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***Toxoplasma*: the next 100 years**

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Abstract

It has been 100 years since *Toxoplasma gondii* was initially described in Tunis by Nicolle and Manceaux (1908) in the tissues of the gundi (*Ctenodoactylus gundi*) and in Brazil by Splendore (1908) in the tissues of a rabbit. *T. gondii* is a ubiquitous, Apicomplexan parasite of warm blooded animals that can cause several clinical syndromes including encephalitis, chorioretinitis and congenital infection. Due to the extensive repertoire of applicable experimental techniques available for this pathogen it has become a model organism for the study of intracellular pathogens. Data obtained from genome wide expression studies, including ChIP on chip and proteomics surveys, are refining our understanding of the genetic networks involved in the developmental biology of this pathogen as well as the interactions of the parasite with its host. This review will address recent advances in our understanding of the developmental biology and host pathogen relationships of *T. gondii*.

Keywords

Toxoplasma gondii; Apicomplexa; cell biology; genetics; epigenetics; proteomics

1. Introduction to the parasite

Toxoplasma gondii was first identified over 100 years ago in the tissues of birds and mammals. Nicolle and Manceaux (1908) reported the first description of the asexual tachyzoite forms in the gundi, a North African rodent [1]. Splendore, in Brazil, simultaneously (1908) identified the parasite in the tissue of the rabbit [2]. Nicolle and Manceux named the genus *Toxoplasma* for its bow-like shape (from Greek: toxo= bow or arc; plasma=creature). Other forms of *Toxoplasma* including tissue cysts were also recognized, but it was not until the 1960s and 1970s that the parasite was recognized to be a coccidian parasite. The cat was identified as the definitive host by several groups working independently including Dubey and Frenkel (1970) [3]. Further details of the history of the discovery of the pathogen are described in recent reviews [4,5].

T. gondii is a ubiquitous protozoan parasite estimated to infect about one third of the world's human population. It is able to infect all warm-blooded animals and is a significant zoonotic and veterinary pathogen. In humans and veterinary hosts, *T. gondii* is frequently associated with congenital infection and abortion. *T. gondii* has received intensive scrutiny as an opportunistic pathogen associated with encephalitis or systemic infections in the immunocompromised, particularly individuals with HIV/AIDS.

The parasite can be transmitted by vertical transmission of the rapidly growing tachyzoite form, if an immunologically naïve mother acquires a new infection during pregnancy. In humans, *T. gondii* is most commonly acquired by oral ingestion of tissue cysts containing bradyzoites. Finally *T. gondii* can be acquired by ingestion of oocysts containing sporozoites that are the product of sexual cycle in cat intestines. Oocysts are very resistant to harsh environmental conditions and are highly infectious.

Once cysts or oocysts are ingested, they invade host cells and differentiate into tachyzoites. *T. gondii* is an obligate intracellular parasite and cannot be propagated axenically. Tachyzoite forms divide rapidly within host cells and are thought to be responsible for the clinical manifestations of infection. Tachyzoites are generally not readily transmitted due to their relative sensitivity to environmental conditions. Tachyzoites differentiate into latent bradyzoite forms, which are surrounded by a carbohydrate-rich cyst wall within the parasitophorous vacuole. This differentiation can be increased by exposure of the organism to stress conditions such as an immune response to the tachyzoites. These tissue cysts can persist indefinitely for the life of the host, perhaps due to a cycle of reaction and re-infection. If an individual becomes immunocompromised these tissue cysts serve as a reservoir from which disseminated or local infections can develop. The cysts have a predilection for neural and muscle tissue as well as the eye, with most cases of reactivation disease presenting as encephalitis or chorioretinitis.

Classically, consumption of undercooked meat, particularly pork and lamb has been ascribed to be the major risk factor for acquisition of toxoplasmosis. Improved animal husbandry practices as well as increased awareness of the risks of cooking undercooked meat have resulted in decreased prevalence of toxoplasmosis world-wide [6]. As illustrated by an outbreak in Victoria, Canada [7] and a better understanding of the epidemiology of toxoplasmosis in South America [8,9], oocysts transmitted via water or other environmental sources is a significant source of *T. gondii* infection. The association of *T. gondii* with waterborne outbreaks has led to its classification as a National Institute of Allergy and Infectious Diseases (NIAID) Category B priority agent.

T. gondii is unusual in that its propagation does not require passage through the definitive host (felids in whose intestinal tissues the sexual cycle occurs). Thus *T. gondii* can propagate clonally through intermediate hosts by ingestion of undercooked meats containing tissue cysts with bradyzoite forms. Analysis using enzyme zymodemes and single nucleotide polymorphisms (SNPs) has revealed that most *T. gondii* isolates from North America and Europe can be grouped into one of three genotypes [10,11]. Expansion of these lineages can be dated to about 10,000 years ago and is most likely related to the rapid expansion of the parasite when domestication of animals by humans became commonplace [12]. Type I strains grow rapidly in tissue culture and are highly virulent in mice. These strains seem to be more frequently associated with ocular toxoplasmosis and acute outbreaks [13]. Type II and Type III isolates are significantly less virulent in mice, and readily form cysts in vitro. Type II strains are most commonly isolated from clinical cases of toxoplasmosis, and it is not known if this reflects their being more common or that if these strains have inherent, but uncharacterized, differences that make them more likely to reactivate in human hosts. Recent data suggests that in many other parts of the world other genotypes predominant [14] and that these other genotypes may represent zoonotic isolates related to non-domesticated animal species.

Although these isolates have features of clonal expansion [15], in general their genotypes are more variable, suggesting that the sexual life cycle has been more important in the evolution of these *T. gondii* isolates [9,16].

2. Unique aspects of *T. gondii* cell biology

As an obligate intracellular parasite, *T. gondii* must successfully invade host cells and create a hospitable environment in which it can acquire nutrients, yet avoid killing by its host cell. The characteristic apical secretory organelles for which Apicomplexa were named, the micronemes, rhoptries and dense granules are specialized for the invasion and remodeling of the host. Elegant studies in the past two decades have revealed that the micronemes secrete a collection of adhesion proteins termed MICs that mediate host cell entry [17–19]. Rhoptry proteins and lipids, with the assistance of microneme protein AMA1 form the moving junction during invasion [20], eventually resulting in a parasitophorous vacuole that enables efficient procurement of nutrients and evasion of host immune defenses [21,22]. Proteomic analysis of the rhoptries has resulted in the identification of rhoptry neck proteins (RONs) in addition to traditional rhoptry proteins (ROPs) [23]. These RONs and ROPs are involved in different aspects of formation of the moving junction during invasion [20,24]. ROPs are also injected into the host cell resulting in extensive modification of host gene expression and signaling pathways [22].

The parasitophorous vacuole membrane is modified extensively by the parasite and contains multiple proteins that interact with host cell organelles including the host cell mitochondria and endoplasmic reticulum. The parasite modulates host signaling pathways including the apoptosis pathway and co-opts specialized aspects of vesicular transport machinery related to lipid acquisition. Finally, dense granule proteins appear to facilitate formation of specialized tubules that enable nutrient acquisition by the parasite [25] and some of these proteins, most notably GRA7 are also secreted into host cells [25].

Toxoplasma gondii, like most Apicomplexa, possess a novel nonphotosynthetic chloroplast – like organelle called the apicoplast. As an essential organelle, the apicoplast has been a topic of intensive interest both because of its biological uniqueness and its potential as a novel chemotherapeutic target. Antibiotics including clindamycin, tetracyclines, chloramphenicol and quinolones target apicoplast processes. The functions of the apicoplast include synthesis of heme, lipid biosynthesis and the isoprenoid pathway. The isoprenoid pathway is important in synthesis of the signaling molecule abscissic acid that regulates calcium dependent signaling in host invasion and egress [26].

3. Insights from the *T. gondii* genome

The availability of inexpensive DNA sequencing has revolutionized *T. gondii* research by making available genome sequences and thousands of ESTs (expressed sequence tags) from different life-cycle stages publicly accessible. Experimental data are gathered together at the website (<http://www.toxodb.org>). Currently 12x genome sequence is available for ME49, a commonly used Type II strain, which was the first *T. gondii* strain sequenced. Subsequently GT-1 (a Type I strain able to complete the entire life cycle) and VEG (a Type III strain also able to complete the life cycle) were sequenced. Alignments of the sequences of these three strains are now available as genetic maps and a single nucleotide polymorphism (SNP) analysis of these 3 major genotypes. Sequence for chromosome Ia and Ib from RH strain, a commonly used Type I laboratory strain, is also available [27].

Apidb.org is a coordinated effort to gather data for several Apicomplexa including *T. gondii*, *Plasmodium* species, *Cryptosporidium* as well as data from other sources [28,29]. These data include genome sequences, EST and other microarray expression data and proteomics data

with the goal of facilitating comparative research. Recent data on the *T. gondii* proteome (Albert Einstein Biodefense Proteomics Center) suggest that improved gene prediction models for the Apicomplexa are needed, and the multiple types of experimental data available will be invaluable for annotation of the genome.

4. The post genomics era

The availability of the genome sequence and ESTs, has directly facilitated the application of high throughput techniques to the study of *T. gondii* biology. Chief among these has been the study of gene expression. The patterns of gene expression during development were studied using SAGE (serial analysis of gene expression) [30] and then DNA microarrays. Early efforts with expression arrays have been reviewed [31]. As technology has improved and evolved, more sophisticated and comprehensive studies have been performed.

Expression profiles of *T. gondii* were first tested using cDNA arrays and were instrumental in identifying genes linked to virulence. Although limited in their representation of genes, the initial arrays provided new insights into biological processes including differences in gene expression during bradyzoite differentiation [31,32]. As the ME49 genome was completed, a new Affymetrix gene array was designed by the *T. gondii* community (www.toxodb.org) and initial results of studies with this array are available at <http://www.toxodb.org> on a gene by gene basis. These arrays are now being used for numerous applications, including expression analysis of progeny of genetic crosses [33,34], expression analysis of phenotypic mutants and comparisons of gene expression of different genotypes of *T. gondii* (www.toxodb.org). These studies promise to provide new insights into the biology of toxoplasmosis.

One of the most remarkable discoveries has been that *T. gondii* injects various signaling molecules into the host cell resulting in extensive remodeling of the host cell gene expression profile and metabolic pathways. The gene products so far are primarily rhoptry and dense granule proteins, and are implicated in organelle recruitment, modulation of host cell apoptotic pathways, as well as direct regulation of host gene transcription within the nucleus. Two of these genes, ROP16 and ROP18, are kinases that are secreted into the host cell cytoplasm during invasion [33–35]. Phosphatases are also secreted into the host cell cytoplasm [36]. Although the molecular targets of these parasite-encoded signaling molecules are unknown, genetic studies have firmly linked these genes to parasite virulence and critical phenotypic differences between the 3 major genotypes [33–35]. ROP18 localizes to the parasitophorous vacuole [37] whereas both ROP16 and a phosphatase are secreted from rhoptries and are trafficked to the host cell nucleus after parasite invasion. These molecules are reminiscent of the many effector molecules secreted by bacteria that alter host cell responses to infection [22]. The study of parasite-encoded molecules is in its infancy, but promise fascinating insights into the parasite host relationship.

The parallel completion of the genomes of the mouse and human has enabled interrogations of the host cell response in response to *T. gondii* infection. As more parasite effectors are identified, the host cell response of mutant parasites can also be studied. *T. gondii* infection results in significant alterations of the host cell transcriptome with changes in activation of immune regulatory pathways, upregulation of glycolysis and lipid pathways [38]. The nuclear localization of ROP16 and at least one phosphatase suggest that they mediate a direct effect on host transcription. These studies provide a direct link between metabolic activation and virulence of parasites within mammalian hosts.

Several groups have used host cell arrays to examine how host cell gene expression is modulated by parasite infection. ROP16 alters STAT signaling in the nucleus [35] and cytokine responses to different *T. gondii* genotypes varies. Other groups have identified key transcription factors such as the HIF1 (hypoxia induced factor) that regulate the pathways that are altered

in the host [39]. Human cell division autoantigen (CDA1) has been identified as a host protein involved in mediating the effects of the kinase inhibitor Compound 1 on triggering host cell changes that lead to bradyzoite formation [40]. Type 1 strains are resistant to the effects of compound 1 and do not exhibit induction of CDA1 [40], illustrating the complex interplay between parasite and host genes.

Further studies at the host pathogen interface promise to lead to further insights into the host immune response to toxoplasmosis. Epidemiological studies have linked toxoplasmosis to a variety of neuropsychiatric conditions [41], and as our knowledge of the host-parasite relationship improves, we are likely to develop further insights into how toxoplasmosis may modulate host gene expression or neural signaling pathways to act as contributor to other multifactorial diseases such as schizophrenia.

5. Epigenetics and epigenomics in *T. gondii* development

More recently whole genome arrays have been used to understand the role for epigenomic modifications in gene expression ([42]; Gissot, Weiss and Kim, unpublished). These arrays have the advantage that they are unbiased and therefore have the potential for new gene discovery as well as understanding the relationship of epigenetic modifications to gene expression. Although many phenotypic differences in *T. gondii* are genetically encoded, the parasite's developmental program and other response to changes in its environment are also likely to be epigenetically regulated. Parasite virulence in animals and propensity toward bradyzoite formation are influenced by prior propagation conditions. Epigenetic gene regulation provides a mechanism by which an organism can maintain a type of short term memory of its most recent environment, and can respond quickly to changing conditions. As *T. gondii* does not appear to have detectable DNA cytosine methylation [43], modification of chromatin structure, particularly posttranslational modification of histones is likely to be essential for coordinated regulation of gene expression.

Histones are conserved in *T. gondii* and H2A, H2B, H3, H4 as well as atypical histones have been cloned and characterized[44,45]. Further, histone modifying enzymes and modifications are conserved, with many commercial antibodies having cross reactivity to *T. gondii* histones [42,45–48]. Chromatin remodeling is biologically significant for developmental transitions but also may represent novel drug targets. Drugs like apicidin have activity against the protozoa [49] and microarray analysis reveals perturbation of bradyzoite gene expression in response to these inhibitors [31]. Several chromatin remodelers have been characterized. These include the histone acetyl transferases GCN5A and GCN5B (H3 [50] ; Myst (H4 [51])); histone lysine methylases (SET8; H4K20[48]); and histone deacetylases [47]. In general these enzymes have been shown to be enzymatically active with similar, although not identical, specificity to orthologues in other species.

Peaks of histone modifications H3K4me3, H3K9ac, and H4ac are coincident in the genome, and represent epigenetic modifications that correlate strongly with gene activation [42,47]. These marks can be used to predict regions of the genome that drive gene expression [42]. Correlation of these marks with cDNA expression enables a comprehensive survey of genes that are active and the epigenetic marks that identify active genes. Initial studies suggest that H3K9ac and H4ac marks become associated with bradyzoite genes and are no longer associated with tachyzoite-specific genes after bradyzoite differentiation [47]. The kinetics of the changes in chromatin modifications during stage specific regulation is an ongoing area of great interest and several groups are also attempting to identify chromatin marks that correlate with gene repression ([42,47,48]; Gissot & Kim, unpublished data).

Chromatin immunoprecipitation (ChIP) analysis of individual loci has been reported for individual epitope tagged chromatin remodelers ([47,48] and genome-wide ChIP-chip

experiments are feasible ([42,47,48]; Gissot, Weiss and Kim, unpublished). ChIP-chip analysis enables rapid identification of genes that are differentially expressed or regulated in the 3 major genotypes. While the majority of genes appear to be expressed similarly in the genotypes, a minority of genes are expressed differentially, have different promoters or other differences that make them worthy of further investigation. Further ChIP-chip and cDNA expression studies can be performed on progeny of the Type Ix III cross (GT-1xCTG; [52]) or Type IIxIII cross (ME49xCTG; [53]) to quickly ascertain the significance of their contributions to a phenotype of interest. Genome analysis of the repertoire of potential proteins involved in chromatin remodeling has been performed [45,54,55], but for most the function of these proteins has not been tested. A comprehensive analysis of the promoters associated with these regulators with gene expression analysis will enable the identification of co-regulated genes.

Recently a family of plant-like transcription factors of the AP2 family were identified using a bioinformatics approach [56]. The AP2 family members can be grouped according to their conserved DNA-binding domains and several appear to be conserved in several species of Apicomplexa [54]. In *P. falciparum*, where the prolonged 48 h life cycle has enabled an hourly monitoring of gene expression, expression of AP2 family members is stage specific and clusters with groups of genes expressed at specific times within the cell cycle [56]. Using bioinformatics several groups have demonstrated that clustered gene groups from *P. falciparum* have candidate motifs in their upstream regions [57–59]. *T. gondii* has over 50 members of the AP2 family, most of which are expressed in *T. gondii* tachyzoites (Gissot and Kim, unpublished). Intriguingly, AP2 family members are known to regulate developmental transitions and stress-response in plants.

6. Proteomics

Understanding the protein repertoire or differences in protein expression is also an essential aspect of elucidating biological phenomena. Proteomics efforts in *T. gondii* have included general surveys of the proteome of *T. gondii* tachyzoites (Wastling group data is available at www.toxodb.org) as well as other significant sub-proteomes such as membrane associated proteins (Albert Einstein College of Medicine Biodefense Proteomics Center, <http://toro.aecom.yu.edu/biodefense/>) and the cytoskeleton ([60]; Albert Einstein College of Medicine Biodefense Proteomics Center <http://toro.aecom.yu.edu/biodefense/>). There have also been focused studies characterizing particular cellular compartments or types of proteins. It has become clear with these studies that many of the gene prediction algorithms are imperfect. Thus, in addition to providing important insights into the protein repertoire, the cataloguing of proteins expressed by *T. gondii* provides a unique opportunity to refine gene annotations and gene prediction algorithms with experimental data (Weiss, Kim and Fiser, unpublished).

Parasite subproteomes have been particularly informative. The excreted secreted antigen protein profile consists of microneme and dense granule proteins. This repertoire of secreted proteins is surprisingly complex with evidence for multiple redundant adhesive complexes whose components undergo extensive proteolysis during organelle maturation and host cell invasion [61,62]. The rhoptry proteome was defined after subcellular fractionation of rhoptry organelles followed by a multiplatform proteomic analysis [23]. These studies identified numerous new candidate rhoptry proteins including the kinases and phosphatases discussed above that probably mediate the extensive remodeling of host gene expression and metabolism in response to parasites infection.

More recent studies have identified a more extensive and previously unappreciated set of glycosylated proteins. Glycosylation is probably significant for interactions with host cells as well as intracellular signaling [63,64]. Among the glycosylated proteins are rhoptry proteins, microneme proteins, and components of the glideosome, the molecular motor with which *T.*

gondii actively invades host cells [63,64]. Glycosylated proteins are also a major component of the cyst wall that forms during the stress-induced differentiation of bradyzoite forms [65, 66]. Proteomic analysis of these unique and biologically significant sub-proteomes will complement the genome wide studies currently underway.

7. Metabolomics, the next “omics” on the block

As intracellular parasites that undergo complex developmental changes within different hosts, *T. gondii* must constantly monitor and respond to environmental changes. How it senses these changes is unknown, but the parasite must possess the ability to sample changes in nutrients and other small molecules. In addition, the parasite must acquire many nutritional factors from its host and reprogram its gene expression profile in response to changes in available nutrients. An important “omic” that is likely to bridge data from expression arrays and proteomics is metabolomics. Correlations between the metabolites that the parasite sees in its host cells with global views of gene expression and protein profiles may lead to new insights into how the parasite senses and interacts with host cells while within the parasitophorous vacuole. For example, it was recently demonstrated that in *T. gondii* the phytohormone abscisic acid induced formation of the second-messenger cyclic ADP ribose, stimulated calcium-dependent protein secretion, and induced parasite egress from the infected host cell in a density-dependent manner [26].

Many cofactors involved in epigenetic changes or signal transduction are byproducts of essential metabolic pathways such as the amino acid, lipid, purine and polyamine pathways. *T. gondii* has acquired the ability to alter host metabolic pathways. Despite efforts, it cannot be cultivated axenically, and must require as yet unknown factors from its host. Identification of these molecules will further our understanding of the biology of the parasite and its intracellular life style.

8. Challenges for the future: moving beyond “omics” into systems

The availability of high throughput technologies for sequencing, proteomics, transcriptomics, epigenomics and metabolomics has changed the nature of research approaches from hypothesis driven to hypothesis generating. Frequently one asks how does protein or gene expression change in response to a different condition, stimulus or among different genotypes. The challenge is to then integrate these many sources of data into novel hypothesized that can be tested by conventional lines of experimental, by computational modeling or by additional high-throughput experiments. In the face of the huge amounts of data generated by the “omics” efforts, novel approaches will be needed to integrate data and generate platforms for data analysis.

Remarkable progress has been made in understanding the life cycle of *T. gondii* in the 100 years since its discovery, but many mysteries remain about its developmental cycle and its intimate relationship with its hosts. Integration of the many sources of genomic, proteomic and metabolomic data promises fascinating insights into how *T. gondii* has evolved into such a successful parasite.

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