Toxoplasma gondii Differentiation: Effects of Modifying Lactate Dehydrogenase and Argonaute Expression Patterns

Urszula Liwak
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Toxoplasma gondii Differentiation: Effects of Modifying Lactate Dehydrogenase and Argonaute Expression Patterns

by

Urszula Liwak

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2009

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ABSTRACT

Conversion between two growth stages, tachyzoites and bradyzoites, plays a key role in the pathogenesis of *Toxoplasma gondii*. Current studies aimed to elucidate the roles of lactate dehydrogenase (TgLDH) and argonaute (TgAgo) in relation to conversion. The over-expression of TgLDH1 or TgLDH2 did not change the overall enzymatic activity or increase growth of tachyzoites. However, a significant *in vitro* differentiation into bradyzoites was detected. These findings suggest that TgLDH1 and TgLDH2 have important physiological functions, in addition to being glycolytic enzymes and differentiation markers. Furthermore, altered TgAgo expression increased bradyzoite formation and interfered with the parasite’s ability to successfully perform double stranded induced gene silencing. The change in TgAgo expression also affected the levels of putative dicer proteins suggesting a direct correlation between TgAgo and an RNAi-like mechanism.
DEDICATION

I would like to dedicate this thesis to my loving parents
ACKNOWLEDGEMENTS

First of all, I would like to thank my research supervisor, Dr. Sirinart Ananvoranich, for all her guidance and support throughout the years. I appreciate all the time and effort she has spent with me and the motivation and confidence that she provided me with. I would also like to thank my committee members, Dr. Siyaram Pandey and Dr. Andrew Swan for their help and suggestions throughout my studies.

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<td>Ago</td>
<td>argonaute</td>
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<tr>
<td>AgoKO</td>
<td>argonaute knockout</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
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<tr>
<td>ChFP</td>
<td>cherry fluorescent protein</td>
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<tr>
<td>DHFR-TS</td>
<td>dihydrofolate reductase-thymidylate synthase</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dsRNA</td>
<td>double stranded RNA</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GFP</td>
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<td>HFF</td>
<td>human foreskin fibroblast</td>
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<td>HXGPRT</td>
<td>hypoxanthine-xanthine-guanine phosphoribosyltransferase</td>
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<td>LacZ</td>
<td>β-galactosidase</td>
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<td>lactate dehydrogenase</td>
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<td>Luc</td>
<td>luciferase</td>
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<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization – time of flight</td>
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<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MPA</td>
<td>mycophenolic acid</td>
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<td>messenger RNA</td>
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<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<td>PTGS</td>
<td>posttranscriptional gene silencing</td>
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<td>RdRP</td>
<td>RNA dependent RNA polymerase</td>
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<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<td>red fluorescent protein</td>
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<td>Rho</td>
<td>rhodamine</td>
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<td>RISC</td>
<td>RNA induced silencing complex</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
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<td>TCA</td>
<td>tricarboxylic acid</td>
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<tr>
<td>TUB</td>
<td>β-tubulin</td>
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<td>UPRT</td>
<td>uracil phosphoribosyltransferase</td>
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Chapter I

Introduction

1.1 Significance of Toxoplasma gondii and disease

Toxoplasma gondii is an intracellular protozoan parasite and a member of the Apicomplexa phylum, which is characterized by the presence of apical secretory organelles including micronemes, rhoptries, and dense granules (Dubey et al., 1998). T. gondii causes significant disease in a variety of mammals and birds and is known to infect approximately one third of the world’s population, which may lead to the development of toxoplasmosis.

T. gondii infections can be acquired either through ingestion of viable tissue cysts in under-cooked meat or through ingestion of water contaminated with oocysts from infected cat faeces (Frenkel, 1973; Dubey et al., 1970). Initial infection of individuals with a healthy immune system, are normally asymptomatic because the rapidly multiplying tachyzoite stage of the parasite can be cleared by the host immune response. When infection is acquired during pregnancy however, in utero toxoplasmosis can occur, resulting in harm to the fetus such as abortion, severe birth defects, or fetal death (Guerina et al., 1994). Tachyzoites can undergo a stage interconversion into the slowly multiplying, encysted bradyzoites, which can remain dormant for the lifespan of the host, causing chronic toxoplasmosis (Ferguson et al., 2002; Lindsay et al., 1991). Toxoplasmosis can prove fatal when infected patients become immuno-compromised, such as HIV/AIDS patients (Wong and Remington, 1993; Luft and Remington, 1992),
wherein the bradyzoites are released from the tissue cysts and differentiate back into tachyzoites giving rise to a recurrent infection and making this an opportunistic parasite. The ability of the parasite to interconvert between these two stages is thought to play a key role in the pathogenesis of the parasite. The process of interconversion in humans and other non-definitive hosts is poorly understood (Reiter-Owona et al., 2000). To date, no effective treatment for chronic toxoplasmosis has been developed. Therefore, studies of *T. gondii* in cell culture facilitate the search for possible drug targets and the development of preventative strategies and therapeutic agents for toxoplasmosis.

1.2 *Toxoplasma gondii* as an apicomplexan model

The apicomplexan parasites include many pathogenic protozoans, such as *Plasmodium* and *Cryptosporidium*, which cause a variety of diseases in humans. For instance, the *Plasmodium* parasite is known to infect human red blood cells causing malaria, a serious and possibly fatal disease (Cowman and Crabb, 2002), while *Cryptosporidium* causes a diarrhoeal illness called cryptosporidiosis that is also associated with immuno-compromised individuals (Abrahamsen et al., 2004). Studies of these parasites have proven challenging due to the difficulty of culturing and maintaining them in the laboratory. *T. gondii* however, is the most experimentally tractable parasite since it is easily propagated and cultured (Kim and Weiss, 2004). Thus, *T. gondii* has become a major model for the study of apicomplexans due to the ease of genetically manipulating the parasite for the study of basic biology and host response (Roos et al., 1994; Black and Boothroyd, 1998). The 80 Mb haploid genome of *T. gondii* consists of 14 chromosomes. Recent sequencing projects have provided a great amount of
information that has been deposited into the *T. gondii* database further enhancing research efforts (ToxoDB: http://www.toxodb.org; Kissinger *et al.*, 2003).

### 1.3 Genetic manipulations of *Toxoplasma gondii*

A variety of tools have been developed for genetically manipulating *T. gondii*, thus enhancing the study of gene functions and molecular events. In order to introduce DNA and RNA into *T. gondii*, electroporation has become a method of choice, in which an electric current is applied to the parasite creating pores in the membrane, allowing for the nucleic acids to be incorporated.

#### 1.3.1 Transient and stable transfections

Transient gene expression occurs when the transforming plasmid lacks a *T. gondii* origin of replication, or fails to be maintained in the genome resulting in gene expression that lasts for a short amount of time (Roos *et al.*, 1994). Reporter genes such as β-galactosidase (LacZ), chloramphenicol acetyltransferase (CAT), and luciferase (Luc) have been employed to indicate that the plasmid introduction was successful, with highest efficiency occurring when the expression is driven by *T. gondii* promoters such as those of surface antigens, rhoptry proteins, and β-tubulin (Roos *et al.*, 1994; Soldati and Boothroyd, 1995).

Stable transformation, on the other hand, results from either homologous or random integration of the transgenes, and leads to parasites that permanently express the selectable marker. Transformation vectors containing sequences from the dihydrofolate
reductase-thymidylate synthase gene (DHFR-TS) have been developed for random integration (Donald and Roos, 1994).

1.3.2 **Endogenous selectable markers for stable transfections**

There are a variety of selectable markers that can be used for generating stable transformants. Two important selectable markers that are highly utilized in the present studies include the hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and uracil phosphoribosyltransferase (UPRT) genes, which are part of the purine and pyrimidine pathways, respectively, and provide the substrates for DNA and RNA biosynthesis (Iltzsch, 1993; Chaudhary et al., 2004).

1.3.2.1 **Hypoxanthine-xanthine-guanine phosphoribosyltransferase**

Similar to many intracellular pathogens, *T. gondii* does not contain a *de novo* purine nucleotide biosynthesis pathway. Therefore, the parasite relies on the purine salvage pathway to provide purine nucleotides that are required for growth (Krug et al., 1989). Hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT, E.C. 2.4.2.8), an essential gene of the purine salvage pathway, catalyzes the conversion of hypoxanthine, xanthine, and guanine into inosine monophosphate (IMP), xanthine monophosphate (XMP), and guanine monophosphate (GMP), respectively (Fig. 1.1). In the absence of HXGPRT or xanthine, IMP dehydrogenase can synthesize XMP. When utilizing HXGPRT as a positive selectable marker; mycophenolic acid (MPA) and xanthine are added to the culture media. IMP dehydrogenase is inhibited by MPA, thus synthesis of XMP and GMP, which is required for parasite survival, is prevented.
However, parasites containing HXGPRT can utilize the added xanthine to synthesize XMP and survive. Therefore, parasites lacking the HXGPRT gene, RHΔHX, are electroporated with plasmids containing the HXGPRT gene and therefore, only parasites that incorporate and express HXGPRT can survive under MPA and xanthine selection. Furthermore, HXGPRT can also be utilized for negative selection. In this case, HXGPRT catalyzes the conversion of 6-thioxanthine (6-TX) in the media, into 6-thioxanthosine-5’-phosphate, which causes toxicity to the parasite, thus only parasites that lack the HXGPRT gene can survive (Chaudhary et al., 2004; Donald and Roos, 1993).
Figure 1.1 Purine salvage pathway of *Toxoplasma gondii*. The dominant enzymes of the purine salvage pathways are adenosine kinase (AK) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT). The other enzymes are numbered: (1) adenosine deaminase; (2) purine nucleoside phosphorylase; (3) adenine deaminase; (4) AMP deaminase; (5) IMP dehydrogenase; (6) GMP synthetase; (7) adenylosuccinate synthetase; (8) adenylosuccinate lyase. HC represents host cell cytoplasm; PV refers to the parasitophorous vacuole; PC refers to the protist cytoplasm. This image was modified from Chaudhary *et al.*, 2004.
1.3.2.2 Uracil phosphoribosyltransferase

Uracil phosphoribosyltransferase (UPRT, E.C. 2.4.2.19) is an enzyme responsible for the incorporation and conversion of uracil into uracil monophosphate (UMP) in the pyrimidine salvage pathway (Fig. 1.2) (Schwartzman and Pfefferkorn, 1981). Since T. gondii is capable of synthesizing pyrimidines de novo, UPRT is not an essential enzyme for the parasite. UPRT has the ability to recognize pyrimidines, as well as cytotoxic analogs of pyrimidines. Thus, UPRT can also incorporate and convert 5-fluorouracil into 5-fluorouridine which is further synthesized into 5-fluorouridine monophosphate (F-dUMP) which prevents the synthesis of thymidine monophosphate, resulting in lethality to the parasite (Iltzsch and Tankersley, 1994). Generation of parasites lacking the UPRT gene allows for survival, since the 5-fluorouracil cannot be incorporated and metabolized. Mammalian cells do not have the ability to take up uracil from the media, therefore studies involving UPRT can also be utilized for measuring the parasite’s ability to incorporate radioactively labelled uracil, [5,6-\textsuperscript{3}H]-uracil in order to measure viability (Pfefferkorn and Pfefferkorn, 1977) and gene regulation (Al-Anouti et al., 2003).
Figure 1.2 *De novo* pyrimidine biosynthesis and pyrimidine salvage pathways of *Toxoplasma gondii*. The synthesis of UMP, the precursor for pyrimidines, is shown where the UPRT enzyme (1) is part of the salvage pathway. This image was obtained from Fox and Bzik, 2002.
1.4 *Toxoplasma gondii* parasite types and host cells

Molecular analysis suggests that all currently identified strains of *Toxoplasma gondii* can be classified into three common clonal lineages; types I, II, and III (Howe and Sibley, 1995; Sibley and Boothroyd, 1992). Type I strains, such as RH and GT1, are highly virulent strains that cause 100% lethality in mice with a single infectious organism and have a limited ability to form cysts in culture and in animals. Also, Type I strains are fairly uncommon, where only a small group of congenital toxoplasmosis patients and opportunistic infections in AIDS patients have been identified. On the other hand, Type II strains, such as ME49 and PLK, and Type III strains, such as VEG, are less virulent in mice and cause lethality with more than 1000 parasites. Type II strains have been found to be the most prevalent strain in clinical isolates and may cause mild infection in healthy individuals but severe disease in AIDS patients (Khan *et al.*, 2009; Sibley, 2009). Furthermore, type I strains are commonly used in laboratories for *in vitro* studies due to their rapid replication rate and efficiency of lysing host cells, allowing for isolation of large amounts of tachyzoites.

The proliferative form of the parasite is the tachyzoite, which can be maintained in many mammalian cells. The infection ability of tachyzoites is greater in adhesive cell types rather than in suspension cultures, possibly due to the higher probability of contact between parasites and adherent monolayers as compared to suspended cells. Therefore, human foreskin fibroblasts (HFF) are typically used in routine cell culture. HFF cells are convenient due to their ease of passage and their morphology which consists of a large plasma membrane allowing for multiple cycles of replication of *T. gondii* to occur before lysis. Finally, HFF cells are fairly resistant to metabolic inhibitors due to their inability to
grow past confluence, thus allowing for drug selection of transfected parasites (Roos et al., 1994).

1.5 General cell biology of Toxoplasma gondii

1.5.1 Sexual cycle in the definitive host

Toxoplasma gondii has a complex life cycle involving two phases, the sexual cycle in the feline definitive host and the asexual cycle in any intermediate host, such as cattle and humans, which is summarized schematically in Figure 1.3. The sexual cycle occurs only in intestinal epithelial cells and begins with the feline ingesting tissue cysts from infected animals such as rats (Frenkel, 1973). The parasites are then released in the digestive system where they proceed through gametogenesis which consequently leads to the formation of a diploid zygote known as an oocyst. The oocysts are excreted in the feces, and upon exposure to oxygen and ambient temperature, sporogony occurs wherein two meiotic divisions and one mitotic division produce eight haploid sporozoites divided into two sporocysts which remain stable and infectious for months to a year in the soil, even in harsh climates (Frenkel, 1973).

1.5.2 Asexual cycle in intermediate hosts

Infectious oocysts in the soil or viable tissue cysts in undercooked meat provide an entry into humans and animals. Upon entry, the sporozoites or bradyzoites infect the intestinal epithelium and differentiate into tachyzoites, a process also known as interconversion. The tachyzoites travel throughout the body via the blood stream, causing initial infection by T. gondii which is typically asymptomatic or may cause mild flu-like
symptoms. The asexual cycle occurs within the host cell where the parasite multiplies by endodyogeny forming two daughter cells in each division, eventually causing the host cell to rupture and allowing the parasite to infect neighbouring cells (Sheffield and Melton, 1968; reviewed in Black and Boothroyd, 2000). After the host’s immune response is triggered, the tachyzoites convert into the slowly multiplying bradyzoites where they are protected within tissue cysts for the remainder of the host’s lifespan (Ferguson et al., 2002; Lindsay et al., 1991).
Figure 1.3  The life cycle of *Toxoplasma gondii*. The life cycle of *T. gondii* consists of a sexual cycle within intestinal cells of felines, and an asexual cycle that occurs in mammals, birds, and virtually any nucleated cell. Infection is often acquired through accidental ingestion of eliminated oocysts. Acute infection is represented by the quickly replicating tachyzoites, while chronic infection is representative of encysted bradyzoites that remain dormant for the lifespan of the host. This image was obtained from Dubey, 1986.
1.5.2.1 Tachyzoites

The asexual cycle of *T. gondii* consists of two different stages of the parasite including tachyzoites and bradyzoites, which differ in many aspects. Tachyzoites are the rapidly dividing form of the parasite which cause initial infection. They are often crescent shaped with an approximate length of 6 μm and width of 2 μm. Tachyzoites have the ability to infect almost any nucleated cell by attaching to the host cell membrane and directly penetrating the cell where they become encased in the parasitophorous vacuole (PV) composed of membranes from both the parasite and host cell (Dobrowolski and Sibley, 1996). The asexual cycle occurs within the host cell where the parasite multiplies by endodyogeny forming two daughter cells in each division (Sheffield and Melton, 1968). Tachyzoites continue to replicate until they eventually rupture the host cell and are released, where they can infect neighboring cells.

1.5.2.2 Bradyzoites

Tachyzoites differentiate into bradyzoites upon a response from the host’s immune system. Bradyzoites are the slowly multiplying form of the parasite that generate tissue cysts within the host, defining the chronic stage of infection. Bradyzoite tissue cysts are commonly found in the central nervous system as well as in the muscles ranging from a size of 50 μm to 70 μm containing 1000-2000 bradyzoites that are crescent shaped with a size of 7 μm by 1.5 μm (Dubey *et al.*, 1998; Fortier *et al.*, 1996; Sims *et al.*, 1989). The cyst wall, composed of β-(1,4) linked N-acetylglucosamine residues called chitin, a derivative of glucose, surrounds the bradyzoites and is responsible for protecting the parasite from the host’s immune response. This cyst wall is easily identified in culture by
fluorescently labeled lectins in *Dolichos biflorus* which bind the glycoprotein CST1 (Zhang *et al*., 2001; Boothroyd *et al*., 1997).

Ultrastructurally, bradyzoites differ from tachyzoites since bradyzoites have a posteriorly located nucleus, honeycombed rhoptries, and several amylopectin granules for glucose storage; whereas tachyzoites have a centrally located nucleus, electron dense rhoptries and very few amylopectin granules (Ferguson *et al*., 1987, 1989). Furthermore, bradyzoites are more slender than tachyzoites, and tachyzoites are more sensitive to destruction by proteolytic enzymes (Jacobs *et al*., 1960). The differences in structure and organelle location can be observed in Figure 1.4.
Figure 1.4  Schematic representation of *T. gondii* tachyzoites and bradyzoites. The asexual cycle consists of two life stages; the tachyzoites (left) and the bradyzoites (right), which differ both structurally and metabolically. Tachyzoites are quickly replicating while bradyzoites are the slower multiplying, encysted form found during chronic infection. This image was obtained from Dubey *et al.*, 1998.
1.5.2.3 Tachyzoite to bradyzoite interconversion

Interconversion between the two developmental stages is often associated with altered environmental conditions and is typically a factor in pathogenesis. The reversible process of interconversion between tachyzoites and bradyzoites plays a key role in the pathogenesis of *T. gondii* because chronic infection is characterized by the differentiation of tachyzoites into bradyzoites found in tissue cysts, and recurrence of infection is characterized by the reconversion of bradyzoites into tachyzoites in immunocompromised individuals (Dubey *et al*., 1998). The mechanisms behind the interconversion process are poorly understood.

There are many limitations in obtaining large amounts of encysted bradyzoites *in vivo*, therefore, mimicking the interconversion *in vitro* allows one to study the process in detail and gain a better understanding of the mechanisms involved. The stress exerted by the host’s immune response triggers the formation of bradyzoites, thus a variety of stress conditions have been applied to culture bradyzoites *in vitro*. The *in vitro* cysts have been shown to be similar to *in vivo* cysts isolated from brains of chronically infected mice in that they are both resistant to pepsin treatment (Popiel *et al*., 1996; Knoll and Boothroyd, 1998).

In order to initiate differentiation *in vitro*, that is, conversion from tachyzoites to bradyzoites, a variety of methods can be utilized that exert stress on the intracellular parasites. These methods include culturing the infected monolayers in the presence of basic (pH 8.0-8.2) media (Soete *et al*., 1994; Weiss *et al*., 1995; Weiss and Kim, 2000), interferon (IFN)-γ (Bohne *et al*., 1993), mitochondrial inhibitors (Bohne *et al*., 1994;
Tomavo and Boothroyd, 1995), a trisubstituted pyrrole molecule (compound 1) (Radke et al., 2006) and by increasing the levels of cyclic nucleotides (Kirkman et al., 2001).

The most common method used to initiate in vitro differentiation is to increase the pH of the culture media to a pH of 8.0-8.2 and culture under ambient CO₂, since bradyzoite formation is most consistent and frequent under this condition (Soete et al., 1994; Weiss et al., 1995). Alkaline culturing media can result in a direct effect on T. gondii, as well as an indirect effect from host cells under stress, which subsequently triggers stage conversion. The major benefit of using alkaline media is that the bradyzoites formed have the most defined bradyzoite phenotype that can be achieved in culture (Radke et al., 2006).

It has been observed that proinflammatory cytokines including interferon-gamma (IFN-γ) are required for resistance against T. gondii (Suzuki et al., 1988; Bohne et al., 1993). These cytokines are related to mechanisms involving production of nitric oxide (NO) and tryptophan starvation, which may lead to a limitation in parasite replication, thus leading to formation of bradyzoites. Production of nitric oxide in T. gondii is associated with its ability to react with the iron-sulfur centers present in proteins that are part of the electron transport chain in aerobic respiration, thus halting the process and leading to differentiation. Furthermore, sodium nitroprusside (SNP), a nitric oxide donor, has also been shown to induce bradyzoite formation (Bohne et al., 1994).

Other drugs and chemicals have shown ability to initiate bradyzoite formation in vitro. In particular, inhibitors of mitochondrial function such as antimycin A (inhibitor of the electron transport chain), myxothiazol (inhibitor of coenzyme Q-cytochrome c reductase in the electron transport chain), and oligomycin (inhibitor of mitochondrial
ATP synthetase function) result in a reduction in growth and formation of bradyzoites (Bohne et al., 1993, 1994; Soete et al., 1993, 1994).

Nutrient deprivation has also been utilized in differentiation studies. *T. gondii* lacks the enzymes required for *de novo* arginine biosynthesis, thus it requires arginine from the host cell. Consequently, arginine starvation has shown to lead to bradyzoite formation. Finally, increasing cyclic nucleotides can also result in differentiation *in vitro*. It has been determined that increasing the levels of cyclic GMP (cGMP) and cyclic AMP (cAMP) leads to differentiation. This is conjunction with using the NO donor sodium nitroprusside, since this also triggers increased levels of cAMP, and in turn leads to differentiation (Kirkman et al., 2001). Therefore, using the above mentioned methods, it is possible to study the differentiation process *in vitro* to gain a better understanding of the mechanisms involved.

1.6 Energy production and consumption in *Toxoplasma gondii*

Intracellular parasites of the Apicomplexa phylum, including *Toxoplasma gondii*, rely on eukaryotic host cells for survival. The exclusive replication within host cells suggests that the metabolism of the host is important for parasite survival and that parasites have evolved metabolic pathways that reflect their intracellular nature. Thus, studies of these pathways are important for determining possible targets for drug development (Kim and Weiss, 2007).

*Toxoplasma gondii* not only changes its rate of multiplication and morphology between the tachyzoites and bradyzoites, but it also changes its metabolism upon changes in environmental conditions. Tachyzoites rely on both aerobic and anaerobic respiration, whereas bradyzoites rely heavily on anaerobic respiration. As previously noted, certain
mitochondrial inhibitors such as antimycin A, myxothiazol, and oligomycin result in an increase in expression of antigens specific to the bradyzoite stage which further suggests a relation between mitochondrial function and stage conversion (Bohne et al., 1993, 1994; Soete et al., 1993, 1994). Also, some enzymes of the tricarboxylic acid (TCA) cycle are not present in bradyzoite parasites (Denton et al., 1996), providing further evidence that mitochondrial oxidative phosphorylation is likely impaired in bradyzoites and that bradyzoites rely heavily on glycolysis for energy production (Fig. 1.5). Moreover, interference with the electron transport chain occurs when activated macrophages produce nitric oxide leading to an increase bradyzoite formation (Bohne et al., 1994) which further suggests that the host may attempt to remove the parasite by interfering with the energy generating pathways in order to control proliferation. In order to compensate, the parasite converts into the slow-replicating, less energy-consuming, encysted form, thus increasing its viability. Furthermore, *T. gondii* bradyzoites contain amylopectin, a polymer of glucose, granules that are not present in high amounts in tachyzoites, providing energy storage for the encysted and dormant bradyzoites. It has been suggested that amylopectin granules provide energy for the conversion back into tachyzoites by the degradation into glucose which provides metabolic substrates and intermediates for either glycolysis or mitochondrial oxidative phosphorylation (Kim and Weiss, 2007).

Although tachyzoites and bradyzoites have equally functional glycolytic pathways (Denton et al., 1996), a unique set of glycolytic enzymes are responsible for the energy production of each differentiation stage (Fleige et al., 2007; Tomavo, 2001). With the exception of hexokinase and glucose-6-phosphate isomerase, each glycolytic enzyme is coded by at least two independent genes whose mRNAs are differentially regulated
It has been shown that three isoenzymes of glycolysis, lactate dehydrogenase, glucose 6-phosphate isomerase, and enolase, are stage-specifically expressed in *T. gondii* (Yang and Parmley, 1997; Dzierszinski *et al*., 1999).

Since glycolysis is the predominant energy-generating pathway in bradyzoites, the stage-specific expression of these enzymes suggests that they contain features that are not available with the tachyzoite isoenzymes. It is also likely that the isoenzymes are required for the adaptation of the parasite to differing environments and there may be a relationship between energy production and stage conversion.
Figure 1.5  The metabolism of *Toxoplasma gondii*. Lactate dehydrogenase is shown as the last committed enzyme of glycolysis in *T. gondii*. This schematic also demonstrates the relationship between glycolysis and amylopectin granules. This image was obtained from Weiss and Kim, 2007.
Toxoplasma gondii lactate dehydrogenase

*T. gondii* contains two isoforms of lactate dehydrogenase (TgLDH1 and TgLDH2, L-lactate:NAD\(^+\) oxidoreductase, EC 1.1.1.27), the last committed enzyme of the glycolytic pathway (Fig. 1.5). The expression of genes encoding TgLDH1 and TgLDH2 are differentially regulated and commonly used as markers of the differentiation stage: TgLDH1 is the marker of the tachyzoite stage, while TgLDH2 is that of the bradyzoite stage (Yang and Parmley, 1997). Both TgLDH1 and TgLDH2 catalyze the interconversion of pyruvate to lactate and have a preference for the co-enzyme nicotinamide adenine dinucleotide (NAD\(^+\)) (Kavanagh *et al.*, 2004).

The mRNA of TgLDH1 has been reported to be present in both the tachyzoite and bradyzoite stages, however the protein product is only found in tachyzoites, suggesting that the expression of TgLDH1 may occur at the post-transcriptional or post-translational level. The TgLDH2 mRNA and protein product, on the other hand, are only present in bradyzoites, suggesting that the expression is controlled at the transcriptional level. TgLDH1 and TgLDH2 have 64% nucleotide sequence identity and 71.4% amino acid sequence identity, with predicted molecular masses of 35,550 and 35,342 Da, respectively, and predicted isoelectric points of 5.96 and 7.08, respectively (Yang and Parmley, 1997). As compared to LDH proteins in other apicomplexan parasites, *T. gondii* LDH1 and LDH2 share 46.5% and 48.5% amino acid identity with the LDH from *Plasmodium falciparum*, respectively (Bzik *et al.*, 1993). Structural and biochemical analyses showed that the active sites of TgLDH1 and TgLDH2 carry a pentapeptide insertion (Kavanagh *et al.*, 2004; Bzik *et al.*, 1993), which is similar to the LDH in *P. falciparum* and is lacking in the human LDH isoenzyme. *T. gondii* LDH2 and *P. falciparum* LDH have the same insertion (KSDKE) whereas the insertion differs in
TgLDH1 (KPDSE) (Yang and Parmley, 1997). This pentapeptide insertion makes TgLDH1 and TgLDH2 ideal targets for the development of anti-parasitic agents to combat both acute and recurrent toxoplasmosis. By binding to this pentapeptide insertion, small molecules/ligands would interfere with the activity of TgLDH1 and TgLDH2. More importantly, the inhibition of TgLDH2 might confer a highly potent anti-parasitic effect because the encysted and slow-replicating bradyzoites lack a functional TCA cycle and respiratory chain (Denton et al., 1996), and would consequently depend greatly on the activity of TgLDH2 for energy production under anaerobic respiration. Moreover, previous work showed that the physiological level of TgLDH1 and TgLDH2 expression is required for the parasites to maintain their normal growth and to allow for differentiation (Al-Anouti et al., 2004). This evidence thus indicates that the activity along with the temporal regulation of TgLDH1 and TgLDH2 expression could be used as potential drug targets.

### 1.8 RNA biochemistry and gene regulation

Posttranscriptional gene silencing (PTGS), or RNA interference (RNAi), is an evolutionary conserved mechanism wherein RNA plays a role in sequence specific degradation of mRNA. It is thought that the native role of RNAi was to inhibit retrotransposon movement, to silence repeated DNA sequences, and to defend against viral infection (Mello and Conte, 2004). Today, RNAi has become an important tool for regulating gene expression and has potential for being a therapeutic technique for the treatment of infectious diseases and cancers.
1.8.1 Discovery of RNA interference

RNA induced gene silencing is a novel gene regulatory mechanism that may either suppress transcription or cause sequence-specific degradation; a process known as posttranscriptional gene silencing or RNA interference. Initially, PTGS was discovered in plants, but was quickly followed by a similar discovery in a variety of eukaryotes including protozoa, nematodes, insects, as well as mouse and human cell lines. The initial discovery in plants occurred by a search for transgenic petunia flowers that had a deep purple color after over-expressing chalcone synthase, an enzyme required for production of anthocyanin pigments. Unexpectedly, the transgenic petunia plants lost both the endogenous and transgene activity, and the phenomenon was coined cosuppression (Van Blokland et al., 1994; van der Krol et al., 1990; Napoli et al., 1990). It was later discovered that introduction of sense and antisense transgenes could initiate PTGS in plants and other eukaryotes (Francesco et al., 2001).

The discovery of PTGS in plants led to studies in other eukaryotic organisms where the ability of double stranded RNA (dsRNA) to induce gene silencing in the nematode Caenorhabditis elegans was demonstrated (Fire et al., 1998). In nematodes, the unc22 gene is responsible for a myofilament protein that, when present in lowered amounts, causes a twitching phenotype. Researchers injected the nematode with dsRNA corresponding to the unc22 gene and observed the twitching phenotype that increased in the progeny (Fire et al., 1998). These findings paved the way for discoveries of RNA interference in many eukaryotic organisms, as well as for the discovery of the proteins involved in the pathway, and most importantly, the use of RNAi to easily create knockdown mutants.
1.8.2 General mechanism of an RNA interference pathway

Elucidating the mechanism of RNA interference began with genetic screens of *Neurospora crassa*, *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* to search for mutants defective in RNAi. This led to the discovery of many essential proteins to the pathway indicated as initiators, effectors, and amplifiers.

The process of RNAi begins with the cleavage of long double stranded RNA into small interfering RNAs (siRNAs) consisting of 21-25 nucleotides. Identification in *Drosophila* of an enzyme that contains an RNaseIII domain which was known to have specificity for dsRNA led to the discovery of dicer (Bernstein et al., 2001). Dicer cleaves dsRNAs into short uniformly sized siRNAs which contain 3’ overhangs of 2 to 3 nucleotides and 5’-phosphate and 3’-hydroxyl termini (Elbashir et al., 2001), which is termed the initiating step. Dicer enzymes consist of four domains including a helicase domain, RNaseIII domain, dsRNA binding domain, and a PAZ domain. The RNaseIII domain is responsible for the cleavage action of dicer. The next step is the effector step, where the siRNAs bind an RNA-induced silencing complex (RISC), which undergoes activation by ATP exposing the antisense strand of the siRNA, allowing it to identify and cleave mRNA molecules at 10-11 nt from the 3’ terminus of the guide RNA (Elbashir et al., 2001) leading to mRNA degradation (Hammond et al., 2000), translational inhibition (Pillai et al., 2005), or heterochromatin formation (Verdel et al., 2004). The main protein part of the RISC complex that is responsible for the binding to and cleaving of homologous mRNA is the argonaute protein, consisting of a PIWI and a PAZ domain (Hammond et al., 2000). Therefore, the identification of argonaute in an organism implies that a mechanism similar to RNAi occurs in the organism. Finally, mutation studies in *Drosophila* of an RNA-dependent RNA polymerase (RdRP) protein showed
that RNAi activity was affected, leading to evidence that this protein plays a role in amplifying the RNAi signal by generating dsRNA through utilization of the siRNA as primer molecules (Lipardi et al., 2001; Sijen et al., 2001).

Along with siRNAs, microRNAs have been identified in many organisms such as D. melanogaster, C. elegans, plants, and humans, and function in RNAi pathways responsible for preventing mobile transposable elements from disrupting the genomes, as well as having important roles in development. microRNAs are typically generated from single stem-loop precursor structures (Hutvágner et al., 2001). Both microRNAs and siRNAs are processed by dicer proteins; however siRNAs bind the RISC complex, whereas microRNAs bind other microribonucleoprotein complexes, both of which contain a member of the PIWI/PAZ family (Fig. 1.5).
Figure 1.6  Pathways of post-transcriptional gene regulation. The fates of siRNA derived from antisense transcription, viral infection, and transfection, and miRNA from a primary mRNA transcript are shown, which result in either mRNA cleavage or translational inhibition. This image was obtained from Filipowicz et al., 2005.
1.8.3 Argonaute family of proteins

An important core component of the RISC complex has been identified as an argonaute protein that is responsible for the cleavage of mRNA transcripts guided by siRNAs (Song et al., 2004; Liu et al., 2004). Argonaute proteins consist of two structural domains: a central PAZ domain and a C-terminal PIWI domain, where the PAZ domain binds siRNA and the PIWI domain cleaves mRNA. Structural studies of argonaute from *Pyrococcus furiosus* indicated similarities between the PIWI domain and the RNase-H family of ribonucleases providing further evidence that argonaute is responsible for cleavage (Song et al., 2004).

Along with functions in RNAi, argonaute proteins have also been shown to function in developmental processes of many organisms (Carmell et al., 2002; Jaronczyk et al., 2005). Studies in *Drosophila* and *C. elegans* of reduced expression of argonaute resulted in defective germlines leading to speculation that there are evolutionarily conserved roles in germline stem cell maintenance (Cox et al., 2000; Tijsterman et al., 2002).

Studies of the role of argonaute in *Trypanosoma brucei* has suggested that protozoans contain an RNAi pathway that resembles that of multi-cellular eukaryotic organisms (Ullu et al., 2004). The *T. brucei* genome contains two argonaute genes, TbAgo1 and TbPiwi, where only TbAgo1 functions in RNAi (Durand-Dubief and Bastin, 2003). Further studies on TbAgo1 determined that it is involved in controlling siRNA and retrotransposon transcript levels, as well as having a role in chromosome segregation and mitotic spindle assembly (Shi et al., 2004; Durand-Dubief and Bastin, 2003).

Searching genomic databases has identified both dicer and argonaute homologs in other protozoans. Specifically, *Toxoplasma gondii* argonaute (TgAgo) has recently been
identified. It was found that TgAgo is a 58 kDa protein containing a conserved PIWI domain, and a non-conserved PAZ domain (Fig. 1.6), that participates in double stranded induced gene silencing in *T. gondii* (Al Riyahi *et al.*, 2006). However, TgAgo’s role in either an RNA interference pathway or in other cellular pathways is still unknown. Therefore, further studies are required to elucidate the role of argonaute in *T. gondii*. 

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Figure 1.7  **Alignment of Toxoplasma gondii argonaute.** Alignments of structural domains of *T. gondii* argonaute protein (TgAgo) with that of: *Arabidopsis thaliana* argonaute 1 (AtAgo1, accession number: U91995), *Homo sapiens* argonaute 2(EIF2C2 (HsAgo2, accession number: NP_036286), *Trypanosoma brucei* argonaute 1 (TbAgo1, accession number: AAR10810), and *Archaeoglobus fulgidus* Piwi protein (AfPiwi, PDB ID: 1W9H). The domain locations and sizes are estimated and solid lines indicate amino acid sequences with no recognized domains. This image was obtained from Al Riyahi *et al.*, 2006.
1.9 Objectives

I. The current studies aimed to elucidate the role of the glycolytic enzyme lactate dehydrogenase (TgLDH) within the differentiation process. To do so, transgenic parasites over-expressing either TgLDH1 or TgLDH2 were generated. Phenotypical analyses were conducted to measure changes in growth under tachyzoite and bradyzoite conditions, overall TgLDH enzymatic activity, and differentiation ability to determine if over-expression of TgLDH’s affected any of the above process.

II. It was of interest to study if argonaute (TgAgo) played a role in differentiation. A fusion between TgAgo and a cherry fluorescent protein was generated and over-expressed in *T. gondii*. The differentiation ability of this strain was compared to that of a parental strain and a TgAgo knockout strain.

III. The relationship between TgAgo and a possible gene silencing pathway of *T. gondii* was studied. The transgenic parasites containing altered expression of TgAgo were tested in ability to perform double stranded induced gene silencing, and as well, the expression of putative dicer proteins was monitored upon alterations in TgAgo expression.
Chapter II

Materials and Methods

2.1 Plasmid design and constructs

2.1.1 Construction of pTUB8Myc_His_TgLDH

The coding sequences of TgLDH1 and TgLDH2 (GenBank accession nos. U35118 and U23207) were amplified from pMAL_LDH1 and pMAL_LDH2 (plasmids obtained from Dr. S. Parmley, Palo Alto Medical Foundation) using oligonucleotide primers, 5'LDHxNsiI: GATTTCA GAATTCGGATCC and 3'LDHxPacI: CCCTTAATTAAGTGCCAAGCTTAAGATC to generate PCR products with NsiI on their 5'-terminus and PacI on their 3'-terminus. The PCR products were subsequently cloned into the Toxoplasma-expression plasmid, pTUB8Myc_His_X-HX (gift from Dr. D. Soldati, University of Geneva), so that the expression of TgLDH1 or TgLDH2 was under the control of a modified tubulin (TUB) promoter and the recombinant proteins were led by Myc- and His-epitope tags on their N-terminus to give ~39 kDa and ~40 kDa protein products, respectively. The constructs were analyzed by restriction endonucleases and confirmed by nucleotide sequencing reactions.
2.1.2 Construction of pTUB8_TgAgo_ChFP plasmid

The open reading frame of TgAgo (GenBank accession no. DQ196314) was PCR amplified using the oligonucleotide primers Nhe1on5TgAgo: AAAGCTAGCAAATGATCATGCCTCCCCGAAATCG and Bgl2on3TgAgo: AAAAGATCTACAGAACACCATCGGGGTC to generate a fragment with NheI on the 5’ terminus and BglII on the 3’ terminus. The fragment was cloned into a BlueScript pkS plasmid (Strategene) for blue/white screening, and the correct plasmid was confirmed by restriction digestion using XhoI and PvuII. The TgAgo coding sequence was obtained by digestion with NheI and BglII and was further cloned into the Toxoplasma-expression plasmid, pTub8_ChFP (Dr. John Murray, University of Pennsylvania), and verified using restriction digestion analysis and DNA sequencing. The expression of TgAgo, fused to a cherry fluorescent protein on the C-terminal, was under the control of a modified TUB promoter producing a protein product of ~85 kDa.

2.2 General molecular biological techniques for plasmid generation

2.2.1 Transformation of Top10 Escherichia coli cells

General molecular biological techniques were used for the transformation of E. coli cells, isolation of plasmid DNA, and restriction digestions of plasmids for confirmation of constructs. Briefly, all plasmids were transformed into Top10 E. coli subcloning-grade competent cells by the heat shock method. Competent cells were incubated with plasmid DNA on ice for 20 min, followed by heat pulsing at 42°C for 45 s, and incubating on ice for another 2 min. Next, 400 µL of pre-heated Luria-Burtani (LB) medium (10 g/L tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) was added, and the reaction was incubated for 30 min at 37°C with agitation. The cells were then plated on
an LB agar plate with the appropriate antibiotic (10 g/L tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 15 g/L agar, 1mg/L antibiotic) and grown at 37°C overnight.

2.2.2 Plasmid isolation by minipreps

Plasmids were isolated by growing a bacterial colony in 3 mL of LB broth supplemented with antibiotic at 37°C overnight with agitation. Half of the culture was centrifuged at 12,000 rpm for 1 min and the pellet was resuspended in 100 µL of 25mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 µL of 0.2N NaOH, and 1% SDS. Furthermore, 150 µL of 3 M NaOAc (pH 5.2) was added, the sample was mixed by inversion, and centrifuged at 12,000 rpm for 15 min. The clear supernatant was combined with 400 µL of TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 30 s, and centrifuged at 12,000 rpm for 5 min at room temperature. The plasmid DNA was precipitated with 800 µL of 95% ethanol and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed with 75% ethanol, air dried, resuspended in 30 µL of TE buffer containing 20 µg/ml RNase A and incubated at 37°C for 15 min.

2.2.3 Restriction digestion

Plasmids were digested with the appropriate restriction enzymes for a minimum of 4 h at 37°C. The fragments were visualized on 1% (w/v) agarose gels (0.4 g agarose in 40 mL of 1X TAE buffer, and 0.02% (w/v) ethidium bromide). The samples were mixed with gel loading buffer (6x: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in water) and the gel was electrophoresed for 30 min at 130 V in the Miniature Horizontal Gel System MLB-06 (Tyler Research Instruments) with 1X
TAE buffer. The gel was visualized and imaged using the Alphaimager Imaging System with AlphaEase software.

2.3 Parasite cultures

Human foreskin fibroblasts (HFF) and the laboratory strains, RH and RHΔHX, which are type I strains of *T. gondii*, were obtained from National Institutes of Health AIDS Research and Reference Reagent Program. HFF cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% cosmic calf serum (Hyclone) and antibiotic-antimycotic (Invitrogen) and used for *T. gondii* infection. The infected HFF monolayers were maintained using Minimum Essential Medium (MEM) (Invitrogen) supplemented with 1% dialyzed calf serum (Hyclone) and antibiotic-antimycotic. The RHΔHX parasite strain was chosen because of the ability to generate transgenic parasites using hypoxanthine-xanthine-guanine phosphoribosyltransferase as a selectable marker (Donald *et al.*, 1996). Using a previously described method (Roos *et al.*, 1994), transgenic parasites were generated by electroporation of RHΔHX with plasmids containing HXGPRT and selected and maintained in the culture media containing 25 µg/ml mycophenolic acid and 50 µg/ml xanthine. RH parasites were electroporated with plasmids containing chloramphenicol acetyltransferase (CAT) as a selectable marker and maintained in media containing 20 µM chloramphenicol. Freshly released parasites were subcultured every few days until further needed.

2.4 Transfection of *Toxoplasma gondii* by electroporation

Freshly lysed parasites were centrifuged at 1,500 rpm for 15 min and the pellet was resuspended in 400 µL electroporation buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM
K$_2$HPO$_4$/KH$_2$PO$_4$ (pH 7.6), 2 mM EDTA, 5 mM MgCl$_2$, 2 mM ATP, 5 mM glutathione). 10 µg of plasmid DNA was added in 4 mm electroporation cuvettes and pulsed at a voltage of 1,500 V, resistance of 25 Ω, and a capacitance of 25 µF using the BTX model 600 Electro Cell Manipulator (Genetronics). The parasites were then added to a confluent plate of HFF cells after a 15 min incubation period, and grown for 24 h followed by addition of the appropriate selection.

### 2.5 Isolation of genomic DNA

Genomic DNA was isolated using the DNAzol Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, parasite pellets were resuspended in 500 µL of DNAzol Reagent and lysed by gentle pipetting, followed by centrifugation for 10 min at 10,000 rpm at room temperature, and the viscous supernatant was transferred to a fresh tube. The DNA was precipitated by the addition of 250 µL of 100% ethanol, mixing by inversion, and storing at room temperature for 3 min, followed by centrifugation at 4,000 rpm for 2 min at room temperature to pellet the DNA. The DNA precipitate was washed twice with 800 µL of 75% ethanol, air dried for 15 s, and resuspended in 200 µL of 8 mM NaOH and 32 µL of 0.1 M HEPES.

### 2.6 Genotyping of *Toxoplasma gondii*

Isolated genomic DNA was subject to amplification by PCR using the primers specific to GRA6 and SAG2; GRA6-F1: TTTCCGAGCAGGTGACCT and GRA6-R1x: TCGCCGAAGAGTTGACATAG and SAG2.F2: ATTCTCATGCCTCCGCTTC and SAG2.R: AACGTTTCACGAAGGCACAC. The PCR reaction consisted of 25 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s,
with a final extension at 72°C for 5 min. PCR products were digested with \textit{MseI} and \textit{HhaI} for GRA6 and SAG2, respectively, and visualized on a polyacrylamide gel (4.8 mL 30% acrylamide, 4.8 mL H$_2$O, 2.4 mL 5X tris-borate EDTA (TBE)), where TBE is 54 g/L Tris base, 27.5 g/L boric acid, 20 mL/L of 0.5 M EDTA, (pH 8.0).

2.7 Lactate dehydrogenase enzymatic activity assay

Enzymatic assays were performed as previously described (Al-Anouti \textit{et al}., 2004; Dando \textit{et al}., 2001; Kavanagh \textit{et al}., 2004). Briefly, freshly released tachyzoites were harvested and lysed in 100 mM Tris (pH 7.4) and 0.5 mM PMSF protease inhibitor. Protein concentrations were determined using the Bradford reagent (BioRad Laboratories, Inc.). Reactions were carried out at 25°C in a reaction buffer containing 100 mM Tris (pH 7.4), 0.1 mg/ml NADH (Sigma-Aldrich), and 5 µg of cell-free lysates. Reactions were initiated by the addition of 1.6 mM pyruvate (Sigma-Aldrich) and monitored at 340 nm for the change in absorbance of NADH ($\varepsilon = 6220$ M$^{-1}$cm$^{-1}$). The assays were performed in triplicate, and average values were presented with their standard deviations.

2.8 Production of double stranded RNA

T7 promoter sites were incorporated into either side of the UPRT gene using the primers T7AntisenseUP: TAATACGACTCTATAGGGTTCCAAAGTACCGGT and T7promoterGG: TAATACGACTCATATAGG. The PCR reaction consisted of 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min 30 s, with a final extension at 72°C for 5 min. The PCR template was then subjected to an \textit{in vitro} transcription reaction (200 u of T7 polymerase, 2.5 mM rNTPs, 50 u Murine
RNase inhibitor, 10 mM DTT, 1/100 dilution pyrophosphatase) for 4 h at 37°C. The reaction was quenched with 2 u RQ1 RNase-Free DNase (Promega Corp.) for 15 min at 37°C. The sample, visualized on a 1% agarose gel, was subjected to phenol:chloroform precipitation, and quantified by spectrophotometry (Genesys 10uv Thermo Electron Corporation) by measuring absorbance at 260 nm and 280 nm.

2.9 RNA interference assay

Electroporation was used to introduce dsRNA corresponding to UPRT into parasites which were then infected onto HFF cells grown in a 24-well plate, and allowed to grow for 24 h. The media was replaced with appropriate selection media, and 1 µCi of \(^{1}\)H\(^{3}\) Uracil was added to each well. HFF cells with only electroporation buffer and no parasites were used as a control. After incubation at 37°C for another 4 h, the plate was chilled at -20°C for 2 min, followed by addition of 1 mL of 0.6 M trichloroacetic acid (TCA) to the existing media. The monolayer was fixed for at least 1 h at 4°C. The wells were washed repeatedly for approximately 4 h with ddH\(_2\)O and gentle agitation. Scintillation counting was performed on each wash sample containing 250 µL of sample and 3 mL of scintillation fluid. Once wash readings were approximately 50-300 cpm, the ddH\(_2\)O was replaced with 0.5 mL of 0.1 N NaOH and incubated for 1 h at 37°C. Scintillation counting was performed on 250 µL of each sample with 3 mL of scintillation fluid.

2.10 Antibodies

The rabbit anti-red fluorescence protein (RFP) antibody was obtained from Biovision (Mountain View, CA). The mouse anti-Myc antibody (9E10) and the mouse
anti-Tub (12G10) antibodies were developed by J. Michael Bishop, and Joseph Frankel and E. Marlo Nelsen, respectively, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Iowa City, IA). The rabbit anti-LDH1 and rabbit anti-LDH2 antibodies were obtained from Dr. Stephen Parmley (Palo Alto Medical Foundation). The rabbit anti-RNaseIII antibody was produced using a peptide derived from the N-terminal of the signature domain of an RNaseIII domain and obtained from ProSci Incorporated (Poway, CA). The rabbit anti-Bag1 antibody was obtained from Dr. Louis Weiss. Horseradish peroxidase-conjugated goat anti-mouse and horseradish peroxidase-conjugated goat anti-rabbit were purchased from Rockland (Gilbertsville, PA). Rhodamine-conjugated sheep anti-mouse was purchased from Rockland (Gilbertsville, PA). Finally, FITC-conjugated *Dolichos biflorus* agglutinin was obtained from Sigma-Aldrich.

### 2.11 Growth assays and immunofluorescence assays

To monitor the tachyzoite growth, the parasites were infected onto HFF monolayers grown on coverslips and kept under 37°C and 5% CO₂ for 24 and 48 hours (Al-Anouti *et al.*, 2004). To induce stage conversion and measure bradyzoite growth, the infected monolayers were grown under atmospheric CO₂ and in RMPI-1640 media (Sigma-Aldrich) supplemented with 50 mM HEPES (pH 8.2), 5% dialyzed serum, and antibiotic-antimycotic for 5 days (Soete *et al.*, 1993, Tomavo, 2001). After 5 days, the alkaline stress was removed and parasites were grown under tachyzoite conditions for another 48 h to measure the interconversion ability.
Immunofluorescence assays were performed by fixing the infected monolayers grown on coverslips with 3% paraformaldehyde. The nuclei of intracellular parasites and host cells were visualized by staining with 100 μM Hoechst (Sigma-Aldrich). FITC-conjugated *Dolichos biflorus* agglutinin (1:300) was used for the visualization of cyst structures. The parasites, from an average of 100 vacuoles or cysts per coverslip, were counted in triplicate to determine the number of parasites per vacuole or cyst.

For other immunofluorescence assays, the paraformaldehyde fixed monolayers were permeabilized with 0.25% Triton X-100 in PBS, and non-specific sites were blocked using 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. The slides were incubated with the primary antibodies: mouse anti-Myc (1:500), rabbit anti-TgLDH1 (1:1,000) or rabbit anti-TgLDH2 (1:1,000). Coverslips were then washed 3 times for 5 min each in PBS and incubated with the secondary antibodies: rhodamine-conjugated sheep anti-mouse (1:800) or rhodamine-conjugated goat anti-rabbit (1:800). All images were taken with a Q-imaging CCD camera on a Leica DMIRB microscope using the Northern Eclipse software.

### 2.12 Immunodetection

Western blot analyses were performed using the lysates prepared from freshly lysed parasites in 100 mM Tris-HCl (pH 7.6) containing 0.5 mM phenylmethylsulfonylfluoride (PMSF) protease inhibitor (Al-Anouti *et al*., 2004). Briefly, cell pellets were resuspended in PBS, sonicated, and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was separated from the cellular debris. Protein concentration was measured using the Bradford reagent according to manufacturer’s
instructions. Cell lysates were denatured in 5X SDS protein loading dye, resolved on a 12% SDS-PAGE at 120 V for 1 h in a 1X SDS-PAGE running buffer (25 mM Tris-base (pH 8.3), 250 mM glycine, and 0.1% SDS) using the vertical gel electrophoresis system from BRL (Bethesda Research Laboratories), and transferred to a nitrocellulose membrane (Pall Corporation) at 80 V for 1.5 h in transfer buffer (20% methanol, 39 mM glycine, 48 mM Tris-base, 0.037% SDS, (pH 8.3)). Following the transfer, blots were blocked in 5% non-fat milk in 1X Tris-buffered saline containing Tween®-20 (TBST; 0.068 M NaCl, 8.3 mM Tris-base (pH 7.6), 0.1% Tween) for 1 h at room temperature. Membranes were then probed with the specific primary antibodies in 2% non-fat milk in TBST for 1 h at room temperature. The primary antibodies used were: mouse anti-Myc (1:1,000), mouse anti-Tub (1:1,000), rabbit anti-LDH1 (1:2,000), rabbit anti-LDH2 (1:2,000), rabbit anti-Bag1 (1:250), rabbit anti-RFP (1:250), and rabbit anti-RNaseIII (1:4,000). The blots were washed 3 times for 5 min, incubated in the appropriate secondary antibody for 45 min at room temperature, and washed again 3 times for 5 min each. The secondary antibodies used were: horseradish peroxidase-conjugated goat anti-mouse (1:10,000) and horseradish peroxidase-conjugated goat anti-rabbit (1:20,000). Chemiluminescent detection was performed using the chemiluminescent HRP substrate kit (Millipore).

2.13 Immunoprecipitation assays

In order to immunoprecipitate putative dicer proteins or TgAgo_ChFP, rabbit anti-RNaseIII and rabbit anti-RFP were used, respectively. Protein-A agarose beads (10 µL beads/500 µL reaction) were incubated with the primary antibody on a rotator for 1 h at room temperature. The beads were collected by centrifugation at 4,000 rpm for 30 s and
the supernatant removed. The appropriate parasites were lysed in either polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, 0.5% Nonidet P40, 1 mM DTT, 2 mM vanadyl ribonuclease, 10% SDS) for dicer immunoprecipitation, or a Tris buffer (50 mM Tris-HCl, pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1% SDS) for TgAgo_ChFP immunoprecipitation and incubated with the antibody bound beads for 4 h at 4°C while rotating. The beads were washed 3 times for 5 min at 4°C with wash buffers, (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, 0.5% Nonidet P40, 1 mM DTT, 2 mM vanadyl ribonuclease, 10% SDS, 100 mM NaCl) and (50 mM Tris-HCl, pH 7.6), 0.1% Triton X-100, 150 mM NaCl, 0.1% SDS) for dicer and TgAgo_ChFP, respectively. The bound proteins were eluted by addition of 30 µL SDS-loading dye and boiled for 5 min. The samples were analyzed by western blot analysis and coomassie staining.

2.14 Mass spectrometry

The sample was lysed, electrophoresed by SDS-PAGE, and immunoblotted onto a PVDF membrane as previously described. The lane was cut into three sections and western blot analysis was performed on the two outer sections using rabbit anti-RFP (1:250) and horseradish peroxidase conjugated goat anti-rabbit (1:20,000). The bands were visualized using 3 mg of the colorimetric substrate DAB (3,3′-diaminobenzidine) in 20 mL of 0.05 M Tris-HCl (pH 7.2), and 20 µL of 3% H₂O₂, and matched to the unused section. The band with the corresponding size was excised using a sterile blade in a laminar flow hood. The band was washed 3 times each with methanol and ddH₂O, and blocked with 1 mL of 0.5% polyvinylpyrrolidone-360 (Sigma) containing 100 mM acetic acid for 30 min at 37°C, followed by 5 washes with ddH₂O. Tryptic digestion was performed with 260 ng of sequencing grade trypsin (Promega Corp.) resuspended in 50
mM ammonium bicarbonate (pH 8.0) to a final concentration of 13ng/µL, at 37°C overnight with agitation. The reaction was quenched by addition of formic acid at a final concentration of 1%. Additional peptides were released by sonicating the membrane for 3 min in 300 µL of ddH₂O and the solution was concentrated to approximately 10 µL by speed vacuuming. The tryptic peptides were analyzed by mass spectrometry using a matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometer (Applied BioSystems Voyager-DE™ PRO BioSpectrometry™ Workstation; Applied BioSystems, Framingham, MA). The peptides were crystallized on a MALDI target plate (Applied BioSystems) using an α-cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich, Inc.) in 60% acetonitrile, 1% formic acid. The tryptic peptide ion masses were compared to those generated by an in silico tryptic digestion of the TgAgo_ChFP protein using the MS-Digest program of Protein Prospector (UCSF; University of California, San Francisco, CA).
Chapter III

Results

This chapter is divided into two parts. Part I focuses on the role of the lactate dehydrogenase enzyme of *T. gondii* in the process of interconversion. Transgenic parasites were generated by over-expressing either TgLDH1 or TgLDH2 in tachyzoites and the phenotypes were monitored when the parasites were grown in the presence of alkaline stress. Part II focuses on the argonaute protein of *T. gondii*, and its role in interconversion and post-transcriptional gene regulation. The studies were aimed at determining the differentiation and gene knockdown abilities of a knockout strain of TgAgo and a transgenic strain over-expressing TgAgo.

PART I: Physiological characterization of *Toxoplasma gondii*’s lactate dehydrogenases

3.1 Genotyping of *Toxoplasma gondii* strains

When compared to type II strains of *Toxoplasma gondii*, type I strains have been shown to be a more virulent, lethal type of parasite, which show a minimal ability to differentiate into bradyzoites *in vitro* (Sibley and Boothroyd, 1992). To ensure that the parental RH and RHΔHX strains used in these studies were type I strains, and were not contaminated with any type II strains, genotyping reactions were performed. In order to
genotype the *T. gondii* strains as type I or type II, restriction fragment length polymorphisms (RFLP) of PCR amplified gene products were used. The genes encoding SAG2 and GRA6 have been identified by Howe *et al.* (1997) and Fazaeli *et al.* (2000), respectively, to contain several polymorphisms that allow for genomic typing between the three types. The oligonucleotide primers used for PCR amplification were designed by Khan *et al.* (2005). 200 bp of the 3’ terminus of SAG2 was amplified and digested with *HhaI*. Type I does not contain the restriction site and therefore the product is the same size as the PCR fragment. On the other hand, type II parasites contain the *HhaI* digestion site in the PCR amplified region, thus 2 fragments are produced from the digestion (Fig. 3.1A). The GRA6 gene was PCR amplified to produce a fragment of 346 bp, which was then digested with *MseI*. Type I parasites contain the *MseI* site close to the 3’ end generating 2 fragments of 261 bp and 85 bp. Type II parasites, on the other hand, contain the *MseI* site in the middle of the fragment generating 2 products of 162 bp and 182 bp (Fig. 3.1B). Only patterns specific to type I were detected, thus confirming that the parental strains used were of type I origin and were not contaminated with any type II parasites.
Figure 3.1  Genotyping of two laboratory strains of *Toxoplasma gondii*, RH and RHΔHX. (A) SAG2 and (B) GRA6 primers were used to generate PCR products which were then digested with *Hha*I and *Mse*I, respectively. The undigested and digested samples were resolved on a 12% polyacrylamide gel.
3.2 Over-expressing recombinant TgLDHs in *Toxoplasma gondii*

To study the effect of TgLDH1 and TgLDH2 on differentiation, clonal parasites that stably over-expressed either TgLDH1 or TgLDH2 were generated. Those selected for the study were named RML1-A & -B and RML2-A to -D, respectively where R indicates the RHΔHX parental strain, M indicates the Myc tag, and L1 and L2 indicate over-expressed TgLDH1 and TgLDH2, respectively.

To determine levels of recombinant TgLDH1 and TgLDH2 expression in the tachyzoite stage, western blot analysis was employed. Figure 3.2A indicated that tachyzoites of the parental parasite (RHΔHX) strain expressed detectable levels of TgLDH1, but not TgLDH2, which is in agreement with previous observations (Yang and Parmley, 1995, 1997). Endogenous TgLDH1 has a molecular weight of ~35 kDa, and was detected in all samples prepared from the selected clones as well as in the RHΔHX strain, using an anti-TgLDH1 antibody. In the RML1-A and RML1-B strains, recombinant TgLDH1 protein was detected by both anti-Myc and anti-TgLDH1 antibodies, which resolved at ~39 kDa. As expected, recombinant TgLDH2 (~40 kDa) was only detected in the four clonal RML2 (A to D) strains using anti-TgLDH2 and anti-Myc antibodies, while no endogenous levels of TgLDH2 were detected in any of the strains, since tachyzoites do not express TgLDH2 (Fig. 3.2A).

To normalize the expression of the recombinant proteins in the transgenic clonal parasites, the expression levels, as detected by anti-TgLDH1 or anti-TgLDH2, were quantified against those of the internal control, tubulin (Fig. 3.2B). Between the clonal TgLDH1 strains, RML1-A exhibited a higher level of recombinant TgLDH1 than RML1-B. Among the clonal TgLDH2 strains, RML2-C exhibited the lowest amount of recombinant TgLDH2 and RML2-A and RML2-D had the highest levels. It was noted
Figure 3.2  Expression of the recombinant proteins in the selected clones. (A) Western blot analyses of recombinant *Toxoplasma gondii* lactate dehydrogenase (TgLDH1 and TgLDH2) expression. Anti-Myc, -TgLDH1, -TgLDH2 and -Tub antibodies were used in revealing the corresponding proteins as indicated on the left. Estimated sizes of the detected bands are indicated on the right. (B) Comparative levels of the recombinant TgLDH proteins among the clonal strains was obtained following densitometric measurements of the band intensities and normalized against those of tubulin (Tub).
that there was no direct relationship between the level of recombinant protein expression and the endogenous level of TgLDH1 expression.

Prior to phenotypical analyses, immunofluorescence assays were performed to ensure that recombinant TgLDH1 and TgLDH2 proteins were expressed uniformly in each clone (Fig. 3.3). The expression of recombinant proteins was uniform in all strains as detected by an anti-Myc primary antibody and a rhodamine-conjugated anti-mouse secondary antibody. Figure 3.3 shows the recombinant protein in the RML1-B strain. The results were similar for all other clones. It was observed that recombinant TgLDH1 and TgLDH2 proteins localized to the cytosol, which is in agreement with previous studies (Ferguson et al., 2002).
Figure 3.3  Expression of TgLDH1 in tachyzoites. Immunofluorescence detection of Myc-tagged *Toxoplasma gondii* lactate dehydrogenase 1 (TgLDH1) in the tachyzoites of RML1-B. Nuclei of the HFF host and the tachyzoites were stained with Hoechst, as indicated by an arrowhead and an arrow, respectively. The scale represents 20 microns.
3.3 Effect of TgLDH1 or TgLDH2 over-expression on overall enzymatic activity of TgLDH

To determine if the varied levels of over-expression altered the overall activity of TgLDH, an LDH enzymatic assay was performed by measuring the change in NADH absorbance at 340 nm when pyruvate was added to freshly lysed parasites (Table 3.1). The overall TgLDH activity of the parental tachyzoites, RHΔHX, was found to be 1473 ± 4 nmol. min\(^{-1}\).mg\(^{-1}\). The over-expression of TgLDH1 in the RML1-A and RML1-B strains displayed similar activity as parental, 1428 ± 107 and 1933 ± 241 nmol. min\(^{-1}\).mg\(^{-1}\), respectively. The RML2-A, RML2-B, RML2-C, and RML2-D strains with over-expressed TgLDH2, with activities of 1260 ± 287, 1072 ± 210, 1009 ± 185, and 1167 ± 132 nmol. min\(^{-1}\).mg\(^{-1}\), respectively, also resembled the activity of the parental strain. Therefore, varied levels of over-expression did not decrease nor increase the overall enzymatic activity of TgLDH.
Table 3.1  Overall enzymatic activity of TgLDH in transgenic and parental strains. Enzymatic activity of parental and transgenic clones was monitored by the change in absorbance of NADH at 340 nm. The values and standard deviations are given in nmol.min⁻¹.mg⁻¹.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Activities (nmol.min⁻¹.mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHΔHX</td>
<td>1473 ± 4</td>
</tr>
<tr>
<td>RML1-A</td>
<td>1428 ± 107</td>
</tr>
<tr>
<td>RML1-B</td>
<td>1933 ± 241</td>
</tr>
<tr>
<td>RML2-A</td>
<td>1260 ± 287</td>
</tr>
<tr>
<td>RML2-B</td>
<td>1072 ± 210</td>
</tr>
<tr>
<td>RML2-C</td>
<td>1009 ± 185</td>
</tr>
<tr>
<td>RML2-D</td>
<td>1167 ± 132</td>
</tr>
</tbody>
</table>
3.4 Phenotypical analyses of transgenic parasites over-expressing TgLDH1 and TgLDH2

Clonal RML1-B, RML2-A, and RML2-D strains were selected for further phenotypical analyses since there was no apparent correlation between the levels of over-expression, localization and the overall TgLDH activity.

3.4.1 Growth of transgenic parasites under tachyzoite conditions

Growth of tachyzoites was monitored to determine if over-expression of TgLDH caused any changes to occur. Growth assays were conducted using clonal parasites from early and late passages, where the former were from less than three passages and the latter from six passages, to ensure that the results were consistent, since it has previously been reported that serial passages of transgenic *T. gondii* cultures may cause a reduction in the expression of recombinant proteins (Van et al., 2007).

The parasites were infected under tachyzoite conditions, and the number of parasites in the vacuoles was monitored from the early (Fig. 3.4 A and B) and late passages (Fig. 3.4 C and D) after 24 and 48 hours. The infected monolayers were fixed after 24 or 48 hours and stained with Hoechst to facilitate counting. At 24 hours post-infection, 50% of vacuoles formed by all strains, including RML1-B, RML2-A, RML2-D, and RHΔHX, contained 8 parasites. At 48 hours post-infection, all strains showed a similar growth pattern of having about 50% of vacuoles with more than 32 parasites, and the early and late passages exhibited the same trend. Therefore, the over-expression of recombinant TgLDH1 or TgLDH2 proteins had minimal effect on the growth of tachyzoites, as compared to the parental strain, under tested conditions.
Figure 3.4  Tachyzoites’ growth analyses. Comparative growth analysis of parasites from an early passage (A and B) and late passage (C and D) is displayed as the fraction (%) of vacuoles containing 2, 4, 8, 16, or >32 parasites. An average of 100 vacuoles were counted from each strain at 24 (A and C) or 48 (B and D) hours post-infection, in triplicate.
3.4.2 Differentiation of transgenic parasites using alkaline media

The expression of TgLDH1 and TgLDH2 proteins has been widely used as a marker of parasite differentiation (Weiss and Kim, 2000; Yang and Parmley, 1997), and previous studies have shown that knockdown of TgLDHs caused a decrease in differentiation ability (Al-Anouti et al., 2004). Thus, it was important to investigate whether TgLDH1 or TgLDH2 over-expression also affected the parasite’s ability to differentiate into bradyzoites.

Parasites were infected onto HFF cells, and the monolayers were cultured under alkaline conditions (pH 8.2) for 5 days, which has been shown to cause differentiation (Boothroyd et al., 1997; Soete et al., 1993). To ensure that the cyst structure formation correlated with the presence of bradyzoite markers, western blot analysis was performed. Parasites cultured under alkaline conditions were compared to those cultured under tachyzoite conditions for the presence of the bradyzoite-specific antigen 1 (BAG1, 30 kDa) using the anti-BAG1 antibody (Fig. 3.5; Bohne et al., 1995). A mixed population strain of TgLDH2 over-expressed parasites, RML2-mix, was used as a test in western blot analysis, while PLKΔHX, a type II parasite known to readily differentiate, was used as a positive control. Figure 3.5 shows that BAG1 is detected in the lysate of the bradyzoite strains of both RML2-mix and PLKΔHX, while it is not detected in the lysate of the tachyzoite samples, further confirming that differentiation did occur and can be correlated with cyst wall formation that appears in parasites cultured for 5 days in alkaline media.
Figure 3.5  Expression of the bradyzoite marker BAG1. Western blot analysis of *Toxoplasma gondii* tachyzoites (tz) and bradyzoites (bz) of a type II strain, PLKHX, and a transgenic line over-expressing TgLDH2, RML2-mix. Anti-BAG1 was used to reveal the BAG1 protein at 30 kDa in the bradyzoite samples only. Tubulin was used as a loading control.
To determine if cyst formation occurred in the transgenic clones, levels of recombinant proteins in the clonal parasites were evaluated using an anti-Myc primary antibody and a rhodamine-conjugated sheep anti-mouse secondary antibody; while cyst wall formation was detected using FITC-conjugated Dolichos lectin. Figure 3.6 indicates that a detectable cyst wall was observed in the RML2-A strain, which was similar to all other transgenic strains, and that recombinant protein expression correlated with the cyst wall. The formation of cysts was counted in all strains and quantified against the total number of vacuoles. It was observed that the parental RHΔHX strain could differentiate, with ~14% of vacuoles forming cyst structures under conditions tested. Notably, all clonal strains, including RML1-B, RML2A and RML2-D, showed an enhanced ability to differentiate and form cyst structures. From all transgenic parasite clones counted, over half of the vacuoles exhibited distinctive cyst structures, demonstrating that TgLDH over-expression caused an increase from ~14% (parental) to ~60% (transgenic) in cyst formation (Fig. 3.7). To ensure that differentiation and cyst formation was not an artifact derived from the expression of a recombinant protein, an unrelated protein, Myc-tagged GFP, was over-expressed and differentiation was monitored. It was observed that the GFP over-expression resulted in ~12% of cyst formation, which was similar to the parental strain, suggesting that differentiation was not enhanced by an unrelated protein. This finding confirmed that the increase in differentiation was directly correlated to TgLDH over-expression.
Figure 3.6  Differentiation of parasites over-expressing TgLDH by alkaline stress. Immunofluorescence image shows the cyst structure of the RML2-A strain which was similar to all other transgenic parasites. The nuclei were stained with Hoechst. The arrowhead indicates the nucleus of the HFF host cell, while the arrow indicates the nuclei of the bradyzoites. The recombinant Myc-tagged TgLDH2 was revealed by an anti-Myc primary antibody and a rhodamine-conjugated sheep anti-mouse secondary antibody. The cyst structure was revealed using FITC-conjugated Dolichos lectin staining. The scale represents 15 microns.
Figure 3.7   Quantification of cyst formation in transgenic and parental parasites. Quantitative analyses of cyst formation were conducted in the differentiated RML1-B, RML2-D, RML2-A, and RHΔHX strains. The fraction (%) of vacuoles that formed cysts was calculated from at least 100 vacuoles and from three independent assays.
3.4.3 Growth of transgenic parasites under alkaline conditions

To evaluate parasite growth under alkaline conditions, the number of parasites in each vacuole, after 5 days of culturing in alkaline media (pH 8.2), was counted. Figure 3.8 shows that the parental RHΔHX strain retained a sufficient ability to multiply under alkaline conditions and formed 2, 4, 8, and 16 parasites per vacuole at 5 days post-infection. The distribution of these vacuoles was almost equal (20-30%). In the clonal RML1-B and RML2-D strains, the majority (~50-60%) of the vacuoles contained 2 parasites, suggesting that alkaline conditions halted parasite multiplication at an early division stage. On the other hand, the RML2-A strain exhibited a unique growth pattern blending the characteristic of the parental parasites in having an even distribution of the number of vacuoles containing either 2, 4, and 8 parasites per vacuole, but not 16 parasites per vacuole, while having 50-60% differentiated vacuoles.
Figure 3.8 Growth analyses of *Toxoplasma gondii* under alkaline conditions. Comparative growth analyses of parasites that were cultured under alkaline conditions for 5 days is displayed as the fraction (%) of vacuoles containing 2, 4, 8, 16, or >32 parasites. An average of 100 vacuoles was counted for all strains in triplicate.
3.4.4 Cyst formation of transgenic parasites under tachyzoite conditions

To determine if over-expressing TgLDH1 or TgLDH2 could enhance differentiation of *T. gondii* in the absence of a stress, the parasites were grown under tachyzoite conditions and stained with Dolichos-FITC to measure cyst formation. It was observed that no cyst structures were formed in all strains, indicating that TgLDH did not enhance cyst formation under tachyzoite conditions (data not shown).

3.4.5 Cyst formation of transgenic parasites after the removal of stress

It was further determined whether the over-expression of TgLDH1 or TgLDH2 altered the bradyzoite’s ability to reconvert into tachyzoites after the alkaline stress was removed. Parasites from all clonal strains, along with the parental, RHΔHX, were grown under alkaline media, as previously described. After 5 days, the parasites were returned to tachyzoite conditions and grown for 48 hours. The parasites were fixed, and stained with Dolichos-FITC for visualization of cysts at both time points. It was determined that both parental and transgenic clones could successfully reconvert into tachyzoites after 48 hours. After 5 days in alkaline media, RHΔHX formed 18.3 ± 4.2% cysts. When reintroduced back into tachyzoite conditions for 48 hours, a total of 7.2 ± 0.2% of vacuoles remained encysted, indicating that some vacuoles were unable to revert into tachyzoites. On the other hand, after 5 days in alkaline media, transgenic clones formed an average of 49.6 ± 3.8% cysts. After being reintroduced back into tachyzoite conditions for 48 hours, 8.5 ± 0.3% of vacuoles remained encysted (Fig. 3.9). Therefore, although TgLDH1 or TgLDH2 over-expression may play a role in enhancing the
differentiation process under stressful conditions, it does not cause the parasites to remain encysted after the stress is removed.

Overall, all clonal parasites expressing either of the recombinant TgLDH proteins could easily differentiate and slowly multiply in comparison to the parental parasite under alkaline conditions. Also, the majority of the bradyzoites were able to reconvert into tachyzoites once the stress was removed. These findings suggest that TgLDH plays an important role in the differentiation process of *Toxoplasma gondii*. 
Figure 3.9  Cyst formation after removal of stress. Quantification of cysts after culturing in alkaline media for 5 days, and returning the parasites to tachyzoite conditions for 48 h. An average of 100 vacuoles was counted in triplicate. The average values for RML1-B, RML2A and RML2-D are shown as transgenic clones.
PART II: The role of argonaute in *Toxoplasma gondii*

3.5 Over-expression of *T. gondii* argonaute fused to a cherry fluorescent protein

To investigate the physiological function of TgAgo, four strains of parasites were used: (i) the parental RH and RHΔHX strains, (ii) a previously characterized TgAgo null mutant (obtained from Dr. Boothroyd, Stanford University School of Medicine), and (iii) a transgenic line constitutively over-expressing a TgAgo_ChFP fusion.

To detect the presence and level of TgAgo_ChFP in *T. gondii*, western blot analysis was used. Since cherry fluorescent protein is a derivative of the red fluorescent protein (RFP), the antibody against RFP was used to detect the fusion. The molecular weight of TgAgo is ~58.5 kDa, while that of ChFP is ~27 kDa, generating a fusion product of ~85.5 kDa, which was detected on the blot. The lysate of the parental RH parasite was used as a negative control, while a GFP_ChFP fusion protein was used as a positive control and was detected at ~54 kDa (Fig. 3.10). A band at ~85 kDa was revealed only in the TgAgo_ChFP parasites, but a band at ~27 kDa was also seen indicating that degradation or cleavage of ChFP had occurred.
Figure 3.10  Expression of TgAgo_ChFP in transgenic parasites. Western blot analysis was used to detect the TgAgo_ChFP fusion protein revealed by an anti-RFP antibody. RH parasites were used as a negative control, while GFP_ChFP was used as a positive control. The estimated sizes are indicated on the right.
Following identification of the fusion protein by western blot analysis, fluorescence microscopy was used to verify that the fusion protein was producing cherry fluorescence and that the localization of the fusion was similar to argonaute proteins in other organisms. Upon monitoring cherry fluorescence, it was observed that the protein localized mainly to the cytosol when compared to Hoechst staining of the nuclei, which is in agreement with previous studies (Fig. 3.11; Sen and Blau, 2005).

Furthermore, mass spectrometry was utilized to unambiguously identify the fusion protein. The lysate containing the fusion protein was electrophoresed and electroblotted onto a PVDF membrane and the band was identified by a colorimetric substrate of horseradish peroxidase (HRP). The band was excised, digested with trypsin, and analyzed by mass spectrometry (data not shown). The identification of the peptides was not clear since many of the peptides were the same size as autolysis peaks of trypsin. There were a few bands that seemed promising; however the amount of protein present was limited so the protein identification was not sufficient to fully confirm the fusion.
Figure 3.11 Expression and localization of TgAgo_ChFP in tachyzoites. Fluorescent microscopy was used to detect TgAgo_ChFP in the tachyzoite parasites. The nuclei of the parasites were stained with Hoechst. The scale represents 10 microns.
3.6 Interconversion ability of Toxoplasma gondii strains with altered TgAgo expression

Database mining for TgAgo revealed that the expression of TgAgo in type I parasites is elevated as compared to type II based on mRNA expression profiles (Fig. 3.12). This observation led to the hypothesis that argonaute may play a role as a negative regulator in the differentiation process since type I parasites differentiate less than type II. That is, higher levels of argonaute may decrease the ability of the parasite to differentiate into bradyzoites. This hypothesis was tested by comparing the differentiation ability of transgenic parasites containing TgAgo_ChFP with the knockout (AgoKO) strain and parental parasites under alkaline conditions.

Immunofluorescence assays were used to monitor cyst formation in all the strains, where the cyst wall was detected by the FITC-conjugated Dolichos lectin. Many cyst structures were detected in both the over-expressed TgAgo_ChFP clonal strain and the AgoKO strain when compared to a parental, RHΔHX, strain (Fig. 3.13). Cyst formation was quantified by counting the number of vacuoles that formed cysts under tested conditions in all three strains (Fig. 3.14). The parental strain formed ~14% of cysts, whereas the AgoKO strain formed ~70% of cysts, and the transgenic clone formed ~40% of cysts. Therefore, TgAgo may play a role in the interconversion process, but the definition of that role is still unknown.
Figure 3.12 Expression profile of TgAgo. The mRNA levels of TgAgo were determined by microarray analyses in all three types of parasites. This data was provided to ToxoDB pre-publication by Paul Davis and David Roos (http://toxodb.org; Oct. 2008).
Figure 3.13  Differentiation of parasites with altered TgAgo levels. Parasites were cultured for 5 days under alkaline conditions and subjected to immunofluorescence assays. Cysts are revealed by Dolichos-FITC, nuclei are revealed using Hoechst staining, and TgAgo_ChFP was identified by the cherry fluorescence. The scale represents 20 microns.
Figure 3.14  Quantification of cyst formation in strains with altered TgAgo levels. Quantitative analyses of cyst formation were conducted for the differentiated TgAgo_ChFP, AgoKO, and RHΔHX. The fraction (%) of vacuoles that formed cysts was calculated from at least 100 vacuoles and from three independent assays.
3.7 The role of TgAgo in an RNA interference-like pathway

The discovery of an argonaute gene in *T. gondii* has suggested that an RNAi-like pathway exists in the parasite, and early experiments have shown that gene knockdown is observed upon introduction of double stranded RNA specific to the UPRT and HXGPRT genes (Al-Anouti *et al.*, 2004). To determine if argonaute plays a specific role in this knockdown phenomenon, the following studies aimed to monitor any changes in gene knockdown ability when argonaute is either over-expressed or knocked out relative to a parental control.

3.7.1 Measuring RNAi activity in RH parental parasites

To determine the maximum knockdown that could be observed in *T. gondii*, RH parental parasites were used as a positive control. UPRT dsRNA was generated and increasing amounts were electroporated into parasites that were infected onto HFF monolayers. After 24 hours, radioactive uracil was added to the media, parasites were grown for another 4 hours, and the amount of incorporated radioactivity was measured. If knockdown of UPRT was successful, then the parasites would lose the ability to incorporate radioactive uracil. As a control, the ability of parasites to incorporate uracil was measured in parasites that were electroporated with 0 μg of dsRNA and the resulting level of radioactivity was set as 100%. Figure 3.15 shows that the introduction of ~22 μg of RNA by electroporation resulted in a maximum knockdown of ~60%. Any increase in RNA concentration greater than 22 μg caused a plateau effect.
Figure 3.15  RNA interference assay of RH parental parasites with a range of dsRNA concentrations. Knockdown of UPRT was measured using radioactively labelled uracil. Increasing amounts of UPRT dsRNA was electroporated into RH parasites and the percentage of knockdown is shown as compared to control with 0 μg dsRNA. The experiment was repeated in triplicate and the values are given with their standard deviations.
3.7.2 Measuring RNAi activity in parasites containing altered levels of TgAgo

To determine if TgAgo plays a role in the gene knockdown phenomenon observed in *T. gondii*, the UPRT dsRNA was electroporated into both the AgoKO and TgAgo_ChFP transgenic parasite lines, and the knockdown was measured using radioactive uracil as previously described. As expected, the AgoKO line showed no ability to cause knockdown of UPRT (Fig. 3.16A). The amount of uracil that was incorporated was similar to the control parasites that did not contain dsRNA. Unexpectedly, the TgAgo_ChFP transgenic line also showed an inability to cause gene knockdown of UPRT (Fig 3.16B). Similar to the AgoKO strain, the TgAgo_ChFP strain had values close to control with increasing dsRNA concentrations.
Figure 3.16 RNAi activity of parasites with altered levels of TgAgo. Double stranded UPRT was electroporated into (A) AgoKO and (B) TgAgo_ChFP, and an RNAi assay was performed using radioactively labelled UPRT. The percentage of knockdown as compared to control is shown relative to the amount of UPRT dsRNA electroporated. The experiment was repeated in triplicate and the values are given with their standard deviations.
3.8 The effect of altered TgAgo expression on levels of putative dicer proteins

In order to study if the alteration of TgAgo expression affected the levels of putative dicer proteins, western blot analysis was conducted. Putative dicer proteins were revealed using an antibody created against a conserved peptide sequence of the RNaseIII domain of dicer (rabbit anti-RNaseIII). Figure 3.17A shows 3 reactive bands when probed with anti-RNaseIII at ~120, ~70, and ~40 kDa. RH parasites were used as a control for natural protein levels. The levels of the 3 bands were quantified against those of tubulin (Fig. 3.17B). When over-expressing TgAgo_ChFP, there is a slight increase of the ~120 kDa band from levels in RH, but a drastic decrease in the AgoKO strain. When comparing the ~70 kDa band, the levels in RH are higher than in the other two strains, with AgoKO having the lowest levels. Alternatively, when comparing the ~40 kDa band, the levels in RH are the lowest of the three strains, with AgoKO having the highest levels. These results suggest that putative dicer proteins may be up-regulated or down-regulated depending on expression of TgAgo.
Figure 3.17  Expression of RNaseIII containing proteins in strains with altered TgAgo levels. (A) Western blot analysis of RH, TgAgo_ChFP, and AgoKO strains with anti-RNaseIII antibody. Tubulin was used as a loading control, and approximate sizes are indicated on the right. (B) Quantitative analysis of putative dicer proteins as compared to tubulin was obtained by densitometry for all three strains.
To identify the putative dicer bands, immunoprecipitation (IP) reactions were performed using anti-RNaseIII bound to protein-A agarose beads. The process consisted of a pre-clearing step to remove non-specific proteins that bind to the protein-A agarose. Figure 3.18 shows the coomassie stain of the pre-clear and IP samples. Many bands were observed in the pre-clear sample, while only the heavy chain of the antibody was observed in the IP sample. After western blot analysis using anti-RNaseIII (Fig. 3.18), two bands were revealed at ~40 and ~120 kDa in both the pre-immune sample and RH lysate that was used as a positive control. In the IP sample, a thick band was observed at ~55 kDa that was specific to the heavy-chain of the antibody, but no bands specific to anti-RNaseIII were observed. This suggests that the protein was bound to the beads, and removed in the wash steps. Therefore, the ability to pull-down and identify putative dicer proteins was not successful. Thus, further optimization of the experiment is necessary to successfully characterize the putative dicer proteins.
Figure 3.18 Identification of putative dicer proteins by immunoprecipitation. Immunoprecipitation was used to pull-down putative dicer proteins by the anti-RNaseIII antibody bound to protein-A agarose beads. (A) A coomassie stain shows many bands in the pre-clear sample, while only the heavy-chain of the antibody is seen in the IP sample. (B) Western blot analysis with anti-RNaseIII indicates that putative dicer proteins were observed in the pre-clear sample that were similar to the RH lysate sample, however no bands were pulled down in the IP sample. Approximate sizes are indicated on the right.
Chapter IV

Discussion and Future Work

*Toxoplasma gondii* is a widespread pathogen that infects approximately one-third of the world’s population. Typically asymptomatic in healthy individuals, *T. gondii* can remain dormant for the lifespan of the host as encysted bradyzoites. However, if the host becomes immuno-compromised due to illness such as AIDS, *T. gondii* can reconvert from its dormant stage into the highly virulent tachyzoite stage and cause a recurrence of infection that may prove fatal (Ferguson *et al.*, 2002; Lindsay *et al.*, 1991; Luft and Remington, 1992). The interconversion between bradyzoites and tachyzoites is thought to be an important process in the pathogenesis of the parasite; however studies of the process have proven unsuccessful in determining the mechanisms behind interconversion and thus remains poorly understood (Reiter-Owona *et al.*, 2000). The current studies have focused on lactate dehydrogenase and argonaute, and the roles they may play in this process. Elucidation of the mechanism of interconversion may eventually lead to development of possible drug targets that would prevent the parasite from entering a dormant stage, thus preventing chronic toxoplasmosis.

Previous studies on *T. gondii* lactate dehydrogenase indicated that knockdown of TgLDH resulted in variable growth rates under both tachyzoite and bradyzoite conditions. More importantly, the differentiation ability was impaired *in vitro* and the parasites were unable to form tissue cysts in a murine model system. The parasites in the mice were not as virulent, and the mice survived a challenge with parental parasites that would typically
cause 100% mortality. These results suggested that TgLDH is important for both the cell cycle and differentiation ability of *T. gondii* (Al-Anouti *et al.*, 2004). Therefore, it was of interest to study the effect of over-expressing TgLDH in tachyzoites, and measuring the resulting phenotypes in relation to growth and differentiation ability.

Furthermore, it was also of interest to study the role of the argonaute protein in a putative gene knockdown pathway. In other organisms, posttranscriptional gene silencing, known as RNA interference, has offered insight into the importance of the pathway, as well as provided a genetic tool for studying the organism. The identification of *T. gondii* argonaute, and the knockdown phenomenon generated by introduction of dsRNA leads to speculations that an RNAi-like pathway exists in *T. gondii* (Al Riyahi *et al.*, 2006). Thus, current studies aimed to measure the effect of differentially expressing TgAgo on the knockdown phenomenon, to better understand the phenomenon within the parasite. Moreover, argonaute proteins have been identified as key players in other cellular functions in many organisms (Carmell *et al.*, 2002; Jaronczyk *et al.*, 2005). Therefore, the studies also revolved around the parasites ability to successfully differentiate *in vitro* upon the over-expression or knockout of TgAgo.

### 4.1 Validation of parental parasites as type I parasites

In order to further characterize the role of TgLDH’s in *T. gondii*, transgenic lines over-expressing Myc-tagged TgLDH1 and Myc-tagged TgLDH2 were generated. The parental parasites, RH and RHΔHX, used for generation were first subjected to genotyping studies to ensure that there was no contamination with any type II parasites.
that may have occurred during routine culture. This was important because the present studies involved the formation and quantification of bradyzoites in vitro, and since type II parasites have an increased ability to differentiate, any contamination would have skewed the results (Sibley and Boothroyd, 1992).

Single nucleotide polymorphisms (SNPs) are DNA sequence variations where one basepair in the genomic sequence is altered between different strains or types of an organism. These SNPs allow for genotyping studies that involve restriction fragment length polymorphisms. In this case, sequences of the genome that contain SNPs are amplified by PCR, and further digested using restriction endonucleases. Since one type of the parasite will contain the correct restriction site, digestion will occur. However, the presence of an SNP in another type of parasite will eliminate the restriction site thus preventing digestion. This results in varying digestion patterns in differing types of parasites. Two genes, GRA6 and SAG2, have been shown to contain SNPs, and were thus used in these studies to validate the typing of the parasite (Fig. 3.1). The resulting banding patterns were compared with those identified for type I and type II parasites and it was concluded that only type I parasites were present (Khan et al., 2005). Since differentiation studies revealed a relatively high number of cyst structures in parental parasites when using alkaline media, as compared to the low values found in literature (Radke et al., 2006), it suggests that these parasites have type II characteristics. However, since genotyping confirmed the parasites as type I, the high values were possibly due to the method of quantification. Since the same method of quantification was used for all experimental strains, the values were comparable to each other.
4.2 Transgenic parasites over-expressing TgLDHs were successfully generated

*T. gondii* lactate dehydrogenase (TgLDH) is an important enzyme in the glycolysis pathway that catalyzes the interconversion of pyruvate to lactate. After an immune response, the parasites differentiate into bradyzoites, which are the slowly multiplying, encysted form that primarily utilize glycolysis for energy production. To study the importance of TgLDH’s on bradyzoite formation, transgenic parasites over-expressing either TgLDH1 or TgLDH2 were used. The clonal parasites were analysed by western blot analysis for the presence of TgLDH (Fig. 3.2A). The clonal parasites expressed either TgLDH1 (~39 kDa) or TgLDH2 (~40 kDa) whose larger sizes corresponded to addition of the Myc-tag. The light bands found in the anti-LDH2 panel were the residual signals resulting from successive anti-Myc and anti-LDH2 antibody incubations. The blot was first revealed using an anti-Myc antibody and then stripped prior to anti-LDH2 incubation (Fig. 3.2A).

The levels of over-expressed TgLDHs in all clones were quantified against those of tubulin (Fig. 3.2B). Between the clonal TgLDH1 strains, RML1-A exhibited a higher level of recombinant TgLDH1 than RML1-B. Among the clonal TgLDH2 strains, RML2-C exhibited the lowest amount of recombinant TgLDH2 and RML2-A and RML2-D had the highest levels. It was noted that there was no direct relationship between the level of recombinant protein expression and the endogenous level of TgLDH1 expression. That is, varying amounts of over-expressed protein was observed that did not depend on or relate to endogenous levels of TgLDH1. The varied levels of recombinant TgLDH1 and TgLDH2 expression in the clonal strains might be the result of the integration frequency and location where the random gene integration took place, which is quite
common in *T. gondii* (Donald *et al.*, 1996; Striepen *et al.*, 1998). If there was a higher frequency of integration in one clone versus another, the levels of over-expression would have increased regardless of endogenous levels of TgLDH1. It is also important to note that if random integration had occurred within a genomic sequence coding for a specific gene, the phenotypes observed may result from the loss of a different gene. However, this is unlikely since the possibility of the integration interrupting the same gene in each clone is improbable, and so the same phenotype that was observed in multiple clones is likely due to TgLDH over-expression. It was also noted that no endogenous levels of TgLDH2 appeared when probed with anti-LDH2, thus ensuring that the parasites were in fact in the tachyzoite stage and not in the bradyzoite stage. Furthermore, the level and localization of the over-expressed proteins in tachyzoites was detected by immunofluorescence assays (Fig. 3.3). Only clone RML1-B is shown, however all other clones exhibited the same result. The over-expressed proteins were revealed using rhodamine-conjugated mouse anti-Myc so that the over-expressed proteins, in particular TgLDH1, could be identified apart from endogenous TgLDH1. It was observed that the over-expressed proteins could successfully be revealed with anti-Myc and that the localization was in the cytosol, which is in agreement with previous studies (Ferguson *et al.*, 2002).
4.3 TgLDH over-expression did not alter the overall TgLDH enzymatic activity or growth of parasites under tachyzoite conditions

Due to the distinctive over-expression, it was expected that a change in the overall TgLDH enzymatic activity would occur. The enzymatic activity was determined by measuring the absorbance of NADH after addition of pyruvate to lysates which were compared to parental and host cell controls. Unexpectedly, neither an increase nor decrease in overall TgLDH activity was detected (Table 3.1). It has been suggested that the active TgLDH enzyme is formed upon the assembly of a homotetrameric complex (Dando et al., 2001). If the recombinant proteins were non-functional, due to mis-folding or mutations, the proteins would have interfered with the assembly of wild-type TgLDH complexes and would have decreased the overall TgLDH activity. It is also likely that the mis-folded proteins would have been degraded and given rise to truncated and unstable forms of the recombinant proteins. If this was the case, multiple bands with varying molecular weights would have been detected during western blot analyses. However, only single bands were detected when either the anti-Myc or anti-TgLDH antibodies were used (Fig. 3.2A) suggesting that stable proteins were present. On the other hand, if the recombinant proteins were functional and able to form ternary complexes, an increase in the overall enzymatic activity would have been observed. However, since an increase was not observed, it is possible that the recombinant proteins were sequestered upon over-expression and thus were unable to participate in formation of homotetrameric complexes. This sequestering phenomenon has been observed in many organisms upon over-expression of proteins, in particular, the formation of inclusion bodies in bacteria is quite common and leads to inactive proteins (Kiefhaber et al., 1991; Mukhopadhyay,
1997). If sequestered, the recombinant proteins would have been protected from degradation as well as from the formation of active ternary complexes, while still remaining in the cytosol (Fig. 3.3) and maintaining the enzymatic activity as seen in parental.

The growth of the transgenic tachyzoites was monitored at 24 and 48 hours post-infection from parasites that underwent less than three passages (early) or six passages (late) in culture (Fig. 3.4 A-D) to ensure that long passage in culture did not alter the expression of recombinant proteins and hence the effect of these proteins as suggested by Van et al. (2007). It was observed that the growth of the parasites from early and late passages was unaffected and mirrored the growth of the parental strain. The finding that the growth and enzymatic activity were unaffected with over-expression of either TgLDH1 or TgLDH2 suggests that the proteins did not increase the rate of glycolysis and energy production which would be associated with a higher growth rate. Thus, it is likely that the proteins are playing a different role within the parasites.

4.4 The over-expression of TgLDHs increases differentiation under alkaline media stress

The conversion between the rapidly dividing tachyzoites and the slowly growing, encysted bradyzoites is an important event in the parasite life cycle. TgLDH1 and TgLDH2 proteins have been used as markers for tachyzoites and bradyzoites, respectively (Weiss and Kim, 2000; Yang and Parmley, 1997). The TgLDH1 mRNA is still present in bradyzoites, however the protein is replaced by TgLDH2. Thus, it was important to study
the effect of TgLDH1 and TgLDH2 over-expression on differentiation. Since the process of differentiation is poorly understood, it was necessary to verify that the observed cyst formation correlated to the presence of bradyzoite markers. The bradyzoite specific antigen-1 (BAG1) was used as a control for bradyzoite formation (Bohne et al., 1995), and tubulin was used as a loading control. As a positive control, the PLKΔHX strain was used, which is a type II laboratory strain that has a greater ability to differentiate as compared to type I parasites. The RML2-mix strain is the original strain that was electroporated with TgLDH2, and used to clone individual parasites. This strain was used as a test for differentiation. Western blot analysis indicated that the marker appeared in bradyzoite samples that were grown in alkaline media for 5 days and was not present in tachyzoite samples (Fig. 3.5). This indicated that when levels of cysts were visualized in transgenic parasites, the results correlated to bradyzoite formation.

A fluorescently labelled (FITC) Dolichos-lectin was used to monitor cyst formation (Fig. 3.6), which exhibited similar characteristics as observed by Dzierszinski et al. (2004). Cyst formation was quantified by manually counting the number of vacuoles that formed cyst walls (Fig. 3.7). It was observed that transgenic parasites over-expressing either TgLDH1 or TgLDH2 formed nearly 50% more cyst structures than the parental RHΔHX. Over-expressing an unrelated GFP protein produced ~12% of cyst, suggesting that the increased differentiation was a direct result of over-expressed TgLDHs. Furthermore, the growth of parasites under alkaline conditions was halted in the transgenic clones, which is characteristic of cyst formation (Fig. 3.8). It is not understood, however, why both TgLDH1 and TgLDH2 have a similar effect on the parasite’s ability to differentiate. It is likely that post-transcriptional regulation is
important for the temporal and spatial regulation of TgLDH’s and over-expression may cause interference in this regulation. Also, it is not known if differentiation causes the regulation of TgLDH’s or if the regulation affects the differentiation process. By testing the parasite’s ability to spontaneously form cysts, without any alkaline stress, it was determined that the regulation likely relies on the differentiation process since an increase in spontaneous cyst formation was not observed. Also, the parasite’s ability to convert back into tachyzoites once the stress was removed further suggests that TgLDHs may play a role in increasing cyst development under stress, but do not cause the parasites to remain encysted (Fig. 3.9). It is important to note that the parental parasites form almost 20% of cysts after 5 days in alkaline media. After the parasites are returned to tachyzoite conditions for 48 hours, ~8% of vacuoles are still encysted. Transgenic parasites, on the other hand, formed nearly 50% of encysted vacuoles after 5 days in alkaline media. Once again, after returning the parasites to tachyzoite conditions for 48 hours, ~10% of vacuoles remain encysted. Therefore, the presence of either over-expressed TgLDH1 or TgLDH2 increased the rate of reconversion from bradyzoites to tachyzoites. Thus, not only is the conversion into bradyzoites increased under stress, but the reconversion into tachyzoites once the stress is removed is also increased.

In conclusion, these studies are the first to suggest an important physiological function of TgLDH, in addition to being a glycolytic enzyme. To further determine the domains responsible for the function, one would perform deletion studies in which the substrate-, coenzyme-binding domains, or other truncated proteins can be assayed for their effects on the differentiation efficiency. Also, immunoprecipitation assays could be performed to detect any proteins that the TgLDHs are interacting with to attempt to
elucidate any other pathways in which TgLDHs may have roles. Finally, it would be interesting to measure the ability of these transgenic parasites to infect mice and form tissue cysts, to determine the differentiation ability in vivo.

4.5 TgAgo_ChFP was successfully generated and localized

The discovery of *Toxoplasma gondii* argonaute (TgAgo; Al Riyahi *et al.*, 2006) suggested that there may be a functional RNA interference (RNAi) pathway within *T. gondii*. It is also likely that TgAgo may play a role in other cellular functions in *T. gondii* as it does in many organisms (Carmell *et al.*, 2002; Jaronczyk *et al.*, 2005). To study the role of TgAgo, a fusion with cherry fluorescent protein (ChFP) was generated (Appendix A). ChFP was derived from a red fluorescent protein as a monomeric protein that excites at 587 nm and emits at 610 nm. The benefits of using ChFP rather than GFP or RFP are that ChFP has a fast rate of maturation, high photostability, resistance to pH changes, and the ability to form stable N-terminal fusions within a cell (Shaner *et al.*, 2004). Therefore, localization of TgAgo in the parasites is not likely to be compromised by the presence of ChFP.

Parasites harbouring the TgAgo_ChFP fusion were identified both with western blot analyses and by fluorescence microscopy. The anti-RFP antibody was able to be utilized since the ChFP protein is derived from RFP and the antibody was shown to cross react. The western blot revealed a band at ~85 kDa as was expected since the molecular weight of TgAgo is ~58.5kDa, while that of ChFP is ~27kDa (Fig. 3.10). There were two bands that were visualised for the TgAgo_ChFP sample that were nearly the same size. It
is possible that the faint higher band is a phosphorylated form of TgAgo. It has been shown that phosphorylation of human argonaute 2 plays a regulatory role in localizing argonaute 2 into processing bodies where degradation of messenger RNA occurs (Zeng et al., 2008). Further studies of TgAgo would have to be performed to determine if phosphorylation is occurring for example by utilizing antibodies specific to phosphorylated forms of proteins. On the other hand, the band shift may also be the result of a modification of ChFP or a possible degradation form of the fusion; however degradation is unlikely since the difference in size is minimal. Fluorescence microscopy indicated that TgAgo_ChFP localizes mainly to the cytosol when compared to staining of the nuclei. This finding is in agreement with localization studies of argonaute in other organisms. The proper localization suggests that TgAgo_ChFP functions appropriately since it has been shown that localization is related to function (Sen and Blau, 2005). The expression level and localization of the fusion protein could not be related to endogenous levels of argonaute due to a lack of antibody towards the endogenous protein.

Further characterization of TgAgo_ChFP was attempted by using mass spectrometry. The lysate of transgenic parasites was run on a western blot, transferred to a PVDF membrane, and the band corresponding to TgAgo_ChFP was excised and trypsin digested. Unfortunately, the autolysis peaks of trypsin overlapped with peptide masses of TgAgo_ChFP, so identification was unsuccessful. In order to properly utilize mass spectrometry, another enzyme, such as GluC, could be used for digestion to avoid the overlapping of peaks. These peak sizes would differ since GluC cleaves after glutamic or aspartic acid residues, unlike trypsin which cleaves after lysine and arginine residues, thus these peaks would not overlap with important TgAgo_ChFP peaks. Another possibility is
to enrich for the protein of interest by immunoprecipitation. This would increase the amount of protein available for digestion by proteases. If the number of generated peptides is increased, then the intensity of trypsin autolysis peaks decreases, allowing for isolation of specific peptides of interest and identification of the amino acid sequences. Immunoprecipitation of TgAgo_ChFP was attempted using the anti-RFP antibody bound to protein-A agarose beads, however the binding between the antibody and protein is weak, thus preventing successful immunoprecipitation. Preliminary data suggested that either no protein bound, or very low levels bound (data not shown). The former is more likely since silver staining of the SDS-PAGE gel did not show a band appearing at the expected size and this method of staining is very sensitive where its detection is in the nanogram scale (Chevallet et al., 2006).

4.6 Differential expression of TgAgo alters the interconversion ability of T. gondii

Database mining revealed that mRNA levels of TgAgo vary among the three types of parasites, wherein type I parasites contain the highest levels of TgAgo (Fig. 3.12). Since type I parasites do not differentiate very well, it was hypothesized that TgAgo may play a role as a negative regulator of the process. That is, higher levels of TgAgo may somehow prevent the parasite from successfully differentiating into bradyzoites. This hypothesis was tested by studying the differentiation ability of three strains of parasites: transgenic parasites over-expressing TgAgo_ChFP, a null mutant of TgAgo (AgoKO), and a parental RHΔHX strain. It was observed that both the TgAgo_ChFP and AgoKO strains could differentiate more than RHΔHX. The cyst structures observed with FITC-conjugated Dolichos-lectin are similar to the cyst structures observed in over-expressed
TgLDH parasites (Fig. 3.6 and 3.13). Quantification of the cyst structures was performed as previously described, and showed that AgoKO parasites formed ~65% of cysts versus ~14% in the parental RHΔHX (Fig. 3.14). This result suggests that TgAgo plays a significant role by preventing parasites from differentiating. One concern is from the generation of the AgoKO strain. The TgAgo genomic sequence is ~4,500 bp in length. In order to generate the knockout, approximately a 6.5 kb region of sequence was deleted. A putative long chain fatty acid CoA ligase gene and two hypothetical proteins are located on either end of TgAgo. Deletion of any part of these genes may be attributed to the phenotypes observed. Therefore, these studies must be performed in another knockout strain to be generated by deleting a smaller fragment of TgAgo which would ensure that no other putative or hypothetical genes are interrupted.

It was unexpected that the over-expression of TgAgo_ChFP formed just under 40% of cysts that correlate with cherry fluorescence (Fig. 3.13, 3.14). Moreover, it was expected that over-expression of the fusion would result in the differentiation ability to resemble or be lower than parental levels. These results suggest that over-expressing TgAgo may somehow counter-effect the protection against cyst formation. It is possible that the large ChFP tag may interfere with proper TgAgo function if it interrupts proper associations with other proteins, thus causing differentiation to increase. Furthermore, recent database mining has revealed new available data for microarray studies of TgAgo that show similar levels of TgAgo between type I and type II parasites (http://toxodb.org; April 2009), as opposed to the higher levels in type I that were observed in previous database mining efforts. These new experiments had used two different strains of parasites for each type to show reproducibility within a lineage. Also, these experiments
were performed with better controls in that all parasites were harvested together at a similar confluence, the RNA samples were prepared at the same time, and all hybridizations were performed by one technician. The current studies were based on the previous microarray results that suggested that TgAgo may play a role in differentiation. The altered differentiation ability that was observed between the mutant TgAgo strains fits the initial hypothesis. Thus although the new data indicates that levels of TgAgo do not actually differ, TgAgo likely has a role in the pathway. To further validate the levels of TgAgo mRNA, real time PCR should be performed so that more definitive correlations between TgAgo expression and differentiation can be made.

4.7 Argonaute plays a role in a putative gene knockdown pathway in *T. gondii*

Identification of key players of the RNA interference pathway in the apicomplexan *T. brucei* led to elucidation of a similar pathway in *T. gondii*. Recently, an argonaute protein was identified in *T. gondii*, and gene knockdown was observed after introduction of double stranded RNA (Al-Anouti *et al*., 2004). It was of interest to identify a direct correlation between TgAgo and dsRNA induced gene knockdown. The over-expressed and knockout lines of TgAgo were utilized to determine if the ability to knockdown UPRT was affected. RH parasites were used as a control for knockdown levels, and it was determined that a maximum knockdown of ~60% was observed when using ~20 µg of dsRNA (Fig. 3.15). The same experiment was repeated using the AgoKO and TgAgo_ChFP strains where a range of dsRNA concentrations were electroporated into the parasites. It was observed that no significant knockdown was seen
in either strain (Fig. 3.16). Knockout or knockdown of argonaute in other organisms resulted in a large reduction of RNAi activity suggesting that argonaute plays a key role in RNAi (Durand-Dubief and Bastin, 2003). The lack of RNAi activity in the AgoKO strain suggests that TgAgo also plays a key role in the gene knockdown pathway of *T. gondii*. If the knockdown had been a result of non-specific degradation, then knocking out TgAgo would not have affected the results. However, since knockdown was no longer visible in the AgoKO strain, it is suggested that TgAgo plays a role and the degradation is sequence specific. In the over-expressed strain, an inability to successfully knockdown UPRT was also observed. It is possible that the large cherry tag interferes with RISC formation that may be required for a functional RNAi pathway. To overcome this problem, a different tag such as a Myc or FLAG tag should be used that is smaller in size and would not interfere with protein-protein interactions. An N-terminal fusion that contains the cherry fluorescent protein on the N-terminal of TgAgo was generated to determine if the position of cherry caused the fusion to act as an inhibitory protein in proper RISC formation (Appendix C). Therefore, this fusion should be tested in the future in its ability to differentiate and perform double stranded induced gene silencing. It would also be interesting to determine if the observations made in these studies are the direct result of altered levels of endogenous argonaute. Several clones of the fusion parasite should be tested since random integration may interfere with other genes causing some of the mentioned phenotypes. Thus it is necessary to ensure that the results with the fusion strain were not merely artifacts.

4.8 Altered levels of TgAgo affect the expression levels of putative dicer proteins

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It was of interest to determine if the expression of putative dicer proteins differed in parasites that had altered TgAgo levels. To do so, an antibody raised against the RNaseIII domain of dicer was used in western blot analysis (Fig. 3.17). It was observed that the expression of two putative dicer proteins at ~120 kDa and ~70 kDa was downregulated in the AgoKO strain, while expression of a putative dicer protein at ~40 kDa was upregulated in the AgKO strain, as compared to parental. These results suggest that the proteins may be involved in the same pathway since expression of one affects the expression of the other, or that upregulation of an RNaseIII domain containing protein is compensating for the loss of argonaute. Full identification of the putative dicer proteins was attempted by immunoprecipitation assays which would be used in conjunction with mass spectrometry, unfortunately, very low levels were observed in the pre-clear sample and no proteins were pulled down (Fig. 3.18). Optimization and possible enrichment experiments are required in further analyses to elucidate the proteins and their functions. Also, putative dicer proteins, as detected by database mining can be cloned and over-expressed in *T. gondii* to measure the resulting phenotypes to further determine if the protein products participate in an RNAi-like mechanism.

### 4.9 Conclusions

Studies of the pathologically important *T. gondii* focused on the lactate dehydrogenase enzyme in the interconversion pathway, and on argonaute’s possible role in both interconversion and gene knockdown. It was observed that although the over-expression of TgLDH1 or TgLDH2 did not change the overall enzymatic activity,
increase growth of tachyzoites, or yield a more virulent parasite strain, a significant *in vitro* differentiation to bradyzoites was detected after increased TgLDH1 or TgLDH2 expression. It was also observed that TgAgo may play a role in differentiation since mis-regulation of the protein expression results in an increase in bradyzoite formation. Finally, the knockout of TgAgo failed to produce an RNAi response suggesting that TgAgo plays a critical role in the pathway since the pathway cannot occur without its presence. The lack of gene knockdown in the over-expressed TgAgo_ChFP strain may be due to the large cherry tag interfering with RISC formation, thus halting the process. Finally, altered levels of argonaute expression significantly alter expression of putative dicer proteins suggesting that these proteins may participate in the same pathway.
Appendices

Appendix A

A.1 Generation and identification of *Toxoplasma gondii* argonaute fused to a cherry fluorescent protein

In order to study the argonaute protein in *Toxoplasma gondii* (TgAgo) four strains of parasites were used: parental RH or RHΔHX strains, a TgAgo null mutant (obtained from Dr. Boothroyd, Stanford University School of Medicine), and a transgenic line over-expressing TgAgo_ChFP. The TgAgo_ChFP fusion protein was generated by utilizing a plasmid that contained the cherry fluorescence protein driven by a *T. gondii* tubulin promoter (pTub_eGFP_mChFP). The plasmid was digested with *Nhe*I and *Bgl*II to excise the GFP gene. The vector backbone was then extracted from an agarose gel, and used for the ligation reaction.

A.2 Preparation of TgAgo coding sequence

To acquire the coding sequence of TgAgo containing restriction sites that were compatible with the vector, the sequence was PCR amplified using oligonucleotides which incorporated the *Nhe*I and *Bgl*II sites on either end, and the PCR fragment was ligated into a pBluescript plasmid. This plasmid was transformed into *E. coli* cells and used for blue/white screening to identify clones that contained the target gene. The plasmid was confirmed by digestion with *Pvu*II and *Xho*I (Fig. A.1) and further digested with *Nhe*I and *Bgl*II (Fig. A.1) to obtain the TgAgo coding sequence which was extracted from an agarose gel, ligated with the pTub_ChFP vector, and transformed into *E. coli*. PCR amplification of an 800 bp fragment...
using internal oligonucleotides confirmed the presence of TgAgo in the isolated plasmids (Fig. A.1).

A.3 Confirmation of the pTubTgAgo_ChFP plasmid by restriction digestion and DNA sequencing

To ensure that TgAgo had been ligated into the vector in the correct orientation, restriction digestions were performed using \textit{XhoI} and \textit{PvuII} (Fig A.2), which produced the correct banding patterns. The plasmid DNA was sent to Genome Quebec Innovation Centre (Montreal, Quebec) for DNA sequencing analysis.
Figure A.1 Isolation of TgAgo and generation of TgAgo_ChFP. (A) Confirmation of the pBlueScript_TgAgo plasmid by digestion of the plasmid with PvuII and XhoI. The undigested plasmid was run as a control. (B) The pBlusk_TgAgo plasmid was doubly digested with NheI and BglII to isolate and extract the TgAgo coding sequence at 1581 bp which was further used for ligation reactions. (C) The pTubTgAgo_ChFP plasmid was used as a template for PCR amplification using internal oligonucleotides that amplify an 800 bp fragment from TgAgo. The pTubGFP_mChFP plasmid was used as a negative control. Approximate sizes are indicated on the left.
Figure A.2  Confirmation of pTubTgAgo_ChFP plasmid by restriction digestion. (A) XhoI and (B) PvuII enzymes were used to confirm the construction of the plasmid. Approximate sizes are indicated on the left. The correct banding patterns were obtained.
# Appendix B

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Appendix C

Figure C.1 Identification of an N-terminal fusion of ChFP_TgAgo. Western blot analysis was used to identify the expression of the N-terminal fusion of ChFP_TgAgo in *T. gondii*. The anti-RFP antibody was used to reveal a band at 85 kDa that corresponded to the fusion. The band at 27 kDa corresponds to a cherry cleavage and RH was used as a negative control.
References


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NAME: Urszula Liwak

PLACE OF BIRTH: Janow Lubelski, Poland

YEAR OF BIRTH: 1985

EDUCATION
University of Windsor, Windsor, ON, Canada
2007 – Present MSc Candidate, Biochemistry

University of Windsor, Windsor, ON, Canada
2003 – 2007 BSc, Honours Biochemistry and Biotechnology – Psychology minor
