THE USE AND IMPACT OF DNA MICROARRAYS IN *Toxoplasma gondii* RESEARCH - A REVIEW

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ABSTRACT

Toxoplasma gondii is arguably the most successful parasite worldwide. The organism is thought to be capable of infecting all warm-blooded animals including humans and can be found in most regions of the world. Infection with the parasite can result in a wide spectrum of clinical signs depending on the host animal species. *Toxoplasma* infection can be fatal for some species of sea mammals and marsupials, as they have largely evolved separately from the parasite. In other species, such as humans and some farm livestock species, congenital infection is common, resulting in disease within the developing foetus. Thus the parasite is of significant medical and veterinary importance worldwide. *Toxoplasma* is also of immense interest to biologists researching host–parasite relationships and looking to understand why this parasite has so successfully adapted to and exploited such a diverse range of hosts. The mechanisms by which *Toxoplasma* grows within its host cell, encysts, and interacts with the host's immune system are important questions. Here, we will discuss how the use of DNA microarrays in transcriptional profiling, genotyping, and epigenetic experiments has impacted our understanding of these processes. Finally, we will discuss how these advances relate to toxoplasmosis and how future research on toxoplasmosis can benefit from DNA microarrays.

Keywords: DNA-Microarrays, *Toxoplasma gondii*, Toxoplasmosis, Host–parasite relationships, Parasitology.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular Apicomplexan parasite that can infect a wide range of warmblooded animals including humans[1]. *Toxoplasma gondii* was initially discovered by accident, in 1908, by a scientist, Charles Nicolle, who was working in North Africa and searching for a reservoir of *Leishmania* in a native rodent, *Ctenodactylus gundi*[2]. The gundis live in the foothills and mountains of Southern Tunisia and were commonly used to study *Leishmania* at the Pasteur Institute in Tunis. The name *Toxoplasma* means 'arc form' in Greek and was named according to the crescent-shaped morphology of the tachyzoite and bradyzoite stages of the organism observed by the scientists. At about the same time, Alfonso Splendore working in Sao Paulo discovered a similar parasite in rabbits[3].

This pathogen is one of the most common in humans due to many contributing factors that include: (1) its complex life cycle allows it to be transmitted both sexually via felid fecal matter and asexually via carnivorism. (2) *Toxoplasma* has an extremely wide host cell tropism that includes most nucleated cells. (3) In humans and other intermediate hosts, *Toxoplasma* develops into a chronic infection that cannot be eliminated by the host's immune response or by currently used drugs. In most cases, chronic infections are largely asymptomatic unless the host becomes immune compromised. Together, these and other properties have allowed Toxoplasma to achieve infection rates that range from ~23% in the USA[4] to 50–70% in France[5,6].

In humans and other intermediate hosts, infections are the result of digesting parasites shed in felid feces or present in undercooked meat[6]. Both infection routes result in the infection of intestinal cells after which the parasites develop into tachyzoites, which are the fastgrowing, disseminating form of the parasite. Tachyzoites replicate within intestinal cells where they stimulate recruitment of neutrophils and dendritic cells. The parasite can then infect these immune cells and use them to disseminate throughout their hosts[7,8]. Once parasite reach their target tissue they respond to the resulting IFNγ-based Th1 response by transforming into bradyzoites. Ultimately, bradyzoites will form quiescent tissue cysts that do not cause any significant disease[9]. Bradyzoite conversion is a critical step in the parasite's life cycle since bradyzoites are impervious to immune-mediated destruction, are relatively non-immunogenic, and are the infectious form of the parasite during horizontal transmission (e.g. digestion of undercooked meat). Thus, it is critical that tachyzoites evade IFNγ-induced death while they convert to bradyzoites. The molecular details underlying each of these processes are largely unknown but are important because these data could lead to the development of new drugs to treat the infection.

The past decade has seen important developments in the molecular tools to study *Toxoplasma gondii*. These include the development of transfection technologies[10-12], sequencing of both host and parasite genomes (see www.toxodb.org), increasing use and refinement of highthroughput genomic and proteomic technologies[13-19], sensitive whole animal imaging[20-22], and large-scale mutagenesis-based screens[23-24]. These technologies and

approaches have been instrumental in increasing our understanding of *Toxoplasma* replication within its host cell, bradyzoite development, and virulence mechanisms. In this review, we will focus our discussion on how the use of DNA microarrays and other high-throughput transcriptome analysis contributed to these developments and the implications these findings have for toxoplamosis.

The role of host cell transcription in *Toxoplasma gondii* growth

A common requirement for intracellular pathogens is they must scavenge nutrients from their hosts while avoiding innate host defense mechanisms[25]. *Toxoplasma* is no different and how it replicates within a host cell has been the focus of intense investigation by several laboratories. Biochemical and cell-biological-based assays demonstrated that parasites modify host microtubule and intermediate filament organization[26-28], inhibit host cell apoptosis[29-30], upregulate pro-inflammatory cytokines[31-34], and scavenge purine nucleosides, cholesterol, and other nutrients from their host cells[35,36].

To examine the molecular basis for these changes, DNA microarrays have been analyzed to identify changes in host gene expression following infection[11,19,37]. These studies indicated that changes in host transcription were extremely widespread. These changes came in at least two distinct waves with the first wave being induced within 2 hours and included a large number of pro-inflammatory response genes[11]. Besides the inflammatory response genes, the first wave of gene expression also included genes (EGR1, EGR2, c-jun, and jun-B) that encode transcription factors commonly activated in response to cellular stresses. These data suggests that activation of these genes helps the infected host cell withstand the stress of a *Toxoplasma* infection. Upregulation of these genes is not a general feature of a cell's response to infection since these genes were not modulated in host cells infected with either *Trypanosoma cruzi*[38] or the closely related Apicomplexan parasite, *Neospora caninum*[39]. This result indicated that parasite activation of these transcription factors is accomplished through a *Toxoplasma*-derived molecule that interacts with a specific host protein. One mechanism by which *Toxoplasma* can specifically signal to its host cell is by the release of proteins from the rhoptries, which are specialized secretory organelles that contain proteins secreted into the host cytoplasm and nucleus, in a manner analogous to bacterial Type III secretion systems[40]. Consistent with rhoptries being key regulators of host cell functions, upregulation of EGR2 and, likely the other immediate early response host transcription factors, is mediated by a rhoptry factor[39].

The second wave of gene expression included genes that encode proteins that function in a diverse set of cellular processes. Most striking from these studies was the finding that glucose, mevalonate, and iron metabolic genes were upregulated specifically by *Toxoplasma*[13]. This was intriguing because these genes function in pathways related to some of *Toxoplasma*'s auxotrophies. Thus, their upregulation may be necessary to increase levels these nutrients for the parasite to scavenge. Expression of the glycolytic and iron genes (as well as other genes also observed in the microarray experiments) is regulated by a common transcription factor named hypoxia inducible factor 1 (HIF1)[41,42]. HIF1 is a heterodimer composed of α and β subunits and stabilization of HIF1α is the rate-limiting step to its activation. Consistent with the array data, *Toxoplasma* increased HIF1α proteins levels, activated HIF1-dependent transcription, and required HIF1α for parasite growth[43].

Host cell cycle modulation is a common cellular target for many types of pathogens[44]. When both proliferating and non-proliferating cells are infected with *Toxoplasma*, genes commonly associated with cell growth are differentially modulated[13,15]. These data suggested that like other intracellular pathogens, *Toxoplasma* is actively modulating the host's cell cycle. This hypothesis was tested by several groups who demonstrated that parasite infection leads to changes in host cell cycle progression and causes cells to arrest at the G2/M border[18,46,47]. *Toxoplasma*'s effect on the host cell cycle was cell type independent, which was consistent with the microarray data noting differential expression of these genes in various types of infected host cells. Surprisingly, both replicating and senescent cells were similarly affected. The specific parasite factor(s) that regulate the host cell cycle is unknown but at least one appears to be a secreted factor larger than 10 kDa[47]. The importance of such an extrinsic-acting factor is unknown but could be to optimize neighboring cells for infection.

Besides regulating expression of metabolic and cell cycle genes, anti-apoptotic transcripts are a third major class of genes upregulated in *Toxoplasma*-infected cells. Given that apoptosis of cells infected with viruses and bacteria is one host defense against infection[48,49], it is logical that *Toxoplasma* would actively prevent host cell apoptosis and appears to do so by interfering with both extrinsic and intrinsic induction of apoptosis[50]. The extrinsic pathway is activated by death signals such as TNFα and FAS and is dependent on Caspase 8. *Toxoplasma* interferes with this process by blocking Caspase 8 activity[51]. In contrast, parasite modulation of the intrinsic pathway, which is activated by intracellular stress and the subsequent release of cytochrome C from the mitochondrion, is dependent on the host cell transcription factor NF-κB[52]. NF-κB is a family of five different proteins and several of these have been observed to be activated in parasite-infected host cells[53-55]. The NF-κB-regulated genes required for parasite inhibition of host cell apoptosis are unknown but candidates include the antiapoptotic gene bcl2 as well as several members of the IAP family.

Turning on and off the switches that regulate bradyzoite development

Bradyzoite development is a complex process in which the parasite expresses enzymes that allow it to form a cyst wall while dramatically altering its metabolism and immunological characteristics[9]. These changes are important because bradyzoite-containing cysts are impervious to host defenses and currently prescribed anti-toxoplasmotic drugs. In addition, bradyzoite differentiation is a critical step in the parasite's life cycle since cysts are a transmissible form of the parasite during horizontal transfer. Molecular characterization of the genes encoding bradyzoite-specific antigens indicated that their stage-specific expression was due, in large part, to increasing the abundance of the transcripts that encode them[56-58]. It was not until the transcriptomes of bradyzoites and tachyzoites were directly compared by either comparative EST sequencing or SAGE analysis that the extent of these changes were realized[59-61]. Although these studies were critical in allowing us to appreciate the complexity of the transcriptional changes that take place during development, the laborious nature of preparing and analyzing EST and SAGE libraries limited their ability to assess the dynamic nature of bradyzoite development.

In contrast, an important advantage of DNA microarrays is that they can readily examine multiple time points and conditions[61]. As a first step, microarrays spotted with the cDNAs used for the bradyzoite EST sequencing project[60] were generated and used to compare the transcriptional responses that take place at various time points following induction of differentiation[15]. Although these first generation microarrays were spotted with fewer than 650 unique genes, they demonstrated that the microarrays could be used to discover additional bradyzoite-specific genes. Besides gene discovery, DNA microarrays can also be used to map transcriptional pathways. As an example, the transcriptional response of wild-type parasites and bradyzoite differentiation mutants were compared after stimulating the parasites to undergo differentiation. The resulting microarray data demonstrated that the transcriptional pathways induced during development were hierarchal[62,63].

The full complexity associated with differentiation was demonstrated using full-genome *Toxoplasma* microarrays that compared the transcriptional responses of three distinct *Toxoplasma* strains to a drug that induces bradyzoite development[64]. Analysis of the 5' proximal promoters of some bradyzoite-specific genes identified a short 6–8 bp sequence that conferred stage-specific expression. Electrophoretic mobility shift assays indicated that a parasite nuclear factor binds this promoter element64. This was a significant finding because *Toxoplasma*'s genome (and those of other Apicomplexan parasites) lacked genes that shared homology with most known transcription factors. Recently, however, a novel transcription factor family (ApiAP2) was identified in *Plasmodium falciparum*[65] and subsequent sequence analysis indicated that *Toxoplasma*'s genome possesses approximately 40 ApiAP2 family members[66]. Given the lack of other known transcription factors and the large number ApiAP2 proteins, it is likely that one or more members of this family will be involved in bradyzoite development.

Post-translational modifications of histones (e.g. acetylation and methylation) are a widespread epigenetic mechanism critical for regulating gene expression[67]. Due to a lack of experimentally confirmed bradyzoiteinducing transcription factors, investigators began testing whether bradyzoite-specific gene expression could be regulated by epigenetic remodeling. These experiments demonstrated that the promoters of bradyzoite-specific genes in parasites growing under tachyzoite conditions have low levels of acetylated histones and become more extensively modified after exposure to bradyzoite-inducing conditions[68,69].

Histone acetylation is a reversible modification controlled by enzymes that add (histone acetyltransferases (HATs)) or remove (histone deacetylases (HDACs)) acetyl groups from specific lysine residues in the various histones. The importance of histone acetylation was demonstrated by inhibiting HDAC3, a histone H4 deacetylase. Treatment of parasites with a HDAC3 inhibitor (FR235222) induced tachyzoite to bradyzoite differentiation. DNA complexes co-immunoprecipitated using α-acetyl-H4 antibodies and hybridized to a high-resolution *Toxoplasma* DNA microarray demonstrated that the promoters of many but not all bradyzoite genes were hyper-acetylated on histone H4[70]. But the fact that not all bradyzoite-specific genes harbored this modification suggests that other histone modifications may be important for regulating these genes. Altogether, these data paint a picture in which differentiation is controlled transcriptionally by both DNA binding proteins and by epigenetic-based histone modifications. But how all of these changes come together to convert a tachyzoite to a bradyzoite remains unclear.

Besides epigenetic control of *Toxoplasma* gene expression during bradyzoite differentiation, HATs are expressed in tachyzoites[71,72] and the expression of some tachyzoite-specific genes are epigenetically regulated[69,73]. An extremely high-resolution DNA oligonucleotide Toxoplasma microarray representing a well-annotated region of Chromosome Ib was used in CHIP-on-chip assays to characterize the organization of active and silent promoters in tachyzoites[74]. This study demonstrated that the location and organization of specific modifications of acetylated and methylated histones within the genome could predict not only whether a promoter was active but the 5′–3′ orientation of the gene it was regulating.

As an intracellular pathogen, the interplay between the parasite and host cell is likely to have an impact on all aspects of the parasite life cycle including bradyzoite development. An example of this interplay came from the observation that a novel drug named Compound 1 stimulated bradyzoite development in specific

Toxoplasma strains[75]. Multifactorial microarray analysis of RNA isolated from Compound-1-treated, mock-infected, or parasite-infected host cells led to the discovery that overexpression of a human gene named CDA1 was sufficient to promote bradyzoite development. CDA1 encodes a protein whose overexpression leads to cell cycle arrest suggesting the status of the host's cell cycle determines if a parasite will undergo bradyzoite development. This is an intriguing hypothesis given the observations that bradyzoites appear to preferentially develop in cells such as neurons and muscle cells that have exited from the cell cycle[76-78].

Virulence

The population structure of *Toxoplasma* is extremely clonal and the genotypes of the majority of *Toxoplasma* strains isolated in North America and Europe group into one of three clonal lineages (types I, II, and III)[79,80]. In mice, type I strains are highly virulent while the other two are significantly less so[81]. Although all *Toxoplasma* strain types can cause disease in human infections, type II strains are more commonly associated with congenital infections and toxoplasmic encephalitis while type I and other atypical strains are more commonly associated with postnatally acquired infections that lead to ocular disease[82,83]. Understanding the basis for differences between *Toxoplasma* strain types is important for two reasons. First, optimal treatment options to either prevent or cure reactivated infections may be dictated by the parasite's genotype. Second, optimal vaccine design necessitates identifying non-polymorphic antigens.

Toxoplasma virulence is a multi-step, complicated process comprised of transmission, dissemination, host immune evasion, encystation, and reactivation. Although it was commonly accepted that multiple parasite genes would be important for virulence, the first experimental data that this was true came from the finding that a cross between either two avirulent genotypes (types II and III) resulted in virulent progeny[84]. Quantitative trait locus (QTL) mapping of these progeny identified five virulence loci[85]. Thus far, two of these virulence genes have been identified as ROP16 and ROP18. These virulence genes encode rhoptry kinases that are secreted into infected host cells. In an independent study, QTL mapping of progeny from a type I/III cross also identified ROP18 as a virulence gene[86]. One way that the different strains may affect virulence is by differentially modulating host gene expression[14]. Based on human DNA microarray analysis, over 3,000 host genes were differentially expressed in cells infected with progeny from the type II/III cross[14]. Expression QTL mapping of these differences in host gene expression indicated that at least one locus on each parasite chromosome is responsible for differential expression of a portion of these host genes. Modulation of the largest number of these genes (~1,100 genes) mapped to a single locus on Chromosome VIIb and ROP16 was determined to be the gene responsible for these differences in host gene expression. Pathway analysis indicated that many ROP16- modulated genes were targets of the STAT3/STAT6 transcription factors. How ROP16 specifically regulates STAT3/ STAT6-dependent expression is unknown but infection with parasites harboring the type III allele of ROP16 (which is identical to the allele in type I strains) leads to sustained activation of STAT3/STAT6 [12]. Given ROP16's role in virulence it is therefore tempting to speculate that sustained STAT3/STAT6 activation causes an overproduction of proinflammatory cytokines that can induces immune-mediated tissue destruction.

NK and T-cell-derived IFNγ is the critical cytokine in protection against infections with all *Toxoplasma* strains[87]. This cytokine protects against *Toxoplasma* infections by upregulating the expression of inducible nitric oxide synthase, indoleamine dioxygenase, and a family of IFNγ-regulated GTPases that degrade the parasitophorous vacuole (reviewed in[88] Regardless of its effectiveness, some parasites can evade IFNγ-mediated killing and develop into bradyzoites. One possible mechanism by which the parasite avoids IFNγ is to disable IFNγ-induced signaling. Indeed, microarray and cell biological assays demonstrated that IFNγ-induced transcription is abrogated in cells previously infected with *Toxoplasma*[89,90]. In contrast to the polymorphic ROP16 and ROP18 virulence factors, *Toxoplasma*'s effects on IFNγ-dependent transcription are strain independent[90]. The mechanism underlying parasite abrogation of IFNγ-stimulated transcription is still unclear but does not appear to involve blocking nuclear localization of STAT1, which is a key IFNγ-regulated transcription factor. However, infection upregulates the expression of members of the suppressor of cytokine signaling (SOCS) family that function by preventing sustained STAT1 activation or levels[91]. Thus, the parasite may utilize a feedback regulatory mechanism to reduce IFNγ-dependent signaling. It should be noted that others have failed to find evidence of SOCS-mediated inhibition of IFNγ signaling since they did not detect differences in STAT1 protein levels in mock- or parasite-infected cells[89,92]. Instead, these studies suggest that the parasite affects the ability for STAT1 to bind DNA.

Implications for toxoplasmosis

As a parasite with a potentially devastating clinical outcome, an important goal of toxoplasmosis research is the development of new drugs and treatments. There are two major reasons that new drugs are needed to treat *Toxoplasma* infections. First, the drugs currently used to treat *Toxoplasma* infections are poorly tolerated, have severe side effects, and cannot act against bradyzoites[93,94]. Second, there are reports that *Toxoplasma* is developing resistance to the current generation of drugs[95,96]. How resistance to these drugs has developed is not known but is critical to understand because it will lead to improved drug design and will increase our understanding of the biological functions of these drug targets. One way to understanding mechanisms of resistance is to compare the transcriptional profiles of wild-type and resistant

parasites grown in the absence or presence of the drug. Such studies in bacterial resistance have demonstrated that pathogen responses to antibiotics are multifactorial and complex[97]. Whether the same will be true in *Toxoplasma* is unclear, but data from these types of experiments will likely impact new anti-*Toxoplasma* drug design.

SUMMARY

Over the past decade, the application of host and parasite microarrays have allowed *Toxoplasma* researchers to make great strides in understanding how *Toxoplasma* grows, differentiates, and causes disease. The majority of these experiments have thus far focused on tissue culture-based experimental systems or death-as-endpoint virulence studies. Relative to these systems, our understanding of how *Toxoplasma* interacts with and causes disease has been lagging. But the techniques and technologies that these other studies have pioneered (e.g. microarrays, QTL screening, and epigenetic mapping) coupled with highthroughput DNA sequencing and proteomics, will allow ocular toxoplasmosis researchers to make important and rapid advances in the very near future.

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