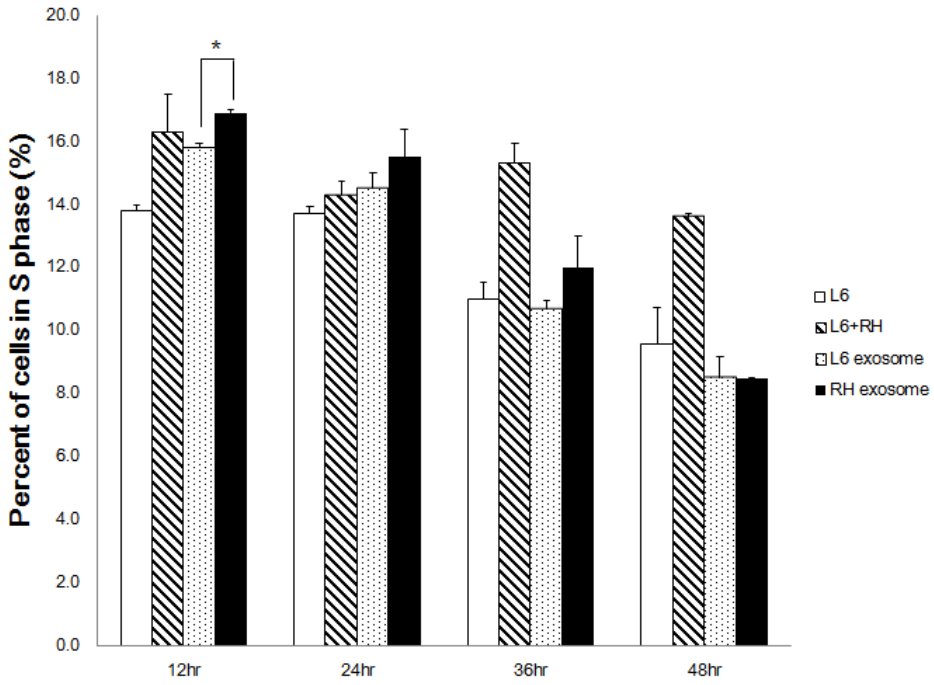

Figure 7. Effects of RH and L6 exosome on cell cycle S phase.

Changes of the percent of cells in the S phase at 12, 24, 36, and 48 hour post *T. gondii* infection or exosomes treatment. Only minute difference is observed between cells treated with L6 exosome or RH exosome at 12 hour post treatment ($*P = 0.028$).

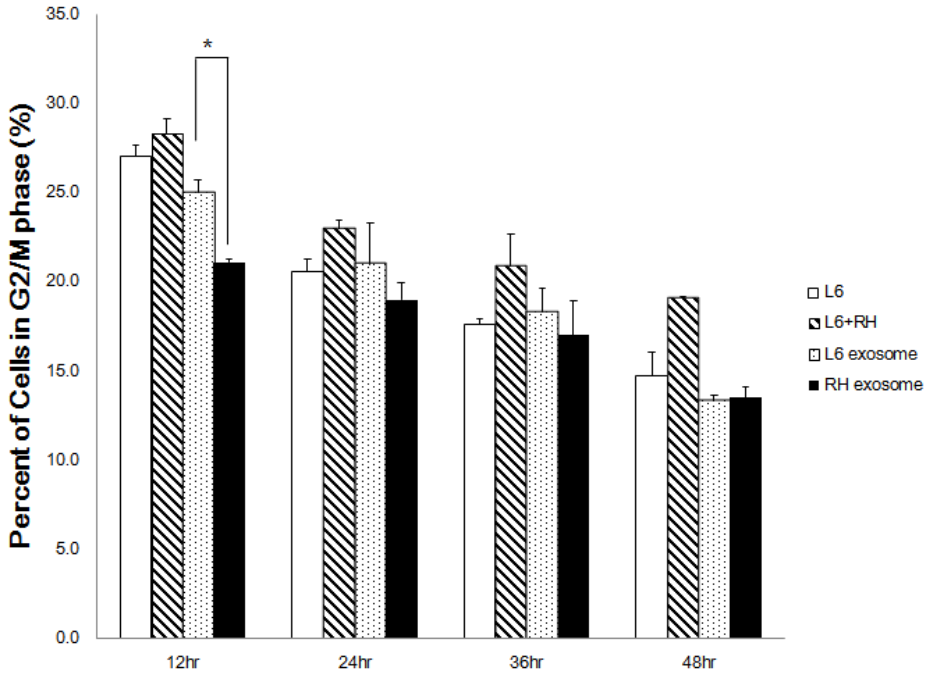

Figure 8. Effects of RH and L6 exosome on cell cycle G2/M phase.

Changes of the percent of cells in the G2/M phase at 12, 24, 36, and 48 hour post *T. gondii* infection or exosomes treatment. Cells treated with RH exosome showed decrease in G2/M phase compared to those treated with L6 exosome at 12 hour post treatment ($*P = 0.030$).

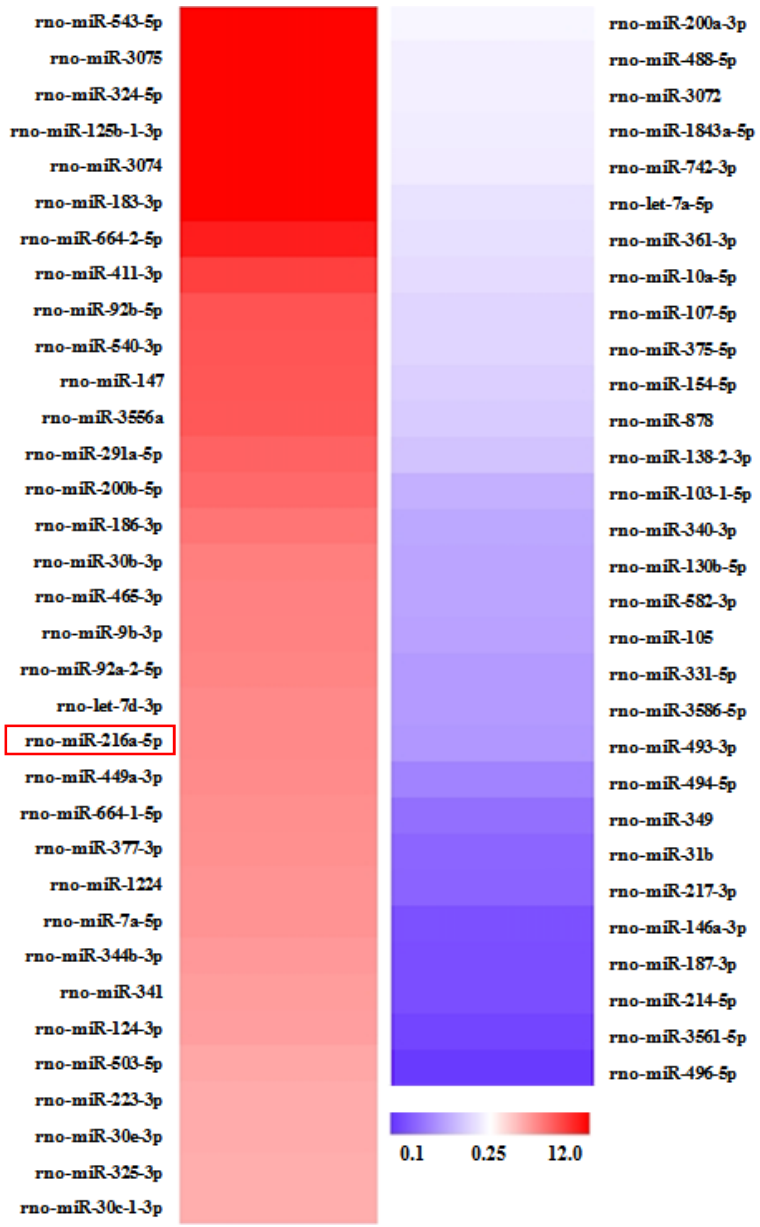

Figure 9. Significantly altered exosomal miRNAs. Significantly differently expressed miRNAs in RH exosome compared to those in L6 exosome and their altered signal intensities.

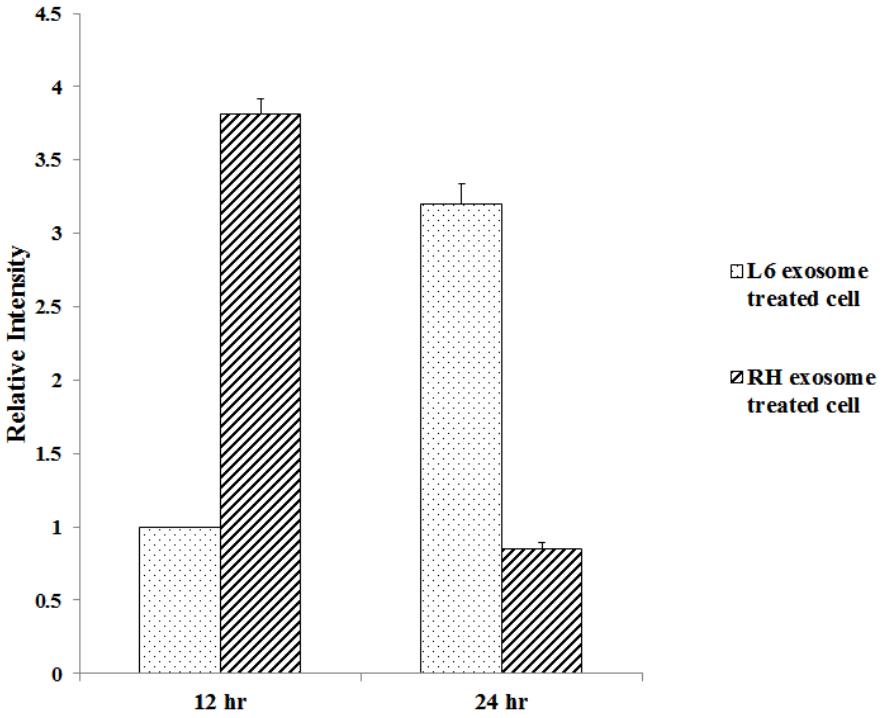

Figure 10. Temporal changes of *hmgb1* expression in RH and L6 exosome treated L6 cells.

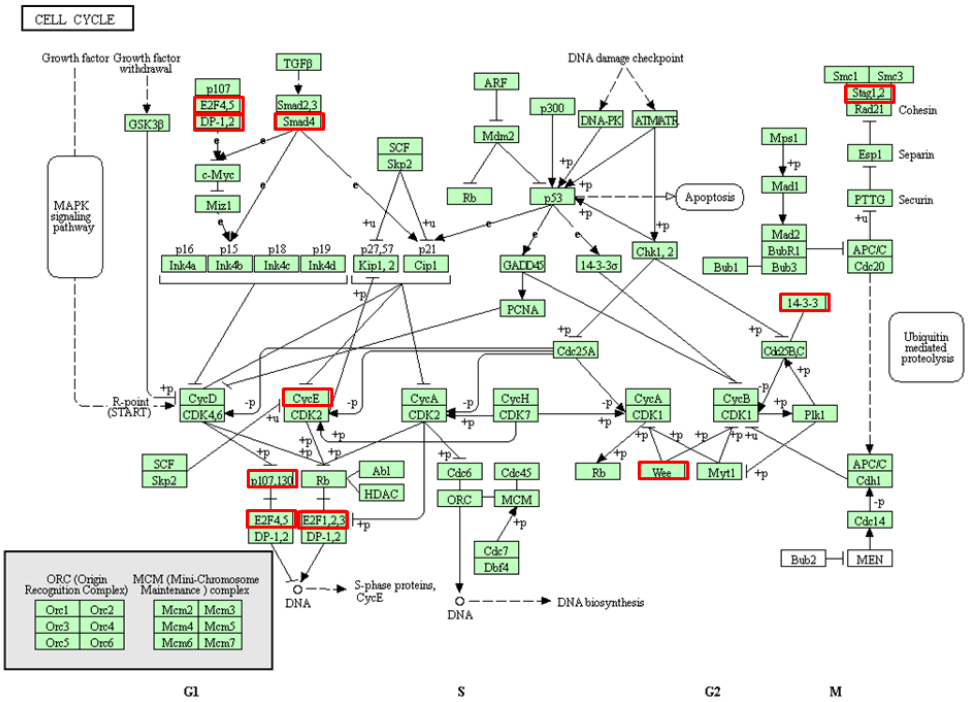

Figure 11. The possible cell cycle associated mRNAs regulated by miRNAs. The analysis and presentations were performed using web-based tool (http://david.ncifcrf.gov).

Table 1. Altered mRNAs associated with significantly differently expressed miRNAs

Changed Time Point	Expression	Gene Name	Related miRNA
12 hour	down-regulated	*Zfp780b*	rno-miR-223-3p
		Shank2	rno-miR-7a-5p
		Ly86	rno-miR-3075
		Mapk4	rno-miR-92a-2-5p
		RGD1359158	rno-miR-124-3p
		LOC685067	rno-miR-488-5p
	up-regulated	*Fbxo24*	rno-miR-3072
		Igsf11	rno-miR-146a-3p
		Rup2	rno-miR-214-5p
		Spib	rno-miR-361-3p
24 hour	down-regulated	*Armc9*	rno-miR-664-2-5p
		Asic4	rno-miR-92a-2-5p
		Folr2	rno-miR-325-3p
		Hmg111	rno-miR-200b-5p
		Hmgb1	rno-miR-216a-5p
		Tmcc3	rno-miR-146a-3p
		Ptgdrl	rno-miR-3556a
		Ptprn2	rno-miR-92a-2-5p
		LOC68516	rno-miR-92a-2-5p
	up-regulated	*Usp29*	rno-miR-214-5p
		B3gnt5	rno-miR-146a-3p
		Gcsam	rno-miR-214-5p
		Ildr2	rno-miR-124-3p
		Mgarp	rno-miR-200a-3p
		RGD15641	rno-miR-3586-5p

Table 2. The significantly differently expressed mRNAs with gene ontology term related with cell cycle or cell proliferation

Changed Time Point	Gene ontology	Gene Name
12 hour	Cell cycle	*Rprm*
		RGD1565862
	Cell proliferation	*Aldh1a2*
		Apoe
		Ccl24
		Cd40lg
		Fzd9
		Ltf
		Rarres1
		Rc3h1
		Reg1a
		RGD156122
		Rps9
		Tf
24 hour	Cell cycle	*Capn3*
		Epgn
		Hmgb1
		Ins2
		Olr1514
		Scimp
		Sox4
		LOC102550816
	Negative regulation of cell proliferation	*Mmp9*
		Pla2g2a

Changed Time Point	Gene ontology	Gene Name
24 hour	Negative regulatioin of cell proliferation	*Sox4*
	Positive regulation of stem cell proliferation	*Cxcl1*
		Hmgb1

DISCUSSION

We have shown that *T. gondii* infection of L6 cells resulted in decreased host cell proliferation and cell cycle alteration. L6 cells incubated with the exosomes from *T. gondii*-infected L6 cells also showed similar changes on cell cycle and proliferation, although transiently. To investigate whether miRNAs contained in the exosome could mediate these changes, microarray analysis was performed on the exosomal miRNAs and mRNAs of the exosome treated L6 cells. The functions of the mRNAs and the relation between miRNAs and mRNAs were elucidated by using the multiple web-based databases. As a result, we propose the miRNA, rno-miR-216a-5p, and its target mRNA of *Hmgb1* as the possible mediators of the cell cycle and cell proliferation alteration.

The cell cycle dysregulation phenomenon caused by the infectious organism is already well recognized. Many viruses alter the cell cycle of the infected host cells [34]. Among the protozoan parasites, *Theileria* species are famous for inducing

infected cells to proliferate [44]. Several studies already showed *T. gondii* could also change the host cell cycle [38]. For example, *T. gondii* infection induced the G1/S transition of the human trophoblasts and fibroblasts, followed by the G2 arrest. As a result, the host cell proliferation was suppressed, and host cells at the G2/M stage increased [35]. Another study showed that *T. gondii* infection caused human fibroblasts to enter S phase. This change appeared not only in directly invaded cells but also in neighboring cells. The filtered culture media from *T. gondii*-infected cell cultures caused the same changes, suggesting soluble factors mediate such parasite-host interactions [36]. These changes are regarded as beneficial for the parasite because *T. gondii* more easily invades cells in the S stage [37].

In our study, the proportion of cells in the S and G2/M phase was increased following *T. gondii* infection of the L6 cells. In addition, the host cell growth was suppressed compared to that of the L6 cells. The phase-contrast images of the *T. gondii*-infected cells showed continued intracellular replication of *T. gondii* at 24 hour and 48 hour post infection. The cell lysis was evident from 72 hour post infection and thereafter. Thus, the decreased cell growth in the early period till 48 hour probably

represents suppressed proliferation of directly invaded cells and neighboring cells rather than cell lysis. The declining cell number after 72 hour post infection is thought to be associated with cell lysis.

To verify our hypothesis that the exosomes can mediate these functional alterations in cell growth, we directly added the RH exosome and the L6 exosome in the exosome-depleted culture media and observed cell growth patterns and cell cycle distributions. The uninfected L6 cells grown with the RH tachyzoite exosomes exhibited similar growth patterns with the *T. gondii*-infected L6 cells. The cell numbers were significantly lower than that of the L6 cells grown in culture media only or with the L6 exosome. These differences in cell number were evident at 36 hour after the exosome treatment and disappeared at 48 hour. Contrast to these differences in cell number, the cell cycle status did not differ much among the L6 cells grown with RH exosome, L6 cell exosome, and culture media only. Only a minute increase of S phase and decrease of G2/M phase were observed in cells grown with RH exosome at 12 hour after addition of the exosome. However, the difference was soon weakened and disappeared at 48 hour after the

exosome treatment.

We could observe not only *T. gondii* infection itself but also the exosomes derived from the infected cells caused the same alterations in cell growth, i.e., suppression of proliferation and the tendency to stay in S phase. These changes were only transient. Alterations in the cell cycle appeared early, if any, and those in the cell proliferation appeared more lately as 36 hour after the exosome addition. Considering that exosomes act to negatively modulate the mRNA of the target genes, we postulated that the changes in the mRNA and protein profiles occur in very early period, and later the cell cycle mechanism changes and finally the cell number differed as a final phenotype. Considering that the doubling time of the L6 cells is about 22 hour [45], the changes which appeared at 36 hour can be the results of actions of exosomes in early period as before 12 hour.

In contrast to the effects of direct infection of *T. gondii*, the effects of exosome treatment was only transient. When the L6 cells were infected by *T. gondii*, probably the infected cells continuously secreted the RH exosome. In contrast, the uninfected L6 cells grown with RH exosome were exposed to the exosomes only in the early period. As cells continue to

proliferate, the initial environment with high quantities of RH exosomes would have been replaced by the exosomes secreted from the uninfected cells. This probably could explain the transient effects of the exosomes shown in this study.

Finally, we tried to find out whether the miRNAs capable of regulating cell cycle or cell proliferation are really contained in the RH exosome. The targets mRNAs of the exosomal miRNAs were predicted using web-based tools. Furthermore, the expression of mRNAs in the exosome-treated L6 cells was analyzed to confirm the expression of the predicted target mRNAs is really altered. Using two separate microarray analysis, a total of 701 exosomal miRNAs and 22259 cellular mRNAs were screened. Using two different approaches, only one miRNA-mRNA pair, rno-miR-216a-5p and Hmgb1, remained as biologically valid. The real-time PCR once more confirmed the changes of expression level in Hmgb1 in exosome-treated L6 cells.

HMG protein, high mobility group protein, was discovered in 1973 from the calf thymus chromatin as a nuclear protein [46]. Under contemporary nomenclature, HMG proteins are classified into 3 superfamilies, HMGA, HMGB and HMGN.

HMGB1 protein is the most highly expressed protein among all the HMG family proteins. HMGB1 is known to locate in the nucleus, cytosol and cellular membrane. HMGB1 is also secreted into and exists in the extracellular spaces. Nuclear HMGB1 is known to have DNA bending and binding activities and thus regulates nuclear functions. Extracellular HMGB1 is known to function as chemokine and cytokine and involved in many cellular processes such as cell differentiation, migration, proliferation and tissue regeneration [47].

The functions of HMGB1 protein in L6 cells can be estimated indirectly from other studies. One study reported that HMGB1 and its receptor RAGE (receptor for advanced glycation end products) stimulated L6 myoblast differentiation and inhibited its proliferation. When HMGB1/RAGE signal pathway was blocked, the L6 cell proliferation increased and apoptosis decreased [48]. *HMGB1* mRNA expression level was higher in RH exosome-treated cells than L6 exosome-treated cells at 12 hour and its level declined at 24 hour. We propose that this could be the underlying molecular events associated with transient decreased cell proliferation in RH exosome-treated cells. Further studies including other genes regulating cell cycle or proliferation would elucidate the significance of

altered HMGB1 level in RH exosome-treated L6 cells.

In the previous studies using the exsosomes derived from *T. gondii* infected cells, the immunological property of the exosomes were mainly investigated. Cells with immunologic function, such as dendritic cell or macrophage, were usually used in the experiment. The effect of exosome on inflammatory response and the immunological protection were usually investigated [21,23,24]. In contrast, we focused on the cell to cell communication as a function of exosome in *T.gondii* infection.

In conclusion, we identified that *T. gondii* infection alters the cell proliferation mechanisms of the L6 cells and that the exosomes secreted by *T. gondii*-infected cells can mediate such changes to neighboring cells. Microarray analyses of the exosomal miRNAs and cellular mRNAs of the exosome-treated cells suggested that miRNA-mRNA pair, rno-miR-216a-5p and *Hmgb1*, could be the possible mediators of the altered cell proliferation. Further studies are needed to confirm the actual

role of rno-miR-216a-5p and *Hmgb1* in regulation of the L6 myoblast cell cycle and proliferation.

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국문 초록

톡소포자충이 감염된 세포는 숙주 세포의 세포 주기와 세포 증식에 변화가 생긴다. 이런 변화는 톡소 포자충에 직접 감염된 세포뿐만 이 아니라 감염된 세포 주변의 다른 세포에서도 일어나는 것으로 알려져 있다. 그러한 변화를 매개하는 물질은 가열하면 그러한 작용을 잃어버리고 10 킬로 달톤(kDa)이상의 분자량을 가지는 것으로 알려져있으나 그 정확한 실체는 아직 확인되지 않은 상태이다. 이에 본 연구에서는 톡소포자충이 감염된 숙주 세포에서 분비된 엑소좀이 그러한 변화를 일으킬 수 있는지 확인하고자 하였다.

랫의 근육모세포인 L6 세포와 톡소포자충의 RH 주를 실험에 이용하였다. L6 세포를 톡소포자충에 감염시킨 그룹과 그렇지 않은 그룹으로 구분하여 톡소포자충이 감염되었을 때 L6 세포의 세포 증식과 세포 주기에 어떤 변화가 생기는지 확인하였다. 트리판 블루(trypan blue)로 염색된 세포를 공초점 현미경으로 관찰하여 세포 증식 패턴을 파악하고 유세포 분석을 통하여 세포 주기를 확인하였다. 톡소포자충에 감염된 L6 세포는 세포 증식이 저하되었고 세포 주기 중 S 주기 혹은 G2/M 주기에 있는 세포가 증가하였다.

이후 톡소포자충이 감염된 L6 세포와 감염되지 않은 L6 세포에서 각각 초원심분리기를 이용하여 엑소좀을 분리하였다. 감염되지 않은 L6 세포에서 나온 엑소좀 (L6 엑소좀)을 처치하였을 때에 비해 톡소 포자충이 감염된 L6 세포에서 나온 엑소좀 (RH 엑소좀)을 처치하였을 때, 12 시간 째 세포 주기에서 S 주기가 증가하고 36 시간째 세포 증식이 저하되는 것이 확인되었다. 이러한 변화는 일시적이었으며 48 시간 째에는 세포 주기와 세포 증식의 변화가 모두 소실되었다. L6 세포의 증식주기가 22 시간임을 고려하면, 엑소좀의 영향으로 12 시간 째 세포 주기의 변화가 일어나고 이런 변화가 시간이 지나 36 시간째에 세포 증식을 저하시키는 결과로 나타난 것으로 생각된다. 실험 결과가 일시적이었던 이유는 실험 초기에는 실험적으로 처치한 엑소좀이 많지만 시간이 갈수록 주변의 감염되지 않은 L6 세포 들이 분비하는 엑소좀으로 대치되면서 생기는 효과로 생각된다.

엑소좀 안에 포함된 마이크로 알엔에이(miRNA)들이 이런 변화를 유도할 수 있을지 확인하기 위하여 마이크로 어레이 분석을 시행하였다. L6 엑소좀과 RH 엑소좀에 포함된 마이크로 알엔에이와 엑소좀을 처치한 L6 세포에서 분리한 메신저 알엔에이(mRNA)를 이용하여 발현량이 유의하게 차이나는 마이크로 알엔에이와 메신저 알엔에이를 확인하였다. 이후 웹 기반의 데이터베이스 분석을 시행하여 마이크로 알엔에이와

메신저 알엔이이의 연관성을 확인하였고 확인된 메신저 알엔에이가 세포 주기나 세포 증식에 영향을 미치는지 확인하였다. 결과적으로 하나의 마이크로 알엔에이-메신저 알엔에이 조합, rno-miR-216a-5p and *Hmgb1*, 을 확인하였으며 이는 엑소좀이 숙주 세포의 세포 증식에 미치는 영향을 매개할 수 있을 것으로 생각된다.

결론적으로 본 논문은 톡소 포자충에 감염된 세포에서 분비된 엑소좀이 주변의 다른 세포에 영향을 미치고 S 주기에 있는 세포가 증가하고 세포 증식이 저하되는 것을 확인하였다. 또한 *Hmgb1* 유전자를 이러한 변화를 유도할 수 있는 후보 유전자로 제시하고자 한다

핵심어: 톡소포자충, L6 세포, 엑소좀, microRNA, 세포 주기, 세포 증식, Hmgb1, miR-216a-5p

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