Echinococcus multilocularis as an experimental model in stem cell research and molecular host-parasite interaction

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SUMMARY

Totipotent somatic stem cells (neoblasts) are key players in the biology of flatworms and account for their amazing regenerative capability and developmental plasticity. During recent years, considerable progress has been made in elucidating molecular features of neoblasts from free-living flatworms, whereas their role in parasitic species has so far merely been addressed by descriptive studies. Very recently, however, significant advances have been made in the *in vitro* culture of neoblasts from the cestode *Echinococcus multilocularis*. The isolated cells proved capable of generating mature metacestode vesicles under laboratory conditions in a manner that closely resembles the oncosphere-metacestode transition during natural infections. Using the established neoblast cultivation protocols, combined with targeted manipulation of *Echinococcus* genes by RNA-interference, several fundamental questions of host-dependent parasite development can now be addressed. Here, I give an overview of current cultivation techniques for *E. multilocularis* neoblasts and present experimental approaches to study their function. Furthermore, I introduce the *E. multilocularis* genome sequencing project that is presently in an advanced stage. The combined input of data from the *E. multilocularis* sequencing project, stem cell cultivation, and recently initiated attempts to genetically manipulate *Echinococcus* will provide an ideal platform for hypothesis-driven research into cestode development in the next years.

Key words: *Echinococcus*, cestode, parasite, genome, regeneration, neoblast, stem cell, antigen B, host-parasite interaction, *in vitro* cultivation.

INTRODUCTION

Having largely been neglected by developmental biologists until about 15 years ago, flatworms have recently made a great career as invertebrate models in studying molecular and cellular aspects of animal development. This renewed interest was mainly fuelled by the amazing regeneration capacity of free-living turbellarians such as *Dugesia japonica* and *Schmidtea mediterranea* (also called planarians) which can rebuild an entire organism from sliced body-pieces of as few as 10,000 cells (for recent reviews see Sanchez-Alvarado, 2006; Sanchez-Alvarado and Tsonis, 2006; Rossi *et al*. 2008; Saló *et al*. 2009), a trait that is absent in classical invertebrate models such as *Drosophila melanogaster* or *Caenorhabditis elegans*. Planarians owe this regeneration capacity to a population of totipotent somatic stem cells, called neoblasts, that are distributed over the entire body of the animal and decisively contribute to wound repair and tissue regeneration upon physical damage. Neoblasts are the only planarian cell type that exhibits mitotic activity and can differentiate into all types of somatic cells. Apart from their important role in tissue regeneration, neoblasts are also involved in the enormous developmental plasticity of planarians, which can grow or shrink under favourable or poor environmental conditions, respectively, simply by increasing or decreasing the overall body cell number (Sanchez-Alvarado and Kang, 2005; Saló, 2006).

Apart from free-living turbellarians, the vast majority of species in the phylum Platyhelminthes belong to the three large parasitic lineages of the cestodes (tapeworms), the trematodes (flukes) and the monogeneans (Olson, 2008). Just like in their free-living relatives, totipotent stem cells are believed to play an important role in the developmental biology of parasitic flatworms, where they are called 'germinal cells', 'germinative cells', regenerative cells' or sometimes just 'stem cells' (reviewed by Reuter and Kreshchenko, 2004). Despite this different nomenclature, it can be expected that the basic mechanisms of self-renewal and differentiation are largely shared between the neoblasts of the free-living species and totipotent stem cells of parasitic flatworms. Furthermore, totipotent stem cells must surely have played a master role in the evolution of the complex and highly fascinating life-cycles of parasitic flatworms, yet again revealing a tremendous developmental plasticity. Hence, although free-living flatworms are generally better accessible from

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the experimental point of view, the full evolutionary potential and flexibility of flatworm totipotent stem cells can only be adequately addressed by also including parasitic model systems in developmental studies. Furthermore, respective investigations should also lead to fundamental knowledge concerning flatworm parasitism and the evolution of complex life-cycles. While, during regeneration, neoblasts of free-living species have to process cell-autonomous and environmental data that derive from a homologous planarian environment, their parasitic counterparts are also subject to regulation by heterologous host signals. Particularly in the case of asexually multiplying larvae, like intra-snail stages of the trematode *Schistosoma mansoni* or metacestodes of several cyclophyllidean tapeworms, which can in fact be viewed as a special kind of xeno-transplants, proliferation and pattern formation of the parasitic tissue is most probably to a large degree governed by the host cytokine micro-environment that is present at the site of infection and generated during the host immune response. How these parasitic flatworm species integrate host-derived signals into their 'inherited' network of stem cell control will be a clue in understanding the complex host-parasite interaction mechanisms in flatworm infections.

Although the past decades have witnessed several important advances concerning *in vitro* cultivation techniques for larval or adult stages of trematodes and cestodes (reviewed by Coustau and Yoshino, 2000; Siles-Lucas and Hemphill, 2002; Brehm and Spiliotis, 2008*a*), significant breakthrough in the cultivation of isolated parasite cells, including totipotent stem cells, has so far only been reported for the cestode *Echinococcus multilocularis*. Respective studies were one of the main foci of my laboratory and in this article I will give an overview of the present status of *E. multilocularis* cell cultivation techniques. According to the suggestion of Reuter and Kreshchenko (2004), I will use the term 'neoblast' instead of 'germinal cell' throughout the article whenever totipotent or pluripotent *Echinococcus* cells are addressed. I will integrate current knowledge on the regulation of planarian neoblasts since these data will surely have a great impact on future studies in the parasitic systems. I will also present data concerning *E. multilocularis* signalling systems that are involved in the molecular interaction with the host, and might play a role in stem cell regulation as well. Finally, I will briefly address how studies on *Echinococcus* neoblasts could shed more light on so far unanswered questions concerning the function of trans-splicing in this organism, or the generation of genetic variability during larval growth. A brief outline of the present status of the *E. multilocularis* whole genome sequencing project and approaches to genetically manipulate parasite larvae will be given at the end of the article.

THE LONG WAY TO *ECHINOCOCCUS* NEOBLAST CULTURES

During the golden age of ultrastructural studies on cestodes in the 1950–1980s, the presence of undifferentiated 'germinal cells' has frequently been described in a variety of adult and larval tissues. Morphologically, these cells could be clearly distinguished from their differentiated neighbours in having a large nucleus with a prominent nucleolus, surrounded by scant, basophilic cytoplasm, that contained few mitochondria as the only organelles (Heath and Lawrence 1976; Mehlhorn *et al*. 1983; Swiderski, 1983; Rybicka, 1966; Sakamoto and Sugimura, 1970; Slais, 1973; Sakamoto, 1981, 1982). Mostly by studying the cellular dynamics of proliferating cells through a combination of electron microscopy and the uptake of radioactively labelled thymidine, a relatively clear picture emerged that 'germinal cells', like their neoblast counterparts in planarians, are the only mitotically active cells in cestodes that give rise to all other differentiated cells (reviewed by Reuter and Kreshchenko, 2004). According to these early studies, neoblasts are usually located in the neck region of the adult's scolex and initiate the formation of proglottides from the germinative area. Within proglottides, neoblasts are then involved in the formation of the genital anlagen and the genital apparatus (see references in Reuter and Kreshchenko, 2004). After gamete formation and fertilization, neoblasts arise early during embryogenesis, actively synthesize RNA during the entire process of oncosphere formation and are usually located at one pole of the late bilateral symmetrical oncosphere in differing numbers (between 6 and 12), depending on the species (Rybicka, 1966; Slais, 1973; Swiderski, 1983; Mlocicki *et al*. 2006). Studies on the early development of *E. multilocularis* in experimentally infected mice then showed that the neoblasts are the only oncosphere cells that contribute to metacestode formation and that hooks and muscles completely disappear within a few days after infection (Sakamoto and Sugimura, 1970; Vogel, 1977; Mehlhorn *et al*. 1983). Hence, the transition from oncosphere to metacestode in *Echinococcus* spp. is, as in other tapeworms, a true metamorphosis. Although only very few investigations on the formation of protoscoleces within metacestode vesicles and hydatid cysts of *E. multilocularis* and *E. granulosus* have so far been undertaken, it can be expected that the development of this larval stage is also induced by neoblasts that are part of the germinal layer. At least preliminary evidence that this is indeed the case has been obtained for *E. granulosus* (Galindo *et al*. 2003).

Due to the decisive role of neoblasts in driving the life-cycle of *Echinococcus* and other cestodes, several attempts toward their cultivation *in vitro* have already been undertaken in the 1980s (reviewed in

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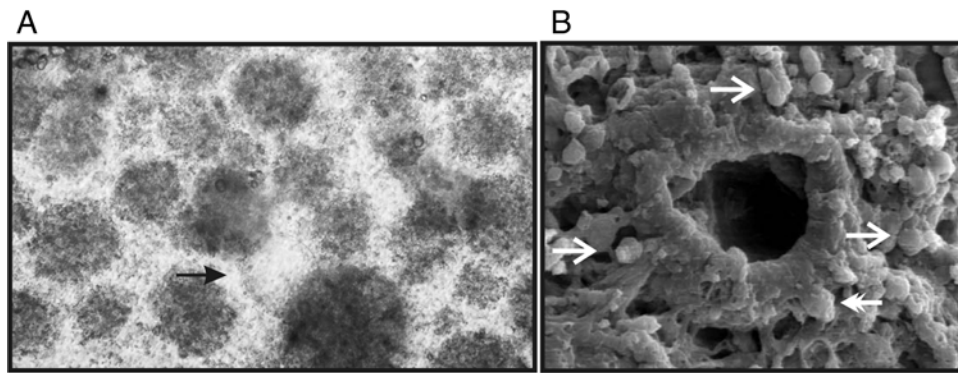

Fig. 1. *In vitro* regeneration of *E. multilocularis* metacestode vesicles from primary cells. (A) Displayed is a typical culture after 3–4 weeks of cultivation. Note the extensive cell aggregates in which metacestode tissue is formed around central cavities. A complete, young metacestode vesicle is present close to the middle (arrow). (B) Scanning electron microscopic image of regenerating cell-aggregate (detail). During fixation, a developing vesicle was ruptured, thus revealing the central cavity surrounded by the growing germinal layer. Arrows indicate small cells that most probably represent stem cells. Double pointed arrow indicates a differentiating cell that fuses with the growing germinal layer. For further images of *E. multilocularis* primary cells, please refer to Spiliotis *et al*. (2008).

Brehm and Spiliotis, 2008*a*). However, these early studies were confounded by the fact that parasite material from experimentally infected mice had been used as a source for parasite cells, and that it was not known at that time that *Echinococcus* cells are highly sensitive towards reactive oxygen species that are typically produced during *in vitro* culture. As a consequence, the isolated parasite cells were frequently overgrown in culture by contaminating host cells (Brehm and Spiliotis, 2008*a*). First steps towards a solution of this problem have been made by Hemphill and Gottstein (1995) as well as Jura *et al*. (1996) through the development of *in vitro* cultivation systems for *Echinococcus* metacestode vesicles. Although these systems still relied upon co-cultivation of *Echinococcus* tissue with host feeder-cells (Caco-2 colon carcinoma cells or primary rat hepatocytes), they provided the first systems by which parasite vesicles could be produced that, after vigorous washing, were almost free of host-cell contamination. A decisive step was then made by Spiliotis *et al*. (2004) who established the first system for axenic cultivation of *E. multilocularis* vesicles. This study showed that parasite vesicles can be kept in the absence of host cells, provided that the medium contained reducing substances, and that the cultures were maintained under a nitrogen atmosphere, thus eliminating the formation of reactive oxygen species (Spiliotis *et al*. 2004; Brehm and Spiliotis, 2008*a*). Since, even under these conditions, parasite vesicles only proliferated when host cell-conditioned medium was applied, the axenic cultivation system was also the first to demonstrate unequivocally that host cell-produced growth factors support parasite development and proliferation (Spiliotis *et al*. 2004). One of the most important outcomes of this study was that the axenic vesicles now provided an excellent source to set up parasite cell cultures essentially free of host contamination. After modifying the axenic cultivation system towards the so-called ‘large-scale liquid culture system’, by which significant numbers of axenic vesicles can be produced, Spiliotis and Brehm (2009) were able to isolate, through tryptic digestion, primary parasite cells in sufficient quantity to establish *in vitro* cultures. Using flow cytometry, Spiliotis, M. *et al*. (2008) showed that at least 30% of freshly established primary cells were in the S- and G2-phase of the cell cycle and, thus, represented neoblasts. Furthermore, the typical cestode neoblast morphology of a large fraction of *Echinococcus* primary cells was demonstrated in this study by transmission electron microscopy (Spiliotis *et al*. 2008). Under ideal growth conditions, the *Echinococcus* neoblasts proliferated in culture and formed cell aggregates which later developed small internal cavities (Fig. 1). After 5 weeks of incubation, the young vesicles enlarged and the parasite tissue surrounding the cavities contained both neoblasts and differentiated cells. At this time, the acellular laminated layer (LL), which is the typical distal border of mature metacestode vesicles acting as a physical barrier to separate host and parasite tissue in the later phase of the infection, was not yet formed. After 6 weeks of incubation, the LL was present, indicating the formation of fully mature metacestode vesicles. When injected into the peritoneal cavity of mice, these vesicles yielded high loads of parasite material, including metacestode tissue and protoscoleces, indicating totipotency of the cells that were initially set up in the primary culture system (Spiliotis *et al*. 2008). Hence, the enormous regenerative potential of free-living flatworms can also be observed for parasitic species and, in the case of *E. multilocularis*, the complete regeneration of metacestode vesicles was obtained from dispersed cells that were not even part of an intact tissue.

On the one hand, the *E. multilocularis* neoblast culture system should be a highly useful tool to study

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metastasis formation which often occurs during prolonged infections of the intermediate host (Mehlhorn *et al*. 1983). On the other hand, the pattern of parasite tissue formation from neoblasts in the *in vitro* regeneration system (Spiliotis *et al*. 2008) closely resembles the different phases of metacestode development as observed after infecting laboratory mice with oncospheres (Rausch, 1954; Sakamoto and Sugimura, 1970; Vogel, 1977). Hence, despite the different origin of *Echinococcus* neoblasts in the regeneration system (metacestode-derived) and in natural infections (oncosphere-derived), there seems to be no qualitative difference in how they react to comparable environmental conditions. I therefore suggest that metacestode-derived *Echinococcus* neoblasts are useful tools to study a wide variety of developmental transitions and processes, including the formation of metastases, the oncosphere-metacestode-metamorphosis, the formation of protoscoleces from the germinal layer, or even the production of proglottides by adult worms. The main challenge for the future will be to find suitable experimental *in vitro* conditions for each of these different contexts and settings in order to obtain data that are applicable to the *in vivo* situation.

ECHINOCOCCUS NEOBLASTS – WHAT NEXT?

The establishment of the neoblast regeneration system is, of course, only the first step into hypothesis-driven research on *Echinococcus* development. Next, it will be important to identify stem cell markers in order to track the parasite's neoblast population during vesicle generation by immunohistochemical methods and flow cytometry. This is especially important as studies on planarians have already shown that several sub-types of neoblast exist, some of which might be 'true' totipotent stem cells whereas others probably represent cells that are in a transition state and bound to differentiate (Eisenhoffer *et al*. 2008; Higuchi *et al*. 2007; Rieger *et al*. 1999). The situation could be similar in *Echinococcus* since ultrastructural studies have regularly revealed the co-occurrence of so-called 'light-stained' and 'dark-stained' undifferentiated cells in developing metacestode tissue (Sakamoto and Sugimura, 1970; Sakamoto, 1981, 1982). Dark-stained undifferentiated cells apparently contain numerous polyribosomes in the cytoplasm and, according to Sakamoto, T. and Sugimura, M. (1970), are differentiating into so-called 'asteroid transforming cells' or fuse with the syncytial germinal layer during asexual multiplication, whereas light-stained undifferentiated cells appear to be precursors of the dark-stained type. Hence, like in the neoblast populations of planarians, one type of *Echinococcus* neoblasts, the light-stained cells, might represent the true stem cell lineage that is self-sustained during larval development and that, by asymmetrical cell division, gives rise to dark-stained cells that either directly fuse with the growing syncytial tissue, or undergo limited rounds of self-amplification prior to differentiation into other cell types.

Classical methods of staining neoblasts in planarians involved labelling of S-phase cells through incorporation of the thymidine analogue 5′-bromo-desoxy-uridine (BrdU) or the utilization of anti-phospho-histone H3 antibodies in immunohistochemistry (Saló and Baguna, 2002; Nimeth *et al*. 2004). The first method has already been employed by us to identify proliferating cells in *E. multilocularis* primary cell populations (Spiliotis *et al*. 2008) and, at least in preliminary Western blot experiments, we could detect phospho-histone H3 in *Echinococcus* metacestode vesicles (Hemer, S. and Brehm, K., unpublished results). While these techniques will be suitable for detecting proliferating cells in the *Echinococcus* neoblast regeneration system, it will not be possible to differentiate between different subpopulations of stem cells. Towards this end, it will be necessary to employ antibodies against stem cell-specific markers, of which several have been identified in planarian neoblasts (Table 1). The list includes the VasA orthologue *DjvlgA* (a DEAD-box family RNA helicase; Shibata *et al*. 1999), a member of the minichromosome maintenance protein family, *DjMCM2*, that is involved in the formation of pre-replication complexes during G1-S transition (Salvetti *et al*. 2000), and the proliferating cell nuclear antigen *DjPCNA* (Ito *et al*. 2001). Interestingly, BLAST analyses against the first draft version of the genome predict that close orthologues to all those factors are also expressed by *E. multilocularis* (Table 1) and that, due to highly conserved domains, antibodies directed against the planarian proteins might also cross-react with the cestode factors. Using such antibodies in combination with anti-phospho-histone H3 antibodies and BrdU labelling in immuno-histochemical studies will most probably reveal a picture of *Echinococcus* stem cell dynamics that cannot be obtained by ultrastructural investigations alone. Mostly by using RNA interference (RNAi) techniques, a series of additional proteins have been identified that, by different cellular and molecular mechanisms, are involved in planarian stem cell maintenance (Table 1). Although not exclusively restricted to neoblasts, and thus not suitable as direct markers, it can be expected that their functions are also conserved in stem cell populations of parasitic flatworms. In the first draft version of the *Echinococcus* genome, orthologues of the majority of these factors are present (Table 1) and, by adapting RNAi and transgenic techniques to the *Echinococcus* neoblast regeneration system (see below), it should be possible to discern how far the molecular mechanisms of stem cell renewal and differentiation overlap, or differ, in planarians and cestodes.

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Table 1. Possible stem cell markers and regulators encoded by the *E. multilocularis* genome.

Protein	Species	Homology	Function	Expression	Reference	contig	e-value
DjvlgA	*D. japonica*	DEAD box RNA helicase	translation regulation	SSC, GSC CB	Shibata et al. 1999	8817	3e-133
DjvlgB	*D. japonica*	DEAD box RNA helicase	translation regulation	GSC CB	Shibata et al. 1999	8817	3e-108
DjMCM2	*D. japonica*	minichr. maint. prot.	replication initiation	SSC, GSC	Salvetti et al. 2000	4550	4e-232
DjPCNA	*D. japonica*	proliferating cell nuclear AG	DNA replication	SSC, GSC	Ito et al. 2001	6820	1e-065
SMEDWI-2	*S. mediterranea*	PIWI/Ago orthologue	piRNA expression	SSC, GSC	Reddien et al. 2005	–	–
SMEDWI-3	*S. mediterranea*	PIWI/Ago orthologue	piRNA expression	SSC, GSC	Palakodeti et al. 2008	6139	3e-011
DjCBC-1	*D. japonica*	DEAD box RNA helicase	translational regulation	SSC, GSC CB	Yoshida et al. 2007	9079	1e-170
Djnos	*D. japonica*	NANOS-orthologue	translational regulation	GSC CB	Sato et al. 2006	9653	9e-012
Bruli	*S. mediterranea*	BRUNO-orthologue	translation initiation	SSC, GSC	Guo et al. 2006	0960	6e-027
DjPum	*D. japonica*	Pumilio-orthologue	translational repression	SSC	Salvetti et al. 2005	3014	2e-049
PTEN-1/2	*S. mediterranea*	PTEN-orthologue	PI3K/Akt- signalling	SSC, GSC diff. cells	Oviedo et al. 2008	7253	2e-021
Smedinx-11	*S. mediterranea*	innexin	gap junction	SSC diff. cells	Oviedo and Levin 2007	3006	2e-054
Spoltud-1	*S. polychroa*	TUDOR-orthologue	mt rRNA localization	SSC, GSC CB	Solana et al. 2009	?	?

Homology searches against the publicly available, first assembly of the *E. multilocularis* genome have been performed to identify orthologues of known stem cell regulatory proteins of free-living flatworms. Indicated are the protein designations (protein), the species where they have been identified as well as homologies and putative functions. Expression patterns refer to somatic stem cells (SSC), germline stem cells (GSC) and differentiated cells (diff. cells). 'CB' indicates that the protein specifically locates to chromatoid bodies in planarians. 'Contig' indicates the contig number (first assembly version) where the closest orthologue is located on the *E. multilocularis* genome. Dashes (–) indicate that no orthologue is present in the first assembly version. Question marks indicate that proteins with TUDOR-domains are encoded but that overall e-values are low.

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One striking morphological feature that differs between flatworm neoblasts appears to be the occurrence of so-called 'chromatoid bodies' that are typically present in stem cells of free-living species but have, so far, never been reported for parasitic species (Reuter and Kreshchenko, 2004). Chromatoid bodies are composed of amorphous material and locate close to the nucleus of undifferentiated and differentiating planarian neoblasts as well as germ cells. Since chromatoid body-like structures are also present in germ cells of vertebrate and other invertebrate species such as *D. melanogaster* or *C. elegans*, an involvement of these structures in establishing and maintaining an undifferentiated, totipotent state of stem cells has already been suggested in early ultrastructural studies on planarians (Hori, 1982). Various RNA-binding proteins such as DEAD-box RNA helicases (e.g. VasA) or members of the PIWI/Argonaute family map to the chromatoid body of many different species, including planarians (Table 1), and there is accumulating evidence that micro-RNA and RNA-decay pathways converge to exactly this structure in germ cells (Kotaja and Sassone-Corsi, 2007). It is presently hard to understand why structures like chromatoid bodies, conserved in totipotent stem cells across phyla as different as arthropods, chordates, nemathelminths, and (free-living) flatworms should not be present in stem cells of the parasitic flatworm lineages, particularly since proteins that are typically located to these structures appear to be conserved in these organisms (Table 1). It might be that chromatoid body-like structures have so far merely been overlooked in ultrastructural studies on parasitic flatworms since their particular role in stem cell maintenance has only recently been established. Alternatively, since the formation of the chromatoid body appears to be linked to the cell cycle (Yokota, 2008), the neoblasts of proliferating parasite tissue might preferentially be in a state that is free of chromatoid bodies whereas 'resting', non-proliferating stem cells are equipped with these structures. At least in his ultrastructural studies on *Echinococcus* oncospheres, Swiderski (1983) described numerous 'chromatin aggregates beneath the nuclear envelope' in undifferentiated cells that were 'often surrounded by small spherical granules of high electron density'. Whether these structures represent the *Echinococcus* type of chromatoid bodies still remains to be elucidated.

A highly interesting observation of potential relevance for chromatoid body-like structures in cestodes has been made by Fernandez *et al.* (2002) during the establishment of cDNA libraries from *E. granulosus* protoscoleces and metacestode tissue. These authors reported that a considerable part ($> 60\%$) of cDNA clones from conventional libraries represented polyadenylated, mitochondrial (mt) transcripts coding for ribosomal subunits of the 55S (mt) type. During a sabbatical in my laboratory, C. Fernandez later made the same observation for *E. multilocularis* cDNA libraries (Fernandez, C. and Brehm, K., unpublished observation). Interestingly, polyadenylated ribosome-encoding mt transcripts are also present in *D. melanogaster* germ plasm which is inherited by cell lineages that give rise to germ cells (Kobayashi *et al.* 2005). Furthermore, they have been identified in germinal granules of *Xenopus* embryos (Kashikawa *et al.* 2001), and they are used in ejaculated mammalian sperm for protein translation during the final maturation steps prior to fertilization (Villegas *et al.* 2002; Gur and Breitbart, 2008). In *Drosophila*, the *TUDOR* protein was found to be essential for the transport of mt polyA-RNAs to polar granules (Amikura *et al.* 2001*a*; Kobayashi *et al.* 2005) and RNAi treatment against the planarian *TUDOR* orthologue, *Spoltud-1*, resulted in an efficient depletion of neoblasts, indicating that similar mechanisms are also relevant for stem cell maintenance in free-living flatworms (Solana *et al.* 2009). Indeed, polyadenylated mt RNAs have already been specifically localized to chromatoid bodies in stem cells of planarian polyclad embryos (Sato *et al.* 2001). Taken together, these data point to an important role of mt polyA-RNAs in stem cell maintenance in different animal phyla and, due to the predominance of respective transcripts in the *Echinococcus* polyA transcript pool, they are probably also relevant for cestodes. This offers an excellent opportunity to identify *Echinococcus* neoblasts by using the characterized mt polyA-RNAs (Fernandez *et al.* 2002) as probes in *in situ* hybridization experiments, as previously carried out in planarians (Sato et al. 2001). Moreover, not only are polyadenylated mt RNAs present in cytoplasmic structures of *Drosophila* and mammalian germ cells, they also produce 55S ribosomal subunits that are located in germinal granules and mediate the translation of a subset of nuclear mRNAs (Amikura *et al.* 2001*b*, 2005; Gur and Breitbart, 2008). Hence, by using antibodies against mt 55S ribosomes in immuno-histochemistry, not only neoblasts but also chromatoid body-like structures could be located in *Echinococcus* cell preparations. Finally, since antibiotics directed against prokaryotic (and mt) ribosomes, such as chloramphenicol or the aminoglycoside kasugamycin, have already been successfully used to affect stem cell function in *Drosophila* (Amikura *et al.* 2005), they might also be used to study *Echinococcus* neoblast function *in vitro*, and even to inhibit parasite development *in vivo* during an infection. In view of these data, it might well be that the highly deleterious effects of the macrolide antibiotic clarithromycin on *in vitro* cultivated *E. multilocularis* metacestode vesicles, recently reported by Mathis *et al.* (2005), were not only due to an inhibition of ribosomes in mitochondria, but also to stem cell-specific effects on cytosolically active mt ribosomes. In a similar way,

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the specific depletion of neoblasts of the germinative area could account for the profound anthelminthic effects of several different aminoglycoside antibiotics on strobilar stages of *Taenia* spp. (Botero, 1970) and *Hymenolepis nana* (Maki and Yanagisawa, 1985).

THE *ECHINOCOCCUS* METACESTODE CYTOKINE MICROENVIRONMENT AND HOST-PARASITE CROSS-COMMUNICATION

While stem cell maintenance in planarians is mainly regulated by cell-autonomous factors such as VasA-like RNA helicases and other components of chromatoid bodies, neoblast proliferation and differentiation during the regeneration process is also governed by surrounding planarian cells that provide an appropriate cytokine microenvironment to ensure correct pattern formation and integration of the newly formed tissues into established body structures. Respective signals involve, for example, cytokines of the bone morphogenetic protein (BMP)-family that are required for proper formation of the dorsoventral axis during planarian regeneration (Molina *et al*. 2007) or ligands of the wingless-related (Wnt)-family that are involved in determining anterior-posterior polarity (Tanaka and Weidinger, 2008). As already mentioned, the situation is somewhat different for the developing *E. multilocularis* metacestode that is embedded in liver tissue of the host and surrounded by immune effector cells (Fig. 2). On the one hand, it is expected that the proliferating *Echinococcus* tissue secretes parasite-derived cytokines and hormones into the freshly forming cavities and into the surrounding host medium. In this context, we are currently studying three members of the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP)-family that are encoded by the *E. multilocularis* genome and are all expressed in the early developing metacestode (Table 2). On the other hand, in addition to *Echinococcus* cytokines the primary site of infection should also contain host-derived cytokines and hormones in high concentrations (Fig. 2). BMP-cytokines are, for example, regularly present in liver tissue where they regulate extracellular matrix production, regeneration and iron homeostasis (Xu *et al*. 2006: Babitt *et al*. 2007; Kinoshita *et al*. 2007; Sugimoto *et al*. 2007; Truksa *et al*. 2007). EGF- and FGF-family cytokines are strongly expressed and secreted by hepatocytes during regeneration processes (Fausto, 2000), which might be induced once damage has been inflicted on liver tissue either by the oncosphere or by the ongoing immune response. Insulin concentrations in mammalian hosts are highest where the portal vein meets the liver parenchyma (Shojaee-Moradie *et al*. 2000), and this is exactly the site where the oncosphere gains entry to its preferred target organ. TGF-β and related activin- and inhibin-cytokines as well as several

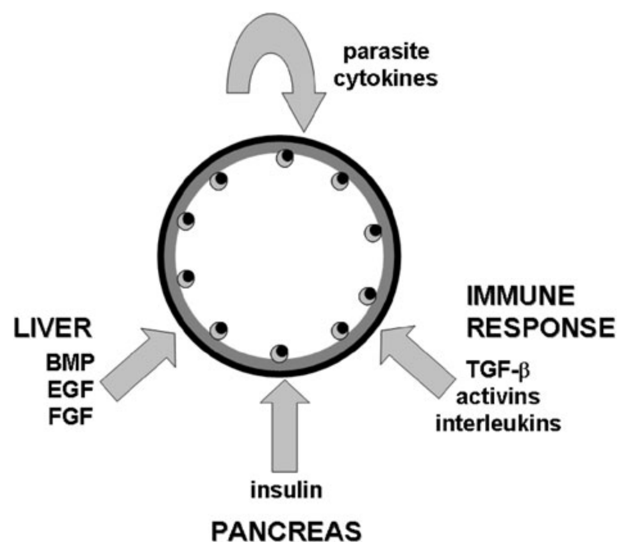

Fig. 2. The hormone- and cytokine-microenvironment for metacestode growth. Within the liver, young metacestode vesicles are subject to regulation by parasite-derived cytokines but also to host-derived signalling factors present in the liver or generated during the immune response. Not displayed are lipophilic hormones that are present at the site of infection and might bind to nuclear hormone receptors of the parasite.

interleukins (IL-10, IL-6, IL-4, IL-5 in the case of echinococcosis) are secreted in significant quantities by host cells in the vicinity of the developing parasite (Wellinghausen *et al*. 1999; Harraga *et al*. 2003). This is similar to the situation in cancer initiation, where the immune response around cancer stem cells and the released cytokine milieu stimulate cell proliferation (Tysnes and Bjerkvig, 2007); thus, the parasite-induced immune response around establishing metacestode vesicles, supported by hepatocyte-derived cytokines and hormones, could have an influence on parasite neoblast proliferation and tissue formation.

For this to occur, the parasite has to be equipped with signalling systems that are able to respond to host-derived cytokines, which in fact seems to be the case. During recent years, we were able to characterize a variety of evolutionarily conserved signalling systems in *E. multilocularis* that are structurally and functionally closely related to insulin-, EGF-, FGF-, and TGF-β/BMP-signalling systems of mammals (Table 2; Brehm *et al*. 2006; Brehm and Spiliotis, 2008*b*). The list includes insulin- and EGF-receptor-like receptor tyrosine kinases (RTKs) (Konrad *et al*. 2003; Spiliotis *et al*. 2003) as well as a series of downstream-acting factors of the MAP kinase cascade such as orthologues to Ras-like small GTPases, a Raf-like mitogen activated protein kinase kinase kinase (MAPKKK), an Erk-like MAPK, and a member of the p38-family of MAPKs (Spiliotis and Brehm 2004; Spiliotis *et al*. 2005, 2006; Gelmedin *et al*. 2008). Encoded by the parasite's genome are an additional insulin-receptor-like RTK, EmIR2, an

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Table 2. Receptor tyrosine- and serine/threonine-kinase signalling pathways expressed by the *E. multilocularis* metacestode.

	Protein	Function	contig	Reference
RTK-signalling				
Receptors	EmIR	InsR-like RTK	0721–0723	Konrad *et al.* 2003
	EmIR2	InsR-like RTK	9496–9498	Hemer, Brehm, unpub.
	EmER	EGFR-like RTK	2029–2031	Spiliotis *et al.* 2003
	EmFR	FGFR-like RTK	7936	Schäfer, Brehm, unpub.
Ligands	EmIns	insulin-like peptide	5296	Hemer, Brehm, unpub.
IST*	EmRas	Ras-like GTPase	0973	Spiliotis *et al.* 2005
	EmRal	Ras-like GTPase	4874	Spiliotis and Brehm, 2004
	EmRaf	Raf-like MAPKKK	8498–8723	Spiliotis *et al.* 2005
	EmTAK	TAK-like MAPKKK	0604	Zavala-Gongora, Brehm, unpub.
	EmMKK1	MKK3-like MAPKK	0132	Gelmedin *et al.* 2009
	EmMKK2	MEK-like MAPKK	0803	Gelmedin *et al.* 2009
	EmMPK1	Erk-like MAPK	4907	Spiliotis *et al.* 2006
	EmMPK2	p38-like MAPK	7471	Gelmedin *et al.* 2008
	EmMPK3	JNK-like MAPK	8178	Riedl, Brehm, unpub.
TGF-β/BMP-signalling				
Receptors	EmTR1	type I TGF-β RSTK	8724–8723	Zavala-Gongora *et al.* 2006
	EmTR2	type II TGF-β RSTK	5086	Bernthaler *et al.* submitted
	EmTR3	type I TGF-β RSTK	6522	Bernthaler *et al.* submitted
	EmTR4	type I TGF-β RSTK	4932	Epping, Brehm, unpub.
Ligands	EmBMP1	BMP-like cytokine	19290	Zavala-Gongora, Brehm, unpub.
	EmBMP2	BMP-like cytokine	6836	Epping, Brehm, unpub.
	EmAct	activin/inhibin-like	5988	Epping, Brehm, unpub.
IST*	EmSmadA	AR-Smad	5519	Zavala-Gongora *et al.* 2003
	EmSmadB	BR-Smad	2557	Zavala-Gongora *et al.* 2003
	EmSmadC	AR-Smad	1658	Zavala-Gongora *et al.* 2008
	EmSmadD	Co-Smad	7303–7306	Zavala-Gongora *et al.* 2008
	EmSmadE	BR-Smad	2920	Epping, Brehm, unpub.
	EmSKIP	transcr. coregul.	3339–3340	Gelmedin *et al.* 2005

* intracellular signal transducers

FGF-receptor-like RTK, EmFR, a JUN-kinase orthologue, EmMPK3, and two MAPKKs, EmMKK1 and 2, that are currently investigated in our laboratory (Table 2). Regarding TGF-β/BMP-signalling, we have identified and characterized a type I receptor serine/threonine kinase of the Alk1 family, EmTR1 (Zavala-Gongora *et al.* 2006), three receptor-activated Smad (R-Smad) transcription factors, EmSmadA-C, a common-mediator Smad (Co-Smad), EmSmadD, and a member of the SNW/Skip family of transcriptional co-regulators that interact with several of the *Echinococcus* Smads (Zavala-Gongora *et al.* 2003, 2008; Gelmedin *et al.* 2005). This list will soon be complemented by a type II – family RSTK, two type I RSTKs, and an additional BR-Smad (Epping, K., Bernthaler, P. and Brehm, K., unpublished results). Evidence that the *Echinococcus* signalling systems are indeed able to respond to corresponding host signals has been obtained in three cases. First, we demonstrated that the insulin-receptor-like RTK EmIR can interact with host-derived insulin (Konrad *et al.* 2003) which is especially interesting as we already observed significant effects of insulin on the formation of metacestode vesicles from primary cells *in vitro* (Konrad, C., Hemer, S. and Brehm, K., unpublished results). Second, the RSTK EmTR1 interacted with mammalian BMP2 upon expression in HEK-293 cells (Zavala-Gongora *et al.* 2006). Third, exogenous addition of human EGF to *in vitro* cultivated metacestode vesicles resulted in a stimulation of the parasite's MAPK cascade, as demonstrated by the induced phosphorylation of the Erk-like MAPK, EmMPK1, and this was most probably mediated by the EGF-receptor-like RTK EmER (Spiliotis *et al.* 2006). Preliminary evidence that host-derived FGF and TGF-β also stimulate corresponding receptors in *E. multilocularis* has recently been obtained in our laboratory (Epping, K., Schäfer, T. and Brehm, K., unpublished results). Intense biochemical cross-communication between cytokine–cytokine receptor systems of invertebrates and vertebrates is not restricted to *Echinococcus* since it has already been observed for other organisms such as the trematode *S. mansoni* (EGF-, insulin-, and TGF-β-signalling; Beall and Pearce, 2001; Vicogne *et al.* 2004; Khayath *et al.* 2007) and the nematode *Brugia malayi* (TGF-β-signalling; Gomez-Escobar *et al.* 2000). Indeed, a picture is currently emerging in which hormonal cross-communication between tissue-dwelling

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Table 3. Predicted Wnt, Delta/Notch and Hh signalling components in the *E. multilocuaris* genome.

	Protein	Homology	contig	e-value*
Wnt-signalling				
Receptors	EmFZD1	frizzled-like	10587	4e-090
	EmFZD2	frizzled-like	11101	1e-054
	EmFZD3	frizzled-like	6084	7e-040
	EmFZD4	frizzled-like	6510	3e-029
Ligands	EmWNT1	wingless-related	4636	8e-035
	EmWNT2	wingless-related	4905	1e-030
	EmWNT3	wingless related	3665	1e-028
	EmWNT4	wingless-related	9583–9584	2e-012
IST	EmDVL1	dishevelled-like	6792–6797	9e-030
	EmDVL2	dishevelled-like	0172–0173	3e-029
	EmGSK3	glyc. synth. Kinase	8737	4e-061
	EmCTN	beta catenin	5389	1e-117
Delta/Notch-signalling				
Receptors	EmNTL1	Notch-like	4599	1e-022
	EmNTL2	Notch-like	4244	2e-011
Ligands	EmJGL	jagged-like	1614	4e-022
IST	EmSOH	suppr. Of hairless	0007	2e-035
Hedgehog-signalling				
Receptors	EmPTC	patched-like	5552–5555	8e-016
	EmSMO	smoothened-like	6477	8e-013
Ligands	EmHH	hedgehog-like	2725	5e-009

* e-value in comparison with the closest human orthologue.

helminth parasites and host signalling-systems appear to be the rule, rather than an exception.

Using the neoblast regeneration system and transgenic techniques (see below), one of the major challenges of the next years will be to determine the contribution of each of the characterized RTK- and RSTK-signalling systems, and corresponding host cytokines, to stem cell maintenance and pattern formation in *Echinococcus* larvae. In order to obtain a comprehensive picture, components of three additional evolutionary conserved signalling systems, Delta/Notch-, Hedgehog (Hh)-, and Wnt-signalling, should be included since these have also been demonstrated to play important roles in stem cell maintenance, body patterning, and cell fate determination (Guo and Wang, 2009). In the available *E. multilocularis* genome information, genes encoding respective ligands, receptors and intracellular signal transducers were identified (Table 3), indicating that all three systems are operative in the parasite. Unlike the situation with insulin-, EGF- and TGF-β/BMP-like cytokines, it has not yet been established whether vertebrate ligands of the Delta/Notch, Hh and Wnt-signalling pathways are capable of interacting with corresponding receptors of invertebrates. Furthermore, it is not known whether significant amounts of these ligands are expressed in adult liver, the primary site of *Echinococcus* infection, or whether they contribute to the cytokine microenvironment for parasite development. However, due to considerable intracellular cross-interaction with MAPK cascade components and Smad transcription factors, as regularly observed in other stem cell systems (Guo and Wang, 2009), the parasite's Delta/Notch-, Hh- and Wnt pathways may significantly modify the processing of host-signals that are transmitted through RTKs and RSTKs. Furthermore, through homologous interactions with the *Echinococcus* frizzled-, Notch-, and patched-like receptors, these ligands are of course expected to contribute to metacestode pattern formation and differentiation.

To my knowledge, it has not yet been investigated whether cytokines of the adaptive or the innate immune system of the host can influence *Echinococcus* development *in vitro*. Particularly Th2-cytokines such as interleukin 4 (IL-4) as well as anti-inflammatory IL-10 are secreted in considerable amounts during active echinococcosis (Wellinghausen *et al*. 1999), and IL-10 has been demonstrated to be produced by host immune cells in the vicinity of the developing parasite (Harraga *et al*. 2003). Although direct effects of host-derived interleukins or interferons on *Echinococcus* cells cannot be excluded, the situation will surely be different from the above outlined mechanism of hormonal host-parasite cross-communication involving insulin-, EGF, FGF- and TGF-β-like cytokines. In contrast to the latter hormones and cytokines (and corresponding receptors) which have arisen very early in animal evolution and are thus still present in metazoans of all phyla, ILs and IL-receptors of the adaptive immune system have first evolved in jawed vertebrates and are therefore absent in invertebrates (Kaiser *et al*. 2004). At least in the present version of

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the *E. multilocularis* genome, I could find no indication for the presence of IL-or IL-receptor orthologues. Furthermore, components of the Jak/STAT (Janus-kinase/signal transducers and activators of transcription)- or the NFκB (nuclear factor kappa-light chain enhancer of activated B cells)-pathways which, in vertebrates, are collectively mediating IL-, tumor necrosis factor- and interferon-signalling (Guo and Wang, 2009), appear to be completely absent in the *Echinococcus* genome. Hence, on the basis of these results, it is highly unlikely that *E. multilocularis* stem cells directly respond to cytokines other than TGF-β, which are released as part of innate or adaptive immune responses, or that they release interleukin orthologues in order to affect the immune system.

RELATED QUESTIONS OF INTEREST

What is the stem cell niche in the Echinococcus *metacestode?*

In order to control properly stem cell renewal, asymmetric division, and differentiation of stem cell progeny, close range interactions of stem cells with their local tissue microenvironment, called the stem cell 'niche', are usually necessary. Depending on the system, this can involve direct interactions between stem cells and extracellular matrix components or cell-cell interactions between stem cells and surrounding differentiated cells (Scadden, 2006; Drummond-Barbosa 2008; Morrison and Spradling 2008). In the case of the *Echinococcus* oncosphere, this raises the question whether differentiated, somatic cells surrounding the ten neoblasts of the oncosphere (Swiderski 1983) have an influence on early pattern formation in the liver and thus act as a niche, or whether the neoblasts 'alone' can form early vesicles solely dependent on the local cytokine environment. On one hand, *in vivo* infection experiments conducted by Rausch (1954) and Vogel (1977) indicated that the differentiated part of the oncosphere might be dispensable for proper neoblast function since hooks, muscle and glandular cells disappeared rapidly once the oncosphere had reached the site of infection in the liver tissue. In these studies, groups of few neoblasts, either surrounded by host cells (Vogel, 1977) or by a necrotic zone of host (or parasite) cells (Rausch, 1954), were observed within the liver tissue 24 h post infection. On the other hand, *in vitro* studies on early metacestode development from activated oncospheres of *E. granulosus* (Harris *et al.* 1989) and other cestodes (Slais, 1973) showed that neoblast proliferation and the formation of a central cavity within the oncosphere regularly occurred while the stem cells were still surrounded by differentiated cells. Furthermore, in our primary cell cultivation system, neoblasts and differentiated cells readily formed cell aggregates (already after 2 days of incubation), and during expansion of the central cavity via fusion of stem cell progeny with the growing syncytial layer, the neoblasts were still surrounded by a mixture of other cells until the laminated layer appeared (Spiliotis *et al.* 2008). To clarify the situation, it will be necessary to set up *in vitro* cultures of highly purified neoblast populations, without additional 'differentiated' cells, and to study whether those are able to form metacestode vesicles in the same manner as mixed cultures do. Once *Echinococcus* neoblast surface markers are identified, the generation of such purified neoblast populations might be accomplished using FACS (fluorescent activated cell sorting) technology. The outcome of these experiments will not only be relevant for developmental mechanisms of the metacestode at the initial site of infection. Previous studies have already suggested that the dissemination of parasite stem cells can lead to metastasis formation in secondary organs (Mehlhorn *et al.* 1983; Ali-Khan *et al.* 1983), and this is definitely more likely if neoblasts can form vesicles without being part of a niche of differentiated *Echinococcus* cells. Finally, establishing a cell line of constantly dividing, deregulated *Echinococcus* neoblasts via genetic manipulation will surely be facilitated if there is no need to support cell proliferation by direct contact with differentiated cells of the same organism.

What is the mutation rate in Echinococcus *neoblasts?*

The asexual multiplication mode of *E. multilocularis* (and other *Echinococcus* species) within the intermediate host is remarkable since, in theory, metacestode tissue that derives from just one oncosphere can produce a group of genetically heterogeneous protoscoleces that are passed on to the definitive host. Any neutral mutation that occurs in particular neoblasts during asymmetrical division will finally show up in protoscoleces that are produced by the respective region of metacestode tissue, whereas protoscoleces produced from neoblasts in other regions will not carry this mutation (although possibly others). Furthermore, mutations (mostly dominant ones) that confer a selective advantage to the respective neoblast over other stem cells will be positively selected, leading to a larger proportion of protoscolex variants carrying this particular mutation that are passed to the definitive host. Thus, depending on the mutation rate in neoblast populations, fully developed metacestode tissue and protoscoleces deriving from this tissue might either be genetically homogeneous (low mutation rate) or to a certain degree heterogeneous (higher mutation rates). If heterogeneity occurs, the milieu within the intermediate host will positively select for mutations that lead to better adaptation, which might explain the relatively broad spectrum of intermediate hosts to which *E. multilocularis* is able to adapt.

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Interestingly, evidence for the generation of genetically heterogenous protoscolex populations in single cysts of the related dog tapeworm *E. granulosus* has already been obtained. In these studies, Arend *et al*. (2004) and Haag *et al*. (2004) observed a larger than expected number of alleles encoding antigen B, one of the most abundant proteins in hydatid fluid, in protoscoleces from single hydatid cysts, originally considered to be a genetically homogeneous clone since they derive from a single oncosphere. Due to the antigenic nature of the encoded protein and the fact that genetic heterogeneity was not observed for other gene loci such as *mdh*, coding for the 'housekeeping factor' malate dehydrogenase, these authors speculated that the most abundant antigen B alleles might have been positively selected during asexual multiplication of the parasite within the intermediate host (Arend *et al*. 2004; Haag *et al*. 2004). Although studies on antigen B heterogeneity have not yet been reported for *E. multilocularis*, at least circumstantial evidence for the selection of particular mutations in neoblasts has been obtained. In both *E. multilocularis* and *Taenia crassiceps*, the two taenid cestode species whose metacestode stage can be kept in the laboratory through serial passages in the peritoneum of mice, it is well known that isolates (or 'strains') gradually tend to lose the capacity of (proto)-scolex production, the longer they are kept in the somewhat 'artificial' peritoneal environment. If protoscoleces from such a long-term *E. multilocularis* laboratory isolate are used to infect a definitive host or exclusively (without metacestode tissue) for intraperitoneal infection of mice, the capacity of protoscolex-production can be restored. Taken together, these data indicate that neoblast populations which lost their ability to produce protoscoleces due to specific mutations are favoured over 'wild-type' neoblasts when kept for prolonged periods of time under conditions in which the energy-consuming process of protoscolex production is not necessary (i.e. peritoneal passage). From a technical point of view, care should therefore be taken when the physiology and cell biology of *E. multilocularis* is studied using long-term laboratory 'strains', since this tissue is 'peritoneum-selected' whereas, under natural conditions, the liver is the primary target organ. Concerning neoblast mutation rates in *E. multilocularis*, it might therefore be worthwhile to compare allele frequencies of selected genes in long-term laboratory strains and fresh isolates. With regard to immune selection processes, an interesting line of investigation would be the injection of genetically characterized neoblast populations into the peritoneum of permissive mouse strains that exhibit differing immune responses, followed by analyses on which parasite alleles are selected under these specific conditions. The antigen B cluster (see below) would surely be one locus to investigate first in this context. Another mechanism of interest is the possible selection of drug-resistant neoblasts upon prolonged treatment of intermediate hosts with sub-lethal doses of anthelminthics. In *E. granulosus*, Morris and Taylor (1990) previously observed that cysts isolated from the peritoneal cavity of laboratory mice after long-term treatment with the benzimidazole carbamate, albendazole, exhibited much lower sensitivity to the drug than cysts from untreated control mice, indicating positive selection of mutations that confer drug-resistance. In the *E. multilocularis* system, this could be investigated by studying allelic differences in the genes encoding the three β-tubulin isoforms Tub1-3 (Brehm *et al*. 2000*b*), the cellular targets of benzimidazole carbamates, in parasite tissue deriving from drug-treated mice versus untreated control tissue. Finally, as an alternative (or in addition) to the accumulation of mutations in parasite stem cells, epigenetic effects could account for alterations in the physiology of laboratory strains when compared to fresh isolates. Epigenetic regulatory mechanisms such as DNA methylation at CpG islands or histone modifications are well known in regulating stem cell functions (Gan *et al*. 2007; Delcuve *et al*. 2009) and, involving cancer stem cells, play a dominant role in the adaptation of neoplastic tissue to differing environmental conditions (van Staveren *et al*. 2009). The *E. multilocularis* genome contains a number of genes encoding factors of potential relevance for epigenetic control, such as histone acetyl-transferases, protein-arginine-methyltransferases, histone deacetylases, histone demethylases, and at least one DNA (cytosine-5) methyltransferase (Brehm, K., unpublished data), which are all of interest in this context. Furthermore, particularly surrounding residues that are of relevance for epigenetic modifications, the parasite's histones are highly homologous to mammalian histones, thus facilitating the use of commercially available, modification-specific antibodies in investigations on epigenetic regulatory mechanisms during parasite development.

Is trans-splicing associated with neoblast function?

mRNA trans-splicing is a mechanism of transcript processing by which a small mini-exon, called the spliced leader (SL), donated by a small nuclear RNA (the SL-RNA) that structurally resembles the U1 snRNA, is spliced *in trans* to the 5′ end of a variety of cellular transcripts (Brehm *et al*. 2000*a*, 2002, 2003, 2006 and references therein). Apparently absent in several prominent lineages such as mammals or insects, this type of transcript processing is used by primitive chordates (tunicates), cnidarians, some protozoa, and nematodes (see references in Brehm *et al*. 2000*a*; Cheng *et al*. 2006). Furthermore, it has been described in all major free-living and parasitic flatworm groups, including planarians (Zayas *et al*. 2005), trematodes (Davis *et al*. 1994, 1995) and

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cestodes (Brehm *et al.* 2000*a*, 2002). Although the SLs of all flatworm groups clearly derive from one common ancestor, they do not share homologies with SLs from all other trans-splicing groups, indicating that trans-splicing has evolved several times independently (Brehm *et al.* 2000*a*). While SL trans-splicing in kinetoplastid protozoans and in nematodes is clearly associated with the processing of polycistronic transcripts into individual coding units (Cheng *et al.* 2007), its precise functions in all other groups still remains cryptic. At least in flatworms, no clear-cut evidence has so far been obtained that polycistronic transcription units exist and in my own analyses on the distribution of trans-spliced genes on the *E. multilocularis* genome, no clustering was apparent (data not shown). Since flatworm SLs of all lineages contain a highly conserved AUG codon at the 3′ end, one function of the SL in these organisms might be to provide transcripts with a translational start codon (Brehm *et al.* 2000*a*). Although it has been experimentally demonstrated that the flatworm SL AUG can serve as a translational start point *in vitro* (Cheng *et al.* 2006), only a tiny fraction of transcripts in these organisms appear to utilize this AUG *in vivo* (Brehm *et al.* 2000*a*; Zayas *et al.* 2005; Cheng *et al.* 2006).

Work during recent years clearly indicated that post-transcriptional regulation mechanisms, acting at the level of transcript stability and translation initiation, play a decisive role in stem cell control of all major phyla (Sanchez-Alvarado and Kang, 2005; Gangaraju and Lin, 2009), and these are exactly the proposed functions of trans-splicing apart from resolving polycistronic transcripts. This is due to the fact that trans-spliced transcripts are equipped with a trimethyl-guanosine (TMG) cap, typical for snRNAs, which differs from the 7-monomethyl-guanosine (7mG) cap that characterizes conventionally spliced transcripts. The key factor for translation initiation at trans-spliced and conventionally spliced transcripts is the cap-binding protein eIF4E (eukaryotic translation initiation factor 4E; Lall *et al.* 2004), and eIF4E is also a major target of micro-RNA dependent translational control in various stem cells (Standart and Jackson, 2007). In the nematode *C. elegans*, which co-expresses TMG- and 7mG-capped mRNAs, five eIF4E isoforms are present of which two exclusively bind the 7mG cap while three bind to both 7mG and TMG caps (see references in Lall *et al.* 2004). In the genome of *E. multilocularis*, only one eIF4E orthologue is present that at least binds to the 7mG cap (Schubert, S. and Brehm, K., unpublished data) and most probably also to TMG caps. In the context of micro-RNA-mediated control, it will be interesting to see in future experiments whether TMG- and 7mG-caps are equally or differentially responsive to translational repression.

Another regulatory mechanism of potential interest for flatworm stem cells that converges on eIF4E is the mTOR (mammalian target of rapamycin)-pathway. Best studied in mammals, but also present in invertebrates (Long *et al.* 2004), the mTOR pathway plays a crucial role in regulating cellular growth and proliferation, depending on the action of growth factors and nutrient availability (Jastrzebski *et al.* 2007; Yang *et al.* 2008). Since neoblasts are the only mitotically active cells in flatworms, nutrient-dependent proliferation control should therefore be predominantly acting in these cells. Subject to mTOR regulation via eIF4E in mammals is usually a population of transcripts, which contain an oligo-pyrimidine tract each at the 5′ ends, the so-called TOP mRNAs (Hamilton *et al.* 2006). The pyrimidine tract forms a short hairpin-loop at the mRNAs 5′ end so that translation of TOP transcripts is only possible once eIF4E interacts with another translation initiation factor, eIF4G, at the mRNAs 5′ end. This occurs under conditions of nutrient availability. Under nutrient (particularly amino acid) deprivation, an eIF4E binding protein (E4-BP1) is activated by the mTOR pathway and out-competes eIF4G for binding to eIF4E, thus preventing translation of TOP mRNAs (Hamilton *et al.* 2006; Jastrzebski *et al.* 2007; Yang *et al.* 2008). The majority of TOP mRNAs encode proteins essential for cell cycle progression and proliferation such as several ribosomal proteins, translation initiation- and elongation-factors, cyclin-dependent kinases, or protein degradation factors (Mamane *et al.* 2007), that, as a consequence, are immediately taken out of translation once nutrients become scarce. Interestingly, my own analyses on *Echinococcus* and *Taenia* transcripts revealed that, as in the case of TOP mRNAs, a large number of ribosomal proteins, translation factors (eIF3G, eIF6), proteasome components, or transcription initiation factors are among the trans-spliced fraction (Brehm *et al.* 2000*a*, 2002, 2003; and unpublished results). Furthermore, due to the intrinsic structure of the spliced leader (Brehm *et al.* 2000*a*, 2002), trans-spliced *Echinococcus* transcripts should contain short hairpin-loops at the 5′ end. It is therefore tempting to speculate that trans-spliced transcripts might be the TOP mRNA equivalent of flatworms. In future investigations it will thus be interesting to see whether trans-spliced factors display a neoblast-specific expression pattern or are subject to translational control in neoblasts. Furthermore, since all crucial components of the mTOR pathway are encoded by the parasite's genome (my own unpublished observations), investigations on the effects of rapamycin, a potent inhibitor of the TOR pathway (Dowling *et al.* 2009), on the expression of trans-spliced transcripts and on parasite development should be carried out in order to assess the potential of this important signalling mechanism as a target for anti-parasitic treatment.

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Fig. 3. The *E. multilocularis* antigen B cluster. Database mining with known antigen B sequences against the first *E. multilocularis* genome assembly version revealed the presence of 7 antigen B isoform genes located on 5 adjacent contigs. Two identical (within ORF) copies encoding EmAgB8/3, separated by a gene encoding EmAgB8/5, are present. An additional, imperfectly matching copy is designated EmAgB8/3*. Arrows indicate AgB reading frames and direction of transcription. Numbers below indicate the (minimal) distance between each reading frame on the genome. Double slashes (//) indicate gaps in the genome sequence.

E. MULTILOCULARIS GENOMICS AND GENETIC MANIPULATION

As befits an experimental model system, a whole genome sequencing project for *E. multilocularis* is currently being carried out in co-operation between the Parasite Sequencing Unit of the Wellcome Trust Sanger Centre (Hinxton, Cambridge, UK), led by Matt Berriman, and my group. After initiation of the project in 2004, extensive BAC (bacterial artificial chromosome) libraries were generated from genomic DNA that derived from the natural parasite isolate JAVA (Tappe *et al*. 2007) and, after determination of the parasite's genome size by flow cytometry (Spiliotis *et al*. 2008), capillary sequencing to ~4-fold coverage was carried out. After a first round of assembly, ~19,000 sequence contigs with an average length of 15 kb were obtained which are publicly available under http://www.sanger.ac.uk/Projects/Echinococcus/. BLAST analyses of 50 randomly picked cDNAs from previously generated spliced-leader libraries (Brehm *et al*. 2000*a*) against the available contigs revealed that sequence information for all cDNAs was present although, in some cases, the cDNAs were only partially covered by the corresponding contig (Brehm, K., unpublished results). Hence, although several prominent gaps are still present, at least protein coding information appears to be covered to a great extent by the presently available sequence contigs.

On the basis of extensive sequence variations in alleles encoding antigen B (see above), it has previously been suggested that the genome of the closely related dog tapeworm *E. granulosus* might undergo gene conversion (Haag *et al*. 2004). If also active in *E. multilocularis*, such a mechanism could severely hamper all attempts to properly assemble the genome data. I therefore carried out a preliminary analysis of the distribution of antigen B genes in the available sequence information and found a total of 7 gene copies clustered on 5 adjacent contigs (5712–5716; Fig. 3). In all cases, the genes comprised two exons, separated by introns of 66 to 130 nt, of which the first exon consistently encoded an export directing signal sequence. Two of the gene loci of the antigen B cluster apparently encoded the previously described (Mamuti *et al*. 2006) isoform EmAgB8/3 (100% amino acid sequence identity). Another three encode proteins identical to EmAgB8/5, EmAgB8/4 and EmAgB8/1, while two loci display imperfect matches to previously published sequences for EmAgB8/2 (95% identical, 97% similar residues) and, again, EmAgB8/3 (92%/94%). Although further genomic analyses, supported by EST (expressed sequence tag) data, are surely necessary to assign each of the identified gene loci to reported antigen B isoforms from *E. multilocularis* (Mamuti *et al*. 2006) and *E. granulosus* (Haag *et al*. 2004), the sequence data present in the first assembly of the *E. multilocularis* genome indicate that the antigen B isoforms are encoded by a cluster of 7 genes, that is not interrupted by other predicted genes. Furthermore, the contig information does not contain any indication that antigen B encoding *E. multilocularis* genes are subject to gene conversion, as previously suggested for *E. granulosus* (Haag *et al*. 2004). It should be noted that clustering of *Echinococcus* gene families seems not to be the rule since BLAST analyses concerning the *eg95* family, which encodes host-protective antigens (Chow *et al*. 2001), revealed at least 12 family members that are dispersed over the entire genome (Brehm, unpublished observation). Taken together, the above mentioned analyses as well as the data present in tables 1 and 2, demonstrate that the presently available, preliminary genome information of *E. multilocularis* already serves as a highly useful tool in gene identification.

Following capillary sequencing, additional rounds of unpaired and paired 454 reads as well as extensive sequencing runs using Solexa technology have meanwhile been carried out, amounting to ~140-fold coverage. In the present (unpublished) assembly version, the sequence information is present within 1841 contigs and 597 supercontigs, with 50% of the parasite's genome assembled into 17 scaffolds of more than 1·6 Mb. Final assembly is expected by the end of 2009. Together with the sequence data from the ongoing *T. solium* project (http://www.taeniasolium.unam.mx/advisory.htm), which is also in an advanced stage, a detailed picture of taenid cestode genes and genomes should therefore be available in 2010. Supported by extensive EST data from both *E. multilocularis* and *E. granulosus* (Fernandez *et al*. 2002), available under http://www.nematodes.

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org/NeglectedGenomes/Lopho/LophDB.php and http://www.sanger.ac.uk/cgi-bin/blast/submitblast/ Echinococcus, genome annotation is currently ongoing in the parasite sequencing unit of the Sanger Centre and in my laboratory. Furthermore, we are currently carrying out transcriptome sequencing for a variety of different developmental stages such as early and late metacestode vesicles (with and without brood capsules) as well as dormant and activated protoscoleces, which will eventually be complemented by transcriptome analyses of oncospheres and adult worms. Taken together, these investigations will provide valuable information on developmental stage-specific gene expression patterns covering the entire life-cycle.

The major aim of the *E. multilocularis* genome project is to provide new ideas and concepts for hypothesis-driven research into parasite development, immunological aspects of alveolar echinococcosis, and drug design. To make effective use of this information platform it is necessary to establish robust and reliable technologies for targeted genetic manipulation. We have recently reviewed the approaches that have so far been undertaken towards this aim (Brehm and Spiliotis, 2008*a*), so I will just give a brief outline of what might be achieved in short time. Transient transfection of *in vitro* cultivated cells using liposome-based transfection reagents has been successfully carried out (Spiliotis *et al.* 2008) and should soon be improved in a way that *Echinococcus* proteins can be expressed in primary cells for functional assays. Furthermore, we have demonstrated that the facultative intracellular bacterium *Listeria monocytogenes*, that has already been successfully used to introduce foreign DNA into mammalian cells, can also be used to effectively infect *Echinococcus* cells, thus providing an elegant alternative to lipofection in future attempts to manipulate parasite cells (Brehm and Spiliotis 2008*a*; Spiliotis *et al.* 2008). RNAi – approaches that have recently been established as a useful tool for targeted knock-down of genes in *S. mansoni* (Pearce and Freitas, 2008) should also be applicable to *E. multilocularis* since the genome contains all necessary components (such as the DICER protein; Kiss and Brehm, unpublished results) and first protocols to effectively knock down gene expression in primary cells are already at hand (Spiliotis, M., personal communication). Finally, successful attempts into stable transfection and genomic integration of foreign DNA have been carried out in my laboratory using pantropic virus-constructs (Spiliotis, Kiss, Brehm, unpublished results) in a way very similar to previously reported DNA integration strategies for *S. mansoni* (Kines *et al.* 2008). In combination with the *in vitro* system for parasite regeneration from primary cells (Spiliotis *et al.* 2008), stable integration by virus-based systems should, in principle, produce entirely transgenic parasite strains, and this approach is currently being used in our laboratory. As a result of these first successful attempts, a very positive prognosis can be given that *E. multilocularis* genes and mRNAs can be effectively manipulated for a variety of experimental approaches in the near future.

CONCLUDING REMARKS

All parasitic flatworms have their evolutionary roots in the free-living species (Olson, 2008) and, as outlined in this review, it is expected that basic developmental mechanisms such as the maintenance and differentiation of totipotent stem cells, pattern formation, and signal transmission are largely shared between the parasitic and free-living members of the phylum. Research into the developmental biology of trematodes and cestodes can thus profit immensely from the wealth of data that has already been gathered concerning genetic control of regeneration, asexual multiplication and fecundity in planarians and other free-living flatworm model systems. Furthermore, elaborate methodological approaches such as systematic RNAi-screens of genes involved in stem cell control (Reddien *et al.* 2005), or the utilization of flow cytometry to characterize stem cell populations in planarians (Higuchi *et al.* 2007), should be directly applicable and very helpful in cestode and trematode neoblast research.

In turn, can the free-living flatworm research community take profit from investigations on trematodes and cestodes? I truly think so. As exemplified by the *E. multilocularis* cell culture system (Spiliotis *et al.* 2008), neoblast populations of asexually multiplying larval stages such as cyclophyllidean metacestodes or schistosome sporocysts can be more advantageous for isolated cultivation than their counterparts deriving from free-living species (see Schürmann and Peter, 2001), thus facilitating the establishment of cell lines that are of use for functional analyses on both parasitic and free-living flatworms. The steadily improving methods of genetic manipulation on parasitic flatworm species through virus-based transduction systems (Brehm and Spiliotis, 2008*a*; Kines *et al.* 2008), ballistic gene transfer (Wippersteg *et al.* 2002) or intracellular bacteria (Spiliotis *et al.* 2008) are principally also applicable to free-living species and can broaden the spectrum of methods for functional analyses of planarian genes. Finally, it also appears clear that comparative genomics on model species such as *Schmidtea mediterranea*, *Schistosoma mansoni* and *E. multilocularis*, including analyses of the gain and loss of genes between parasitic and free-living species, should be highly useful in defining the 'core' elements of the flatworm genome responsible for neoblast function.

At present, there is little interaction between the research communities interested in molecular and

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cellular biology of free-living and parasitic flatworms, which is reflected by the fact that planarians have already been used as model systems for genotoxicity testing (Lau *et al*. 2007) or for the development of neuropharmacological (Buttarelli *et al*. 2008) and anti-cancer drugs (Pearson and Sanchez-Alvarado, 2008; Oviedo and Beane, 2009) but, at least to my knowledge, never for the development of anthelminthic drugs against trematode- and cestode-borne diseases. Just as in the case of the nematode research field, where close interactions between the *C. elegans*- and 'parasitic nematode'-research communities are nowadays commonplace (Gilleard 2004), I therefore encourage similar networks among flatworm researchers interested in molecular genetics and developmental control of their organisms. By closing ranks, we significantly broaden our methodological repertoire, our comparative reference base, and our chain of arguments for doing research on flatworms.

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