Strongyloides stercoralis and relatives: recent advances in general and molecular biology

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Abstract

Human strongyloidiasis is a threat to global health, presenting significant challenges in diagnosis and clinical management. The imperative to incorporate strongyoidiasis more fully into control programs for soil-transmitted helminths is increasingly recognized. The unique life cycles of *S. stercoralis* and congeneric species contain both free-living and parasitic generations, and transcriptomic methods have recently identified genes of potential importance to parasitism in these parasites. Proteomics recently revealed stage-specific secreted proteins that appear crucial to the host-parasite interaction. A comprehensive genome sequencing project for *Strongyloides* spp. is now nearing completion. Recent technical advances in transgenesis for *S. stercoralis* and *S. ratti*, including the first establishment of stable transgenic lines, promise to advance functional evaluations of genes expressed in conjunction with crucial life cycle events. Studies employing these methods recently bolstered the hypothesis that *S. stercoralis* uses cellular signaling pathways homologous to three that regulate dauer larval development in *Caenorhabditis elegans* to regulate morphogenesis and development of its infective third-stage larva. The free-living generation of *Strongyloides* makes classical genetics formally possible. Recent advances, such as a genetic map of *S. ratti* and a molecular genetic and karyotypic analysis of sex determination in *S. papillosus*, will greatly facilitate this approach. Advanced methods for study of chemosensation in *C. elegans* were recently applied to discover numerous host attractant molecules that mediate host finding and contact by infective third-stage larvae of *Strongyloides* spp. Finally, nucleic acid-based diagnostic methods have recently come to the fore as alternatives to parasitological and immunodiagnostic techniques.

Keywords

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Introduction and basic biology

Organismal biology and significance to human health

Strongyloides stercoralis is a significant human pathogen and also a parasite of dogs. Estimates of the human infection burden range from 30–100 million people, primarily in tropical and subtropical areas of sub-Saharan Africa, Southeast Asia and South America [1]. It is likely that these are under estimates of the true infection burden due to the poor sensitivity of diagnostic methods hinging upon on direct detection of larval *S. stercoralis* in patient stools. These will probably be revised upward as new molecular diagnostic methods for *S. stercoralis* infection (reviewed below) are incorporated into routine clinical practice [2, 3]. Salient aspects of the *S. stercoralis* life cycle, such as the percutaneous route of infection by infective third-stage larvae (L3i) and their migration from the systemic circulation to alveoli of the lung and subsequently via the tracheae to the digestive tract, invite comparison to the life cycles of hookworms and, excluding the percutaneous route of infection, ascarid roundworms parasitizing humans and dogs [3]. Therefore, uncomplicated *S. stercoralis* infection predictably shares certain clinical manifestations with human and canine hookworm infection, including dermatitis at the point of L3i penetration, pulmonary signs such as dry cough and wheeze due to larval migration through the lung and enteritis including epigastric pain, vomition, dysphagia and diarrhea [1]. However, the life history of *S. stercoralis* differs fundamentally from the life histories of other soil transmitted nematodes in its ability to complete a full generation of free-living development outside the host and, with significant implications for pathogenesis, to undertake the process of autoinfection in which first-stage larvae elaborated by parasitic females develop precociously to autoinfective L3 (L3a) in the intestines of the primary host (Fig. 1). These L3a then penetrate the gut of the primary host, migrate to the lungs via the systemic circulation and, via tracheal migration, establish a new generation of parasitic adults in the intestine. Sequential rounds of autoinfection may occur in an individual host. In immunocompetent hosts, autoinfective generations may occur at a low, well-regulated level, but in hosts immunocompromised by HTLV-1 infection or by treatment with corticosteroids or other immunosuppressive drugs, sequential generations of autoinfection can result in geometric expansion of parasite numbers, invasion of multiple organ systems including lungs, liver and brain and sepsis resulting from systemic release of enteric bacteria. Disseminated hyperinfections of this type may run a fatal course in two to three days [1, 3]. Low, well-regulated levels of autoinfection, allowing gradual replacement of senescent parasitic female worms, have been hypothesized as a mechanism by which immunocompetent hosts maintain exceedingly chronic, subclinical *S. stercoralis* infection, often for decades after the last exposure to L3i [4, 5]. The literature is now replete with clinical reports of patients, frequently elderly migrants from endemic areas, in whom such exceedingly chronic subclinical infections progress to acute, sometimes fatal disseminated hyperinfection as a result of treatment with corticosteroids or other immunosuppressive drugs [6].

Just as the host-phase of *S. stercoralis*' developmental cycle and its clinical manifestations invite comparison to infection by hookworms and other soil transmitted nematodes, the environmental phase of this developmental cycle, which involves a single generation of free

living development with male and female adults (Fig. 1) invites comparison to both free-living nematodes and to soil transmitted parasitic species. It is therefore of significant interest from a standpoint of comparative and evolutionary biology, which are reviewed in detail below. Importantly, crossing between adults of the free-living generation [7] affords *S. stercoralis* an opportunity for sexual recombination not present in the host phase in which only parthenogenetic females are present [8]. As will be discussed below, the free-living generation of *S. stercoralis* and related species also allows both forward and reverse experimental genetics in this species at a level that is thus far impractical in other species of parasitic nematode [9–14•, 8, 15, 16].

Evolutionary biology: *Strongyloides* as a model for the evolution of parasitism in nematodes

The ability of *S. stercoralis* and congeneric parasites to undertake both obligately parasitic and free-living modes of life makes these worms apt subjects for studies of the molecular genetic requirements for vertebrate animal parasitism in nematodes and of the acquisition of these characteristics during evolution from free-living ancestors. It is worth noting that in their homogonic phases of development (Fig. 1), *Strongyloides* spp. are *bona fide* endoparasitic nematodes with the life cycles that superficially resemble those of hookworms, with skin penetrating L3i, postparasitic larval stages that migrate somatically via pathways that include the lung, parasitic adults measuring some 2.0–2.8 mm in length, and, in the case of *S. stercoralis*, an adult lifespan of approximately one year in the host small intestine and progeny that are eliminated of from the host in feces in the form of eggs or hatchling first-stage larvae, which develop either directly or indirectly to L3i in fecally contaminated soil (Fig. 1). Strikingly, the same parasitic adult *Strongyloides* spp. can produce subsets of progeny that, upon elimination from the host, are fated to develop to a generation of males and females (Fig. 1) that have many of the basic biological attributes of obligately free-living nematodes like *C. elegans*. They live in the soil and feed on microbes that are abundant due to fecal contamination. They reproduce sexually and have a lifespan of 72–96 hours. Therefore, the life cycle of *S. stercoralis* and of congeneric parasites contains two distinct adult morphs, one that is parasitic, long-lived and parthenogenetic and another that is free-living, short-lived and dioecious. These life cycles also contain two distinct patterns of larval development in the extrinsic environment. One of these, exhibited by larval progeny of parasitic females, involves sex determination (Fig. 1) and switching between homogonic (directly to L3i and then to parasitic females) and heterogonic (to L3i via a free-living generation of males and females) developmental fates by genetically female larvae (Fig. 1). It is noteworthy that genetically male larvae in this post-parasitic phase of the life cycle all develop to free-living adults (Fig. 1). The other program of larval development in the extrinsic environment occurs in the progeny of free-living males and females. In *S. stercoralis*, all of these progeny are genetically female, and all of them develop to L3i, which persist in the soil and continue their development only when they infect a susceptible mammalian host (Fig. 1). Therefore, the life cycle of *S. stercoralis* and the life cycles of *Strongyloides* spp. generally, offer the opportunity to compare parasitic and non-parasitic morphs of selected life stages, such as parasitic and free-living females, and homogonically and heterogonically developing first- and third-stage larvae that arise from the same genome. The remarkable differences in body plan, ecological niche and developmental fate that such

comparisons reveal at the at the organismal level invite comparisons at the molecular level aimed ideally at determining patterns of gene expression that are unique to and possibly essential for establishing and maintaining the parasitic state. These are discussed further in the segments on developmental biology and comparative transcriptomics below.

Developmental biology: cellular signaling in the infective process

Recent studies of developmental regulation during the infective process in *S. stercoralis* have been driven by the long-standing "dauer hypothesis", which posits that cellular signaling pathways homologous to those regulating dauer larval development in *C. elegans* regulate the formation and further development of L3i in parasitic nematodes [17, 18]. This developmental progression in parasitic nematodes such as *Strongyloides*, filariae and hookworms includes the crucial infective process in which environmental L3i resume their development upon invasion of a permissive mammalian host. Under the dauer hypothesis, these regulatory pathways would include a G protein coupled receptor (GPCR) signaling pathway in amphidial neurons that transduces physicochemical cues of host approach and contact to regulate the synthesis of insulin-like peptides (ILPs) and TGFβ-like peptides, which regulate their respective signaling pathways in numerous tissues in the worm that are remodeled or function in morphogenesis and development of L3i. Parallel insulin- and TGFβ-like signals would act in concert on a downstream dafachronic acid-nuclear hormone receptor signaling pathway to ultimately regulate development by L3I during the infection process. Recent findings have demonstrated that GCPR signaling is likely involved in developmental activation in L3i following host invasion as reflected by resumption of feeding by these larvae [19•] (Fig. 1). 8-bromo-cyclic GMP (8-br-cGMP) stimulated feeding in dose-dependent fashion by L3i cultured in the absence of host-like physico-chemical cues such as elevated temperature (37° C) and the growth promoting components of Dulbecco's Modified Eagles Medium (DMEM). Notably, RNAseq indicated that, concomitant with it's promotion of feeding, 8-br-cGMP also stimulates patterns of abundance of insulin-like peptide encoding transcripts *Ss-ilp-5* and *Ss-ilp-6* in L3i that are highly similar to those seen in L3i following 48 hours' development in susceptible gerbils [20•]. These data are consistent with genetic evidence from *C. elegans* [21] that GPCR signaling acts upstream of ILS to regulate the development of dauer third-stage larvae.

Building upon earlier findings on the insulin-regulated transcription factor, Ss-DAF-16 (originally designted FKTF-1) [22], further evidence that insulin-like signaling regulates L3i development (Fig. 1) appeared recently in a study confirming that *S. stercoralis* has conserved the insulin-regulated phosphoinositol-3-kinase (PI3K) encoded in *C. elegans age-1* and that the PI3K inhibitor LY294002 can block resumption of feeding by L3i grown under host-like culture conditions [23]. A full molecular characterization of the insulin receptor kinase encoding gene *Ss-daf-2* was recently completed [24] and included abundance profiles of its two encoded isoforms in seven life stages of *S. stercoralis*. This study employed transgenesis to identify the intestine as the primary site of *Ss-daf-2* expression.

Recent studies [19•] also tested the hypothesis that insulin-like signaling acts upstream to promote dafachronic acid Ss-DAF-12 signaling in *S. stercoralis*. Dafachronic acid-DAF-12

signaling promotes continuous reproductive development and, presumably, dauer recovery in *C. elegans* [25]. Prior studies had demonstrated that the DAF-12 nuclear hormone receptor is conserved in *Strongyloides stercoralis* and in *Ancylostoma caninum* and that exogenously applied Δ7-dafachronic acid (Δ7-DA) stimulates post-infective development by L3i of both parasites at mid-nanomolar concentrations, similar to its activity range in *C. elegans* [26]. Data from studies of genetic epistasis [27] and experimental data showing that exogenous Δ7-DA suppresses a dauer constitutive loss-of-function mutation in the insulin receptor kinase DAF-2 [25] all support the hypothesis that in *C. elegans* dafachronic acid-DAF-12 signaling regulates dauer larval development downstream of ILS. Surprisingly, however, a kinetic comparison of developmental activation by *S. stercoralis* L3i in response to 200 μM 8-bromo cGMP and 400 nM Δ7-DA showed that Δ7-DA induces the response in cultured L3i more rapidly than 8-bromo cGMP. Also contrary to expectations based on the *C. elegans* paradigm, RNAseq conducted on populations of *S. stercoralis* L3i that had been treated with 400 nM Δ7-DA revealed patterns of *Ss-ilp-5* and *Ss-ilp-6* transcript abundance that were virtually identical to those induced in *S. stercoralis* L3i by treatment with 200 μM 8-bromo cGMP and by developmental activation in a susceptible mammalian host. Results of a third experiment were also contrary to expectations under the *C. elegans* paradigm in showing that treatment with 400 nM Δ7-DA fails to override 10-fold reduction in the feeding response imposed on cultured *S. stercoralis* L3i by the PI3k inhibitor LY294002 [19•]. Overall, recent findings further support the conclusion that GPCR signaling, insulin-like signaling and dafachronic acid Ss-DAF-12 signaling all regulate developmental activation of *S. stercoralis* L3i during the infective process (Fig. 1). Coupled with findings on developmental trends in abundance of transcripts encoding TGFβ-like ligands in S. stercoralis, they also support the recent assessment by Crook [17] that the weight of current evidence supports the "Dauer Hypothesis" as it pertains to GPCR, insulin-like and dafachronic acid-DAF-12 signaling in parasitic nematodes, but not as it pertains to TGFβ signaling, which appears to act in parasitic nematodes in direct opposition to what would be predicted under this hypothesis, prompting the alternate interpretation that TGFβ-like ligands, which in most cases are strongly upregulated in L3i, may serve an immunomodulatory function protecting these invasive larvae as they begin their interactions with the host following infection.

Assembly of the vulva in young parasitic females is crucial to oviposition and therefore to propagation of *Strongyloides* spp. in the environment. A recent histochemical investigation of vulvar assembly in *S. venezuelensis* underscored a central role for the cytoskeleton, and most specifically filamentous actin in this process.

Developmental biology: sex determination

As recently reviewed [12], parasitic female *Strongyloides* spp. reproduce by mitotic parthogenesis, making individuals in the post-parasitic generation genetically identical to their mothers. Strikingly, there is sex determination in this generation such that a proportion of progeny becomes genetically male and develops to free-living adult males, and a proportion remains genetically female and capable of switching between development directly to L3i, which subsequently invade the host, or to free-living females (Fig. 1). Recent findings on the effect of environmental temperature on the infectivity of *S. venezuelensis* larvae in mice [29] probably reflects the practical consequences of skewing this switching

event towards direct development to the L3i by elevated temperature. Karyotypes of *S. ratti* and *S. stercoralis* consist of two pairs of autosomes and a pair of sex (X) chromosomes in females and a single X chromosome in males, all chromosomes being of medium length, reflecting an XX/XO sex determination. By contrast, karyotypes of *S. papillosus* have been alternatively characterized as consisting either of a pair of long (L) chromosomes and a pair of medium length (M) chromosomes or of a single L chromosome, three M chromosomes and a single short chromosome. Nemetschke et al. [12] have recently used molecular genetic techniques to confirm previous speculation that the regions homologous to the X chromosomes of *S. ratti* account for the elongation of L chromosomes of *S. papillosus* and that conflicting descriptions of the karyotype of *S. papillosus* reflect observations before and after a chromatin diminution event giving rise to a male karyotype of 1L3M1S and a female karyotype of 2L2M. Characterization of the eliminated chromosomal region in *S. papillosus* revealed that it contains many genes [12] in contrast to the chromatin diminution observed in *Ascaris* spp. where eliminated chromosomal regions contain a majority of non-coding DNA. Most significantly, Nemetschke et al. [12] observed that remains of the L chromosome undergoing diminution are excluded from mature sperm in *S. papillosus*, thus functionally reconstituting the XX/XO sex determination system seen in the related species *S. ratti* and *S. stercoralis*. Further commentary on this unique sex determination system in *S. papillosus* and the accompanying general analysis of sex determinatrion in *Strongyloides* spp. was published contemporaneously [30].

Host-parasite 'communication'

Since its L3i infect the host percutaneously, *S. stercoralis* and its congeners have evolved a set of behaviors that increase the probability of host contact and as well as sensitivity to a multiplicity of attractive olfactory cues emanating from their respective mammalian hosts. Previously, the only such chemoattractant to have been characterized at the molecular level was urocanic acid a common component of human and other mammalian skin [31]. More recently, Castelletto et al. [32] have undertaken an extensive comparison phylogenetically diverse parasitic nematodes, including entomoparasitic species as well as mammalian parasites in which L3i infect the host by active skin penetration (*S. stercoralis, S. ratti,* and *Nippostrongylus braziliensis*) or by passive ingestion (*Haemonchus contortus*). Overall, host preference appears to be a stronger determinant of specific olfactory sensitivities than phylogeny among the parasitic nematodes studied. For example, L3i of two distantly related rodent parasites, *S. ratti* and *N. braziliensis*, responded to subsets of olfactory cues that were more similar to each other than to the subsets attracting L3i of *S. stercoralis*. In general, L3i of *S. stercoralis* were characterized as fast migrating "cruisers", which increase the probability of host contact by continuous spontaneous movement across horizontal substrates. By contrast, L3i of the passively ingested *H. contortus* were characterized as "ambushers", which exhibit little unstimulated migration but rather ascend blades of grass where they are more liable to be ingested by their grazing ruminant hosts. L3i of *S. stercoralis* were attracted to a wide range of human associated odorants including several of the most active ones, such as 3-heptanol, 1-nonanol and both 2- and 3-methyl-1-butanol, that are also strong chemoattractant for host seeking mosquitoes. That these cues act as volatile odorants as opposed to aqueous gustatory stimuli was underscored by the fact that the L3i

responded to them when they were spotted on the inner side of the assay plate lid as well as when they were applied to the agar layer upon which the worms migrated.

In addition to the direct chemical communication between host and parasite that mediates host finding and contact by L3i, there is substantial evidence (reviewed in [33]) that parasitic females of *S. ratti*, *S. ransomi*, and by extension *Strongyloides* spp. generally, are induced to produce greater proportions of male progeny and a population of female progeny with an increased tendency towards heterogonic (free-living) development in response to a mounting protective immune response by the host. It is striking, therefore, that microarray analyses reveal only modest (< threefold) differences in transcript abundance between parasitic female *S. ratti* recovered at five and nine days of infection from naïve rats and from rats that had been immunized by drug-abbreviated infection [34]. Prominent among the few transcripts upregulated in the presence of the host immune response were those encoding homologs of UNC-87 and UNC-54 in *C. elegans*, both proteins related to muscle function. These findings are certainly consistent with the repair of muscle in the parasitic female damaged by the immune response, as discussed by the authors, but their relation to altered sex determination and developmental fate among the progeny remains obscure. The increased sensitivity and resolution enabled by RNAseq, relative to microarray analysis may help to clarify the molecular basis for this profound influence of host immunity on subsequent developmental fate in the progeny of surviving parasitic females.

Studies at the genomic, transcriptomic and proteomic scales

Genome sequences

At the time of this writing, draft genome sequences for *S. stercoralis*, for the congeners *S. ratti*, *S. papillosus* and *S. venezuelensis* and for the related outgroup *Parastrongyloides trichosuri* are currently under annotation by collaborators at the Wellcome Trust Sanger Center (Hinxton, Cambridge, UK). Current versions of these draft genome sequences are available to the public via file transfer at ftp://ftp.sanger.ac.uk/pub/project/pathogens/HGI/.

The genome of *S. stercoralis* comprises 43 megabases (mb) and 13,114 genes. In general the *S. stercoralis* genome has significantly fewer and smaller introns than *Caenorhabditis elegans* and parasitic nematodes representing Clades I, III and V [35]. For example, the S. stercoralis genome contains 21,058 introns (9.4% of total genomic sequence) with a median length of 52 bp. By contrast, *Haemonchus contortus*, in Clade V along with *C. elegans*, has 171,763 introns (27.7% of the genome) with a median length of 168 bp. Comparable data for *Ascaris suum* in Clade III are 100,624 introns comprising 37.7% of the genome with a median length of 690 bp. These findings suggest a rapid loss of introns during the evolution of parasitic nematodes such as *S. stercoralis* in Clade IV.

The transcriptome

High-capacity or "next-generation" sequencing technology has enabled the recent creation of some robust transcriptomic databases for *S. stercoralis*. One of these, reported by Stoltzfus et al. [20•], contains sequences of polyadenylated RNA from seven life stages of the parasite selected for their key roles in the infective process, in the maintenance of

parasite populations in the host and external environment, and in the property unique to *Strongyloides* and related Clade IV parasites of developmental programming for either free-living or parasitic fates. These stages are: actively reproducing free-living females (FL Females), post-free living first-stage larvae (PFL L1), infectious third-stage larvae (L3i), post-infective third-stage larvae (L3+) recovered 48 hours after inoculation into susceptible gerbils, gravid parasitic females (P Females), predominantly (95%) heterogonically developing post parasitic first-stage larvae (PP L1), and post-parasitic larvae, approximately third-stage, developing heterogonically to free-living adults and predominantly female (PP L3) (Fig. 1). High-quality sequences were derived by Illumina HiSeq technology from 100 bp paired end reads from polyadenylated RNA libraries prepared in biological triplicate from each stage. This resulted in a total of 2.36 billion reads, of which 74% aligned to the 41 megabases of *S. stercoralis* genomic contigs available at the time of the study.

Another recent transcriptomic study of *S. stercoralis* L3i that employed next-generation sequencing techniques [36] emphasized prediction of proteins involved directly in the host parasite interaction as opposed to developmental regulatory molecules intrinsic to the parasite. Of the over 11,000 proteins predicted by transcripts sequenced in this study, most were novel and were predicted to include an abundance of enzymes such as kinases and proteases and over 1,200 excretory secretory proteins. Some of the proteins predicted in this study were potential drug candidates. Next-generation sequencing has also been employed to create transcriptomic resources for other species of *Strongyloides*, such as *S. venezuelensis* [37, 38], which is used widely as a model for immunological studies. Altogether these studies encompassed four developmental stages: eggs/first-stage larvae (L1), environmental L3i, L3i derived from the host lung and parasitic females [37, 38].

Valuable insights into the transcriptomic changes undergone by *S. stercoralis* at crucial life cycle transitions have also been gained recently through microarray analyses. Recent microarray-based studies of *S. ratti*, and earlier studies cited therein stress comparisons of parasitic and non-parasitic morphs of the parasite [39] and also of parasites living in the environments of immune and naive hosts [34]. Ramanathan et al. [40] amassed microarray data from post-parasitic L1 and L3i of *S. stercoralis* that reveal patterns of differential gene expression associated with the acquisition of host infectivity during that transition. These, along with the biological conclusions of the other transcriptomic studies cited above, are discussed in the following segment.

Comparative transcriptomics

The overall rubric of discovering 'genes for parasitism' has driven a series of recent transcriptomic studies of developmental stages in *Strongyloides* spp, initially using profiles of EST abundance and protein expression [41•–43] and, more recently, microarray technology [40] and next-generation sequencing [36, 37, 34, 20•]. Studies focusing explicitly on detecting genes essential for parasitism in *Strongyloides* have taken the approach of identifying expressed sequence tags (ESTs) from *S. ratti* that do not have identifiable homologs in *C. elegans* and that are unique to or significantly up regulated in parasitic females relative to free-living females. This unbiased approach identified a panel of seven ESTs unique to or up regulated parasitic females [42, 43]. Subsequently, these results

were confirmed by qRT-PCR analysis for five of the ESTs, the genes characterized by bioinformatic analyses and the expression dynamics of their encoded mRNAs and, for three candidates, their encoded proteins were described [41•]. Of the three parasitism-associated genes undergoing complete analysis in this study, two, SR00007 and SR00449, encode homologs of chondroitin proteoglycan 3 (CPG-3) in *Ascaris suum* (Table 1). The protein encoded in one of these, SR00449, is expressed in parasitic females of *S. ratti* and is secreted into the worm's surroundings in the host. Transcripts encoding the other CPG-3 homolog, SR00007, are also present in parasitic female *S. ratti*, or in the eggs they carry *en utero*, but these transcripts are not expressed until the post parasitic L1 hatch. A similar pattern of maternal or en utero transcription followed by translation in newly hatched post parasitic L1 occurs in a third parasitism-specific gene in *S. ratti*, SR00984. This gene has significant homology to those encoding small heat shock proteins in *Trichinella pseudospiralis* and in *C. elegans* (Table 1). All three of the parasitism-associated gene products that were characterized by Spinner et al. [41•] are recognized by antibodies in the sera collected from rats in the early phase of *S. ratti* infection prompting the question of whether they may induce a protective response in this host, which effectively suppresses egg output by *S. ratti* within the first 25 days of infection [44]. Although preliminary data suggest that immunization with one or more of these parasitism-associated proteins may decrease the reproductive output of *S. ratti*, further experimentation is required to conclude this [41•]. Nevertheless, this approach demonstrates the power of unbiased transcriptomic screening to identify parasitism-associated gene products for follow-up evaluation as targets for intervention with novel drugs or vaccines. Microarray analysis of transcriptomic differences between post-parasitic L1 and L3i of *S. stercoralis* [40] revealed upregulation of transcriptional 'machinery' in the L1, as would be expected in these rapidly differentiating larvae. Among the transcripts increasing in abundance in the L3i were those encoding the potential chemotherapeutic targets HSP-90 and cytochrome oxidase ucr 2.1, an ortholog of the fatty acid retinol binding protein that formed the basis of an effective vaccine against *Ancylostoma ceylanicum* and the sperm-containing glycoprotein domain that constitutes the target of a vaccine against *Cooperia punctata*, an important trichostrongyle parasite of ruminant livestock. These findings provide a sound rationale for exploring these chemotherapeutic and vaccine strategies in human strongyloidiasis.

Adopting a more hypothesis-driven approach, Biewener et al. [45] used targeted qRT-PCR to demonstrate that in *Strongyloides papillosus*, transcripts encoding an aspartic protease, *Spa*-ASP-2 are upregulated in L3i during the process of skin penetration, and those encoding the lysozyme *Spa*-LYS are downregulated at this crucial point in the infective process. The hypothesis that these transcripts would be regulated at the point of percutaneous migration was based on findings that one isoform of a homologous aspartic protease is preferentially expressed in parasitic females of *S. ratti* [46] and that similar lysozymes are among the excretory-secretory products of the hookworm *Ancylostoma caninum* [47].

While the transcriptomes resulting from the RNAseq study by Stoltzfus et al. [20•] represent a wealth of information on developmentally regulated genes affecting all aspects of S. stercoralis' biology, the study itself emphasized abundance profiles of transcripts homologous to genes in four cellular signaling pathways that regulate dauer larval development in *C. elegans*, this being a hypothetical framework for regulation of L3i

development in parasitic nematodes [18]. Some findings, such as the upregulation of specific G protein-coupled receptor signaling elements in the L3i were expected given the necessity of this larval stage detecting the presence of chemical and thermal cues from the host. Also expected was the presence of insulin-like peptides (ILPs) that are coordinately regulated during development of L3i of *S. stercoralis*. A surprising finding in this aspect of the study was the marked reduction in the number of transcripts encoding different ILPs from 40 in *C. elegans* to only seven in *S. stercoralis*. Four of the seven *S. stercoralis* ILPs may be categorized by whether their transcript abundance rises or falls during post-infective developmental activation of L3i in the host, patterns that would be consistent with agonists or antagonists, respectively, of Ss-DAF-2, the ortholog of the dauer regulatory insulin-like receptor kinase in *C. elegans* [48]. Findings from the RNAseq study by Stoltzfus et al. [20•] confirmed previous observations that transcripts encoding homologs of the TGFβ-like ligand DAF-7, which suppresses dauer formation in *C. elegans*, are actually most abundant in L3i, a pattern that is inconsistent with conservation of *C. elegans*-like DAF-7 function in *Strongyloides*. Finally, transcripts encoding *S. stercoralis* homologs of enzymes catalyzing biosynthesis of dauer-regulatory steroids, dafachronic acids, in *C. elegans* [25] were not coordinately regulated during L3i development.

Salient among the transcriptomes of four key life stages of *S. venezuelensis* [37, 38] are transcripts encoding an expanded repertoire of astacin-like zinc metalloproteases, and a ferrochelatase unique to animal parasitic nematodes, which may catalyze the reverse of the ultimate reaction in heme biosynthesis to scavenge iron from host porphyrins [49]. Also discovered in *S. venezuelensis* was a cluster of transcripts, upregulated in lung-derived L3i, that encode enzymes catalyzing biosynthesis of secreted glycans that are likely to be important in interactions with the intestinal epithelium of the host [37, 38].

The proteome

As of this writing, systematic proteomic studies of *S. stercoralis* have not been undertaken. By contrast, the proteome of *Strongyloides ratti*, particularly the secreted proteome or "secretome", is under active investigation [50•, 51] with particular emphasis on excretory/secretory proteins from the parasite that could modulate immune or other defensive mechanisms in the host intestinal mucosa. The secretome of *S. ratti* comprises 586 proteins, of which 196 are secreted exclusively by L3i [50•]. Prominent in the secretome of L3i are an astacin metalloprotease, likely used to penetrate the host skin, the L3 Nie antigen and a fatty acid retinoid binding protein (Table 1). Among the 79 proteins secreted exclusively by parasitic females are a prolyl oligopeptidase, some small heat shock proteins and a secreted acidic protein. Free-living stages of *S. ratti* uniquely secrete 35 proteins including a lysozyme, a carbohydrate-hydrolyzing enzyme and a saponin-like protein. Most significantly, Soblik et al. [50•] showed that small molecule inhibitors of the secreted prolyl oligopeptidase suppressed motility of cultured parasitic female *S. ratti* in dose dependent fashion, within the low micromolar range, providing evidence that this protein is essential for survival of these stages. Two homologs of *C. elegans* HSP-17, *Sra*-HSP-17.1 and *Sra*-HSP-17.2, which are secreted by parasitic female *S. ratti*, are immunogenic in infected rats, and *Sra*-HSP-17.1 binds to cultured rat epithelial cells, suggesting a role for this protein in the host parasite interaction. Host monocytes exposed to either *Sra*-HSP produced IL-10 but

not TNF-α, suggesting a role for these proteins in directing the host immune response (Table 1). In general, these recent studies [50•, 51] underscore the power of the proteomic approach in identifying potential targets for immunological or chemotherapeutic intervention. Transcriptomic data arising from next-generation sequencing provides a wealth of data that could be used to predict the secretomes of *Strongyloides* spp. A semi-automated computational approach to this process has been developed [52] and verified in *S. ratti* by comparing predictions derived *in silico* to known profiles of excretory-secretory proteins in this parasite.

Linkage mapping

Classical genetic methods, which depend to a large degree on the ability to establish experimental crosses between individuals differing in a trait of interest, have not been used to any significant degree in animal parasitic nematodes because adult males and females of most species are, in practical terms, inaccessible due to their obligate endoparasitic association with the host. One of the great advantages of *Strongyloides* spp. as an experimental model for parasitic nematodes is that all species in the genus are capable of developing through at least one generation of free-living male and female worms. Genetic exchange between these males and females on mating has been confirmed for *S. ratti* [7] and likely pertains throughout the genus. This makes *Strongyloides* spp. the only group of animal parasitic nematodes in which genetic crosses are practical and therefore the only likely subjects for classical genetic study among this important group of pathogens. One prerequisite for locating and ultimately identifying genetic loci encoding traits of interest through crossing is a genetic linkage map. Nemetschke et al. [13•] recently generated such a genetic map for *S. ratti* by crossing two isofemale lines and backcrossing the progeny to create an F2 in which genetic linkage of 74 molecular markers was analyzed. This analysis generated a map predicting three linkage groups, consistent with chromosome number in *S. ratti*, and having an average density of one marker per 1.27 cM. The map also correctly predicts the XX-XO pattern of sex determination in free-living *S. ratti* adults [12]. This map will greatly facilitate classical, and ultimately molecular genetic analysis of crucial traits such as anthelmintic resistance in *S. ratti*, and, by proxy, in other animal parasitic nematodes. The power of such a genetic map as a tool for analyzing genetic cross data and defining the molecular basis of an anthelmintic (oxamniquine) resistance trait through classical genetics was recently demonstrated in the parasitic trematode *Schistosoma mansoni* [53].

Functional genomic methods

The same limitations in accessibility to reproductive adult animal parasitic nematodes that hamper classical genetic study have also limited the application of modern functional genomic techniques such as transgenesis in these worms. As it did for classical genetic study, the availability of a generation of free-living males and females of short duration (two to three days) in the life cycles of *Strongyloides* spp. made it possible, nearly a decade ago, to translate standard methods for transgenesis in *C. elegans* to these parasites as well as the related genus *Parastrongyloides* [54, 9, 10]. Specifically, similarities in both the gross and fine structures of the gonads of *C. elegans* hermaphrodites and free-living female *Strongyloides* made it relatively easy to adapt methods for introducing transgene DNA

constructs by gonadal microinjection of *Strongyloides* spp (Fig. 1). Early on, studies in *Strongyloides* were limited to observations that could be made in transgenic worms of the F1 generation, owing to apparent silencing of transgenes encoded in standard plasmid vectors in the F2 generation and beyond [9]. Recently, however, we have learned that transgenes that are integrated into the chromosomes of *S. ratti* via the *piggyBac* transposon system are inherited stably and maintain their expression indefinitely through alternating rounds of host and culture passage [14•]. This advance resulted in establishment of the first stable transgenic lines of an animal parasitic nematode. In addition to *piggyBac*-mediated chromosomal integration of transgenes, success in this effort hinged on the use of *S. ratti*, which, as a natural parasite of the rat, has a well adapted rodent host in form of the laboratory rat. As all of our prior work on trangenesis had been done with *S. stercoralis*, we showed, preparatory to the study on *piggyBac*-mediated integration, that *S. ratti* could also be transformed by gonadal microinjection of plasmid-based vectors and that 5′ and 3′ regulatory sequences from *S. stercoralis* gave identical patterns of transgene expression in both *S. stercoralis* and *S. ratti* [11]. Potential applications of this approach to stable transgenesis in *S. ratti* and prospects for its translation to *S. stercoralis* are discussed in "Future Directions" below.

Nucleic Acid-based Diagnosis

Free parasite nucleic acids in diagnostic specimens

Relative to parasitological diagnosis of other soil transmitted human helminthiases, the sensitivity of single direct stool examination for larvae of *S. stercoralis* is notably low, and is only moderately improved by methods such as the Baermann technique and Koga's agar plate method, which involve amplification of parasite numbers by rearing through the free-living generation [3]. In addition to the search for more reliable immunodiagnostic methods, the realization that parasite-specific free DNA is voided in the feces of human strongyloidiasis patients [55–60] has sparked keen interest in developing nucleic acid-based diagnostics for this parasitism.

Diagnostic PCR targets and comparisons to parasitological and immune-diagnosis

PCR primer sets for detection of *S. stercoralis*-specific DNA in fecal samples are designed most often to amplify regions of the 18s ribosomal RNA gene [56–58, 60]. Other genes targeted for this purpose in various assay schemes have been the internal transcribed spacer 1 (ITS1) region of the ribosomal RNA gene [55], a region of the 28s ribosomal RNA gene conserved in *S. stercoralis* and five additional *Strongyloides* species [59], the cytochrome c oxidase, subunit 1 gene [60] and the *Strongyloides*-specific repeat sequence AYO28262 [60]. In some cases these primer sets have been incorporated into multiplex PCR-based assays designed to detect not only *S. stercoralis* DNA, but that of other species of soil-transmitted helminth as well [58, 56]. Where evaluations were done, PCR-based assays were virtually 100% specific for *S. stercoralis* [58, 59, 57, 60]. When compared to direct fecal examination, PCR-based methods resulted in a two- to sevenfold increase in detection rates where any samples were positive by direct fecal examination [58, 60]. Similarly, a PCR-based method discovered three infected individuals among 400 patients that were negative by direct fecal examination [56]. However, when compared to more sensitive parasitological

techniques for diagnosis of *S. stercoralis* infection, those involving culture amplification, real-time PCR targeting the 18s ribosomal RNA gene proved highly sensitive with cultures containing high to medium numbers of parasites, but it detected only 15% of cultures with low (1–5 larvae) numbers of parasites per culture [57]. This underscores the reality that PCR is subject some of the same factors, such as discontinuous distribution of parasites (and perhaps parasite DNA) through the sample, that limit the sensitivity of direct microscopic fecal examination. Other factors that limit the sensitivity of PCR-based diagnosis of *S. stercoralis* and other enteric pathogens are the presence of inhibitory chemicals or viral DNA in stool [57]. An encouraging finding from the real-time PCR results in samples with low numbers of larvae per culture was that positive real-time PCR amplifications were characterized by higher Ct values than the results from cultures with moderate to high numbers of larvae per culture, thus indicating that real-time PCR has a semi-quantitative capability that may be valuable in follow-up evaluations of mass chemotherapy programs.

Future Directions

Recent calls for heightened awareness of the global health impact of human strongyloidiasis and for its explicit incorporation into integrated control programs for soil transmitted helminths [2, 1, 3] should stimulate renewed interest in the epidemiology of *S. stercoralis* infection and of the factors predisposing chronic strongyloidiais patients to disseminated hyperinfection. A comprehensive genome project, comprising not only *S. stercoralis*, but also *S. ratti*, *S. papillosus*, *S. venezuelensis* and *Parastrongyloides trichosuri*, is nearing completion, and the annotated genomes of these parasites, with a comparative analysis of them, will be published in the very near future (Berriman et al., In Preparation). It is also likely, and altogether warranted, that the increasing ease and decreasing cost of next-generation sequencing will allow RNAseq to come to the fore as the method of choice for transcriptomic comparisons of life stages of interest. It will be important for individual researchers to make data from such studies publicly available. One crucial transcriptomic comparison, which has not yet been undertaken to the author's knowledge, is between environmental L3i of *S. stercoralis* and infective third-stage larvae arising within susceptible hosts during the process of autoinfection. If a subset of genes regulated preferentially in autoinfective L3 could be identified, these might contain potential targets for intervention to prevent the most serious complications of human strongyloidisis. From a standpoint of basic developmental biology, products of such autoinfection-associated genes might represent essential controls over a process that is unique to *S. stercoralis* infection among all the soil-transmitted helmenthiases.

Recent advances in transgenesis in *S. stercoralis* and *S. ratti*, especially the new capability to prepare stable transgenic lines of parasite should facilitate functional study of many of the putative 'genes for parasitism' identified in the transcriptomic studies discussed above. Gain-of-function studies, which would involve assessing phenotypes associated with over expression of genes of interest, are possible with current techniques for transgenesis. Still lacking for *Strongyloides* spp. are loss-of-function methods such as RNAi or specific gene disruption that have been proven to be such powerful functional genomic tools in other model organisms. One current method for gene editing that is now being applied in *C. elegans* is the CRISPR /Cas9 system, which allows precise targeting of gene sequences for

insertion or deletion of key elements [61•–63]. Methods for heritable transgenesis in *Strongyloides ratti* as discussed above open the formal possibility that this powerful technique can be applied to this species and proof-of-principle for the CRISPR/Cas9 should be a top near-term research priority for the *Strongyloides* community. Similarly, augmentation of double stranded RNA transport proteins in *Strongyloides* spp. by heterologous gene transfer from *C. elegans* is also formally possible and could constitute a means of boosting RNAi sensitivity, which is relatively low in members of this genus.

In the area of classical genetics, the new linkage map for *S. ratti*, which is discussed above, affords the ability to use genetic crosses to map phenotypic traits to specific loci in the genome. It will be of great interest to see this powerful forward genetic approach applied in the near future. Discovery of an array of host-associated chemoattractant molecules represents a significant advance in understanding the process of host finding and contact, a crucial prerequisite to the infective process in *S. stercoralis*. Application of new functional genomic methods to understand how these chemical cues are transduced in the worm will be a key priority in the near future. The trend towards application of nucleic acid-based diagnostic methods to human strongyloidiasis will likely increase in the near future. The recent discovery of parasite specific microRNAs circulating in the plasma of hosts infected with schistosomes [64] and filariae [65] calls for investigation of the possibility that *S. stercoralis*-specific microRNAs also circulate in the plasma of human patients and could be diagnostic markers. Point-of-care diagnosis of *S. stercoralis* infection in human patients could be facilitated in the near future by application of loop-mediated isothermal amplification (LAMP) of parasite-specific nucleic acid markers. This method has already been investigated in other parasitic nematode infections [66, 67]. Finally, the existence of parasitic and non-parasitic morphs (eg. parasitic and free-living females) with radically different body plans, ecological niches, lifespans or developmental fates, all resulting from the same genotype, argues strongly for investigating epigenetic control of developmental fate in *Strongyloides* spp. Gene regulation at this level has not been studied extensively in parasitic nematodes, but recent work on platyhelminths, including schistosomes, indicates a clear role for epigenetic regulation of reproductive biology in those parasites [68–70].

Acknowledgments

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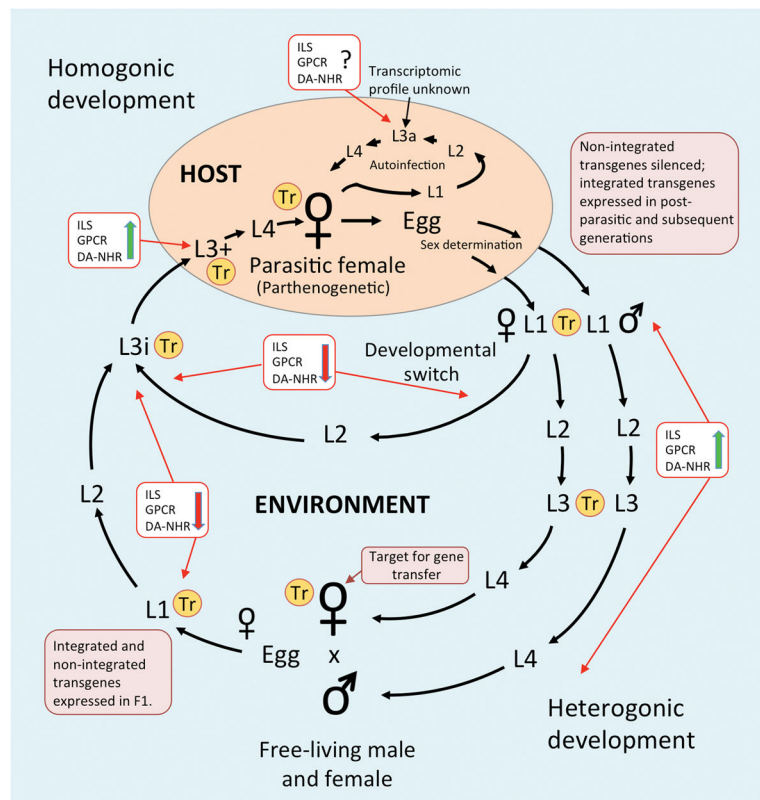
Fig. 1.
Life cycle of *Strongyloides stercoralis* summarizing the current status of transgenesis, significant transcriptomic datasets and hypothetical profiles of signaling through dauer-like regulatory pathways that are supported by current data. Yellow encircled **Tr** denotes a life stage for which transcriptomic datasets, stemming from RNAseq and/or microarray analysis, are available. Purple boxes indicate the free-living female as the effective target for delivery of transgene constructs by gonadal microinjection as well as the expression patterns of integrated and non-integrated transgenes before and after host passage. White boxes give the hypothesized status, based on the weight of current experimental and transcriptomic evidence, of three cellular signal transduction pathways with homologs that regulate dauer larval development in *Caenorhabditis elegans*: **ILS** – Insulin-like signaling; **GPCR** – G protein-coupled receptor signaling; **DA-NHR** – dafachronic acid-nuclear hormone receptor (DAF-12) signaling. Green upward arrows indicate active signaling through the pathways; red downward arrows indicate downregulation of positive signaling or initiation of inhibitory signaling.

Table 1

Summary of genes, gene products and functional pathways ascribed biological significance or potential as drug or vaccine targets based on transcriptomic, proteomic or experimental findings highlighted in this review.

Species	Stage	Gene/Gene product/Pathway	Type of investigation	Putative function / Appliction	Reference
S. ratti	L3i[a]	Astacin metalloprotease		Host tissue invasion	[50•]
		L3 Nie Antigen	Proteomic	Immunogenic; vespid wasp venom family	
		Fatty acid retinol binding protein		Scavenging host lipids	
		Prolyl oligopeptidase		Motility / survival	
		Sra-HSP-17.1		Binds host epithelium; elicits IL-10 production	[51]
	P Female[b]	*Sra*-HSP-17.2		Elicits IL-10 production	
		SR00007 chondroitin proteoglycan	Transcriptomic /EST	Immunogen; function unknown	
		SR00449 chondroitin proteoglycan		Sereted immunogen; function unknown	[41•]
		SR00984 Small heat-shock protein		Immunogen; function unknown	
		Ss-age-1 (ILS[d])	Experimental	All promote developmental activation of cultured L3i in the absence of host-like stimuli.	[24]
		GPCR[e] signaling			[19•]
		Dafachronic acid -Ss-DAF-12 signaling			
S. stercoralis	L3i	HSP-90		Potential chemotherapeutic target	
		Cytochrome oxidase ucr 2.1	Transcriptomic /μArray	Potential vaccine targets	[40]
		Sperm containing glycoprotein domain			
		Fatty acid retinol binding protein-1		Scavenging host lipids	
S. venezuelensis	P Female	Ferrochetalase	Transcriptomic /RNAseq	Iron porphyrin catabolism	[37][38][49]
	LL3[c]	Multiple transcripts		Secretory glycan biosynthesis	[37][38]
S. papillosus	L3i	*Spa-asp-2* Aspartic protease	Experimental	Both upregulated at skin penetration	[45]
		Spa-lys Lysozyme			

[a] Infective third-stage larva

[b] Parasitic female

[c] Post-infective L3 collected from lungs of infected mice, 72-75 hours after inoculation of L3i

[d] Insulin-like signaling

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[e]G protein-coupled receptor

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