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Review

Recent advances in *Echinococcus* genomics and stem cell research

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ABSTRACT

Alveolar and cystic echinococcosis, caused by the metacestode larval stages of the tapeworms *Echinococcus multilocularis* and *Echinococcus granulosus*, respectively, are life-threatening diseases and very difficult to treat. The introduction of benzimidazole-based chemotherapy, which targets parasite β-tubulin, has significantly improved the life-span and prognosis of echinococcosis patients. However, benzimidazoles show only parasitostatic activity, are associated with serious adverse side effects and have to be administered for very long time periods, underlining the need for new drugs. Very recently, the nuclear genomes of *E. multilocularis* and *E. granulosus* have been characterised, revealing a plethora of data for gaining a deeper understanding of host-parasite interaction, parasite development and parasite evolution. Combined with extensive transcriptome analyses of *Echinococcus* life cycle stages these investigations also yielded novel clues for targeted drug design. Recent years also witnessed significant advancements in the molecular and cellular characterisation of the *Echinococcus* 'germinative cell' population, which forms a unique stem cell system that differs from stem cells of other organisms in the expression of several genes associated with the maintenance of pluripotency. As the only parasite cell type capable of undergoing mitosis, the germinative cells are central to all developmental transitions of *Echinococcus* within the host and to parasite expansion via asexual proliferation. In the present article, we will briefly introduce and discuss recent advances in *Echinococcus* genomics and stem cell research in the context of drug design and development. Interestingly, it turns out that benzimidazoles seem to have very limited effects on *Echinococcus* germinative cells, which could explain the high recurrence rates observed after chemotherapeutic treatment of echinococcosis patients. This clearly indicates that future efforts into the development of parasitocidal drugs should also target the parasite's stem cell system.

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1. Introduction

The term echinococcosis is used to describe a group of zoonotic diseases caused by infection with the metacestode larvae of tapeworms of the genus *Echinococcus* (Cestoda: Taeniidae). From the

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medical and veterinary point of view, the two most important species of this genus are *Echinococcus granulosus* (the "dog tapeworm") and *Echinococcus multilocularis* (the "fox tapeworm"), the causative agents of cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively (reviewed by Eckert and Deplazes (2004)).

The life cycle of *Echinococcus* spp. is complex and involves two mammalian hosts (Eckert and Deplazes, 2004; Thompson, 1986). The adult tapeworm develops attached to the small intestine of the definitive hosts, which are typically dogs for *E. granulosus*, and red or arctic foxes for *E. multilocularis*. The adults of *Echinococcus* spp. show a relatively typical cestode morphology, with an anterior scolex containing the attachment organs, and a neck region that generates a short chain of segments (proglottids), each one containing a complete set of male and female reproductive organs. The mature proglottids, containing infective eggs, are released with the faeces of the definitive host. Each egg contains an oncosphere (the first larval stage) that is infective if ingested by the intermediate host. Several ungulate species serve as intermediate hosts for the different lineages of the *E. granulosus* species complex, most of them being domestic species (Eckert and Deplazes, 2004). In contrast, several wild rodent species are the natural intermediate host for *E. multilocularis* (Craig, 2003; Eckert and Deplazes, 2004; Rausch, 1954). Humans can also be an accidental host for *E. granulosus* and *E. multilocularis*, although they are a dead-end for the life cycle of the parasite.

When *Echinococcus* eggs are ingested by the intermediate host, the oncospheres hatch in the intestine, penetrate the intestinal wall and are transported by the bloodstream. Most commonly, primary infections develop in the liver (Brunetti et al., 2010; Eckert and Deplazes, 2004), where the oncosphere metamorphoses into the next larval stage, the metacestode. The metacestodes develop as fluid-filled vesicles, comprising a thin layer of tissue (the germinal layer) covered by a syncitial tegument that secretes an acellular, carbohydrate-rich external layer (the laminated layer). Within the germinal layer, thickenings occur that invaginate into the vesicle, resulting in the formation of brood capsules (Koziol et al., 2013; Leducq and Gabrion, 1992), where eventually protoscoleces are formed, which are the infective larvae for the definitive host. The development of metacestodes as fluid-filled cysts that generate protoscoleces asexually is an evolutionary novelty, and asexual reproduction is very rare among cestodes (Freeman, 1973; Slais, 1973; Trouvé et al., 2003). Protoscoleces remain quiescent, with the scolex invaginated within a small posterior body. When the definitive host ingests an infected intermediate host, it also ingests the protoscoleces, which then evaginate their scolex and attach to the intestine, thus closing the life cycle.

E. multilocularis and *E. granulosus* differ in the morphology and development of the metacestode (Eckert and Deplazes, 2004; Thompson, 1986). In the case of *E. granulosus*, each oncosphere develops into a single vesicle ("unilocular development") that can grow to huge dimensions, exceeding 20 cm in diameter. In the case of *E. multilocularis*, new metacestode vesicles are generated by exogenous budding of the metacestode, which therefore develops as a multilocular labyrinth of interconnected vesicles (Eckert et al., 1983; Rausch, 1954; Sakamoto and Sugimura, 1970; Tappe et al., 2010). In human AE (alveolar echinococcosis), the *E. multilocularis* metacestode tissue therefore grows infiltratively like a malignant tumor into the surrounding liver tissue and, in later stages of the disease, can even form metastases in secondary organs. If not adequately treated, parasite expansion will eventually lead to organ failure and death (Brunetti et al., 2010; Eckert and Deplazes, 2004; Kern, 2010). Although the *E. granulosus* metacestode (the 'hydatid cyst') grows more 'benign' (*i.e.* not infiltratively), steady growth of hydatid cysts within the liver, lungs or the brain can also lead to mechanical pressure and to pathological changes associated with compression or obstruction (Brunetti et al., 2010; Eckert and Deplazes, 2004).

Cure of the disease can only be achieved by surgical removal of metacestode tissue in combination with anti-parasitic chemotherapy or, as possible in some cases of CE (cystic echinococcosis), inactivation of hydatid cysts by minimal invasive procedures such as PAIR (punctuation, aspiration, injection, reaspiration), combined with chemotherapy (Brunetti et al., 2010). However, and particularly in AE, complete surgical removal of parasite material is in most cases not possible due to the fact that the disease has been diagnosed at a late stage so that large regions of the liver are affected. In these cases, chemotherapy is the only treatment option. Currently, both anti-AE and anti-CE chemotherapy rely on benzimidazoles (*e.g.* albendazole, mebendazole) which target parasite β-tubulin, thus preventing the formation of microtubuli (Brehm et al., 2000b; Brunetti et al., 2010; Stojkovic et al., 2009). Since its introduction in 1978, benzimidazole-based chemotherapy has significantly improved disease outcome and prognosis of echinococcosis patients. However, due to the fact that host and parasite β-tubulin are highly similar (>90% identical amino acid residues), benzimidazole therapy is associated with significant adverse side effects (Brunetti et al., 2010). Furthermore, only parasitostatic doses can be given to patients so that benzimidazole chemotherapy has to be administered for long periods of time (up to life-long) and is associated with high recurrence rates (Brunetti et al., 2010; Kern, 2010). Altogether, this underlines the urgent need for novel anti-parasitic compounds that can completely inactivate metacestode tissue instead of just diminishing growth.

In addition to a closer understanding of parasite biology, the host-parasite relationship and parasite evolution, the quest for novel chemotherapeutic targets was surely a major rationale for the initiation of whole genome sequencing projects for *E. multilocularis* and *E. granulosus*, about 10 years ago, which culminated in the release of two highly recognised publications in 2013 (Tsai et al., 2013; Zheng et al., 2013). In the following we will briefly outline the characteristics of *Echinococcus* genomes and how this information, when combined with transcriptomics, can be used for the identification of novel drug targets. Furthermore, we will review recent progress in *Echinococcus* stem cell research which should be highly relevant for the development of parasitocidal chemotherapeutics.

2. Echinococcus genomics

In March 2004 a meeting was held at the Wellcome Trust Sanger Institute (Hinxton, UK) that eventually led to the still ongoing '50 helminth genomes initiative' (Holroyd and Sanchez-Flores, 2012). At this time point, no draft genome sequence of a parasitic helminth had been published, although the projects for the trematodes *Schistosoma mansoni*, *Schistosoma japonicum*, and the nematode *Brugia malayi* were in an advanced stage (Brindley et al., 2009). During the meeting, it was decided to complement these efforts by whole genome sequencing projects for additional nematodes, trematodes and (at that time point) at least one cestode. Although worldwide there are more cases of CE than AE, *E. multilocularis* has been chosen as the species for producing a high-quality reference genome due to the fact that it is much more accessible for *in vitro* cultivation (Brehm and Spiliotis, 2008; Hemphill et al., 2010, 2003; Spiliotis et al., 2008, 2004) and, as it later turned out, for genetic manipulation (Mizukami et al., 2010; Spiliotis et al., 2010). By combining classical capillary sequencing with next generation sequencing (NGS) approaches (454, Illumina) and manual curation, a draft genome was assembled in which 89% of the sequence was contained in 9 chromosome scaffolds with very few gaps, and one chromosome (No. 5) was even complete from telomere to telomere (Tsai et al., 2013). Genetic homogeneity due to inbreeding

was one aspect that greatly facilitated genome assembly and thus contributed to the high quality of the *E. multilocularis* draft genome. In parallel, NGS technology was used to produce draft sequences for *E. granulosus* (G1 genotype; isolate from Uruguay), for Mexican and Chinese isolates of the related tapeworm *Taenia solium*, and for the model tapeworm *Hymenolepis microstoma* which were, however, more fragmented (Tsai et al., 2013). Final assembly of the *E. granulosus* genome was carried out on the framework of the *E. multilocularis* reference genome (Tsai et al., 2013). Later, these cestode whole genome sequencing projects were complemented by efforts of a Chinese/Australian consortium, which produced a second *E. granulosus* draft sequence from a single hydatid cyst (G1 strain) using 454 and Solexa NGS (Zheng et al., 2013). In all three *Echinococcus* genome projects, gene finding and annotation was supported by extensive EST- and NGS-transcriptomic analyses (RNAseq) of several life cycle stages such as oncosphere, metacestode, protoscolex and adult worms (Fernandez et al., 2002; Parkinson et al., 2012; Tsai et al., 2013; Zheng et al., 2013).

Although Tsai et al. (2013) reported nuclear genome sizes of ~115 Mbp for both *E. multilocularis* and *E. granulosus*, the analysis of Zheng et al. (2013) revealed ~150 Mbp for *E. granulosus* which was, however, mostly due to differences in the assembly process that identified a higher proportion of repeats in the Chinese isolate when compared to the assembly of the Uruguayan isolate (Zheng et al., 2013). In any case, and also including data from the *H. microstoma* and *T. solium* sequencing projects, the studied tapeworms have much smaller genomes than the related flukes (about three times) or free-living flatworms (about nine times), which is mostly due to smaller intergenic regions, smaller introns, and a lower content of repeats and mobile genetic elements in tapeworm genomes (Tsai et al., 2013). Depending on the methodology used, between 10.300 and 11.300 genes were predicted in the *Echinococcus* genomes (Tsai et al., 2013; Zheng et al., 2013) and in inter-species comparisons between *E. multilocularis* and *E. granulosus* it is almost impossible to identify genes that are present in one species, but absent in the other. Hence, although due to the different morphology of their metacestode stages *E. multilocularis* and *E. granulosus* are clinically often regarded as 'distinctly different entities' (Stojkovic and Junghanss, 2013), they are highly similar concerning gene structure and gene content. Salient differences were so far only observed in the *Echinococcus*-specific apomucin gene family, of which one copy is highly differentiated between the two species and of which two copies are probably present in *E. granulosus* but absent in *E. multilocularis* (Tsai et al., 2013). This is presumably associated with one of the few clear morphological differences between *E. multilocularis* and *E. granulosus*, the thickness of the laminated layer, since the apomucin gene family encodes important components of this structure (Diaz et al., 2011).

In the genomes of cestodes, but also in those of the related flukes, considerable gene gain and gene loss associated with the adaptation to parasitism has been found. Cestode genomes lack crucial genes or even entire pathways for the *de novo* synthesis of pyrimidines, purines and most amino acids. Furthermore, tapeworms lack the ability to synthesise fatty acids and cholesterol *de novo* (Tsai et al., 2013; Zheng et al., 2013). These essential substances and compounds have to be taken up from the host and consequently, respective genes and gene families have either been expanded or are abundantly expressed. Among the most highly expressed genes in *Echinococcus* metacestodes are, for example, those that encode fatty acid binding proteins and the cestode-specific antigen B family, factors known to be involved in the uptake and transport of lipids (Tsai et al., 2013). Likewise, several amino acid transporters are highly expressed in the metacestode stage (Camicia et al., 2008). One of the most striking expansions in *Echinococcus* has been observed for the heat shock protein 70 (Hsp70) family, of which 22 full copies are present on the genome (2 copies in humans, 6 copies in fruit flies), mostly in sub-telomeric regions (Tsai et al., 2013; Zheng et al., 2013). Hsp70 proteins have been found in the excretory/secretory fraction of tapeworms (Ernani and Teale, 1993; Vargas-Parada et al., 2001) and the expansion of the hsp70 gene family might thus be associated with special functions in host-parasite interaction (*e.g.* immunomodulation), an aspect that clearly requires further investigation. Likewise, many of the cestode-specific and highly expressed antigen families, such as antigen B, the GPI-anchored protein GP50, and the vaccine target EG95 might have a role in immune-evasion processes which, in the case of antigen B, has already been investigated to a certain extent (Siracusano et al., 2008). Surely of high relevance for host-parasite interaction mechanisms is also the presence of evolutionarily conserved signalling systems in *Echinococcus*, such as components of the epidermal growth factor (EGF)-, fibroblast growth factor (FGF)-, transforming growth factor-β (TGF-β)-, and Insulin-signal transduction cascades (Brehm, 2010b; Tsai et al., 2013; Zheng et al., 2013). Host insulin has, for example, recently been demonstrated to stimulate *E. multilocularis* developmental processes via interaction with parasite surface receptors of the Insulin-Receptor family (Hemer et al., 2014), and host-derived EGF is known to induce the *Echinococcus* mitogen activated protein kinase (MAPK) cascade, probably through direct interaction with parasite EGF-receptors (Spiliotis et al., 2006).

Reductions have also been observed in gene complements that are implicated in the patterning of body plans in animals. Cestodes have the most reduced set of homeobox genes of any studied bilaterian animal and specifically lost several ParaHox genes that are ancestrally involved in the specification of a through-gut (Koziol et al., 2009; Tsai et al., 2013). Interestingly, although *E. multilocularis* and *E. granulosus* have a well-developed nervous system (Brownlee et al., 1994; Camicia et al., 2013; Fairweather et al., 1994; Koziol et al., 2013), several homeobox gene families involved in neural development are missing as well (Tsai et al., 2013). Although the *Echinococcus* genomes apparently encode typical sets of Hedgehog- and Notch-signalling components, they do have a reduced set of wingless related (Wnt) ligands (Riddiford and Olson, 2011; Tsai et al., 2013) so that, overall, cestodes have significantly reduced their complement of molecular developmental pathways related to body plan complexity during the evolution of parasitism.

One striking difference in gene expression mechanisms between flatworm parasites and their hosts, which has already been suggested to be exploitable for drug development (Liu et al., 2009), is spliced-leader trans-splicing (SL-TS). Originally described in trypanosomes and nematodes (Hastings, 2005), SL-TS has later also been shown to occur in all parasitic and free-living lineages of flatworms (Brehm et al., 2002, 2000a; Davis et al., 1995; Zayas et al., 2005). In mammals, insects and numerous other phyla, on the other hand, trans-splicing is absent. In SL-TS, the mRNA molecules of so-called 'trans-spliced genes' acquire at their 5′ end an extra exon, the spliced leader, which is donated by a small RNA, the spliced leader RNA, that is encoded elsewhere on the genome by genes that are tandemly arrayed (Brehm et al., 2000a). The transcripts of trans-spliced genes thus all harbor an identical exon at their 5′ end which, in the case of *E. multilocularis*, is 36 bases long and contains a trimethyl-guanosine (TMG) cap, which differs from the 7-methyl-guanosine (7mG) cap present at the 5′ end of usual (non-trans-spliced) mRNAs (Brehm et al., 2000a; Lasda and Blumenthal, 2011). The biological function of trans-splicing remains largely unknown. Certain hypotheses argue that it is an adaptive process for coordinated gene regulation or translational control, others argue the spliced-leader genes might be selfish DNA components that 'hijack' essential nuclear genes until they cannot be eliminated from the genome, leading to an expansion of SL genes in tandem arrays (Blaxter and Liu, 1996; Blumenthal, 2004). Either way, one consequence of trans-splicing is that it

facilitates the evolution of operons in which two or more genes are expressed from one single promoter as a polycistronic mRNA, which is subsequently solved into single translatable units via SL-TS (Lasda and Blumenthal, 2011). Genomic and transcriptomic analyses revealed that ∼13% of all *E. multilocularis* genes are trans-spliced of which hundreds are arranged in polycistrons of up to four genes (Tsai et al., 2013). Among the trans-spliced fraction are numerous genes involved in essential cellular processes such as transcriptional and translational control, splicing or replication (Brehm et al., 2000a; Tsai et al., 2013). Hence, if trans-splicing or the translation of trans-spliced messages could be inhibited, this would surely result in lethal effects for all parasite cells. The development of respective, parasite-specific drugs could prove difficult if they are directed against the splicing process itself, since trans-splicing is carried out by the canonical spliceosome, the components of which are highly conserved between cestodes and mammals (Tsai et al., 2013). However, as already suggested previously (Liu et al., 2009), the eukaryotic translation initiation factor 4E (eIF4E), which initiates translation by binding to the mRNA cap structure, could be a promising target. In mammals, eIF4E only recognises 7mG cap structures, whereas in the trans-splicing schistosomes an eIF4E is present that recognises both 7mG and TMG caps (Liu et al., 2009). The *E. multilocularis* genome contains one single copy gene for eIF4E and it is reasonable to assume that this factor also recognises both types of caps. Hence, structural differences in the cap-binding structures of parasite and host eIF4E, which are likely to exist (Liu et al., 2009), could possibly be exploited for the development of small molecule compounds that target trans-splicing in cestodes.

Other potential drug targets have been identified in *Echinococcus* genomes by bioinformatic genome and transcriptome data mining, including criteria such as expression of the target in the clinically relevant metacestode stage, or the availability of lead compounds that are active against the respective target molecule class as an indicator of 'drugability' (Tsai et al., 2013). The list includes detoxification systems such as the Thioredoxin Glutathione Reductase (TGR) which, in cestodes and trematodes, has merged two enzymatic functions of redox homeostasis in one single enzyme (Bonilla et al., 2008). Most notably, anti-TGR inhibitors have already been shown to be effective against *E. granulosus* larvae at concentrations of 20 μM (Ross et al., 2012). Proteases, G-protein-coupled receptors (GPCR) and ion channels are other groups of "drugable" targets, many of which are expressed in the *E. multilocularis* metacestode (Tsai et al., 2013). Of prominent interest for drug development against echinococcosis should also be the set of 250–300 kinases identified in the *Echinococcus* genome (Brehm, 2014; Tsai et al., 2013). Due to the fact that kinases bind both their substrate and ATP, they are prone to enzymatic inhibition by small molecule compounds and many of the currently available kinase inhibitors actually interfere with the ATP binding pocket (Brehm, 2014). Furthermore, due to their importance in malignant transformation, the biochemistry of kinases is exceptionally well studied and research has already brought up a plethora of inhibitory compounds, many of which are currently in use to treat various forms of cancer (Brehm, 2014). Several prominent kinase inhibitors such as Imatinib, directed against Abl-kinases, BI 2536 (Polo-like kinase) or pyridinylimidazoles (p38 MAPK) have already been shown to exert anti-*Echinococcus* activities *in vitro* by inhibition of the orthologous parasite factors (Gelmedin et al., 2008; Hemer et al., 2014; Schubert et al., 2014) and are thus promising lead compounds for the design of related small molecule compounds that show a high specificity for parasite kinases (Brehm, 2014). Interestingly, genomic and transcriptomic analyses also revealed why some drugs are not effective against larval cestodes. Praziquantel, for example, which is highly active against adult tapeworms, only shows very limited activity against the larval stages (Olson et al., 2012). Voltage-gated calcium channels, the proposed target for praziquantel (Olson et al., 2012), are not expressed in the metacestode stage (Tsai et al., 2013), which might be the reason for reduced praziquantel efficacy against larvae.

Finally, another highly interesting finding that was revealed by genomic analyses is that cestodes employ a considerably modified stem cell system when compared to other flatworms or bilateria. PIWI, VASA, and group 4 Tudor proteins are expressed in germline cells of all metazoans investigated so far where they are associated with maintaining multipotency (the germline multipotency program, GMP; Juliano et al., 2010). Specifically, PIWI proteins as a subgroup of the Argonaute protein family are necessary for the generation of so-called piRNAs which preserve genome integrity and stability by specifically repressing the activity of mobile genetic elements in germline cells (Juliano et al., 2011). For this activity, interactions with the DEAD box RNA helicase VASA, a classical germ cell marker, and group 4 Tudor proteins are necessary (Skinner et al., 2014). Interestingly, in the genomes of cestodes (and trematodes) true orthologs to VASA, PIWI and group 4 Tudor proteins are absent (Tsai et al., 2013; Zheng et al., 2013). Since mobile genetic elements are definitely present in cestodes (Tsai et al., 2013; Zheng et al., 2013), this raises the important question: how can these parasites maintain genome integrity without a canonical piRNA pathway? Investigations into this direction are currently underway and concentrate in particular on the role of Argonaute (the group 4 argonautes) and DEAD box RNA helicase (PL10-like) clades that have expanded in cestodes and trematodes and might have taken over the function of PIWI and VASA (Skinner et al., 2014). Recent research indicated that the *Echinococcus* stem cell system is not only unique in the absence of several classical stem/germ cell markers but it also differs from stem cell systems of the related flukes and free-living flatworms (Koziol et al., 2014). These findings, as well as clear indications that the *Echinococcus* stem cell system is highly relevant for the development of anti-echinococcosis drugs, will be outlined in the following paragraphs.

3. Stem cells and cell renewal in flatworms

Cestodes are part of the phylum Platyhelminthes (flatworms), which comprises a great diversity of free-living groups and parasitic groups. The main parasitic groups (including Cestoda, Monogenea and Trematoda) form the monophyletic clade Neodermata (Baguñà and Riutort, 2004). Despite the great variation found in flatworm groups regarding their morphology, development and life cycles, it is thought that they all share a rather unique system for cell renewal. Indeed, all studies in flatworms have so far shown that differentiated cells are post-mitotic, and the generation of new differentiated cells depends exclusively on the proliferation and differentiation of a population of small undifferentiated stem cells, called the neoblasts (Peter et al., 2004; Reuter and Kreshchenko, 2004; Rink, 2013).

The best studied models are free-living flatworms, especially planarians (order Tricladida) (Rink, 2013; Rossi et al., 2008). In planarians, the neoblasts are located in the parenchyma surrounding the internal organs, but are not found within the organs or in the epidermis. Thus, the population of parenchymal neoblasts proliferates and enters the organs and epidermis, where they differentiate. Furthermore, by means of elegant transplantation experiments, individual neoblasts have been shown to be pluripotent (being able to differentiate into all the somatic cell types found in adult planarians) (Wagner et al., 2011). Recently, neoblast-like stem cells have also been identified in the trematode *S. mansoni*, which shared many characteristics at the molecular level with planarian neoblasts (Collins et al., 2013; Wang et al., 2013). This cell-renewal strategy is in stark contrast to the strategies of cell renewal found for example in adult mammals. Mammalian tissues with high

cellular turnover (such as the epidermis, blood and the intestinal epithelium) contain specific resident stem cells, which have a differentiation potential that is limited to the cell types found in that tissue (Barker et al., 2010; Bryder et al., 2006; Pellettieri and Sanchez Alvarado, 2007). Pluripotent stem cells are thus restricted in mammals to early embryonic stages (Hanna et al., 2010). Furthermore, many mammalian tissues with slow cell turnover depend instead on the self-duplication of differentiated cells (such as hepatocytes in the liver and beta cells in the pancreatic islets) (Dor et al., 2004; Yanger and Stanger, 2011).

Importantly, molecular studies have shown that planarian neoblasts are actually divided into sub-populations expressing different sets of molecular markers, which are likely to have different potentials of self-renewal and differentiation (Eisenhoffer et al., 2008; Reddien, 2013; Rossi et al., 2008; Shibata et al., 2010; van Wolfswinkel et al., 2014). Thus, the morphologically homogeneous neoblasts are actually divided into several cryptic sub-populations, and it is likely that only a specific sub-set of neoblasts are actually pluripotent stem cells, with other neoblasts being already committed to specific fates.

In cestodes, classical studies also identified cells similar to the neoblasts of planarians, which are usually denominated as "germinative cells" (Gustafsson, 1990; Koziol and Castillo, 2011; Reuter and Kreshchenko, 2004). As detailed below, it is likely that, similarly to the case of planarian neoblasts, many morphologically cryptic sub-populations of germinative cells may exist and that only some of them are true stem cells. In classic histological studies, germinative cells have been characterised as small, round or oval cells with a strongly basophilic cytoplasm (due to the abundance of rRNA), few cytoplasmic extensions, a large nucleus and a large and prominent nucleolus (Bolla and Roberts, 1971; Douglas, 1961; Gustafsson, 1976a; Koziol et al., 2010; Loehr and Mead, 1979; Sulgostowska, 1972; Wikgren and Gustafsson, 1971). In the developing adults of selected species of cestodes, good evidence exists that germinative cells are indeed the only proliferating cells, since these are the only cells incorporating tritiated thymidine (^{3}H-T) or the thymidine analog bromodeoxyuridine (BrdU) into their DNA (Bolla and Roberts, 1971; Gustafsson, 1976a; Koziol et al., 2010; Wikgren and Gustafsson, 1971). Pulse and chase experiments with 3-HT and BrdU further provided evidence that many differentiated cells are originated from the pool of germinative cells (Gustafsson, 1976a; Koziol et al., 2010).

At the ultrastructural level, germinative cells have been described in oncospheres, metacestodes and adult cestodes (Bolla and Roberts, 1971; Collin, 1969; Jabbar et al., 2010; Korneva, 2004; Sakamoto and Sugimura, 1970; Swiderski, 1983; Wikgren and Gustafsson, 1971). These cells have a large nucleus with little heterochromatin, scant cytoplasm with abundant free ribosomes, and absent or scarce endoplasmic reticulum and Golgi apparatus. Their morphology is thus very similar to neoblasts from planarians. However, one important exception is that planarian neoblasts contain chromatoid bodies (CBs), which are electron-dense, perinuclear ribonucleoprotein granules (Hay and Coward, 1975; Morita et al., 1969). CBs are molecularly and morphologically similar to the germ granules present in the germ cells of many animals (Auladell et al., 1993; Rossi et al., 2008; Yoshida-Kashikawa et al., 2007). In contrast, CBs have never been described in cestodes. Interestingly, *vasa* and *piwi* have been lost from the genome of cestodes (see above), and VASA and PIWI proteins are a classic component of germ granules in other metazoans (Ewen-Campen et al., 2010; Juliano et al., 2011; Juliano et al., 2010).

4. The germinative cells of *Echinococcus*

Until recently, relatively little was known regarding the germinative cells in *Echinococcus* spp. compared to other cestode species and most studies had been limited to morphological descriptions in the different life stages. Ten undifferentiated (germinative) cells were described to be present in the oncosphere of *E. granulosus* in a detailed electron-microscopical description (Swiderski, 1983) and these cells are thought to be responsible for the metamorphosis into the metacestode stage (Rybicka, 1966; Smyth and McManus, 1989). Germinative cells were also described by classical histological methods in the neck region of *E. granulosus* adults (Gustafsson, 1976b). In the metacestode germinal layer, undifferentiated (germinative) cells had been described at the ultrastructural level for *E. multilocularis* (Sakamoto and Sugimura, 1970). In that study, many differentiated cell types were also described (such as for example the tegumentary cells that form the syncitial tegument covering the germinal layer, muscle cells, glycogen-storage cells, etc.), and these were proposed to originate from the germinative cell pool during development. *E. multilocularis* undifferentiated cells are also presumed to be the origin of the metacestode projections that infiltrate the host tissues and have been proposed to be the source of metastasis by infiltration and detachment into blood and lymphatic vessels (Eckert et al., 1983; Mehlhorn et al., 1983).

Recently, we performed an extensive characterisation of the stem cell system in *E. multilocularis* (Koziol et al., 2014), using recently developed methods for the *in vitro* cultivation of the metacestode larval stage (Brehm and Spiliotis, 2008; Spiliotis et al., 2008, 2004) (in continuation of the pioneering work of Smyth et al. (1966) with protoscoleces), combined with methods for the detection of gene expression patterns at cellular resolution. We identified a pool of cells with typical germinative cell morphology (comprising 20–25% of all cells in the germinal layer) and these were the only cells that were able to proliferate in the germinal layer and in the protoscolex (*i.e.* they were the only ones incorporating the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU), and the only cells found in mitosis; Fig. 1A). Differentiated cells (including tegumental cells, glycogen cells and nerve cells) were identified by morphology but also by means of newly developed molecular markers. Proliferation was never observed in these cells, indicating that they must originate from the differentiation of the pool of proliferating germinative cells. Further evidence in this regard came from EdU pulse-chase and continuous labelling assays, in which various differentiated cell types could be found to be labelled by EdU 7 to 14 days after the pulse started, indicating that they originated from the pool of germinative cells. It is important to note, however, that it is not yet formally possible to rule out the existence of dedifferentiation processes, which could lead to the formation of new germinative cells from differentiated cell types. In planarians, current evidence indicates that dedifferentiation processes, if they exist at all, would only be of minor relevance (Baguñà et al., 1989; Wagner et al., 2011).

Proliferating cells accumulate during early brood capsule and protoscolex development, and sharply decline in number after development is complete (as shown by Galindo et al. (2003) for *E. granulosus*). Interestingly, cell proliferation was quickly increased in protoscoleces after artificially mimicking ingestion by the definitive host (Koziol et al., 2014). This strongly indicates that a large number of germinative cells are present in the mature protoscolex but that they are quiescent or with slow cell cycle kinetics as long as the protoscolex remains within the metacestode vesicles.

The pool of germinative cells in the germinal layer of *E. multilocularis* was relatively homogeneous at the morphological level but could nevertheless be shown to be heterogeneous at the molecular level by analysing the expression of conserved post-transcriptional regulators of stem cells (Koziol et al., 2014). For example, homologs of the post-transcriptional regulator nanos (*em-nos-1* and *em-nos-2*) were expressed in a small proportion of the proliferating germinative cells (<5%) in the germinal layer (Fig. 1C) and were apparently not expressed in the germinative

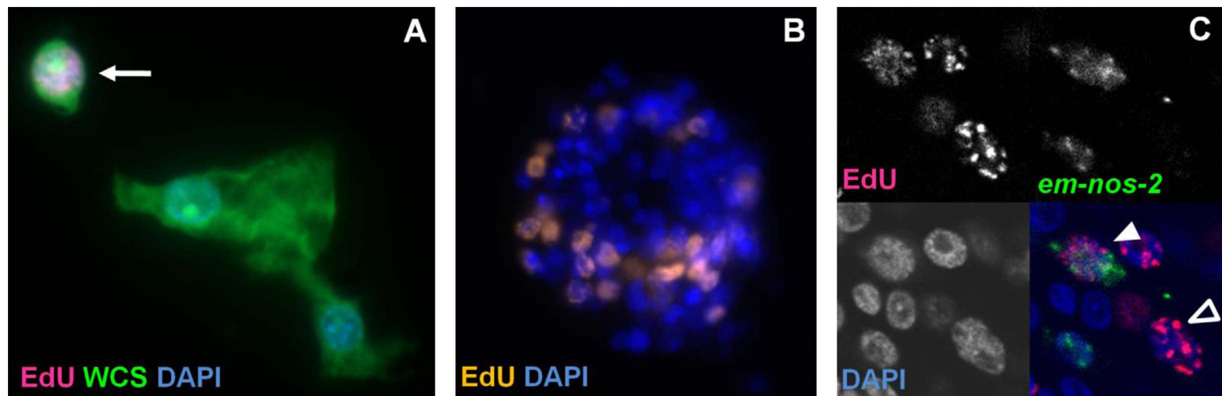

Fig. 1. The *Echinococcus multilocularis* germinative cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
A. Germinative cells are the only proliferating cells in the germinal layer. Metacestode vesicles were incubated *in vitro* with the thymidine analog EdU (50 μM for 5 h in all panels) and after the incubation they were dissociated and fixed as a suspension of single cells. EdU incorporation (magenta) can only be detected in cells with undifferentiated morphology (germinative cells, arrow). Cells are co-stained with 4',6-diamidino-2-phenylindole (DAPI, blue) and a whole-cell stain for better visualisation (WCS, green). B. Primary cell aggregate showing cell proliferation in its periphery (as determined by EdU incorporation). C. Whole-mount *in situ* hybridisation (WMISH) with a probe for the *nanos* homolog *em-nos-2*. Only a subpopulation of the proliferating (EdU-positive) cells express the gene (filled arrowhead), whereas the majority of the EdU-positive cells do not express *em-nos-2* (open arrowhead).

cells in the mid to late stages of protoscolex development. Given the important roles of these post-transcriptional regulators in stem cell and germ cell biology in other animals (Juliano et al., 2010), this indicates the existence of different sub-populations of germinative cells, which could have different self-renewal and differentiation potencies. More generally, many differences are found between the expression patterns of planarian neoblast-specific genes, and the expression of the homologous genes in *E. multilocularis*. This includes wide-spread expression in *E. multilocularis* of homologs of genes that are neoblast-specific in planarians, such as *prohibitin-1* (*phb1*) and histone deacetylase 1 (*hdac1*). These results, combined with the lack of *vasa* and *piwi* orthologs in the genome of *E. multilocularis*, suggest the existence of important functional differences between the germinative cells of cestodes and the neoblasts of planarians.

5. *Echinococcus* germinative cells *in vitro*

Recent methodological advances allowed for the first time the *in vitro* axenic culture of *E. multilocularis* primary cells (reviewed by Brehm and Spiliotis (2008)). These methods were also adapted and successfully applied to the culture of *E. granulosus* primary cells (Albani et al., 2010). The primary cell preparations of *E. multilocularis* are able to regenerate complete metacestode vesicles *in vitro*, demonstrating that at least at the population level these cells must be pluripotent. Furthermore, the early primary cell preparations of *E. multilocularis* form cell aggregates that have been shown to be highly enriched (up to 82%) in germinative cells, and to be specifically depleted of particular differentiated cell types, such as tegumental cells (Koziol et al., 2014) (Fig. 1B). These germinative cells were the only cells that underwent proliferation during regeneration. Thus, *E. multilocularis* primary cell preparations are an ideal system for the functional study of genes and pathways in germinative cells, particularly if used in combination with specific pharmacological inhibitors or with RNA interference methods for gene-specific knockdown (Hemer et al., 2014; Spiliotis et al., 2010). Furthermore, they have been proposed to be an *in vitro* experimental model for the developmental processes that occur during the metamorphosis from oncosphere to metacestode, and during metastasis (Olson et al., 2012). The primary cell culture systems described for *E. granulosus* did not result in complete cyst regeneration but instead seem to be blocked at the point of the formation of cell aggregates (Albani et al., 2010), an early stage of the regeneration process (Spiliotis et al., 2008). It is not clear at this point if this is due to intrinsic differences between the species or due to methodological differences between both culture systems. A continuous cell line has also been obtained from *E. granulosus* (Albani et al., 2013). This cell line appears to have an unstable karyotype but it could be an interesting resource for simple and economic screening of candidate chemotherapeutics.

6. Methods for the specific elimination of *E. multilocularis* germinative cells

Given the role of the germinative cell population as the only known source of new cells for metacestode growth and regeneration, it would be of clear interest to develop methods to deplete them, not only as a research tool but also for the search for new therapeutic options against echinococcosis. Ionising irradiation, the most usual approach in other flatworms (Collins et al., 2013; Eisenhoffer et al., 2008; Pfister et al., 2007; Rossi et al., 2007; Solana et al., 2012) has been shown to be surprisingly ineffective in this regard with *in vitro* cultured metacestode vesicles (Koziol et al., 2014; Pohle et al., 2011). In contrast, treatment with specific inhibitors against enzymes that are important for proliferating cells was more efficient and resulted in strong depletion of the germinative cells. For example, in a proof-of-concept approach, treatment of metacestode vesicles with high concentrations of the ribonucleotide reductase inhibitor hydroxyurea (HU) *in vitro* resulted in a strong depletion in the number of proliferating cells and of total germinative cells. The effect was very specific for the germinative cells, since no difference could be observed in differentiated cell types, as determined using specific molecular markers (Koziol et al., 2014). This experiment further revealed that surviving proliferating cells were able expand in clonal patches that strongly indicated extensive potential to self-renew for individual germinative cells in the germinal layer. The existence of at least some germinative cells with extensive self-renewal capabilities is also expected from the ability of metacestode tissue to be indefinitely passaged *in vivo* by serial transplantation (Norman and Kagan, 1961), making the metacestode stage effectively “immortal”.

In a different study with a more direct connection to possible clinical applications, metacestodes and primary cells of *E. multilocularis* where treated *in vitro* for weeks with low concentrations (5–100 nM) of BI 2536, a highly specific inhibitor for polo-like kinase 1 (PLK-1) (Schubert et al., 2014). PLK-1 is an important

regulator of mitosis, and as such, was validated as an anti-cancer drug target (Archambault and Glover, 2009). Furthermore, the *E. multilocularis* ortholog *emplk-1* is specifically expressed in germinative cells, and upregulated in primary cell cultures (Schubert et al., 2014). Treatment with BI 2536 at concentrations as low as 25 nM resulted in an almost complete elimination of proliferating cells in metacestode vesicles and drastically blocked the regeneration of new metacestode vesicles from primary cells. Interestingly, although growth of metacestodes ceased after treatment with BI 2536, they remained viable for many weeks, and a similar effect was also observed after HU treatment (Koziol et al., 2014). This suggests that specific chemotherapeutic targeting of the germinative cells results *in vitro* in a parasitostatic effect. However, treatments against the germinative cells could be an ideal addition to current anti-parasitic treatments with benzimidazoles, by inhibiting the repair of the damage caused in the germinal layer by chemotherapy and by preventing the formation of new parasite vesicles from surviving parasite tissue.

7. Which signals regulate the *Echinococcus* germinative cells?

Proliferation and differentiation of stem cells are typically regulated by signals from the surrounding cells. Because of the intimate relationship between the metacestode and the host, it is possible that the germinative cells of *Echinococcus* are not only sensing molecules produced by the germinal layer but also from the surrounding tissues of the host. The fact that primary cells from *E. multilocularis* require soluble factors secreted by mammalian feeder cells gives further credit to this hypothesis (Spiliotis et al., 2008, 2010). Because many signalling receptors, ligands, and downstream signalling cascades are evolutionarily conserved between mammals and flatworms (Brehm, 2010b), it is possible that the parasite receptors are able to detect host cytokines and growth factors using cognate receptors homologous to those of the host.

Many such receptors and signalling factors were previously identified by directed molecular cloning (reviewed by Brehm (2010b)), and many more could be identified thanks to the genome sequencing projects for *Echinococcus* spp. (Brehm, 2010a,b; Riddiford and Olson, 2011; Tsai et al., 2013; Zheng et al., 2013), as already outlined above. Previous studies clearly showed that some of these receptors were able to interact with human ligands, including the interaction *in vitro* of the *E. multilocularis* insulin receptors EmIR1 and EmIR2 with human insulin (Hemer et al., 2014; Konrad et al., 2003). Recently, it was shown that physiologically relevant concentrations of insulin added to *in vitro* culture are indeed able to activate the *E. multilocularis* receptors and to promote growth and cell proliferation in metacestode vesicles, as well as the proliferation and regeneration of new vesicles in primary cells (Hemer et al., 2014). Altogether, these results strongly support the hypothesis that the parasite is able to detect host insulin by means of conserved signalling mechanisms. EmIR1 and EmIR2 have different complex expression patterns, including expression in germinative cells and in differentiated cells such as glycogen storage cells (Hemer et al., 2014). Thus, it remains unclear how much of the effect of human insulin on parasite cell proliferation is coming from a direct interaction of insulin with receptors on germinative cells and how much from secondary signals coming from the activation of differentiated cells.

E. multilocularis also has a wide complement of evolutionarily conserved, endogenous signalling ligands that are expressed in the germinal layer, and which could be of importance for the regulation of the germinative cells (Brehm, 2010a,b; Tsai et al., 2013). This thus begs the question: which are the cells that secrete such ligands? Although this question is likely to have multiple answers, depending on the ligands themselves, we would like to point to a possible, previously unsuspected source. We have recently discovered the presence of a discontinuous nerve net in the germinal layer of metacestode vesicles (Koziol et al., 2013). This was an unexpected discovery, since metacestode vesicles are an immotile larval form with a weak layer of disorganised muscle fibers, and therefore with no clear requirement of a nervous system for myomodulation. Furthermore, small populations of nerve cells in the germinal layer could be detected by immunoreactivity against conserved epitopes found in invertebrate neuropeptides but no evidence could be found of serotoninergic or cholinergic elements (Koziol et al., 2013), which are the best characterised myomodulators in cestodes (Maule et al., 2006). We could also detect nerve cells and muscle fibers during early stages of regeneration in primary cell cultures (Koziol et al., 2014). Because of these characteristics, we have hypothesised that this nerve net could actually serve as a neuroendocrine system. Relatedly, possible neuroendocrine elements have previously been described in cestodes, and the neuromuscular system has been suggested to influence the proliferation and differentiation of stem cells in free living flatworms (Reuter and Gustafsson, 1996; Rossi et al., 2012; Witchley et al., 2013).

Finally, during the annotation of the predicted gene complement of tapeworms (Tsai et al., 2013), we also contributed with the *de novo* detection and prediction of candidate neuropeptide-encoding genes in cestodes. We found 22 families of candidate neuropeptides, many of which showed similarity to validated neuropeptides from other flatworms or other invertebrates (Tsai et al., 2013) (see Supplementary information S13 in that publication). Transcription of a subset of these neuropeptide candidate genes could be detected in the germinal layer by RNAseq and RT-PCR. This suggests that some of these neuropeptides could have a biological role, perhaps as peptide hormones, in the metacestode stage.

8. Are *Echinococcus* germinative cells “resistant” to benzimidazoles?

Since the germinative cells are the only *Echinococcus* cells capable of proliferation, they have to be the crucial cell type that mediates the frequently observed recurrence of parasite growth upon interruption of chemotherapeutic treatment in AE patients. Apparently, benzimidazole chemotherapy *in vivo* is in many cases not effective in eliminating the germinative cell population (Brunetti et al., 2010; Kern, 2010). Limited efficacy of benzimidazole treatment against parasite stem cells has also been reported in studies demonstrating that albendazole derivatives only affect germinative (‘undifferentiated’) cells at late time points during *in vitro* treatment of metacestode vesicles (Ingold et al., 1999; Stettler et al., 2003), which was recently verified by Küster et al. (2013) in an *in vivo* mouse model for *E. multilocularis* infections. Indeed, when we tested albendazole on primary cell cultures, which contain a high proportion of germinative cells (Koziol et al., 2014), little or no effects were observed, even at high drug concentrations (Hemer, Schubert, Brehm, unpublished results) which supports the notion that germinative cells are benzimidazole resistant.

What might be the molecular basis for the limited efficacy of benzimidazoles toward germinative cells? It is well established that benzimidazoles act on β-tubulin, an important building block of microtubuli, thus preventing correct assembly and function of the cytoskeleton (Robinson et al., 2004). Although β-tubulins are highly similar between helminths and mammals, few amino acid sequence differences account for a higher affinity of benzimidazoles to helminth β-tubulin than to mammalian β-tubulins. Of particular importance is amino acid residue no. 200, which in mammalian β-tubulins is usually Tyrosine whereas helminth β-tubulins often carry phenylalanine at this position (Robinson et al., 2004).

Table 1
Expression of *E. multilocularis* β-tubulin genes in different developmental stages.

Gene designation[a]	aa200[b]	PC2[c]	PC11	MV−	MV+	PS−	PS+	AW	gAW	Name[d]
EmuJ_000041100	DET**F**CID	3	4	1	0	4	3	71	124	
EmuJ_000069900	DET**Y**CID	6	5	3	80	2	2	306	207	
EmuJ_000202500	DET**F**CID	57	53	343	661	56	53	108	191	*tub*-3
EmuJ_000202600	DET**F**CID	380	411	388	395	189	166	126	409	*tub*-1
EmuJ_000569000	DET**Y**CID	6	2	2	4	1	0	33	49	
EmuJ_000617000	DQT**F**CID	3	6	1	7	4	5	11	18	
EmuJ_000672200	DET**Y**CID	1161	1157	806	1641	745	842	1055	1614	*tub*-2
EmuJ_000955100	DET**Y**CID	1	247	0	6	88	82	29	95	
EmuJ_001081200	DLT**V**ILD	1	4	1	0	7	2	9	21	
EmuJ_001126150	DES**F**TLD	15	15	17	6	20	15	13	16	

[a] Gene designation according to Tsai et al. (2013) as available through GeneDB (http://www.genedb.org/Homepage/Emultilocularis).
[b] Sequence context around amino acid 200 (in bold).
[c] Expression strength in fpkm (fragments per kilobase of exon per million fragments mapped) according to Illumina RNASeq (Tsai et al., 2013) for primary cells after 2 (PC2) and 11 (PC11) days of development, metacestode vesicles without (MV−) and with (MV+) brood capsules, dormant (PS−) and low pH/pepsin-activated protoscoleces (PS+), pre-gravid adult worms (AW) and gravid adult worms (gAW).
[d] Gene name according to Brehm et al. (2000b).

Particularly in studies on the nematode *Haemonchus contortus* it has been established that benzimidazole resistance is associated with Phe to Tyr exchanges at position 200 (Robinson et al., 2004). As shown in Table 1, the *E. multilocularis* genome contains 10 genes encoding β-tubulin subunits. Only 5 of these carry Phe at position 200, one carries Valine, and 4 Tyr. The highest levels of expression throughout the life cycle can be observed for 3 of the parasite's β-tubulin genes which had previously been characterised by Brehm et al. (2000b) and which were designated *tub*-1, *tub*-2, and *tub*-3 (Table 1). Of these, the by far most abundantly expressed isoform in all life cycle stages is *tub*-2, which encodes a potentially resistant β-tubulin (Tyr at position 200). In the metacestode, *tub*-1 and *tub*-3, which both encode β-tubulins that carry Phe at position 200, are well expressed and this is most probably the reason for the general sensitivity of this developmental stage to benzimidazoles. Interestingly, *tub*-2 is highest expressed in primary cell cultures after 2 days of incubation which, according to Koziol et al. (2014), contain a high proportion of germinative cells. *tub*-2 expression then decreases when primary cells develop into metacestode vesicles and rises again when brood capsules are formed (Table 1), which is the expected expression pattern for a gene that is specifically expressed in germinative cells. No such pattern can be observed for *tub*-1 or *tub*-3 or the other *E. multilocularis* β-tubulin genes, most of which could have specific roles in the adult worm (Table 1). These NGS transcriptomic studies are supported by RT-PCR analyses of our lab (Schubert, Brehm, unpublished data) which show that in hydroxyurea- and BI 2536-treated metacestode vesicles (depleted of germinative cells), *tub*-2 expression is strongly decreased, whereas the expression of *tub*-1 and *tub*-3 is unaffected by this treatment. Taken together, these data clearly indicate that *E. multilocularis* germinative cells exclusively express a potentially benzimidazole resistant β-tubulin isoform, which probably accounts for their insensitivity toward the drug. Since *E. granulosus* also expresses a Tub-2 ortholog (100% identical to *E. multilocularis* Tub-2) it can be expected that this species also harbors benzimidazole insensitive germinative cells.

9. Conclusions and outlook

Based on the data discussed above, we consider it important that future efforts toward targeted drug design against echinococcosis concentrate on the parasite's germinative cell system. Promising drug targets do not have to be specifically expressed in germinative cells but they should 'also' be expressed in these cells. If promising targets are exclusively expressed in germinative cells, such as the Polo-like kinase EmPlk1 (Schubert et al., 2014), respective drug treatment can be combined with benzimidazole therapy in order to affect a broad range of *Echinococcus* cells. As an aid for targeted drug design, we are currently carrying out, in cooperation with the Wellcome Trust Sanger Institute, extensive transcription profiling of all *E. multilocularis* genes in various life cycle stages such as primary cells, metacestode vesicles with and without brood capsules, and protoscoleces before and after activation with low pH/pepsin (all preparations in triplicate). These analyses will include transcription profiling for parasite germinative cells. According to previous approaches in planarians which determined neoblast-specific gene expression patterns by subtracting the transcriptome of radiation-treated animals (specifically lacking neoblasts) from the transcriptome of normal animals, we will make use of the recently developed techniques of germinative cell depletion in metacestode vesicles using hydroxyurea and the Plk1 inhibitor BI 2536. Since primary cell cultures after 2 days of incubation are highly enriched in stem cells (Koziol et al., 2014), the transcriptome profile obtained by subtracting germinative cell-free metacestode vesicles from that of normal vesicles can subsequently be verified using the transcription profile of 2-day primary cultures. Altogether, these efforts should be highly effective to generate germinative cell specific transcription profiles in due time.

Concerning *in vitro* screening of compound libraries or specific lead compounds on cultivated parasite material, the crucial role of germinative cells in parasite regeneration stresses the importance of the primary cell cultivation system developed by Spiliotis et al. (2008, 2010). Although drug testing on intact metacestode vesicles, particularly when combined with elegant readout methods as developed by Stadelmann et al. (2010) for medium to high throughput screening, will still be highly important, this system would probably miss all compounds that are specifically acting on germinative cells. Hence, effective drug screening should involve both, the metacestode cultivation method and the primary cell culture systems.

In conclusion, by the characterisation of the nuclear genomes of both medically important *Echinococcus* species, the scientific community has made a great leap forward toward a closer understanding of host-parasite interaction mechanisms, parasite development and parasite evolution. Combined with extensive transcriptome profiling and taking into account recent progress concerning the important role of germinative cells in parasite regeneration, we should be able to identify promising targets for the development of parasitocidal drugs that can be validated in robust cultivation systems for germinative cells and metacestodes. The challenge is now to recruit sufficient funding for drug development against diseases that are still 'neglected'.

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