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Review

The Past, Present, and Future of Genetic Manipulation in *Toxoplasma gondii*

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***Toxoplasma gondii* is a classic model for studying obligate intracellular microorganisms as various genetic manipulation tools have been developed in *T. gondii* over the past 20 years. Here we summarize the major strategies for *T. gondii* genetic manipulation including genetic crosses, insertional mutagenesis, chemical mutagenesis, homologous gene replacement, conditional knockdown techniques, and the recently developed clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system. We evaluate the advantages and limitations of each of these tools in a historical perspective. We also discuss additional applications of modified CRISPR–Cas9 systems for use in *T. gondii*, such as regulation of gene expression, labeling of specific genomic loci, and epigenetic modifications. These approaches have the potential to revolutionize the analysis of *T. gondii* biology and help us to better develop new drugs and vaccines.**

Toxoplasmosis: A Zoonotic Disease in Need of Better Therapeutics

Toxoplasmosis is one of the most important zoonotic parasitic diseases, caused by the obligate intracellular protozoan *T. gondii*, which is capable of infecting all warm-blooded animals including humans [1,2]. It is estimated that one-third of the world's population is chronically infected with *T. gondii* [1,2]. Although *T. gondii* infection is usually asymptomatic in immunocompetent people, it can cause severe complications in immunocompromised individuals. Furthermore, infection during pregnancy can lead to miscarriage, stillbirth, or severe congenital defects including blindness, mental retardation, and hydrocephaly [1,2]. Unfortunately, the strategies used to prevent or cure *T. gondii* infection in humans or livestock are limited and not ideal [3]. An improved understanding of the biology of the parasite will facilitate the identification and characterization of new targets and strategies for intervention. The ability to genetically manipulate the genome of *T. gondii* is central to these advances. Therefore, understanding the molecular pathways of parasite pathogenesis and the ability to dissect gene function at the molecular level are crucial for developing effective vaccination strategies and better therapeutics.

Due to its medical importance and ease of growth in tissue culture, *T. gondii* has received considerable scientific and medical attention and is considered an important model organism for the study of obligate intracellular microorganisms [4]. A range of genetic tools has been developed to analyze gene functions in *T. gondii* [5,6]. Using *T. gondii* as a study model, we recapitulate and describe the currently available molecular genetic systems and potential applications of CRISPR–Cas9 systems in *T. gondii* to provide clues for genetic manipulation in other intracellular microorganisms.

Trends

The transfection technologies first introduced into *Toxoplasma gondii* two decades ago opened the way for molecular manipulation of the parasite.

Numerous genetic tools are now available for *T. gondii*, including a several strategies for conditional knockdown of essential genes.

The recently developed CRISPR–Cas9 system has been adapted to *T. gondii* to allow gene disruption and point mutations and to introduce epitope tags.

We review the advantages and disadvantages of the technologies currently available for genetic manipulation of *T. gondii* and discuss new CRISPR–Cas9-based systems that may be applied to *T. gondii* in the near future.

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Selectable Markers Available to Use with *T. gondii*

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The ease of culturing the infectious stages of the parasite *in vitro*, along with the availability of numerous selectable markers and expression platforms that can be readily introduced into parasites by electroporation, makes highly tractable the study of the biology of *T. gondii* by genetic means (Figure 1). *T. gondii* can be easily propagated in cultured nucleated cells. The efficiency of introducing DNA into *T. gondii* by electroporation can be up to 15%, with less than 20% parasite viability loss during electroporation [7]. In general, most transfected parasites lose newly introduced DNA after a few generations if it is not integrated into the genome. Therefore, numerous selectable markers have been developed to select for stable transformants with heritable genetic changes [8–13]. Chloramphenicol acetyltransferase (CAT) [8], the pyrimethamine-resistant allele of dihydrofolate reductase–thymidylate synthase (*DHFR-TS*) [9], hypoxanthine–xanthine–guanine phosphoribosyltransferase (*HXGPRT*) [10], and bleomycin/phleomycin-binding protein (Ble) are the most commonly used systems for positive selection [11]. The uracil phosphoribosyltransferase (*UPRT*) and *SNR1* (TGME49_290860) loci, conferring resistance to 5-fluorodeoxyribose (FUDR) [12] and sinefungin [13], respectively, on inactivation, are frequently used for negative selections, such as for use in genetic complementation. *HXGPRT* can be used for both positive and negative selection but requires a *HXGPRT*-deficient strain for positive selection [10]. Fluorescent reporter genes can also be used for enriching transformed parasites with fluorescence-activated cell sorting (FACS) [14]. Moreover, selectable markers can be removed from the *T. gondii* genome by Cre recombinase if they are flanked by *loxP* sites [15].

Forward Genetic Tools Available for *T. gondii*

Forward genetics – approaches used to identify the genetic basis of a specific phenotype – have been successfully employed in *T. gondii* in several ways, including genetic crosses, chemical mutagenesis with whole-genome sequencing (WGS), and random insertional mutagenesis. *T. gondii* isolates from Europe and North America have an unusual population structure comprising three dominant clonal lineages (types 1, 2, and 3) [16]. However, with more strains isolated from other parts of the world, it was recently shown by Su *et al.* [17] that the *T. gondii* population structure is more complex than simply three dominant clonal lineages and can be divided into 16 haplogroups within six major clades. Despite being similar genetically, their biological traits can be broadly different [18]. In most laboratory mice, type 1 strains are highly virulent, with $LD_{100} = 1$, whereas type 2 and type 3 strains are less virulent, with $LD_{50} = 10^2$ and 10^5, respectively [16–18]. Additionally, the host genetic background also affects the virulence of *T. gondii*, such as polymorphism of the mouse immunity-related GTPases [19]. Forward genetic approaches based on sexual crosses in cats were first demonstrated by the Pfefferkorn laboratory in 1980 using two drug-resistant lines [20]. Subsequent crosses were created by several laboratories for use in quantitative trait locus (QTL) analysis, which led to the discovery of genes responsible for the phenotypic differences between the types 1, 2, and 3 clonal strains. For example, ROP18 and ROP5 were identified as virulence factors by genetic mapping using progenies derived from 1×3 and 2×3 and 1×2 and 2×3 crosses, respectively [21–24]. Several other genes have also been identified using genetic crosses (e.g., *ROP16* [25], *GRA15* [26], *GRA25* [27]), demonstrating the potential value of this approach. Despite these strengths, this forward genetic approach does not allow the identification of non-polymorphic genes that are conserved in all strains. Another major hurdle for genetic crosses is the use of cats to enable sexual reproduction of the parasites. Performing genetic crosses in cats is rather intractable and requires special handing and biological containment due to the highly infectious oocysts. To overcome this limitation, the development of *in vitro* systems is needed to allow *T. gondii* sexual recombination in cultured cells such as intestinal epithelia cell lines derived from the cats. If successfully developed, such methods would dramatically accelerate genetic-cross studies. Given these limitations, it is unsurprising that only a few crosses have been conducted to date [21–27]. However, with the affordability of WGS in *T. gondii* genetic analysis of progenies derived from crosses became much easier [28].