Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/authorsrights

Author's personal copy

Biotechnology Advances 31 (2013) 1722–1737

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

In vitro cultivation of *Schistosoma japonicum*-parasites and cells

Qing Ye [a], Hui-Fen Dong [b], Christoph G. Grevelding [c], Min Hu [a,*]

[a] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, PR China
[b] Department of Parasitology, School of Basic Medical Science, Wuhan University, Wuhan 430071, Hubei, PR China
[c] Institute for Parasitology, Justus-Liebig-University, 35392 Giessen, Germany

ARTICLE INFO

Article history:
Received 5 May 2013
Received in revised form 6 September 2013
Accepted 8 September 2013
Available online 24 September 2013

Keywords:
Schistosoma japonicum
In vitro cultivation
Cell culture
Parasite culture

ABSTRACT

Schistosomiasis is a serious parasitic zoonosis caused by blood-dwelling flukes of the genus *Schistosoma*. Understanding functions of genes and proteins of this parasite is important for uncovering this pathogen's complex biology, which will provide valuable information to design new strategies for schistosomiasis control. Effective applications of molecular tools reported to investigate schistosome gene function, such as inhibitor studies and transgenesis, rely on the developments of *in vitro* cultivation system of this parasite and cells. Besides the *in vitro* culture studies dealing with *Schistosoma mansoni*, there are also numerous excellent studies about the *in vitro* cultivation of *Schistosoma japonicum*, which were performed by Chinese researchers and published in Chinese journals. Nearly every stage of the life-cycle of *S. japonicum*, including miracidia, mother sporocysts, cercariae, schistosomula, and egg-laying adult worms, was employed for developing *in vitro* cultivation methods, being accompanied by the introduction of several media and supplements that helped to improve culture conditions. It was not only possible to generate mother sporocysts from miracidia *in vitro*, but also to obtain adult worms from cercariae through *in vitro* cultivation. The main obstacles to complete the life cycle of *S. japonicum* in the lab are the transition from mother sporocysts to cercariae, and the production of fertilized and completely developed eggs by adult worms generated *in vitro*. With regard to cells from *S. japonicum*, besides established isolation protocols and morphological observations, media optimizations were conducted by using different chemical reagents, biological supplements and physical treatment. Among these, mutagens like N-methyl-N-nitro-N-nitrosoguanidine and the addition of extracellular matrix were found to be able to induce mitogenic activities. Although enzyme activities or the level of silver-stained nucleolar region associated protein in cultured cells indicated still suboptimal conditions, the achievements made point to the possibility of reaching the aim of establishing cell lines for *S. japonicum*. Both the improvements of the *in vitro* culture of larval and adult worms of *S. japonicum* as well as the access of cells of this parasite provide excellent advances for research on this important parasite in the future.

© 2013 Elsevier Inc. All rights reserved.

Contents

1. Introduction . . . 1723
2. *In vitro* culture of *S. japonicum* parasites . . . 1724
   2.1. Intromolluscan parasite life-stages . . . 1724
      2.1.1. Miracidia and mother sporocysts . . . 1724
      2.1.2. Cercariae . . . 1725
   2.2. Definitive host stages . . . 1725
      2.2.1. Skin-stage and lung-stage schistosomula . . . 1725
      2.2.2. Schistosomula transformed *in vitro* . . . 1725
      2.2.3. Hepatic-portal-phase schistosomula and adult worms . . . 1726

* Corresponding author at: State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei, PR China. Tel.: +86 27 87280032; fax: +86 27 87280408.
E-mail addresses: yeqing198173@gmail.com (Q. Ye), hfdong@whu.edu.cn (H.-F. Dong), Christoph.Grevelding@vetmed.uni-giessen.de (C.G. Grevelding), mhu@mail.hzau.edu.cn (M. Hu).

0734-9750/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biotechadv.2013.09.003

Author's personal copy

3. Cell culture of *S. japonicum* . . . 1727
3.1. Primary cell culture . . . 1727
3.1.1. Cultural methods . . . 1727
3.1.2. Characteristics of cultured cells . . . 1727
3.1.3. Optimization of the culture system for *S. japonicum* cells . . . 1731
3.1.4. Effects of chemical reagents, biological supplements and physical treatment on cultured cells . . . 1731
3.2. Continuous passage cell culture . . . 1733
4. Applications of *in vitro* culture systems for *S. japonicum* . . . 1733
4.1. Application examples of parasite *in vitro* culture . . . 1733
4.2. Application of cell culture . . . 1734
5. Conclusion and perspective . . . 1734
Acknowledgments . . . 1734
References . . . 1734

1. Introduction

Schistosomiasis, a chronic and debilitating parasitic zoonosis, is ranked second after malaria in terms of worldwide public health significance, with more than 200 million people infected. Transmission of schistosomes, the pathogenic trematodes that cause schistosomiasis, is endemic over 70 tropical and subtropical countries (Hotez et al., 2008; Montresor et al., 2012), without well-established control measures. Besides humans, animals are also affected, which contributes to the enormous socioeconomic significance of schistosomiasis (Gryseels, 2012; Huang and Manderson, 2005; King, 2010; Zhou et al., 2012). The complex life-cycle of schistosomes, which includes sexual and asexual reproduction within vertebrate and invertebrate hosts, is a big challenge for the control of this parasitic disease. Paired male and female worms reside in the mesenteric veins of mammalian host, and the females produce a large quantity of eggs every day. Some of the eggs are discharged with the feces, while many become entrapped in the capillaries of the liver and other organs leading to serious pathological consequences within the definitive hosts.

Praziquantel (PZQ), a non-specific broad-spectrum anthelmintic drug, is used as main therapeutic treatment to control schistosomiasis. Due to its low cost and high efficiency, PZQ has been administered as the most frequently used drug to treat schistosomiasis in China, Egypt, Cambodia, Brazil and the other countries (Chen, 2006; da Rocha Pitta et al., 2013). However, since it is only effective in killing adult worms, PZQ is unable to prevent re-infection (Hang et al., 2001; Pica-Mattoccia and Cioli, 2004; Xiao et al., 1985). Moreover, reliance on and extensive use of PZQ also in mass treatment programs create the risk of reduced drug efficacy and emerging resistance, for which first evidence has been obtained (Botros et al., 2005; Doenhoff and Pica-Mattoccia, 2006; Doenhoff et al., 2009; Melman et al., 2009). Therefore, it is a highly desirable goal to develop new effective drugs or vaccines for schistosomiasis control (Beckmann et al., 2012a; Huang et al., 2012; Pitta et al., 2006; Sayed et al., 2008). However, this requires a full understanding of candidate gene/protein function.

Due to tremendous worldwide efforts genome sequencing projects produced the first draft genomes of *Schistosoma mansoni* (see Berriman et al., 2009; Protasio et al., 2012), *S. japonicum* (The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009), and *Schistosoma haematobium* (see Young et al., 2012). Although comprehensive knowledge about the gene repertoire of schistosomes now exists, the functions of most genes are still unknown. This impedes our understanding of this pathogen's complex biology, including its interaction with mammalian and mollusc hosts and the mechanisms involved in parasite development, and its reproduction.

Up to date molecular tools reported to investigate schistosome gene function and importance have mainly included pharmacologic inhibitor studies and transgenesis. Use of pharmacologic inhibitors of targeted proteins has proven to be a better approach to identifying the functions of enzyme- or receptor-encoding genes in schistosomes. These encoded proteins include thioredoxin glutathione reductase (TGR) (Kuntz et al., 2007; Song et al., 2012), protein kinase C (PKC) (Ludtmann et al., 2009), protein kinases related to the reproductive system (Beckmann et al., 2010; Knobloch et al., 2006; Leutner et al., 2011; Long et al., 2010), as well as insulin receptors (You et al., 2010). In a landmark series of reports on the transcriptome of *S. mansoni*, Verjovski-Almeida and colleagues noted the presence of transcripts encoding dicer and piwi/argonaute orthologues, indicating that an intact RNAi pathway may exist in schistosomes (Verjovski-Almeida et al., 2003; Krautz-Peterson and Skelly, 2008; C. Luo et al., 2010; R. Luo et al., 2010). At the same time, Skelly and colleagues established protocols for RNAi in *S. mansoni* using cultured schistosomula (Skelly et al., 2003). Subsequently, excellent studies were performed using RNAi for gene silencing to characterize various genes in cultured schistosomes (Beckmann et al., 2010, 2012b; Boyle et al., 2003; Cheng et al., 2005; Dinguirard and Yoshino, 2006; Kuntz et al., 2007; Osman et al., 2006). With respect to insertional transgenesis, biolistics (Davis et al., 1999; Grevelding, 2006), square-wave electroporation (Correnti et al., 2005; Krautz-Peterson et al., 2007; Morales et al., 2008; Ndegwa et al., 2007; Rinaldi et al., 2008), and soaking procedures (Boyle et al., 2003; Davis et al., 1999; Dinguirard and Yoshino, 2006; Freitas et al., 2007; Grevelding, 2006; Heyers et al., 2003; Rossi et al., 2003; Wippersteg et al., 2002a,b, 2003, 2005) were performed with mRNA, plasmid DNA, reporter-gene constructs or virions, and transient transformation was achieved. Furthermore, progress has also been made on stable transformation of schistosomes, since the genome integration of lentiviruses was demonstrated opening the possibility of gain-of-function and/or loss-of-function analyses in schistosomes (Mann et al., 2011; Rinaldi et al., 2012).

In spite of this progress, the lack of established cell lines, the inability to keep the entire life cycle of schistosome *in vitro*, and the limitations of existing culture techniques for juveniles or adults hamper the progress in the post-genomic era of schistosome research. Different *in vitro* culture systems not only for adult and larval schistosomes but also for isolated cells have been developed. These mainly focus on the *S. mansoni* and *S. japonicum*. Due to its zoonotic nature *S. japonicum* has the greatest impact on human and animal health of all schistosome species. Nevertheless, most of the known *in vitro* culture studies have dealt with *S. mansoni*, with only a few focusing on *S. japonicum* (see Mann et al., 2010; Quack et al., 2010). One of the reasons for this is that most studies about *in vitro* cultivation of *S. japonicum* were performed by Chinese researchers and published in Chinese journal rather than international ones leading to a communication deficit within the international community. This review summarizes relevant research achievements to close existing gaps and to exchange ideas about the improvement of schistosome *in vitro* cultivation. Within this article, we will 1) summarize approaches regarding *in vitro* culture of different developmental stages of *S. japonicum*, 2) review progress towards establishing cell culture systems, 3) discuss the application of established *in vitro* culture systems for functional studies and 4) propose future research directions.

Author's personal copy

2. *In vitro* culture of *S. japonicum* parasites

The earliest *in vitro* culture report on *S. japonicum* in China can be traced back to the 1930s (Chu, 1938; Lee and Chu, 1935). In those systems, adult worms were kept in balanced salt solution to which mammalian serum was added. The male worms survived up to 146 days, whereas females only survived for 60 days in a mixture of Ringer's balanced salt solution and horse serum (Chu, 1938). Moreover, pairing of male and female worms also occurred in this medium but lasted less than 24 h (Chu, 1938). These advances were followed by studies from Japanese scientists who performed *in vitro* cultivation of *S. japonicum* worms in the 1950s (Ito and Komiya, 1955; Ito et al., 1955). Since the 1980s, work was carried out more intensively in China with significant progress in the establishment of various culture systems for the parasites from both intermediate and final hosts (see also Table 1). Larval *S. japonicum* survived *in vitro* and to develop from miracidia to daughter sporocysts, but no infective cercariae could be produced from daughter sporocysts (Bi et al., 1992; Coustau et al., 1997; Mei and Zhou, 1988, 1989). In contrast, definitive host stage worms were maintained *in vitro* starting with schistosomula mechanically transformed from cercariae to adult worms with the ability to lay eggs although these eggs were unfertilized (Hua and Zhou, 1988; Hua et al., 1988; Lin et al., 1983, 1985a; Wang and Zhou, 1987; Wang et al., 1986).

2.1. Intromolluscan parasite life-stages

2.1.1. Miracidia and mother sporocysts

Attempts to culture intromolluscan stage larvae of *S. japonicum* initially employed an axenic culture system. In this system, miracidia hatched from eggs and transformed into mother sporocysts *in vitro*. The best medium for transformation and survival of mother sporocysts was RPMI-1640 solution with a concentration of 5.3 mg/ml supplemented by 10% rabbit serum (Mei and Zhou, 1988, 1989). Abundant germ balls were observed inside of the transformed mother sporocysts

Table 1
In vitro cultivation of different stages of *S. japonicum*.

Stages to start cultivation		Medium for cultivation	Additives	Results of cultivation	Reference
Intramolluscan stage	Miracidium	50% diluted RPMI-1640 adding 10% rabbit serum		Longest survival: 48 days; maximal length: 164 μm.	Mei and Zhou (1989).
	Miracidium	50% diluted RPMI-1640 adding 10% calf serum	5-Hydroxytryptamine	The strongest activity of larvae was detected at 24 h post-treatment, while the maximal length and strongest SDH activity in the parasite were observed 24–48 h post-treatment.	Zhou et al. (2008).
	Miracidium	50% diluted RPMI-1640 adding 10% calf serum	Protein extracts from head–foot or gland tissue of *O. hupensis*	Longest survival: 58 days. The larvae in the experimental group exhibited stronger motility and larger sizes relative to those of the control group. Differential gene expression was also induced by protein extracts.	Zhu et al. (2012).
	Miracidium	SF-900™ II SFM medium	*Sj9* cells as feeder layers	Mother sporocysts were gradually embedded by feeder layer cells after 20 days and were unable to develop to daughter sporocysts. *In vitro*-cultivated mother sporocysts were injected into *O. hupensis* and developed to infective cercariae with same genetic characteristics as wild-type cercariae.	Jiang et al. (2011).
	Cercariae	50% diluted RPMI-1640 adding 10% rabbit serum	Protein extracts from head–foot or gland tissue of *O. hupensis*	The development of cercariae was promoted by protein extracts from head–foot but inhibited by those from gland tissue of *O. hupensis*.	Bi et al. (1992).
Definitive host stage	Skin-stage schistosomula	Medium B		Cultured worms survived for 41 days *in vitro*, and only a small number of parasites developed to cecal conjunction, but failed to develop to liver stage schistosomula.	Lin et al. (1985a).
	In vitro-transformed schistosomula	Medium 841		Mechanically transformed schistosomula survived for more than 110 days in medium 841 and developed to adult female and male worms which paired. The reproductive organs of the adults formed, and the females laid eggs although unfertilized.	Wang and Zhou (1987).
	Lung-stage schistosomula	Medium A Medium B		The schistosomula in medium B were larger sized; earlier emergence of cecal conjunction and reproductive organs compared to those in medium A. The mortality of the worms cultured in medium A was significantly higher than that of the worms kept in medium B up to the 25th day in culture.	Lin et al. (1983).
	Lung-stage schistosomula		ED25 cells as feeder layers	Schistosomula co-cultured with ED25 cells shared more similarities in morphology and tegumental structures with schistosomula directly obtained from infected mice.	Ye et al. (2012).
	18 day-old schistosomula (hepatic-portal- phase)	Medium 841 Medium M-841 M-841 + ghost cells		Parasites in M-841 and M-841 + ghost cells showed lower mortality and bigger size than those in medium 841 after 35 days in culture. The periods required for egg production were different among the worms: 10 days for worms in M-841 + ghost cells, 29 days for worms in M-841 and 25 days for those in 841, respectively.	Hua et al. (1988).
	42 day-old adult worms	Medium 841 Medium 851		Compared to 841, medium 851 induced a higher production of eggs and less abnormal eggs; the period for oviposition was elongated.	Hua and Zhou (1988).
	Adult worms	Medium 851 Medium 891		Regardless of being kept in medium 851 or 891 for several days, the number of eggs produced by adults of *S. japonicum* still decreased, while the rate of abnormal eggs increased.	Yang et al. (1997).

Author's personal copy

when cultured this way for 36 h. The tegument of some mother sporocysts became thick and formed several folds within 24 days, similar to the parasites inside their intermediate host, *Oncomelania hupensis*. Cultured mother sporocysts survived for up to 48 days and nearly doubled in length after 34 days of cultivation (Mei and Zhou, 1988, 1989).

Motility and length of cultured mother sporocysts of *S. mansoni* significantly increased in the presence of 1 μM serotonin (5-hydroxytryptamine; 5-HT) (Boyle et al., 2000). Moreover, the successful emergence of daughter sporocysts from cultured mother sporocysts was supposed to be related to the endogenous stores of 5-HT (Boyle and Yoshino, 2005). Consistent with these findings on *S. mansoni*, supplementing cultures of *S. japonicum* mother sporocysts with 10 μM 5-HT positively affected motility and length of the larvae and the activity of succinate dehydrogenase (SDH) in the parasites. The highest motility among larvae was detected at 24 h post-treatment, while the greatest increase in length and strongest SDH activity in the parasites were detected at 48 h post-treatment. In this system, no production of daughter sporocysts was achieved (Zhou et al., 2008).

In vitro cultivation of intromolluscan stages of *S. mansoni* was promoted by adding *Biomphalaria glabrata* embryonic (*Bge*) cells into the culture system (Hansen, 1976). *B. glabrata* is the intermediate host of *S. mansoni*. Continuous developments from miracidia to daughter sporocysts occurred in the presence of *Bge* cells, and mature, first generation daughter sporocysts emerged from mother sporocysts starting at approximately 30–45 days of initial cultivation (Yoshino and Laursen, 1995), suggesting that host factors are essential for the development of the parasite. Based on this, *Bge* cells were also tested for the *in vitro* culture of *S. japonicum*. Interestingly, the emergence of daughter sporocysts from mother sporocysts of *S. japonicum* co-cultured with *Bge* cells occurred at the 11th week, indicating that *Bge* cells can also play a role in promoting the development of *S. japonicum* sporocysts (Coustau et al., 1997). However, under these conditions the development of *S. japonicum* daughter sporocysts was slower than the development of daughter sporocysts in its primary snail host *O. hupensis*. This confirmed the remarkable host-specificity of *Schistosoma* species (Basch, 1976; Ho, 1963) and prompted first attempts to isolate cells and establish cell lines from *O. hupensis*. Although primary cultures of embryonic cells and liver cells of *O. hupensis* were established (Peng et al., 2003b; Ye et al., 2007), cell lines from *O. hupensis* have not been achieved at this time. Instead of cell lines, protein extracts from head–foot of *O. hupensis* were used to culture mother sporocysts of *S. japonicum* and exhibited positive effects on survival time, motility, and size of mother sporocysts (Zhu et al., 2012). In another study, oocytes of *Spodoptera frugiperda* (Lepidoptera, Noctuidae) (*sf9* cells), were used as feeder layers to culture miracidia of *S. japonicum* (see Jiang et al., 2011). After transformation into mother sporocysts *in vitro* for 24 h, there were some germ balls inside the parasite. The larval motility decreased along with the time in culture and disappeared on day 15. The mother sporocysts were gradually embedded by feeder layer cells after 20 days, unable to develop to daughter sporocysts. After being cultured *in vitro* in the presence of *sf9* cells for 3 days, the mother sporocysts were injected into *O. hupensis* and continued to develop to infective cercariae with the same genetic characteristic as wild type cercariae, providing a new method for *in vitro* studies and/or manipulation of *S. japonicum* larvae (Jiang et al., 2011).

2.1.2. Cercariae

In contrast to *S. japonicum* mother sporocysts, no report on daughter sporocysts *in vitro* culture exists for this species. The only investigation on cercariae culture was started with *S. japonicum* cercariae embryos by using the same medium used for mother sporocyst cultivation (Bi et al., 1992). The length of cercariae embryos increased when protein extracts of the head–foot tissue of *O. hupensis* were added to the medium. However, the addition of liver protein extracts of *O. hupensis* inhibited the development of cercariae embryos indicating that liver components negatively affected the development of this parasite (Bi et al., 1992).

2.2. Definitive host stages

As adult worms develop from cercariae *via* juvenile schistosomula stages within the definitive host, attempts to culture juveniles and adults varied also with respect to the starting stage, but also the scientific aim that was pursued such as promoting development, sexual maturation or fertilization.

2.2.1. Skin-stage and lung-stage schistosomula

Early research on juveniles or adults focused on the screening of suitable media to maintain these stages *in vitro*. Based on their migration behavior, schistosomula were distinguished in three stages comprising the skin-stage, the lung-stage, and the liver-stage (Lin et al., 1985b; Tang et al., 1973). For lung-stage schistosomula, which were isolated from lungs of infected mice on day 3 post infection, two types of modified media (Clegg, 1965; Yasuraoka et al., 1978) termed medium A and medium B, respectively, were used. Both consisted of calf serum, rabbit red blood cells, and antibiotics (penicillin and streptomycin). The difference was that medium A employed Earle's balanced salt solution and lactalbumin hydrolysate, while medium B contained TC 199 basic medium. According to the comparison, schistosomula of *S. japonicum* maintained in medium B grew more rapidly than those in medium A, reached a larger size, and an earlier emergence of the junction of the caeca, which was formed behind the ventral sucker by the union of bifurcated caeca (cecal conjunction), and reproductive organs was observed. The mortality was significantly higher for the worms cultured in medium A (81%) than that of worms in medium B (26.7%) on day 25 of *in vitro* culture suggesting that medium B with richer nutrition was more suitable for maintaining schistosomula *in vitro* (Lin et al., 1983). Based on this, medium B was also used to culture *S. japonicum* skin-stage schistosomula (0.5 h post infection). Under this condition, not only the body size, but also the ultrastructure of the schistosomula cultured *in vitro* was similar to those of *in vivo*-raised parasites obtained from host on day 3 following infection. However, the cultured worms just survived for 41 days *in vitro*, and only a small number of parasites developed cecal conjunction. Furthermore, they never reached liver stage phenotypes suggesting the limited roles of this medium in promoting skin-stage schistosomula to develop to further life stages (Lin et al., 1985a).

2.2.2. Schistosomula transformed in vitro

The main problem to culture skin-stage and lung-stage schistosomula is the limited amount of parasite material that can be obtained from infected hosts. According to a previous report, at most only 30% parasites can be obtained from a lung of an infected mouse on day 3 post infection (Meng et al., 2005). In contrast, 80–90% schistosomula can be obtained if mechanically transformed from cercariae *in vitro* (Chen and Zhou, 1995). To this end *S. japonicum* cercariae were transformed into schistosomula *in vitro* by different methods including syringe passage (Basch, 1981; Colley and Wikel, 1974), fresh serum incubation (Yasuraoka et al., 1978), or PZQ treatment (Xiao et al., 1987). Using syringe passage, more than 80% cercariae lost their tails, which is one of the inducing moments for transformation. Concerning fresh serum incubation, the comparison between rabbit serum and calf serum revealed that within 48 h the rabbit serum induced transformation more easily than the calf serum (Wang et al., 1986). When incubated for 72 h, nearly 100% cercariae were transformed to schistosomula in both sera, in contrast, only 3% cercariae in control group medium (RPM1640) were transformed. Compared to the transformation rate of schistosomula induced by fresh serum for 72 h (98.9%), the transformation rates were 29.8% for serum with inactivated complement, 20.1% for serum without B factor, and 12.5% for serum without complement C3, respectively. Moreover, rabbit serum stored for weeks under −12 °C prompted 62.9% transformation

Author's personal copy

of schistosomula. Serum stored for half year just induced 25.5% transformation. Thus it was proposed that the complement within the serum played a role in the transformation process (Wang et al., 1986).

Independent of being transformed by either serum incubation or syringe passage, transformed schistosomula were both sensitive towards tap water and revealed a negative cercariae hullen reaction (CHR) as well as changes in their ultrastructure that were similar to those observed for schistosomula in mice (Wang et al., 1986). Nearly 97.5% schistosomula discharged the content of preacetabular gland at 72 h post treatment under the influence of serum, while only 89.2% of the mechanically transformed schistosomula discharged their gland content (Wang et al., 1986).

After being transferred to Hank's balance solution (HBS), containing 2% calf serum, tailless cercariae of *S. japonicum* treated with 10 μg/ml PZQ for 0.5 h promptly resumed their normal movement and became more sensitive to tap water than before (Xiao et al., 1987). Between 84.9%–100% schistosomula discharged the contents of their penetration glands at 2–3 days post transferring into HBS. The negative CHR and the disappearance of sensitivity towards complement indicated that tailless cercariae induced by PZQ were similar to post skin-penetration schistosomula.

In order to culture transformed schistosomula *in vitro*, a new medium termed 841 was established. It is a mixture of RPMI 1640 and fetal bovine serum (9:1), containing 10^{-6} mol/l 5-hydroxytryptamine (5-HT), 5×10^{-7} mol/l hypoxanthine, 10^{-6} mol/l hydrocortisone, 0.2 IU/ml insulin, 0.1% lactalbumin hydrolysate, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Wang and Zhou, 1987). Schistosomula mechanically transformed by syringe passage grew more rapidly in 841 medium than those transformed by fresh serum incubation, presenting a higher rate of cecal conjunction ($p < 0.01$) and a greater overall length ($p < 0.01$) after 28 days *in vitro*. In medium 841, the mechanically transformed schistosomula survived for more than 110 days and developed into adult female and male liver-stage worms that paired. Moreover, the reproductive organs of the adults formed, and the females laid eggs although unfertilized. These results provided a promising perspective for *in vitro* cultivation of *S. japonicum* at that time. Subsequently, medium 841 was widely used providing a solid basis for the formulation of new culture media.

Since *in vitro* culture studies in the past revealed significant differences in gene expression profiles between lung stage schistosomula isolated from hosts and those conventionally cultured (Chai et al., 2006), and since host factors such as age, gender, hormones, and/or immune responses were considered to affect growth, development, and gene expression of schistosomes (Gobert et al., 2006), co-culture systems of mechanically transformed schistosomula of *S. japonicum* with different vertebrate host cells were tested (Ye et al., 2012). Among these, human hepatic venous endothelial cells (ED25) turned out to be very suitable and interesting feeder cells. Compared to controls cultured *in vitro* without feeder cells, or co-cultured with other cells, schistosomula co-cultured with ED25 cells shared more similarities in morphology and tegumental structures with schistosomula obtained from infected mice as determined by bright-filed and electron microscopy.

2.2.3. Hepatic-portal-phase schistosomula and adult worms

In order to establish an *in vitro* culture system useful for studying reproductive physiology of *Schistosoma* for *e.g.* drug screening approaches, 18-day old schistosomula obtained from portal vein of the liver (Hua et al., 1988) and 42-day old adult worms (Hua and Zhou, 1988) were employed to perform further experiments, respectively.

Based on medium 841, two further types of media were developed (Hua et al., 1988) and termed modified 841 (M-841) (Table 2) and modified 841 adding ghost cells (M-841 + ghost cells). They were used to culture 18 day old schistosomula. The ghost cells represented erythrocytes treated with 0.38% NaCl showing a lighter color and a bigger size. Although all media supported pairing of males and females, the parasites maintained in M-841 or M-841 + ghost cells showed lower mortality and bigger size than those in medium 841 ($p < 0.05$) after *in vitro* culture for 35 days. There was no difference in the size of the testes and ovaries among the worms maintained in these different media. Furthermore, the ovaries of females in three groups matured, which was proven by fast red staining. However, the periods required to produce eggs differed remarkably among the investigated worm batches: 10 days for worms in M-841 + ghost cells, 29 days for worms in M-841, and 25 days for those in 841, respectively. This suggested that M841 + ghost cells was suitable for further *in vitro* studies on the reproductive physiology of schistosomes. However, the eggs laid by worms cultured under these conditions were abnormal.

RPMI/1640 medium, rabbit serum and human serum were more-favorable for the oviposition, as indicated by the number of eggs laid by females and the proportion of abnormal eggs, than BME medium, bovine serum and sheep serum (Hua and Zhou, 1988). In addition, 5-HT and hypoxanthine were proven not only to increase the number of eggs produced by females, but also to decrease the proportions of abnormal eggs. ATP and caseine hydrolysate just increased the number of eggs and presented no effect on the proportions, while vitamin C had no effect on the number of eggs but reduced the proportion of abnormal eggs. Norepinephrine decreased the number of eggs and showed no effect on egg integrity. Medium 199 was also emphasized for its importance on the oviposition of *S. mansoni* cultured *in vitro*. Therefore, medium 851 was designed by adding ingredients

Table 2
Comparison of components between different modified media.

Components	Concentration			
	841	M-841	851	891
RPMI-1640	**10.4 mg/ml**	**8.32 mg/ml**	**8.32 mg/ml**	**10.4 mg/ml**
Medium 199	–	**2.2 mg/ml**	**2.2 mg/ml**	–
Rabbit serum	10% (v/v)	10% (v/v)	10% (v/v)	10% (v/v)
Lactalbumin hydrolysate	**1 mg/ml**	–	–	**1 mg/ml**
Casein hydrolysate	–	**1 mg/ml**	**1 mg/ml**	–
Insulin	0.2 IU/ml	0.2 U/ml	0.2 U/ml	0.2 IU/ml
Hydrocortisone	10^{-6} mol/l	10^{-6} mol/l	10^{-6} mol/l	10^{-6} mol/l
Hypoxanthine	5×10^{-7} mol/l	5×10^{-7} mol/l	5×10^{-7} mol/l	5×10^{-7} mol/l
Serotonin	10^{-6} mol/l	10^{-6} mol/l	10^{-6} mol/l	10^{-6} mol/l
ATP	–	**0.1 mg/ml**	**0.2 mg/ml**	–
Vitamin C	–	–	**0.01 mg/ml**	–
Thymidine	–	–	–	**1.6×10^{-5} mol/l**
Tryptone	–	–	–	**1 mg/ml**
$NaHCO_3$	**pH 7.4**	**pH 7.6–7.8**	**pH 7.6–7.8**	**pH 7.6–7.8**
Penicillin	100 IU/ml	100 IU/ml	100 IU/ml	100 IU/ml
Streptomycin	100 μg/ml	100 μg/ml	100 μg/ml	100 μg/ml

The bold characters showed differences on concentrations of some components added in the different medium.

Author's personal copy

withapproved positive effect on oviposition compared to medium 841 (Table 2). These ingredients are medium 199 (2.2 g/l), ATP (0.2 g/l),vitamin C (0.01 g/l), and caseine hydrolysate (1 g/l, replacing lactalbumin hydrolysate in 841 medium). The numbers of eggs produced by worms maintained in medium 851 were about 872 per couple within 10 days and 1112 per couple within 23 days, values significantly higher than those produced in medium 841 ($p < 0.05$), which included about 708 per couple within 10 days and 821 per couple within 23 days, respectively. Besides this, the peak egg-producing period for the "851 medium group" was from days 2 to 10 in culture, while this period was from days 3 to 7 using medium 841. Although there was no significant difference between these two groups from the 20th to the 23rd day of cultivation, the proportions of abnormal eggs in medium 851 were significantly lower than those in medium 841 within 14 days ($p < 0.01$) (Hua and Zhou, 1988).

In spite of the positive effects of thymidine, ornithine, prednisolon and tryptone on the oviposition of 42 day-old worms, only thymidine and tryptone significantly reduced the number of abnormal eggs, leading to the establishment of medium 891 by supplementing these two components into medium 841 (Yang et al., 1997) (Table 2). The positive effects of medium 891 on the oviposition of paired worms (increasing egg productions, lowering the number of abnormal eggs, and prolonging the oviposition period) were similar to those of medium 851 within 14 days of cultivation (Yang et al., 1997). Although remarkable progress was achieved, especially with respect to the reduced number of deformed eggs using medium 891, the egg production period *in vitro* ceased from days 10 (medium 851) and 11 (medium 891) on (Hua and Zhou, 1988; Yang et al., 1997). This indicates that the *in vitro* simulation of the *in vivo* situation still requires continuing research input since not all factors necessary to maintain egg production of schistosome couples *in vitro* over a long culture period have been identified yet.

In summary, although *in vitro* maintenance of the whole life cycle of *S. japonicum* has not been achieved yet, the *in vitro* culture systems including various media for parasite culture have been gradually improved and are still under development. What has been achieved up to now provides a good basis for different experimental approaches such as studies on the interactions between host-parasite, development of transgenic approaches (Yuan et al., 2005), or RNAi studies (Wang et al., 2012). However, further improvements of the existing *in vitro* culture systems are needed to achieve the final goal to complete the life cycle of *S. japonicum in vitro* and to provide an optimal technical platform for experimental studies.

3. Cell culture of *S. japonicum*

The cell culture approaches of *S. mansoni* were started in the 1990s (Bayne et al., 1994; Coles and Fitzgerald, 1986; Hobbs et al., 1993; Weller and Wheeldon, 1982), while the attempts to isolate and culture cells from *S. japonicum* were initiated from 1995 (Dong et al., 1995a,b). Numerous studies related to culture conditions and cell lines were performed in the subsequent years.

3.1. Primary cell culture

3.1.1. Cultural methods

The first step required for initiating a cell culture is to isolate primary cells by enzyme digestion directly or let cells migrate from the tissue pieces which were kept in an appropriate environment for a few days. The moist system method, also termed tissue pieces adhesion-method (Wu et al., 1979) and the cold digestion method (Dong et al., 1995b) have been described as two different approaches for cell culture. The moist system method, also termed tissue pieces adhesion-method, was established by a Japanese researcher and described as follows: after being cut into pieces of about 1 mm^3, the tissue fragments were attached to the wall of the culture flask with a little medium and subsequently incubated at 37 °C in an incubator. In order to strengthen the adhesion of the cultured tissues, the flask was turned upside down to remove the access of medium before the flask was put in an incubator. The flask was turned back to its original position 2–4 h later, and sufficient medium was added for continuing the culture. It was suggested that the moist atmosphere could promote the capacity of tissues or cells to attach the wall of the culture flask in this short time period, which was very important for their growth. However, the number of the cells isolated by this approach was limited.

For cold digestion, tissue pieces were digested with 0.25% trypsin under 4 °C for several hours and subsequently centrifuged. After the supernatant was discarded, the precipitate of isolated cells was resuspended in medium and seeded into culture flasks for culturing. This way provides plenty of cells that could be isolated for *in vitro* maintenance, however, the cells couldn't attach to the wall of the flasks leading to gradual degeneration (Dong et al., 1995b).

Primary culture of *S. japonicum* cells was performed by employing schistosomula or adults that were obtained from mice 3–6 weeks post-infection (Dong et al., 1995b). Experiments using a combination of the moist system and cold digestion methods showed beneficial effects compared to the individual methods. Thus more free cells were obtained and the survival time of the cultured cells was extended (Table 3). In the following years, most work on the characterization of these cells was performed on the basis of this combined method.

3.1.2. Characteristics of cultured cells

3.1.2.1. Morphological observations. Along with the attempts to isolate and culture cells of *S. japonicum*, the following morphological observations were made. Cultured cells with a diameter of 1–4 μm derived from *S. japonicum* schistosomula were mainly round and granular ones, some were flagellated, triangle fan-like, polygonal or irregularly shaped (Chen et al., 1997). In contrast to this, most of the cells originating from adult worms were approximately 24 μm × 17 μm in size and polygonous (Dong et al., 1995c). However, no matter whether by bright-field or scanning electron microscopy (SEM), the structures of the cells from juveniles and adults were found to be similar to each other, with some papilla-like projections, villi, concaves, and small holes on their surfaces (Chen et al., 1997). In addition, by using SEM evidence for cell division was obtained among schistosomula cells which survived for 7 months, implying that these cells possessed proliferation potential (Chen et al., 1997).

Using transmission electron microscope (TEM), further analyses on the ultrastructure of cultured cells from *S. japonicum* schistosomula and adults were performed (Figs. 1, 2 and 3) (Dong et al., 2002a). Some differences were observed between these with regard to their shape, ultrastructure of the cell surface, and cell type. Most of the cultured cells from adults were polygonal with papilla-like tubercula, microvilli, and pinocytotic vesicles on their surfaces. These cells were identified as germ cells, vitelline cells, flame cells, multinucleate subtegumental cells, and nerve cells. Cultured schistosomula cells showed a different ultrastructure on their surfaces and were mainly round granular. They could be determined as germ cells, sustentacular cells, flame

Table 3
Effect of cultural methods on cells from *S. japonicum* (see Dong et al., 1995b).

Methods	Worms	Number of trials	Results
Moist system method	Adults	3	Less free cells, but longer survival time of cultured cells lasting for 60 days.
Cold digestion method	Adults Schistosomula	4 2	More free cells, round-shaped; no adherence to tissue culture flasks; degeneration starting from the 3rd day of inoculation.
Combination method	Adults	3	More free cells adhering to flasks; longer survival time of cells lasting for 110 days.

Author's personal copy

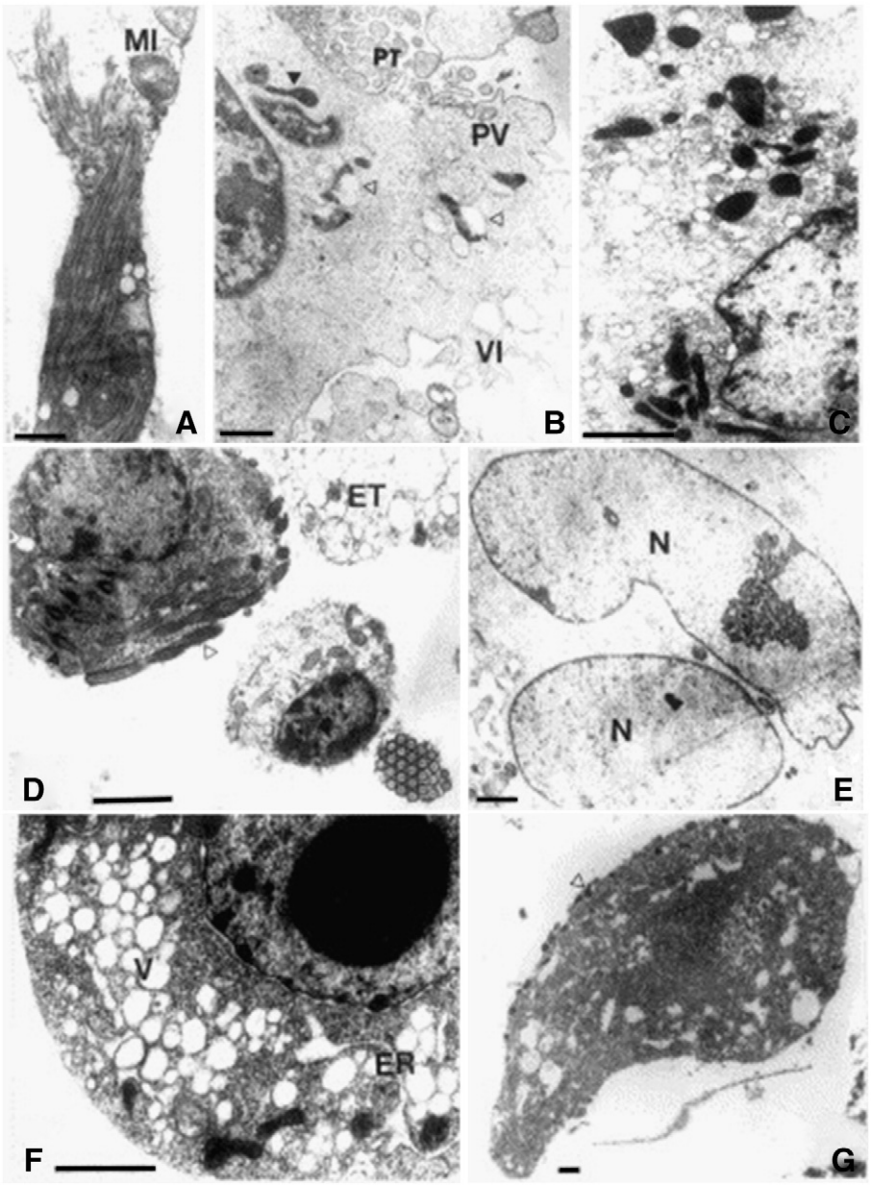

Fig. 1. Ultrastructure of cultures from *Schistosoma japonicum*. Scale bars = 0.5 μm. (A) The flagella of a flagellated cell from schistosomula, cultured for 12 days. MI, mitochondria. (B) The adult cell cultured for 12 days. PT, papilla-like tubercula; VI, microvilli; PV, pinocytotic vesicles; ▲, normal mitochondria; △, degenerated mitochondria. (C) An adult cell cultured for 2 days, with many mitochondria of different shapes in cytoplasm. (D) Flame cells from adult, cultured for 2 days. ▲, Basal part of the cilia; △, free part of the cilia; ET, excretory tubule. (E) A multinucleate subtegumental cell from adult, cultured for 12 days, N, nucleus. (F) Primary oocyte cultured for 2 days. ER, endoplasmic reticula; V, vacuole. (G) Secondary oocyte cultured for 48 days with a cluster of cortical granules (△) near the cell membrane.

cells, nerve cells, mast cells, muscle cells, multinucleate subtegumental cells, interstitial cells, and penetration gland cells. The most interesting finding by TEM was that schistosomula cells obviously kept their capacity to divide since many cells were detected with obvious division characteristics (Fig. 2-F). In contrast to this, strong evidence for cell division in adult cells was not obtained, either by TEM or by bright-field microscopy. This indicated that schistosomula cells possess a division potential, which is interesting with respect to strategies establishing permanent cell lines in the future. Along with increasing culture time, progressive changes were observed in the ultrastructure of schistosomula cells, including an increase of heterochromatin, degeneration of mitochondria, and disappearance of endoplasmic reticula (ER). Based on these findings, the period between days 0–18 of cell cultivation was narrowed down as the best period to induce proliferation of cultured cells derived from schistosomula according to the variation of chromatin.

The ER is an important organelle in animal cells and represents the center of membrane systems. Its morphology, number, and distribution are closely related to the type, function, differentiation, and mitotic phases of the cells (Huang and Song, 1996; Wang and Song, 1999; Zhao and Song, 1995). Due to lack of useful techniques, it was difficult to observe the three-dimensional architecture of whole mount ER membrane systems clearly by conventional method under TEM in the previous studies (Dong et al., 2003). After fixing cultured cells by potassium permanganate (Song, 1994), the three-dimensional architecture of whole mount endoplasmic reticulum membrane systems in the cultured cells of *S. japonicum* was observed under SEM and a small cisternal network composed of membranous tubules and vesicles was well distributed in scarce cytoplasm (Dong et al., 2003). As a cell structure extensively distributed within the cytoplasms of eukaryotic cells, the cytoskeleton (CSL) was considered among others as a platform for signal transduction systems within the cytoplasm (Wang et al., 1993). The CSL of cultured cells isolated from adult *S. japonicum* was primarily analyzed by Coomassie blue and Rhodamin–Phalloidin fluorescence staining (Fan et al., 2001b). It was consisted of intermediate filaments

Author's personal copy

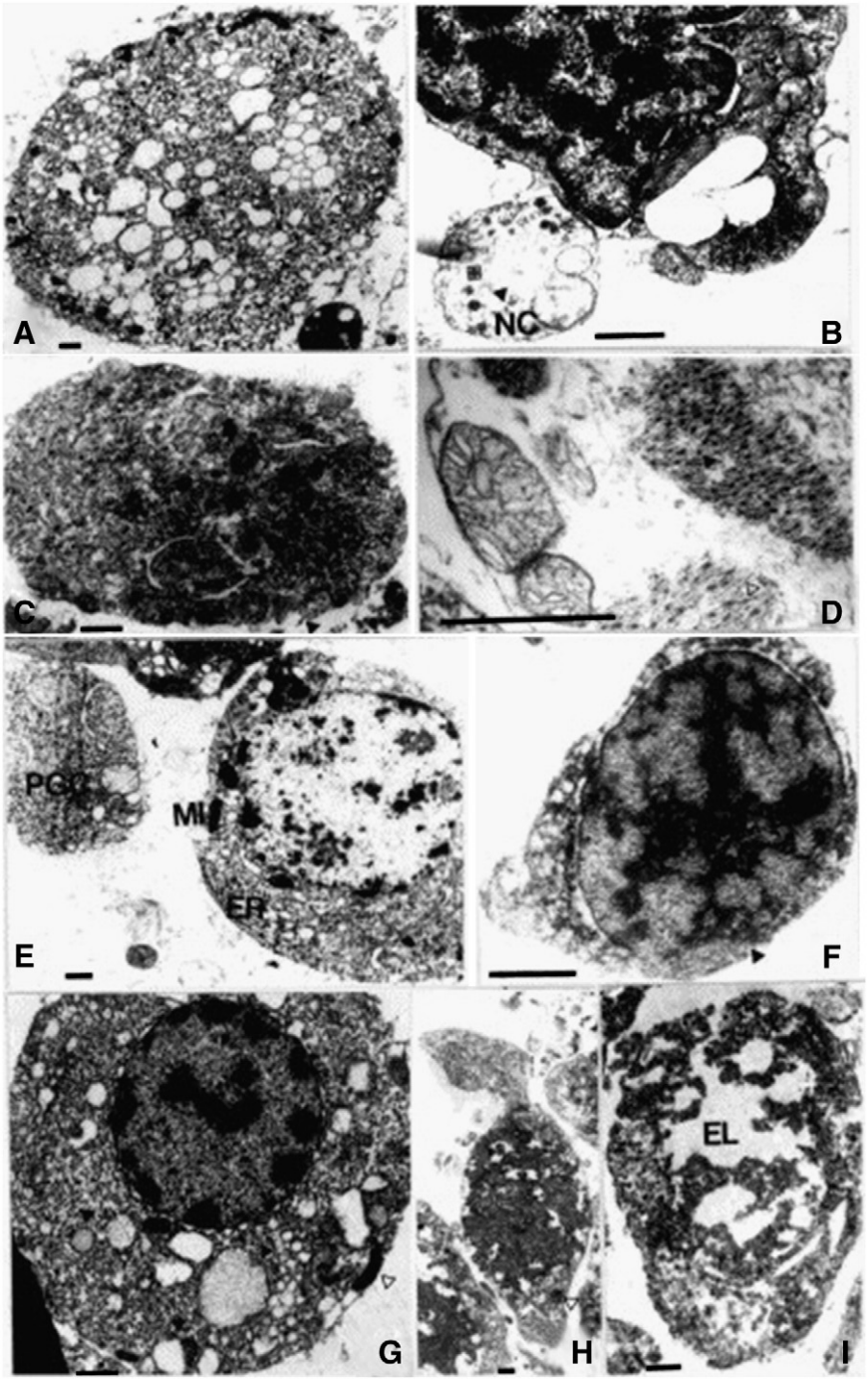

Fig. 2. Ultrastructure of cultures from *Schistosoma japonicum* schistosomula. Scale bars = 0.5 μm. (A) A sustentacular cell cultured for 12 days. △, relicts. (B) A nerve cell (NC) and an interstitial cell (IC) cultured for 6 days. △, Tight junction between the nerve cell and the interstitial cell; ▲, clear vesicle; □, granular vesicle. (C) The mast cell cultured for 12 days. ▲, secretory granule being released. (D) A muscle cell cultured for 12 days. ▲ and △, thick and thin filaments of myofibril. (E) Round granular cells cultured for 2 days. In the right cell, euchromatin was evidently more than heterochromatin and there were mitochondria (MI) and circular endoplasmic reticula (ER). The left was a penetration gland cell (PCG). (F) A division cell, cultured for 12 days. CH, chromosome; ▲, the part of nuclear membrane disappeared. (G) A cell cultured for 24 days. Heterochromatin increased evidently, almost much than euchromatin. In cytoplasm, there were normal mitochondria (△), degenerated mitochondria (▲) in different degrees, enlarged and vacuolar endoplasmic reticula. (H) Cells cultured for 36 days. Heterochromatin was evidently more than euchromatin. Some electron lucent (EL) areas appeared in the cells. There were small mitochondria (△) with low electron density, ribosomes and glycogen granules in cytoplasm. No other organelles were observed. (I) The cell cultured for 54 days. In the severely degenerated cell there were more electron lucent (EL) areas.

and appeared as a tight network-like structure but lacking thick microfilament (MF) bundles (Fan et al., 2001b). Given that the stability of MF bundles was demonstrated to be essential for the transition of proliferated cells from G to S phase (Maness and Walsh, 1982), the absence of the MF bundles in the cultured *S. japonicum* cells derived from adult worms was interpreted as a cause of their inability to divide continuously.

3.1.2.2. Enzyme localization and activities. In addition to the ultrastructural observations of *S. japonicum* cells cultured *in vitro*, the concentration, distribution, and variation of different enzymes within these cells were also investigated by histochemical methods.

Succinic dehydrogenase (SDH) (Kawahara et al., 2009) and lactate dehydrogenase (LDH) (Holbrook et al., 1975) are known to be involved in the energy metabolism of cells. In cultured cells from 26 day-old

Author's personal copy

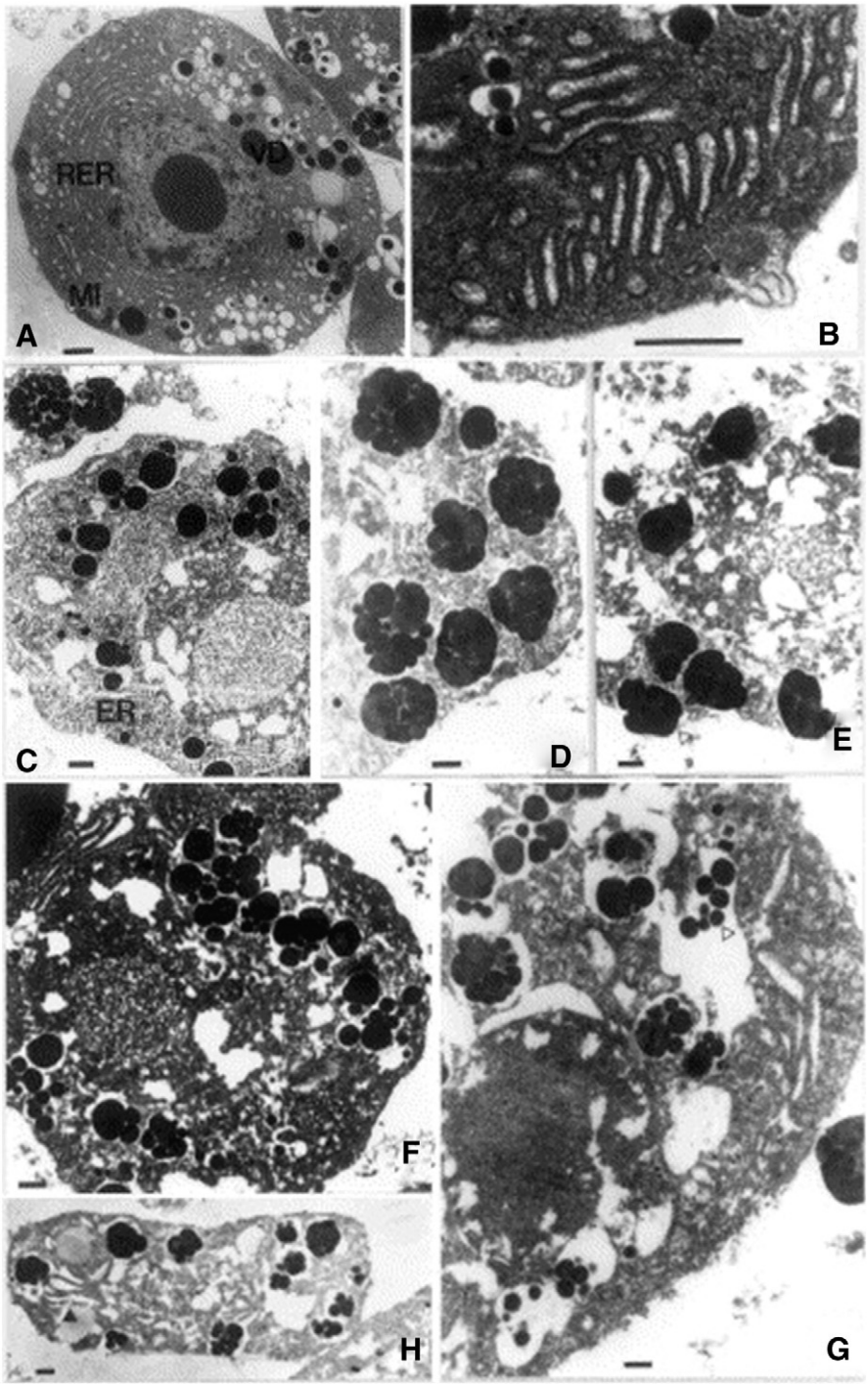

Fig. 3. Ultrastructure of cultured vitelline cells from *Schistosoma japonicum*. Scale bars = 0.5 μm. (A) Immature vitelline cells cultured for 0 day. MI, mitochondria; RER, rough-surfaced endoplasmic reticula; VD, vitelline droplet. (B) An immature vitelline cell cultured for 6 days. The figure shows abundant rough-surfaced endoplasmic reticula with clear ribosomes. (C) Vitelline cells cultured for 18 days. ▲ and △ shows the vitelline droplet of mature and immature vitelline cell, respectively. ER, endoplasmic reticula. (D) A mature vitelline cell cultured for 30 days. (E) A mature vitelline cell cultured for 36 days. △, fused vitelline globules in the vitelline droplet. (F) An immature vitelline cell cultured for 36 days. (G) An immature vitelline cell cultured for 60 days. △, uncovered vitelline globules. (H) Immature vitelline cells cultured for 73 days. ▲, enlarged rough-surfaced endoplasmic reticula.

adult *S. japonicum*, both SDH and LDH were observed within the cytoplasm, of which the former one was mainly distributed around the cell membrane of larger cells and the flagellum of flagellated cells, and the latter one spreads evenly within the cytoplasm (Dong et al., 2002b). Glucose-6-phosphate dehydrogenase (G-6-PD), the rate-limiting enzyme of the pentose phosphate pathway, was also detected to spread evenly within the cytoplasm of cultured cells of *S. japonicum* (see Zhu, 2002), which was similar to that of LDH. The distribution of alkaline phosphatase (AKP) varied with different types of cultured cells of adult *S. japonicum*. For round granular, deltaic fan and multinucleate subtegumental cells, stronger AKP activity was detected within the nucleus, while weaker activity was found within the cytoplasm. For clustered polygonal cells, AKP was mainly distributed in cytoplasm, whereas in flagellated cells, stronger enzyme activity was in both the nucleus and the flagellum part (Liu et al., 2002b). Acidic phosphatase (ACP) was also detected to be very rich in cultured *S. japonicum* cells and distributed particularly around the nucleus and within the cytoplasm of the larger cells (Liu et al., 2002b), which were different from AKP distribution. The activities of SDH, LDH,

Author's personal copy

AKP and ACP in cultured *S. japonicum* cells decreased gradually along with the time of culture (Dong et al., 2002b; Jiang et al., 2004; Zhu, 2002).

Furthermore, the activity of "silver-stained nucleolar region associated protein" (AgNORs), an index cell proliferation (Yao et al., 2001), was higher in cultured cells obtained from 18 day-old schistosomula than those from 24 day adult worms, again indicating the stronger proliferative ability of schistosomula cells (Dong et al., 2004).

3.1.2.3. Carbohydrates and calcium ions in cultured cells. Periodic Acid Schiff (PAS) reaction or Alcian Blue-PAS revealed the distribution and dynamic changes of carbohydrates in the cultured cells of *S. japonicum*. Glycogen, acid mucopolysaccharide, neutral mucopolysaccharide, mucoprotein, glycoprotein and glycolipids are present in cultured cells, and levels of these carbohydrates peaked during the first 2 weeks of the culture, but then gradually declined with the further cultivation (C.J. Liu et al., 2002; Ming et al., 2005).

Free ionic calcium ($[Ca^{2+}]$) is a second messenger playing a pivotal role in the physiology and biochemistry of organisms and the cells (Forsén and Kördel, 1994). The intracellular concentration of free ionic calcium ($[Ca^{2+}]$) is stable at approximately 187.0 ± 10.7 nmol/l on average in resting-stage *S. japonicum* cells which were cultured for 1–3 days (Peng et al., 2003a). This suggests that the stabilized intracellular environment of the cultured cells benefited from the favorable *in vitro* culture system at this early stage of culturing (Peng et al., 2003a).

3.1.3. Optimization of the culture system for S. japonicum cells

According to the previous investigations on the characteristics of cultured cells of *S. japonicum*, not only the transformation of mitochondria and endoplasmic reticula but also the changes of enzymatic activities (SDH, LDH, G-6-PDH, AKP and ACP) were supposed to be useful indicators for assessing the *in vitro* cultural conditions for the cells (Dong et al., 2002a,b; Jiang et al., 2004).

Aiming to optimize culture conditions for *S. japonicum* cells derived from adult worms, several parameters were analyzed for their effects on the growth of cultured cells (Dong et al., 1995a). First, lowering the concentration of serum to 5% improved cell survival. Under these conditions cells survived for more than 150 days, while 20% and 10% sera decreased survival times (between 110 and 150 days). Second, TC-199 medium was found to be better suited for the survival of the cultured cells than RPMI-1640. Finally, antibiotic concentration had no influence on the survival time of the cells, which was consistent to the result of Hobbs et al. (1993), but different from the report of Weller and Wheeldon (1982).

The effects of complex media (TC-199, RPMI-1640, DMEM), extracellular matrix (ECM, including collagen of rat tail, bio-matrix from liver of rabbits and bio-matrix from lung of rabbits), and serum concentration (5%, 10% and 20%) on the activities of SDH, LDH and G-6-PDH in the cells of *S. japonicum* (21-day old) were determined by using orthogonal tests. The results showed that the strongest activities of three enzymes were observed in cells cultured in the presence of the bio-matrixes from liver of rabbits and the medium RPMI-1640 containing 20% bovine serum (Deng et al., 2005). Moreover, three growth-promoting factors, transferrin, nucleotides, and basic fibroblast growth factor (b-FGF) promoted the proliferation of cultured cells of *S. japonicum* schistosomula much more efficiently than the other two substances, non-essential amino acids or vitamin combinations (Gong et al., 2005). Cells treated with these stimulators presented obvious proliferation abilities and much higher AKP activities.

3.1.4. Effects of chemical reagents, biological supplements and physical treatment on cultured cells

Based on the studies of primary cell cultures and the characterization of *in vitro*-cultured cells of *S. japonicum*, experimental efforts were made to prompt the proliferation of these cells. Chemical reagents, biological supplements, and physical treatment were tested aiming at establishing an immortal cell line of *S. japonicum*.

3.1.4.1. Chemical reagents. After treatment with the mitogen phytohemagglutinin (PHA) at a concentration of 333.3 μg/ml, cultured cells from *S. japonicum* adults showed evidence for proliferation. Lymphoblast-like cells emerged among degenerating cells, dumbbell-like cells appeared, and cells in early stages of division were detected. However, unlimited proliferation of the cultured cells did not occur (Dong et al., 1998).

N-methyl-N-nitro-N-nitrosoguanidine (MNNG), a strong mutagen which was shown to cause chromosomal DNA damage and to induce cell transformation leading to immortalized cell lines (Gichner and Veleminsky, 1982) was tested for its capacity to induce cell proliferation (Ming et al., 2006a). Cells treated with 3 μg/ml MNNG for 48 h survived for at least 246 days or more. Not only significant morphological features such as villiae, microridges, ruffles, blebs, concavities, and microvilli were observed on their surfaces, but also many binary and multipolar divisions were found by SEM indicating the positive effects of MNNG on the transformation and proliferation of cells. Transformation in the MNNG treated cells was confirmed by their disordered cytoskeletal arrangement (Fan et al., 2001a). Moreover, both the activities of AKP and ACP, and the carbohydrate metabolism of the cultured cells were improved by the stimulation of MNNG (Liu et al., 2002a; Ming et al., 2006c), which is consistent to the energy requirements of the cells during division. MNNG and liver-matrix protein were synergistic in boosting carbohydrate metabolism in these cells (Ming et al., 2006b).

β-Mercaptoethanol was reported to promote cell viability and to enhance various cell reactions in lymphocytes (Fanger et al., 1970; Ishii et al., 1981a,b) and embryonic carcinoma cells (Oshima, 1978; Rizzino and Sato, 1978) through increasing the synthesis of intracellular glutathione (GSH), which allowed the cells to overcome the oxidative stress that occurs during *in vitro* cultivation (Zmuda and Friedenson, 1983). The addition of β-mercaptoethanol to the medium led to the increase of $[Ca^{2+}]$ in the cultured cells (Peng et al., 2003a). Compared to liver-matrix, β-mercaptoethanol showed stronger effects on the increase of the level of AgNORs and LDH activity in cells during the first two weeks of culture (Peng et al., 2004; Qin et al., 2005). This was reversed from the 3rd week on, proving the long-acting and stable influence of liver-matrix. Unfortunately, no cooperative effect was observed between β-mercaptoethanol and liver-matrix (Peng et al., 2004; Qin et al., 2005).

Finally, an effect of spermidine was tested, which is a cationic amine with functions during growth and proliferation of cells (Pegg, 1986; Tabor and Tabor, 1984). Cultured cells treated with spermidine using a concentration of 75 μM spermidine for 48 h showed the strongest activity of SDH. Higher concentrations of this compound destroyed the cells and the mitochondrial membranes, leading to the decrease of SDH (Yi et al., 2005). The detection of AgNORs suggested the supporting effect of spermidine on the proliferation of cultured cells from *S. japonicum* (see Chen et al., 2006b).

3.1.4.2. Biological supplements. It was proposed that not only non-optimal culture environments, but also the inability of the isolated cells from *S. japonicum* worms to attach to culture flasks was the main reason for the failure to establish cell lines (Dong et al., 1995a). Since extracellular matrix (ECM) proteins showed important regulatory roles for the adhesion of cultured cells (Reid and Jefferson, 1984), three different ECMs, including rat tail collagen, bio-matrix from livers, and lungs of rabbits were employed to cover the wall of culture flasks prior to adding cell suspensions (Zhu et al., 2004). The results showed that rat tail collagen most effectively supported the adhesion of *S. japonicum* cells. Besides this, rat tail collagen also enhanced the heat-resistance of these cells (Chen et al., 2002). However, bio-matrix from rabbit liver was the best ECM supplement with regard to cell morphology, growth, metabolism, division and proliferation (Chen et al., 2006a; Deng et al., 2005). Cells cultured with this ECM supplement had stronger activities of LDH (Zhu et al., 2002), SDH (Zhu et al., 2002), AKP (Yi et al., 2003), and ACP (Yi et al., 2003),

Author's personal copy

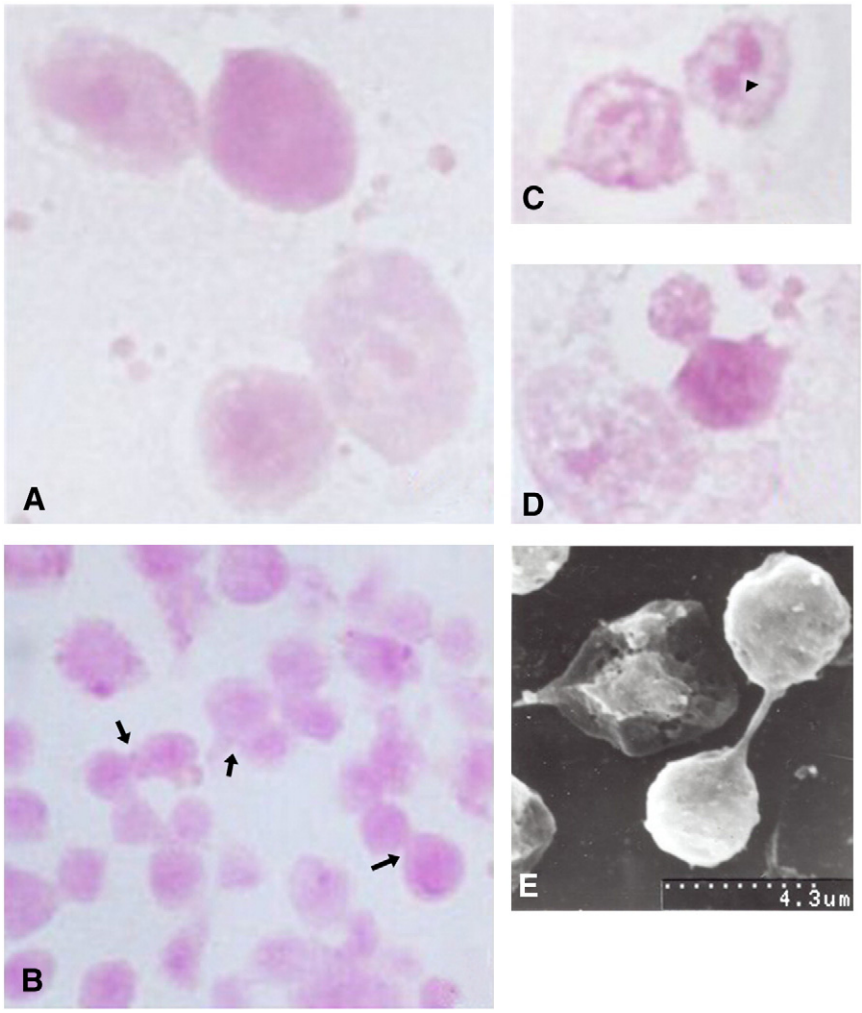

Fig. 4. Morphology of cells from *Schistosoma japonicum* cultured with extracellular cell matrix (ECM). (A–D) Cells cultured for 28 days in the presence of bio-matrix from rabbit livers. A few dividing cells (→) and dividing nuclei (▲) were observed under microscopy after being stained by periodic acid schiff (PAS) reaction. (E) Cells cultured for 60 days in the presence of bio-matrix from rabbit lungs. Binary division was observed by scanning electronic microscope (SEM). (Figs. 1–3 and their legends are from: Dong HF, Chen XB, Ming ZP, Zhong QP, and Jiang MS. Ultrastructure of cultured cells from *Schistosoma japonicum*. Acta Trop 2002a; 82:225-34. We've got a license agreement from Elsevier. License Number: 3140660550247.) Fig. 4: A–D are from: Chen XG, Dong HF, Ming ZP, Zhong QP, Jiang MS, and Hu SQ. Effects of liver matrix on growth of cultured cells from *Schistosoma japonicum*. Chin J Schisto Control 2006a; 18:25-7, in Chinese. E is from: Chen DC, Dong HF, Jiang MS, Zhu JY, and Zhong QP. The function of the bio-matrixes from the rabbit lung and collagen from rat tail on the cultured cells from *Schistosoma japonicum*. Chin J Endemiol 2002; 21:261-3, in Chinese. We've asked the magazines for permission to use the pictures, which was approved in each case.

higher levels of AgNORs (Chen et al., 2006a), stronger abilities to synthesize carbohydrates (Ming et al., 2005), and contained more dividing cells, which were not only in ordinary binary division, but also in tripolar division (Chen et al., 2006a). Moreover, cell divisions occurred as late as day 60 in culture using bio-matrix from rabbit lungs (Chen et al., 2002), while cell divisions using bio-matrix from liver only occurred up to day 28 (Fig. 4) (Chen et al., 2006a).

Feeder cells, which were reported in cell culture previously (Puck et al., 1956), are able to provide the basis for cell–cell and cell–matrix interactions (Ehmann et al., 1998), production of soluble growth factors (Krakauer, 2002; Shimoaka et al., 2002; Tseng et al., 1996), and removal of toxic products from the culture medium (Brodin et al., 1983). Long term culture of cells from 12–21 day-old juvenile worms of *S. mansoni* was reported to accomplish by the use of a feeder layer of irradiated rat liver cells (Bayne and Barnes, 1997). As *S. japonicum*, mouse embryonic fibroblast (MEF) cells were used as feeder layers to culture cells of *S. japonicum* and exhibited stronger effects than ECM, not only on the adherence of cells (compared with rat tail collagen) (Li et al., 2006b), but also on the activities of LDH and ACP (compared with liver matrix) (Xiong et al., 2006). The combination of MEF and ECM (rat tail collagen or liver matrix) was proven to have synergistic effects on the treated cells (Li et al., 2006b; Xiong et al., 2006). The addition of conditioned medium, which was filtered from the medium of nasopharyngeal carcinoma (CNE) cells, led to an increase of LDH activity and AgNOR levels in *S. japonicum* cells (C. Luo et al., 2010; R. Luo et al., 2010).

Paired schistosome females produce hundreds to thousands of eggs per day, which require tens of thousands of vitelline cells to provide egg-shell precursor proteins, as well as yolk and lipids as energy resources for the embryo, vitelline cells. Consequently, vitelline cells were supposed to have a strong ability to divide and to differentiate (Den Hollander and Erasmus, 1984, 1985). Furthermore, strong evidence exists that males fulfill an essential role in controlling female sexual development including mitosis and differentiation of their pairing partners (Grevelding et al., 1997; Kunz, 2001; LoVerde and Chen, 1991). Therefore, proteins extracted from male parasites were employed to culture vitelline cells. They improved the activity of LDH and significantly increased the level of AgNORs in treated cells with a concentration of 100 mg/l (Li et al., 2006a). Moreover, there were much more mature vitelline cells in the treated group compared to the control group, with clear individual vitelline globules inside. The cytoplasms and nuclei of these cells were homogeneously stained, and abundant rough-surfaced endoplasmic reticulum was found in immature vitelline cells of tested group. In the control group, vitelline globules were fused with each other or released and uncovered.

Author's personal copy

Rough-surfaced endoplasmic reticulum in control cells was enlarged and space-vacuolated, and the ribosomes particle dropped from the reticulum. Both results confirmed positive stimulation of development, proliferation, and metabolic activity of the *S. japonicum* vitelline cells by male worm extracts (Zhong et al., 2011).

3.1.4.3. Physical treatment. Since there were some reports of positive effects on cell proliferation induced by electromagnetic field (EMF) (Li et al., 2003; Yang et al., 2003; Zhao et al., 2005), this physical stimulation was introduced into the *in vitro* culture system to assess the effects of EMF application on the activity of LDH in the cultured cells of *S. japonicum*. Although higher level of LDH activity in the cells treated by EMF suggested acceleration of cellular metabolism, no cell proliferation was observed (Huang et al., 2007).

3.2. Continuous passage cell culture

Besides the primary culture, continuous passage of *S. japonicum* cells has been achieved. Successful *in vitro* passage cultivation of cells derived from miracidia, cercariae, and adult worms of *S. japonicum* was reported (Zhang et al., 2002a,b). Briefly, after being collected in modified RPMI-1640 medium with 10% fetal bovine serum (FBS), parasite tissue was disrupted by cutting every individual worm for 2–3 times. Subsequently, the tissue fragments were digested with 250 U crab collagenase at 26 °C (for miracidia and cercariae), or 37 °C (for adults) for 30 min. After centrifugation and washing, the digested products were inoculated into tissue culture flasks in 1 ml medium with 0.5% FBS (low concentration to promote adhesion to the substratum) on the first day and were transferred into fresh medium containing 5% to 20% FBS from the next day on. The emergence of double cells, which was observed within the first 3 days, was considered as a sign of single round replication. Subcultures were made by splitting 1:2 from the 10th culture day on, and they were performed further every 7–14 days. This was cells obtained from miracidia, cercariae, and adults were passaged up to the 6th, 5th and 8th generations, respectively.

Cells isolated from 30-day adult *S. japonicum* worms were also successfully subcultured in 1640-40 medium (RPMI-1640 medium with addition of FBS and several growth factors, which was not provided in detail) (Liu et al., 2006). Briefly, after being treated aseptically, the collected worms were grinded through metal screen cloths with 300 meshes, subsequently digested and filtered. Following the centrifugation at 300 ×*g* for 10 min, the cells were resuspended with RPMI-1640 and were incubated in culture flasks with a density of 1×10^6/ml at 37 °C and 5% CO_2. For *in vitro* cultivation, the medium was changed every 3–5 days. When the cells covered the bottom of the flask, or when the density reached more than three times of the original one, passages were done splitting with the cells 1:2.

Unfortunately, no more details about the specific culture medium (defined as medium1640-40) were provided in these publications (Liu et al., 2006; Zhang et al., 2002a,b) although continuous passage cell cultivations were reported.

Before being subcultured the cells were semi-floating in the medium and gradually aggregated to the middle part of the cultural flask with visible division phenomena, which emerged from the 5th to the 7th day. The survival rates of primary cells, passed cells, as well as the cells recovered from material frozen in dimethyl sulfoxide (DMSO) prior to the 5th generation were all up to 90%, and then decreased to 80% after the 5th generation (Liu et al., 2006). Chromosome karyotype of cells of 5 generations showed diploidy (2n = 8) with 16 chromosomes which were arranged into three groups according to their sizes. Cultured cells of the 4th generation were round, ellipsoid, irregular or spindle-shaped. They were measured 3.0–15.5 μm × 2.5–12.0 μm in size, and the nuclei and cytoplasm of most cells were distinct from each other. All four types of normal morphology and three types of abnormal morphology were observed on the cultured cells of the 5th generation under TEM (Liu et al., 2006).

4. Applications of *in vitro* culture systems for *S. japonicum*

Although not optimal yet, the *in vitro* culture systems available for *S. japonicum* parasitic stages or cells today provide useful platforms for various experimental studies such as protein expression in the homologous system, drug screenings, or functional studies of genes by RNAi and further transgenic approaches.

4.1. Application examples of parasite in vitro culture

Employing established *in vitro* culture systems (Wang and Zhou, 1988; Wang et al., 1987) the effect of PZQ on *S. japonicum* was investigated with a focus on schistosomula since it was known from previous studies in other schistosome species that this juvenile stage is less susceptible to this drug. Cultured schistosomula treated with a low concentration of PZQ (<1 μg/ml) for a short time showed light contractions and weak movements. They recovered completely if transferred to drug-free medium within 48 h. However, at high concentration (>1 μg/ml) PZQ caused strong and sustained contractions of schistosomula, and their movements nearly stopped. Only if transferred to drug-free medium within 6 h, signs of recovery were noted and the schistosomula survived (Wang et al., 1987). In addition, the tegumental ultrastructures of schistosomula treated with PZQ were destroyed in a dose dependent manner according to SEM analysis (Wang and Zhou, 1988).

In addition to assessing the effects of anti-schistosome drugs, the *in vitro* culture system was also employed to study gene functions of *S. japonicum* using RNAi, which was pioneered in *S. man*soni (see Skelly et al., 2003). The first report of RNAi experiments in *S. japonicum* was to knock-down the gene coding for the gynecophoric canal protein of *S. japonicum* (SjGCP) by dsRNA soaking (Cheng et al., 2005). The transcript level of SjGCP in adult couples was reduced by 75% through 7 day treatment of dsRNA. Immunodetection experiments indicated the disappearance of SjGCP from the gynecophoric canal when 100 nM dsRNA was applied. From then on, different stages of schistosomes have been probed with dsRNA or short interfering RNAs (siRNAs) on the basis of *in vitro* cultivation of *S. japonicum*, with efficient and specific interference effects on the target genes, including the mago nashi gene (Zhao et al., 2008), peroxiredoxin genes (Prx-1 nor Prx-2) (Kumagai et al., 2009), Wnt4 (Li et al., 2010), the calcium-regulated heat-stable 24 kDa protein (CRHSP-24)-encoding gene SjCRHSP-24 (Zou et al., 2011), type V collagen (SjColV) gene (Yang et al., 2012), and the small heat shock protein (sHSP)-encoding gene *Sjp40* (Chen et al., 2012).

In vitro culture of *S. japonicum* not only allowed studies on the parasite itself, but also facilitated investigations on molecules related to host-parasite interactions. In a recent study, gene transcription profiles and morphological characters were analyzed in mother sporocysts cultured in the presence of head–foot protein extract of snails (Ye et al., 2012). The expression of several transcripts such as those encoding cytochrome c oxidase subunit 2 (Cox2) and NADH-ubiquinone oxidoreductase (ND1) was significantly up-regulated, whereas dyskeratosis congenita 1 protein (DKC1) was down-regulated by the head–foot protein extract of *O. hupensis* from Hunan province, providing basic information for further research on the interplay between this intramolluscan stage of *S. japonicum* and its host. Based on a comparative co-culture system study to analyze molecular effects during the schistosomula/*O. hupensis* interplay, a suppression subtractive hybridization (SSH) approach comparing transcriptional differences among co-cultured, host group, and control group parasites was performed. The results revealed that four candidate transcripts encoding the cathepsin L precursor (CatL), heat shock protein 70 (Hsp70), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and programmed cell death protein 10 (PCDP 10) were expressed differently among the three groups, which was confirmed by real-time PCR. SDS-PAGE analysis finally showed not only congruent protein patterns, but also interesting differences among the compared schistosomula groups. Although some differences from host-derived

Author's personal copy

schistosomula were still observed, the co-culture with human hepatic vein endothelial cells (ED25) positively influenced parasite morphology and gene expression with respect to their comparability to the situation *in vivo* (Ye et al., 2012).

4.2. Application of cell culture

Schistosome cell cultures have potential applications in the fields of immunology and transgenesis. Although cells isolated from miracidia, cercariae, and adult *S. japonicum* were successfully passaged for several generations (Liu et al., 2006; Zhang et al., 2002a), no more related studies followed except one article focusing on the antigenicity of cells from *S. japonicum* cercariae (Zhang et al., 2002b). Antigens derived from cercarial cells of the fifth passage *in vitro* served to detect circulating antibody in 90.3% of patients with chronic schistosomiasis, whereas the false positive rate was 6.7% in a healthy control group. However, this antigen hasn't been employed in a serodiagnostic test up to the present day.

Beyond that, initial evidence has been obtained for the successful transfection of primary cells from *S. japonicum* by electroporation using a cytomegalovirus (CMV) promoter-GFP construct as vector (Yuan et al., 2005), providing promising perspectives for future investigations.

5. Conclusion and perspective

Today we reflect on a history of 30–40 years of efforts to culture *S. japonicum* in China. Nearly every stage of the parasite was the aim of studies in this direction. These long-lasting efforts were accompanied by the introduction of several media and supplements that helped to improve culture conditions. Thus protocols exist to maintain miracidia, mother sporocysts, cercariae, and egg-laying adult worms. It is not only possible to generate mother sporocysts from miracidia *in vitro*, but also to obtain adult worms from cercariae through *in vitro* cultivation. The main obstacles to complete the life cycle of *S. japonicum* in the lab are the transition from mother sporocysts to cercariae, and the production of fertilized as well as completely developed eggs by adult worms generated *in vitro*. These are tasks requiring some more intensive research input in these early days of the post-genomic era.

With regard to cells from *S. japonicum*, besides established isolation protocols media formulations exist, and it was shown that mutagens like MNNG and ECM were able to induce mitogenic activities. Although enzyme activities or the level of AgNORs in cultured cells indicated still suboptimal conditions, the achievements made point to the possibility of reaching the aim of establishing cell lines for *S. japonicum*.

Also for *S. mansoni* it has not succeeded yet to complete the whole life cycle *in vitro*. The use of *Bge* cells for culturing and the subsequent emergence of daughter sporocysts promoted work in this field, allowing impressive studies on the interplay between *S. mansoni* and *B. glabrata* in subsequent years (Bixler et al., 2001; Coustau et al., 1997; Ivanchenko et al., 1999; Yoshino and Laursen, 1995). Therefore, for work with *S. japonicum* not only the culture of parasites but also that of *O. hupensis* cells should be explored. Besides this, a hypoxic environment, created by gassing with nitrogen in an incubator, proved not only to promote sporocyst production of *S. mansoni* independently of host factors (Bixler et al., 2001; Ivanchenko et al., 1999), but also supported the maintenance of healthy cell cultures over a longer time period (Bayne, 1998; Bayne and Parton, 2005), providing a valuable reference for further endeavors on the *in vitro* cultivation of *S. japonicum* worms and cells. Moreover, genetically modified *S. japonicum* will be important in the future for studying the functions of genes to understand their importance for schistosome biology. This will help to pinpoint potential target molecules for the design of novel anti-schistosomal strategies. In addition to transgenic parasites, schistosome cell transfection with DNA-constructs exhibiting oncogenic potential may represent a feasible approach to produce permanently dividing cells (Quack et al., 2010). To this end results of a new study shed the first light on the presence and function of stem cells in *S. mansoni* (see Collins et al., 2013), which will broaden the perspectives for new approaches to establish cell lines. This is fostered finally by a new method to isolate complete reproductive organs such as ovaries and testes from *S. mansoni* (see Hahnel et al., 2013). This may allow to even pool organ-specific cells and to concentrate cells with proliferative potential serving as suitable sources to start cell-line establishment. Both the improvements of the *in vitro* culture of larval and adult worms of *S. japonicum* as well as the access of cells of this parasite provide excellent advances for research on this important parasite in the future.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant no. 31201897) to QY, and (Grant no. 81273010) to HFD and the "Special Fund for Agro-scientific Research in the Public Interest" (Grant no. 201303037) to MH, and by the Deutsche Forschungsgemeinschaft (Grant no. GR 1549/5-2) to CGG. We are also grateful to Prof. James B. Lok in the Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, for his constructive comments.

References

Basch PF. Intermediate host specificity in *Schistosoma mansoni*. Exp Parasitol 1976;39:150–69.
Basch PF. Cultivation of *Schitosoma mansoni in vitro* I. Establishment of cultures from cercariae and development until pairing. J Parasitol 1981;67:179–85.
Bayne CJ. Invertebrate cell culture consideration: insects, ticks, shellfish, and worms. In: Barnes D, Mather JP, editors. Methods in cell biology. Academic Press; 1998. p. 187–201.
Bayne CJ, Barnes DW. Culture of cells from two life stages of *Schistosoma mansoni*. Cytotechnology 1997;23:205–10.
Bayne CJ, Parton A. Long-term cultures of cells from *Strongylocentrotus purpuratus* and *Schistosoma mansoni*. Bull Mt Desert Biol Lab 2005;44:44–6.
Bayne CJ, Menino JS, Hobbs DJ, Barnes DW. *In vitro* cultivation of cells from larval *Schistosoma mansoni*. J Parasitol 1994;80:29–35.
Beckmann S, Buro C, Dissous C, Hirzmann J, Grevelding CG. The Syk kinase SmTK4 of *Schistosoma mansoni* is involved in the regulation of spermatogenesis and oogenesis. PLoS Pathog 2010;6:e1000769.
Beckmann S, Leutner S, Gouignard N, Dissous C, Grevelding CG. Protein kinases as potential targets for novel anti-schistosomal strategies. Curr Pharm Des 2012a;18:3579–94.
Beckmann S, Quack T, Dissous C, Cailliau K, Lang G, Grevelding CG. Discovery of platyhelminth-specific α/β-integrin families and evidence for their role in reproduction in *Schistosoma mansoni*. PLoS One 2012b;7:e52519.
Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, et al. The genome of the blood fluke *Schistosoma mansoni*. Nature 2009;460:352–8.
Bi XY, Zhou SL, Li Y. Preliminary observation on the growth of cercarial embryo of *Schistosoma japonicum* cultivated *in vitro*. Chin J Schisto Control 1992;49:18–21. [in Chinese].
Bixler LM, Lerner JP, Ivanchenko M, McCormick RS, Barnes DW, Bayne CJ. Axenic culture of *Schistosoma mansoni* sporocysts in low O_2 environments. J Parasitol 2001;87:1167–8.
Botros S, Sayed H, Amer N, El-Ghannam M, Bennett JL, Day TA. Current status of sensitivity to praziquantel in a focus of potential drug resistance in Egypt. Int J Parasitol 2005;35:787–91.
Boyle JP, Yoshino TP. Serotonin-induced muscular activity in *Schistosoma mansoni* larval stages: importance of 5-HT transport and role in daughter sporocyst production. J Parasitol 2005;91:542–50.
Boyle JP, Zaide JV, Yoshino TP. *Schistosoma mansoni*: effects of serotonin and serotonin receptor antagonists on motility and length of primary sporocysts *in vitro*. Exp Parasitol 2000;94:217–26.
Boyle JP, Wu XJ, Shoemaker CB, Yoshino TP. Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Mol Biochem Parasitol 2003;128:205–15.
Brodin T, Olsson L, Sjogren HO. Cloning of human hybridoma, myeloma and lymphoma cell lines using enriched human monocytes as feeder layer. J Immunol Methods 1983;60:1–7.
Chai M, McManus DP, McInnes R, Moertel L, Tran M, Loukas A, et al. Transcriptome profiling of lung schistosomula, *in vitro* cultured schistosomula and adult *Schistosoma japonicum*. Cell Mol Life Sci 2006;63:919–29.
Chen MG. Progress of praziquantel in the treatment of schistosomiasis in foreign countries: a review. Int J Med Parasit Dis 2006;33:20–8. [in Chinese].
Chen P, Zhou S. *In vitro* cultivation of medical parasitology. Beijing: Science Press; 1995 [in Chinese].
Chen XP, Dong HF, Jiang MS, Yang MY, Li Y. Preliminary morphological observation on cultured cells from schistosomula *Schistosoma japonicum*. Acta Acad Med Hebei 1997;18:7–9. [in Chinese].

Author's personal copy

Chen DC, Dong HF, Jiang MS, Zhu JY, Zhong QP. The function of the bio-matrixes from the rabbit lung and collagen from rat tail on the cultured cells from *Schistosoma japonicum*. Chin J Endemiol 2002;21:261–3. [in Chinese].

Chen XG, Dong HF, Ming ZP, Zhong QP, Jiang MS, Hu SQ. Effects of liver matrix on growth of cultured cells from *Schistosoma japonicum*. Chin J Schisto Control 2006a;18:25–7. [in Chinese].

Chen XG, Yi TY, Ming ZP, Zhong QP, Jiang MS, Dong HF. Effects of spermidine on argyrophilic nucleolar organizer region associated proteins in cultured cells from adult *Schistosoma japonicum*. Chin J Endemiol 2006b;25:50–3. [in Chinese].

Chen MF, Mai RJ, Mo QZ, Zhou XH. Effect of RNA interference on small heat shock protein Sjp40 of *Schistosoma japonicum*. J South Med Univ 2012;32:456–61. [in Chinese].

Cheng GF, Lin JJ, Shi Y, Jin YX, Fu ZQ, Jin YM, et al. Dose-dependent inhibition of gynecophoral canal protein gene expression *in vitro* in the schistosome (*Schistosoma japonicum*) by RNA interference. Acta Biochim Biophys Sin (Shanghai) 2005;37: 386–90.

Chu HJ. Certain behavior reactions of *Schistosoma japonicum* and *Clonorchis senensis in vitro*. Chin Med J 1938;2:411–7.

Clegg JA. *In vitro* cultivation of *Schistosoma mansoni*. Exp Parasitol 1965;16:133–47.

Coles GC, Fitzgerald J. Effect of mutagen on cultured *Schistosoma mansoni*. J Helminthol 1986;60:135–42.

Colley DG, Wikel SK. *Schistosoma mansoni*: simplified method for the production of schistosomules. Exp Parasitol 1974;35:44–51.

Collins III JJ, Wang B, Lambrus BG, Tharp ME, Iyer H, Newmark PA. Adult somatic stem cells in the human parasite *Schistosoma mansoni*. Nature 2013;494:476–9.

Correnti JM, Brindley PJ, Pearce EJ. Long-term suppression of cathepsin B levels by RNA interference retards schistosome growth. Mol Biochem Parasitol 2005;143:209–15.

Coustau C, Ataev G, Jourdane J, Yoshino TP. *Schistosoma japonicum*: *in vitro* cultivation of miracidium to daughter sporocyst using a *Biomphalaria glabrata* embryonic cell line. Exp Parasitol 1997;87:77–87.

da Rocha Pitta MG, da Rocha Pitta MG, de Melo Rego MJ, Galdino SL. The evolution of drugs on schistosoma treatment: looking to the past to improve the future. Mini Rev Med Chem 2013;13:493–508.

Davis RE, Parra A, LoVerde PT, Ribeiro E, Glorioso G, Hodgson S. Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. Proc Natl Acad Sci U S A 1999;96:8687–92.

Den Hollander JE, Erasmus DA. *Schistosoma mansoni*: DNA synthesis in males and females from mixed and single-sex infections. Parasitology 1984;88:463–76.

Den Hollander JE, Erasmus DA. *Schistosoma mansoni*: male stimulation, DNA synthesis by the female. Parasitology 1985;91:449–57.

Deng SM, Dong HF, Zhong QP, Ming ZP, Jiang MS, Hu SQ. Studies on culture conditions of cells from *Schistosoma japonicum* by orthogonal test. Chin J Schisto Control 2005;17: 111–5. [in Chinese].

Dinguirard N, Yoshino TP. Potential role of a CD36-like class B scavenger receptor in the binding of modified low-density lipoprotein (acLDL) to the tegumental surface of *Schistosoma mansoni* sporocysts. Mol Biochem Parasitol 2006;146:219–30.

Doenhoff MJ, Pica-Mattoccia L. Praziquantel for the treatment of schistosomiasis: its use for control in areas with endemic disease and prospects for drug resistance. Expert Rev Anti Infect Ther 2006;4:199–210.

Doenhoff MJ, Hagan P, Cioli D, Southgate V, Pica-Mattoccia L, Botros S, et al. Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. Parasitology 2009;136:1825–35.

Dong HF, Jiang MS, Li Y, Ni YH, Chen JQ. Preliminary studies on the cultured conditions of cells from adult *Schistosoma japonicum*. Chin J Schisto Control 1995a;7:257–61. [in Chinese].

Dong HF, Jiang MS, Li Y, Yang MY, Zhou SL. Preliminary studies on the cultural methods of cells from *Schistosoma japonicum*. Acta Hydrobiol Sin 1995b;19: 382–3. [in Chinese].

Dong HF, Jiang MS, Li Y, Yang MY, Zhou SL. Morphological observations on cultured cells from adult *Schistosoma japonicum*. The Festschrift for setting up of Chinese Society of parasitology for 10 years. Beijing: Chinese Science and Technology Press; 1995c. p. 131–3. [in Chinese].

Dong HF, Jiang MS, Chen XP, Yang MY, Zhang PX, Zhou SL. Function of PHA on the proliferation on cultured cells from adult *Schistosoma japonicum*. Chin J Zoonoses 1998;14: 41–4. [in Chinese].

Dong HF, Chen XB, Ming ZP, Zhong QP, Jiang MS. Ultrastructure of cultured cells from *Schistosoma japonicum*. Acta Trop 2002a;82:225–34.

Dong HF, Jiang MS, Liu Q, Zhong QP, Zhu JY. Cytochemical studies on SDH and LDH of cultured cells from adult *Schistosoma japonicum*. Chin J Zoonoses 2002b;18:69–71. [in Chinese].

Dong HF, Zhu JY, Zhong QP, Ming ZP, Jiang MS, Xu MX. The effect of extracellular matrix on whole mount endoplasmic reticulum membrane system in the cultured cells from *Schistosoma japonicum*. Chin J Endemiol 2003;22:485–7. [in Chinese].

Dong HF, Peng Y, Jiang MS, Ming ZP, Zhong QP. Studies on the silver-stained nucleolar organizer region associated protein in cultured cells from *Schistosoma japonicum*. Chin J Zoonoses 2004;20:492–4. [in Chinese].

Ehmann UK, Stevenson MA, Calderwood SK, DeVries JT. Physical connections between feeder cells and recipient normal mammary epithelial cells. Exp Cell Res 1998;243: 76–86.

Fan H, Dong HF, Jiang MS, Ming ZP, Zhong QP. Studies on the effect of MNNG on cytoskeleton of cultured cells from adult *Schistosoma japonicum*. Chin J Endemiol 2001a;20: 401–3. [in Chinese].

Fan H, Dong HF, Jiang MS, Zhong QP, Ming ZP. Studies on cytoskeleton of cultured cells from adult *Schistosoma japonicum*. Chin J Schisto Control 2001b;13:280–1. [in Chinese].

Fanger MW, Hart DA, Wells JV. Enhancement by reducing agent of the transformation of human and rabbit peripheral lymphocytes. J Immunol 1970;105:1043–5.

Forsén S, Kördel J. Calcium in biological systems. In: Bertini I, Gray HB, Lippard SJ, Valentine JS, editors. Bioinorganic Chemistry. California: University Science Books; 1994. p. 107–66.

Freitas TC, Jung E, Pearce EJ. TGF-beta signaling controls embryo development in the parasitic flatworm *Schistosoma mansoni*. PLoS Pathog 2007;3:e52.

Gichner T, Veleminsky J. Genetic effects of N-methyl-N′-nitro-N-nitrosogunidine and its homologs. Mutat Res 1982;99:129–242.

Gobert GN, Chai M, McManus DP. Biology of the schistosome lung-stage schistosomulum. Parasitology 2006;134:453–60.

Gong YF, Zeng QR, Zhang ZP, Lin XC, Cai LT, Yan GW. Preliminary study on the cultural conditions for the cultivation of schistosomula cells of *Schistosoma japonicum in vitro*. Chin J Zoonoses 2005;21:159–63. [in Chinese].

Grevelding CG. Transgenic flatworms. In: Maule AG, Marks NJ, editors. Parasitic flatworms: molecular biology, biochemistry, immunology and physiology. Wallingford: CABI; 2006. p. 149–73.

Grevelding CG, Sommer G, Kunz W. Female-specific gene expression in *Schistosoma mansoni* is regulated by pairing. Parasitology 1997;115:635–40.

Gryseels B. Schistosomiasis. Infect Dis Clin North Am 2012;26:383–97.

Hahnel S, Lu Z, Wilson RA, Grevelding CG, Quack T. Whole-organ isolation approach as a basis for tissue-specific analyses in *Schistosoma mansoni*. PLoS Negl Trop Dis 2013;7: e2336.

Hang PY, Liang YS, Dai JR, Zhu YC, Ning A, Yu BD, et al. Study on susceptibility of *Schistosoma japonicum* in different stages to Praziquantel. Chin J Schisto Control 2001;13:343–6. [in Chinese].

Hansen EL. A cell line from embryos of *Biomphalaria glabrata* (Pulmonata): establishment and characteristics. In: Maramorosch K, editor. Invertebrate tissue culture: research applications. New York: Academic Press; 1976. p. 75–99.

Heyers O, Walduck AK, Brindley PJ, Bleiss W, Lucius R, Dorbic T, et al. *Schistosoma mansoni* miracidia transformed by particle bombardment infect *Biomphalaria glabrata* snails and develop into transgenic sporocysts. Exp Parasitol 2003;105:174–8.

Ho YH. On the host specificity of *Schistosoma japonicum*. Chin Med J 1963;82:405–14.

Hobbs DJ, Fryer SE, Duimstra JR, Hedstrom OR, Brodie AE, Collodi PA, et al. Culture of cells from juvenile worms of *Schistosoma mansoni*. J Parasitol 1993;79:913–21.

Holbrook JJ, Liljas A, Steindel SJ, Rossman MG. Lactate dehydrogenase. In: Boyer PD, editor. The enzymes. New York: Academic Press; 1975. p. 191–292.

Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest 2008;118:1311–21.

Hua XX, Zhou SL. Effects of nutritional factors on oviposition of adult *Schistosoma japonicum* cultured *in vitro*. Acta Zool Sin 1988;34:297–304. [in Chinese].

Hua XX, Li Y, Zhou SL. Growth, development and oviposition of hepatic-portal-phase schistosomula of *Schistosoma japonicum* cultured *in vitro*. Acta Acad Med Hebei 1988;9:195–9. [in Chinese].

Huang YX, Manderson L. The social and economic context and determinants of schistosomiasis japonica. Acta Trop 2005;96:223–31.

Huang JQ, Song JD. Pleomorphism of tridimensional architecture of wholemount endoplasmic reticulum in cultured cells. Acta Anat Sin 1996;27:269–72. [in Chinese].

Huang JJ, Dong HF, Ming ZP, Zhong QP, Jiang MS, Li X, et al. Selection of optimal condition of static electromagnetic field to the cultured cells from *Schistosoma japonicum*. Chin J Zoonoses 2007;23:769–73. [in Chinese].

Huang HH, Rigouin C, Williams DL. The redox biology of schistosome parasites and applications for drug development. Curr Pharm Des 2012;18:3595–611.

Ishii T, Bannai S, Sugita Y. Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol *in vitro*. J Biol Chem 1981a;256:12387–92.

Ishii T, Hishinuma I, Bannai S, Sugita Y. Mechanism of growth promotion of mouse lymphoma L1210 cells *in vitro* by feeder layer or 2-mercaptoethanol. J Cell Physiol 1981b;107: 283–93.

Ito J, Komiya Y. Studies on the survival of *Schistosoma japonicum in vitro*. 2. Survival in a chemically defined medium. Jpn J Parasitol 1955;4:258–61. [in Japanese].

Ito J, Yasuraoka K, Komiya Y. Studies on the survival of *Schistosoma japonicum in vitro*. 1. Survival in blood or serum medium. Jpn J Parasitol 1955;4:12–8. [in Japanese].

Ivanchenko MG, Lerner JP, McCormick RS, Toumadje A, Allen B, Fischer K, et al. Continuous *in vitro* propagation and differentiation of cultures of the intramolluscan stages of the human parasite *Schistosoma mansoni*. Proc Natl Acad Sci U S A 1999;96:4965–70.

Jiang MS, Liu Q, Dong HF, Zhu JY, Zhong QP. Cytochemical studies on dynamic changes of phosphatase in the cultured cells from *Schistosoma japonicum*. Med J Wuhan Univ 2004;25:617–20. [in Chinese].

Jiang Y, Gong RM, Yang Y, Li XH, Liu Y, Zhu CG, et al. Preliminary investigation on *in vitro*-cultivation and manipulation of *Schistosoma japonicum* sporocysts. Int J Med Parasit Dis 2011;38:158–62. [in Chinese].

Kawahara K, Mogi T, Tanaka TQ, Hata M, Miyoshi H, Kita K. Mitochondrial dehydrogenases in the aerobic respiratory chain of the rodent malaria parasite *Plasmodium yoelii yoelii*. J Biochem 2009;145:229–37.

King CH. Parasites and poverty: the case of schistosomiasis. Acta Trop 2010;113:95–104.

Knobloch J, Kunz W, Grevelding CG. Herbimycin A suppresses mitotic activity and egg production of female *Schistosoma mansoni*. Int J Parasitol 2006;36:1261–72.

Krakauer T. Stimulant-dependent modulation of cytokines and chemokines by airway epithelial cells: cross talk between pulmonary epithelial and peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2002;9:126–31.

Krautz-Peterson G, Skelly PJ. *Schistosoma mansoni*: the dicer gene and its expression. Exp Parasitol 2008;118:122–8.

Krautz-Peterson G, Radwanska M, Ndegwa D, Shoemaker CB, Skelly PJ. Optimizing gene suppression in schistosomes using RNA interference. Mol Biochem Parasitol 2007;153: 194–202.

Kumagai T, Osada Y, Ohta N, Kanazawa T. Peroxiredoxin-1 from *Schistosoma japonicum* functions as a scavenger against hydrogen peroxide but not nitric oxide. Mol Biochem Parasitol 2009;164:26–31.

Author's personal copy

Kuntz AN, Davioud-Charvet E, Sayed AA, Califf LL, Dessolin J, Arnér ES, et al. Thioredoxin glutathione reductase from *Schistosoma mansoni*: an essential parasite enzyme and a key drug target. PLoS Med 2007;4:e206.

Kunz W. Schistosome male–female interaction: induction of germ-cell differentiation. Trends Parasitol 2001;17:227–31.

Lee CU, Chu HJ. Simple technique for studying schistosome worms *in vitro*. Proc Soc Exp Biol Med 1935;32:1397–400.

Leutner S, Beckmann S, Grevelding CG. Characterization of the cGMP-dependent protein kinase SmcGK1 of *Schistosoma mansoni*. An Acad Bras Cienc 2011;83: 637–48.

Li F, Jia GL, Zhang RQ, Liu B, Zhao XG. The effects of weak constant magnetic field on the proliferations of human umbilical vein endothelial cells and rabbit aortic endothelial cells. Chin J Pathophysiol 2003;19:772–7. [in Chinese].

Li JL, Jiang MS, Zhong QP, Ming ZP, Dong HF. Selection of optimal concentration of male worm extraction on the cultured vitelline cells from *Schistosoma japonicum*. Chin J Endemiol 2006a;25:482–6. [in Chinese].

Li JL, Xiong F, Zhong QP, Ming ZP, Dong HF, Jiang MS. Effects of mouse embryonic fibroblast feeder layers and combined of mouse embryonic fibroblast and collagen of rat tail on adhesion of cultured cells from *Schistosoma japonicum*. Chin J Endemiol 2006b;25:145–9. [in Chinese].

Li HF, Wang XB, Jin YP, Xia YX, Feng XG, Yang JM, et al. Wnt4, the first member of the Wnt family identified in *Schistosoma japonicum*, regulates worm development by the canonical pathway. Parasitol Res 2010;107:795–805.

Lin JY, Li Y, Zhou SL. Preliminary studies on the *in vitro* cultivation of lung-phase schistosomula of *Schistosoma japonicum*. Chin J Parasitol Parasit Dis 1983;1:164–7. [in Chinese].

Lin JY, Li Y, Zhou SL. *In vitro* cultivation of skin stage schitosomulum of *Schistosoma japonicum* and the observation on the early surface changes of cultured schistosomulum. Natl Med J China 1985a;65:49–50. [in Chinese].

Lin JY, Li Y, Zhou SL. The development of cercariae of *Schistosoma japonicum* in the final-host. Acta Zool Sin 1985b;31:70–6. [in Chinese].

Liu CJ, Dong HF, Jiang MS, Zhong QP, Zhu JY. Cytochemical studies on carbohydrate of cultured cells from adult *Schistosoma japonicum*. Med J Wuhan Univ 2002;23: 202–4. [in Chinese].

Liu Q, Dong HF, Jiang MS, Ming ZP, Zhong QP. Influence of MNNG on phosphatase in the cultured cells from adult *Schistosoma japonicum*. Chin J Schisto Control 2002a;14: 14–6. [in Chinese].

Liu Q, Dong HF, Jiang MS, Zhong QP, Ming ZP. Histochemical observation of SDH, AKP and ACP in cultured cells from *Schistosoma japonicum*. China Public Health 2002b;18: 557–8. [in Chinese].

Liu W, Zeng TB, Zeng QR, Cai C, Zhang ZP, Gong FY, et al. Biological identification on sub-cultivation cells of *Schistosoma japonicum* adult worms *in vitro*. Chin J Parasitol Parasit Dis 2006;24:395–7. [in Chinese].

Long T, Cailliau K, Beckmann S, Browaeys E, Trolet J, Grevelding CG, et al. *Schistosoma mansoni* Polo-like kinase 1: a mitotic kinase with key functions in parasite reproduction. Int J Parasitol 2010;40:1075–86.

LoVerde PT, Chen L. Schistosome female reproductive development. Parasitol Today 1991;7: 303–8.

Ludtmann MH, Rollinson D, Emery AM, Walker AJ. Protein kinase C signalling during miracidium to mother sporocyst development in the helminth parasite, *Schistosoma mansoni*. Int J Parasitol 2009;39:1223–33.

Luo C, Ming ZP, Zhong QP, Ye Q, Jiang MS, Dong HF. Effects of conditioned medium on LDH and AgNORs in cultured schistosomulum cells from *Schistosoma japonicum*. Chin J Schisto Control 2010a;22:552–6. [in Chinese].

Luo R, Xue X, Wang Z, Sun J, Zou Y, Pan W. Analysis and characterization of the genes encoding the dicer and argonaute proteins of *Schistosoma japonicum*. Parasit Vectors 2010b;3:90.

Maness PF, Walsh Jr RC. Dihydrocytochalasin B disorganizes actin cytoarchitecture and inhibits initiation of DNA synthesis in 3 T3 cells. Cell 1982;30:253–62.

Mann VH, Morales ME, Rinaldi G, Brindley PJ. Culture for genetic manipulation of developmental stages of *Schistosoma mansoni*. Parasitology 2010;137: 451–62.

Mann VH, Suttiprapa S, Rinaldi G, Brindley PJ. Establishing transgenic schistosomes. PLoS Negl Trop Dis 2011;5:e1230.

Mei BS, Zhou SL. Effects of osmolarity and pH on the transformation of *Schistosoma japonicum* miracidium to mother sporocyst *in vitro*. Acta Acad Med Hebei 1988;9: 200–5. [in Chinese].

Mei BS, Zhou SL. Effects of nutritive factors on *Schitosoma japonicum* miracidial transformation and mother sporocyst culture *in vitro*. Acta Hydrobiol Sin 1989;13:326–33. [in Chinese].

Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, Wynn NB, et al. Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of *Schistosoma mansoni*. PLoS Neg Trop Dis 2009;3:e504.

Meng W, Wu ZD, Yu XB, Peng ZY, Ruan ZY, Yuan ZQ. Experimental study on the method of collection for the lung-stage schistosomula of *Schistosoma japonicum*. Chin J Zoonoses 2005;21:173–5. [in Chinese].

Ming ZP, Dong HF, Zhong QP, Liu CJ, Jiang MS, Zhang ZR. Dynamic changes of carbohydrate in cultured cells from adult *Schistosoma japonicum* and effects of liver matrix on them. Chin J Endemiol 2005;24:513–6. [in Chinese].

Ming ZP, Dong HF, Zhong QP, Grevelding CG, Jiang MS. The effect of a mutagen (N-methyl-N-nitro-N-nitrosoguanidine) on cultured cells from adult *Schistosoma japonicum*. Parasitol Res 2006a;98:430–7.

Ming ZP, Zhong QP, Dong HF, Jiang MS, Zhang ZR. Effects of combined use of liver matrix and N-methyl-N′-nitro-N-nitrosogunidine on the content and distribution of carbohydrates in the cultured cells from adult *Schistosoma japonicum*. Chin J Zoonoses 2006b;22:259–62. [in Chinese].

Ming ZP, Zhong QP, Dong HF, Zhu JY, Jiang MS. Effects of N-methyl-N′-nitro-N-nitrosogunidine on carbohydrate in cultured cells from adult *Schistosoma japonicum*. Chin J Public Health 2006c;22:50–2. [in Chinese].

Montresor A, Gabrielli AF, Chitsulo L, Ichimori K, Mariotti S, Engels D, et al. Preventive chemotherapy and the fight against neglected tropical diseases. Expert Rev Anti Infect Ther 2012;10:237–42.

Morales ME, Rinaldi G, Gobert GN, Kines KJ, Tort JF, Brindley PJ. RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol Biochem Parasitol 2008;157:160–8.

Ndegwa D, Krautz-Peterson G, Skelly PJ. Protocols for gene silencing in schistosomes. Exp Parasitol 2007;117:284–91.

Oshima R. Stimulation of the clonal growth and differentiation of feeder layer dependent mouse embryonal carcinoma cells by β-mercaptoethanol. Differentiation 1978;11: 149–55.

Osman A, Niles EG, Verjovski-Almeida S, LoVerde PT. *Schistosoma mansoni* TGF-beta receptor II: role in host ligand-induced regulation of a schistosome target gene. PLoS Pathog 2006;2:e54.

Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 1986;234:249–62.

Peng Y, Dong HF, Jiang MS, Zhong QP, Ming ZP. Determination of intercellular free Ca^{2+} concentration in the cultured cells from *Schistosoma japonicum* by using Fura-2/AM. Chin J Schisto Control 2003a;15:139–41. [in Chinese].

Peng Y, Jiang MS, Zhong QP, Gui JF, Dong HF. Preliminary study on primary culture of cells from *Oncomelania hupensis*. Chin J Parasitol Parasit Dis 2003b;21:176–8. [in Chinese].

Peng Y, Dong HF, Jiang MS, Zhong QP, Hu SQ. Effects of the liver matrix and β-mercaptoethanol on the cultured cells from *Schistosoma japonicum*. Chin J Endemiol 2004;23:307–10. [in Chinese].

Pica-Mattoccia L, Cioli D. Sex- and stage-related sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. Int J Parasitol 2004;34:527–33.

Pitta MG, Silva AC, Neves JK, Silva PG, Irmão JI, Malagueño E, et al. New imidazolidinic bioisosters: potential candidates for antischistosomal drugs. Mem Inst Oswaldo Cruz 2006;101:313–6.

Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M, Aslett MA, et al. A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis 2012;6:e1455.

Puck TT, Marcus PI, Cieciura SJ. Clonal growth of mammalian cells *in vitro*; growth characteristics of colonies from single HeLa cells with and without a feeder layer. J Exp Med 1956;103:273–83.

Qin JH, Peng Y, Dong HF, Jiang MS, Ming ZP, Zhong QP. Effects of liver matrix or/and β-mercaptoethanol on cultured cells from *Schistosoma japonicum*. Chin J Public Health 2005;21:1182–3. [in Chinese].

Quack T, Wippersteg V, Grevelding CG. Cell cultures for schistosomes — chances of success or wishful thinking? Int J Parasitol 2010;40:991–1002.

Reid LM, Jefferson DM. Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells. In: Mather JP, editor. Mammalian cell culture. New York: Plenum Publishing Corporation; 1984. p. 239–80.

Rinaldi G, Morales ME, Cancela M, Castillo E, Brindley PJ, Tort JF. Development of functional genomic tools in trematodes: RNA interference and luciferase reporter gene activity in *Fasciola hepatica*. PLoS Negl Trop Dis 2008;2:e260.

Rinaldi G, Eckert SE, Tsai IJ, Suttiprapa S, Kines KJ, Tort JF, et al. Germline transgenesis and insertional mutagenesis in *Schistosoma mansoni* mediated by murine leukemia virus. PLoS Pathog 2012;8:e1002820.

Rizzino A, Sato G. Growth of embryonal carcinoma cells in serum-free medium. Proc Natl Acad Sci U S A 1978;75:1844–8.

Rossi A, Wippersteg V, Klinkert MQ, Grevelding CG. Cloning of 5′ and 3′ flanking regions of the *Schistosoma mansoni* calcineurin A gene and their characterization in transiently transformed parasites. Mol Biochem Parasitol 2003;130:133–8.

Sayed AA, Simeonov A, Thomas CJ, Inglese J, Austin CP, Williams DL. Identification of oxadiazoles as new drug leads for the control of schistosomiasis. Nat Med 2008;14: 407–12.

Shimoaka T, Ogasawara T, Yonamine A, Chikazu D, Kawano H, Nakamura K, et al. Regulation of osteoblast, chondrocyte, and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. J Biol Chem 2002;277:7493–500.

Skelly PJ, Da'dara A, Harn DA. Suppression of cathepsin B expression in *Schistosoma mansoni* by RNA interference. Int J Parasitol 2003;33:363–9.

Song JD. Three-dimensional structure of the endoplasmic reticulum under scanning microscope established by potassium permanganate technology. J Chin Electron Microsc Soc 1994;13:328. [in Chinese].

Song LJ, Li JH, Xie SY, Qian CY, Wang J, Zhang W, et al. Thioredoxin glutathione reductase as a novel drug target: evidence from *Schistosoma japonicum*. PLoS One 2012;7: e31456.

Tabor WC, Tabor H. Polyamines. Annu Rev Biochem 1984;53:749–90.

Tang CC, Tang CT, Tang C. Studies of the migratory route of *Schistosoma japonicum* in its final host. Acta Zool Sin 1973;19:323–36. [in Chinese].

The Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium. The *Schistosoma japonicum* genome reveals features of host–parasite interplay. Nature 2009;460:345–51.

Tseng SC, Kruse FE, Merritt J, Li DQ. Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media. Curr Eye Res 1996;15:973–84.

Verjovski-Almeida S, DeMarco R, Martins EA, Guimarães PE, Ojopi EP, Paquola AC, et al. Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. Nat Genet 2003;35:148–57.

Wang QY, Song JD. A comparative study of the ultrastructure of endoplasmic reticulum in animal cells at different evolutionary levels. Acta Zool Sin 1999;45:23–7. [in Chinese].

Author's personal copy

Wang W, Zhou SL. Studies on the cultivation *in vitro* of *Schistosoma japonicum* schistosomula transformed from cercariae by artificial methods. Acta Zool Sin 1987;33:144–50. [in Chinese].

Wang J, Zhou SL. The observation of praziquantel effecting on the tegumental ultrastructure of schistosomula of *Schistosoma japonicum in vitro*. Acta Hydrobiol Sin 1988;12:286–9. [in Chinese].

Wang W, Li Y, Zhou SL. Studies on artificial transformation of *Schistosoma japonicum* cercariae to schistosomula *in vitro*. Chin J Parasitol Parasit Dis 1986;4:212–4. [in Chinese].

Wang J, Li Y, Zhou SL. An observation of praziquantel effecting on the activity and survival of hepatic-portal-phase Schistosomulae of *Schistosoma japonicum in vitro*. Acta Acad Med Hebei 1987;18:338–42. [in Chinese].

Wang N, Butler JP, Ingber DE. Mechanotransduction across the cell surface and through the cytoskeleton. Science 1993;260:1124.

Wang ZX, Zhao J, Zhou Y, Pan WQ. Study on culture for genetic manipulation of schistosomula of *Schistosoma japonicum*. China Trop Med 2012;12:171–3. [in Chinese].

Weller TH, Wheeldon SK. The cultivation *in vitro* of cells derived from adult *Schistosoma mansoni*. I. Methodology; criteria for evaluation of cultures; and development of media. Am J Trop Med Hyg 1982;31:335–48.

Wippersteg V, Kapp K, Kunz W, Grevelding CG. Characterisation of the cysteine protease ER60 in transgenic *Schistosoma mansoni* larvae. Int J Parasitol 2002a;32:1219–24.

Wippersteg V, Kapp K, Kunz W, Jackstadt WP, Zahner H, Grevelding CG. HSP70-controlled GFP expression in transiently transformed schistosomes. Mol Biochem Parasitol 2002b;120:141–50.

Wippersteg V, Ribeiro F, Liedtke S, Kusel JR, Grevelding CG. The uptake of Texas Red-BSA in the excretory system of schistosomes and its colocalisation with ER60 promoter-induced GFP in transiently transformed adult males. Int J Parasitol 2003;33:1139–43.

Wippersteg V, Sajid M, Walshe D, Khiem D, Salter JP, McKerrow JH, et al. Biolistic transformation of *Schistosoma mansoni* with 5′ flanking regions of two peptidase genes promotes tissue-specific expression. Int J Parasitol 2005;35:583–9.

Wu ZA, Wang YC, Li SD. Cell culture of human tumor. (translation)Beijing: Science Press; 197937–8 [in Chinese].

Xiao SH, Catto BA, Webster LT. Effects of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. J Infect Dis 1985;151:1130–7.

Xiao SH, Xue HC, Le WJ, Qiu LS, Mei JY. Conversion of *Schistosoma japonicum* cercariae to schistosomula under the action of praziquantel. Chin J Parasitol Parasit Dis 1987;5:133–5. [in Chinese].

Xiong F, Li JL, Ming ZP, Zhong QP, Dong HF, Jiang MS. Effect of mouse embryonic fibroblast feeder layer and liver matrix of rabbit on the activities of LDH and ACP in cultured cells from *Schistosoma japonicum*. Chin J Zoonoses 2006;22:502–5. [in Chinese].

Yang J, Li Y, Zhou SL. Studies on the factors which influenced oviposition and maturation of eggs of adult *Schistosoma japonicum in vitro*. Acta Acad Med Hebei 1997;18:308–11. [in Chinese].

Yang W, Xu T, Huo XL, Song T. Effect of extremely low frequency magnetic fields on intracellular free calcium in HepG2 cells. Chin J Ind Hyg Occup Dis 2003;23:332–4. [in Chinese].

Yang YX, Jin YM, Liu PP, Shi YL, Cao YF, Liu JM, et al. RNAi silencing of type V collagen in *Schistosoma japonicum* affects parasite morphology, spawning, and hatching. Parasitol Res 2012;111:1251–7.

Yao ZH, Dong GM, Zhang JK, Yu YX, Liu XC, Liu WX, et al. Analysis on the genetics of Hanta virus S85-46 isolated from Sichuan province. Chin J Epidemiol 2001;22:449–51. [in Chinese].

Yasuraoka K, Irie Y, Hata H. Conversion of schistosome cercariae to schistosomula in serum-supplemented media, and subsequent culture *in vitro*. Jpn J Exp Med 1978;48:53–60. [in Japanese].

Ye Q, Zhu JY, Zhong QP, Jiang MS, Dong HF. Primary culture of the cells from *Oncomelania hupensis* liver. Chin J Parasitol Parasit Dis 2007;25:478–82. [in Chinese].

Ye Q, Zhu JY, Ming ZP, Zhao QP, Grevelding CG, Liu R, et al. Studies on the establishment of a co-culture system of lung stage *Schistosoma japonicum* with host cells. Parasitol Res 2012;111:735–48.

Yi TY, Dong HF, Jiang MS, Zhong QP, Zhang ZR. Influence of matrixes on concentration of alkaline phosphatase and acidic phosphate in cultured cells from adult *Schistosoma japonicum*. Chin J Schisto Control 2003;15:418–21. [in Chinese].

Yi TY, Dong HF, Jiang MS, Ming ZP, Zhong QP. The effects of spermidine on succinate dehydrogenase in the cultured cells from adult *Schistosoma japonicum*. Chin J Parasit Dis Con 2005;18:339–41. [in Chinese].

Yoshino TP, Laursen JR. Production of *Schistosoma mansoni* daughter sporocysts from mother sporocysts maintained in synxenic culture with *Biomphalaria glabrata* embryonic (*Bge*) cells. J Parasitol 1995;81:714–22.

You H, Zhang W, Jones MK, Gobert GN, Mulvenna J, Rees G, et al. Cloning and characterisation of *Schistosoma japonicum* insulin receptors. PLoS One 2010;24(5):e9868.

Young ND, Jex AR, Li B, Liu S, Yang L, Xiong Z, et al. Whole-genome sequence of *Schistosoma haematobium*. Nat Genet 2012;44:221–5.

Yuan XS, Shen JL, Wang XL, Wu XS, Liu DP, Dong HF, et al. *Schistosoma japonicum*: a method for transformation by electroporation. Exp Parasitol 2005;111:244–9.

Zhang ZY, Zeng XF, Li JR, Yi XY, Zhang J, Yan GW, et al. Success *in vitro* cultivation of cells derived from miracida, cercariae and adult of *Schistosoma japonicum*. J Tianjin Agric Coll 2002a;9:5–12. [in Chinese].

Zhang ZY, Zeng XF, Li JR, Yi XY, Zeng QR, Zhang J, et al. Passage cultivation and immunological identification of *Schistosoma japonicum* cercaria cells. Chin J Parasitol Parasit Dis 2002b;20:332–4. [in Chinese].

Zhao YH, Song JD. Scanning electron microscopic studies on the endoplasmic reticulum in the cell cycle. Chin J Phys Med Rehabil 1995;17:165–7. [in Chinese].

Zhao WC, Ma WM, Zhao ZH, Fang ZH, Wu H. Preliminary research on the proliferation and differentiation of rat bone marrow mesenchymal stem cells with exposure to 50 Hz magnetic fields. J Biomed Eng 2005;22:510–3. [in Chinese].

Zhao ZR, Lei L, Liu M, Zhu SC, Ren CP, Wang XN, et al. *Schistosoma japonicum*: inhibition of Mago nashi gene expression by shRNA-mediated RNA interference. Exp Parasitol 2008;119:379–84.

Zhong QP, Li JL, Ming ZP, Jiang MS, Dong HF. Effects of male worm extraction on ultrastructure of cultured vitelline cells from *Schistosoma japonicum*. Chin J Schisto Control 2011;23:424–7. [in Chinese].

Zhou XL, Dong HF, Jiang MS, Li X, Huang JJ. *In vitro* effect of 5-hydroxytryptamine on motility and length of *Schistosoma japonicum* primary sporocysts. Chin J Parasitol Parasit Dis 2008;26:295–8. [in Chinese].

Zhou YB, Liang S, Jiang QW. Factors impacting on progress towards elimination of transmission of schistosomiasis japonica in China. Parasit Vectors 2012;5:275.

Zhu JY. Studies on the effects of three kinds of extracellular matrixes to the cultured cells of *Schistosoma japonicum*. [Master Dissertation] Wuhan University; 200210–21 [in Chinese].

Zhu JY, Dong HF, Jiang MS, Zhong QP, Liu Q. Effects of matrixes on the concentration of SDH and LDH in the cultured cells of *Schistosoma japonicum*. Chin J Parasit Dis Con 2002;15:297–9. [in Chinese].

Zhu JY, Dong HF, Jiang MS, Zhong QP, Zhang ZR, Hu SQ. Effects of extracellular matrix on adhesion of the *in vitro* cultured cells of *Schistosoma japonicum*. Chin J Zoonoses 2004;20:328–31. [in Chinese].

Zhu JY, Ye Q, Zhao QP, Ming ZP, Grevelding CG, Jiang MS, et al. Effects of protein extract from head – foot tissue of *Oncomlania hupensis* on the growth and gene expression of mother sporocysts of *Schistosoma japonicum*. Parasitol Res 2012;110:721–31.

Zmuda J, Friedenson B. Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. J Immunol 1983;130:362–4.

Zou X, Jin YM, Liu PP, Wu QJ, Liu JM, Lin JJ. RNAi silencing of calcium-regulated heat-stable protein of 24 kDa in *Schistosoma japonicum* affects parasite growth. Parasitol Res 2011;108:567–72.