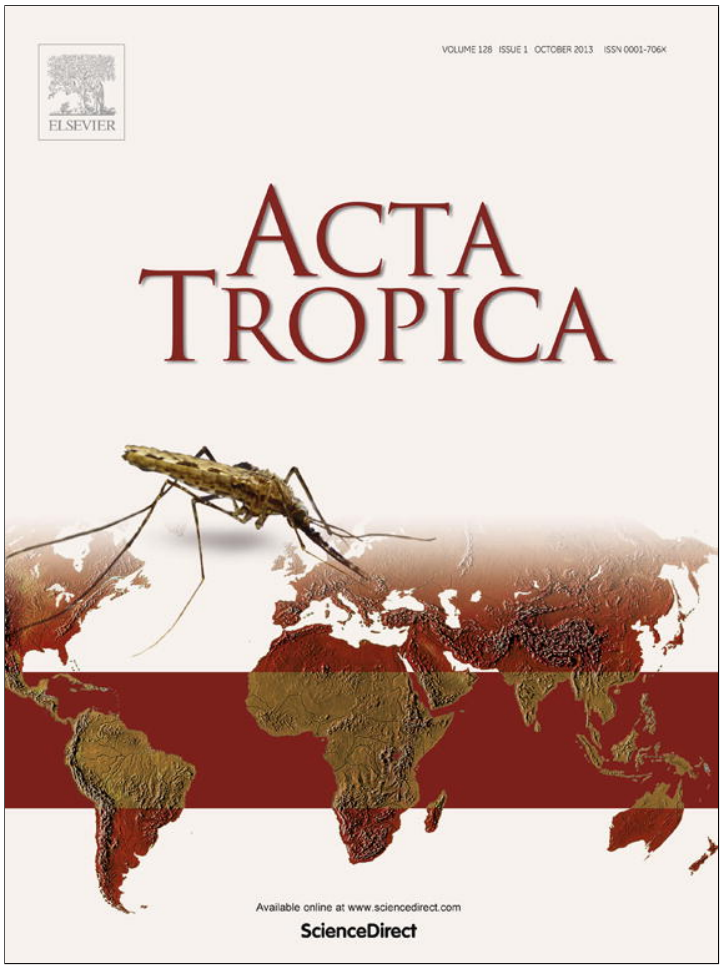

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Development of a cell line from *Echinococcus granulosus* germinal layer

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ABSTRACT

In vitro culture of parasitic helminths provides an important tool to study cell regeneration and physiology, as well as for molecular biology and genetic engineering studies. In the present study, we established in vitro propagation of cells from *Echinococcus granulosus* germinal cyst layer. *E. granulosus* germinal cells grew beyond 100 passages and showed no signs of reduced proliferation capacity. Microscopic analysis revealed that cells grew both attached to the substrate and in suspension, forming three-dimensional structures like mammalian stem cell aggregates. Examination of the chromosome number of attached germinal cells showed a high degree of heteroploidy, suggesting the occurrence of transformation during culture. Monolayer cells survived cryopreservation and were able to proliferate after thawing. Based on the characteristics displayed by *E. granulosus* germinal cells, we establish a cell line from the *E. granulosus* germinal layer. Furthermore, we propose that this cell line could be useful for drug screening and for obtaining parasite material.

1. Introduction

Cystic echinococcosis is a zoonotic infection caused by the larval stage (metacestode) of the parasite *Echinococcus granulosus*, which has a worldwide distribution and important medical and economic impact. The metacestode stage is a fluid-filled bladder which consists of an inner germinal or nucleated layer supported externally by a tough, elastic, acellular laminated layer surrounded by a host-produced fibrous adventitial layer (Eckert and Deplazes, 2004). The germinal layer contains several cell types, including undifferentiated cells with large nuclei and nucleoli, as well as muscle and tegumentary cells. Metacestodes develop in various anatomic sites of the human body from oncospheres released from ingested eggs, but the liver and the lung are the most frequently affected organs (Pawłowski et al., 2001). However, the molecular basis of organ tropism in taeniid cestode infections is presently unknown. Moreover, protoscoleces are able to produce secondary hydatidosis, i.e. they are released predominantly in the abdominal cavity, as a result of spontaneous or trauma-induced cyst rupture, and have the potentiality to differentiate into new cysts (Eckert and Deplazes, 2004).

In vitro culture of parasitic helminths provides an important tool to study cell physiology, molecular biology and co-culture with selected host cells. In addition, it allows identifying targets for parasite chemotherapy, as well as investigating mechanisms of anti-parasitic drug resistance (Roos et al., 1990). Despite their importance, only a few comparable techniques for culturing parasitic helminths have been reported, due to the high complexity of their life-cycles and nutritional requirements that make it difficult to find appropriate growth conditions in the laboratory (Toledo et al., 1997; Cousteau and Yoshino, 2000; Coyne and Brake, 2001).

An interesting topic of study in the developmental biology of flatworms is the population of totipotent somatic stem cells denominated 'neoblasts' in free-living planarians or 'germinal cells' in obligate parasitic trematodes and cestodes (Reuter and Kreshchenko, 2004; Brehm, 2010a). Many studies carried out in planarians have shown that these stem cells possess the capacity to directly differentiate into all somatic cells and are regarded as the only flatworm cell type that is mitotically active (Reuter and Kreshchenko, 2004; Sanchez-Alvarado and Kang, 2005; Rossi et al., 2008; Brehm and Spiliotis, 2008).

Ultrastructural studies have shown that undifferentiated germinal cells form part of the infective oncosphere and that they may contribute to the development of the metacestode during the so-called oncosphere-metacestode metamorphosis (Freeman, 1973; Slais, 1973; Swiderski, 1983). However, its basic biological

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processes remain unknown (Brehm et al., 2006). On the other hand, Galindo et al. (2003) reported that protoscoleces develop from patches of proliferating cells present in the germinal layer which have high proliferating activity and very active protein synthesis, suggesting, at least as preliminary evidence, that the development of this larval stage is also induced by germinal cells that are part of the germinal layer. The in vitro culture of germinal cells should therefore also be a highly relevant tool to study the molecular developmental processes not only during the early establishment of the parasitic oncosphere within the intermediate host (Brehm, 2010b), but also during the formation of protoscoleces.

The first attempts to establish a successful cell culture for *Echinococcus* spp. allegedly failed due to the contamination with host cells (Fiori et al., 1988; Furuya, 1991). Nevertheless, in subsequent studies, appropriate growth conditions and cultures essentially free of host contamination were achieved for *Echinococcus multilocularis* (Yamashita et al., 1997; Spiliotis et al., 2008; Spiliotis and Brehm, 2009). Spiliotis et al. (2008) established a primary cell culture system from cells isolated from cultured metacestode vesicles in vitro. Under reducing conditions in the presence of *Echinococcus* vesicle fluid, the primary cells were maintained in vitro for several months and proliferated. Most interestingly, upon co-culture with host hepatocytes in a trans-well system, mitotically active *Echinococcus* cells formed cell aggregates that subsequently developed central cavities, surrounded by germinal cells. Albani et al. (2010) developed a new method for the establishment of a primary cell culture from the *E. granulosus* germinal layer and described cell growth, metabolic requirements, culture specificity, as well as cell morphology and behavior in culture. *E. granulosus* germinal cells were maintained for at least 4 months, in the presence of reducing agents, hormones and under atmospheric oxygen conditions.

Cell line creation from the germinal layer could represent a suitable in vitro model for the study of the larval stage, because it consists of proliferating cells that can potentially produce new cysts or differentiate into protoscoleces (Yoneva and Mizinska-Boevska, 2001). However, a long-term in vitro culture system of *E. granulosus* germinal cells has not yet been documented. In the present study, we describe the in vitro establishment of a cell line from *E. granulosus* germinal layer.

2. Materials and methods

2.1. In vitro culture of germinal cells

The primary cell culture was obtained from hydatid cysts using the method described in Albani et al. (2010). After 24 h of the establishment of primary cell culture, the non-adherent cells were removed, leaving only the attached cells. As described by Freshney (2005), cultures were splitted using 0.25% of trypsin every 7 days up to a month at a 1:2 dilution rate in 12-well plates and flasks (25 cm^2) and were morphologically characterized (Fig. 1). After a month, some cultures were maintained without splitting up to 4 months to study cellular aggregation (Fig. 1). This experimental procedure was realized regularly, always achieving the long-term proliferating cell type.

The culture medium was supplemented with 10% fetal bovine serum (FBS), 10% hydatid fluid (filtered and conserved at −20 °C), reducing agents (5×10^{-5} M 2-mercaptoethanol and 100 μM L-cysteine), 2 mM L-glutamine (Bio-Rad, USA), 4 mg ml^{-1} glucose (Sigma, USA), 1 mM sodium pyruvate (Sigma, USA), antibiotics (penicillin, streptomycin and gentamicin 100 μg ml^{-1}) and under atmospheric oxygen conditions.

In all cases the final medium pH was 7.5 and the culture medium was changed twice per week. Cell cultures were observed and photographed periodically using an inverted microscope. DAPI staining was employed to nuclei observations. Cell viability was assessed using the trypan blue exclusion test (Redondo et al., 2007).

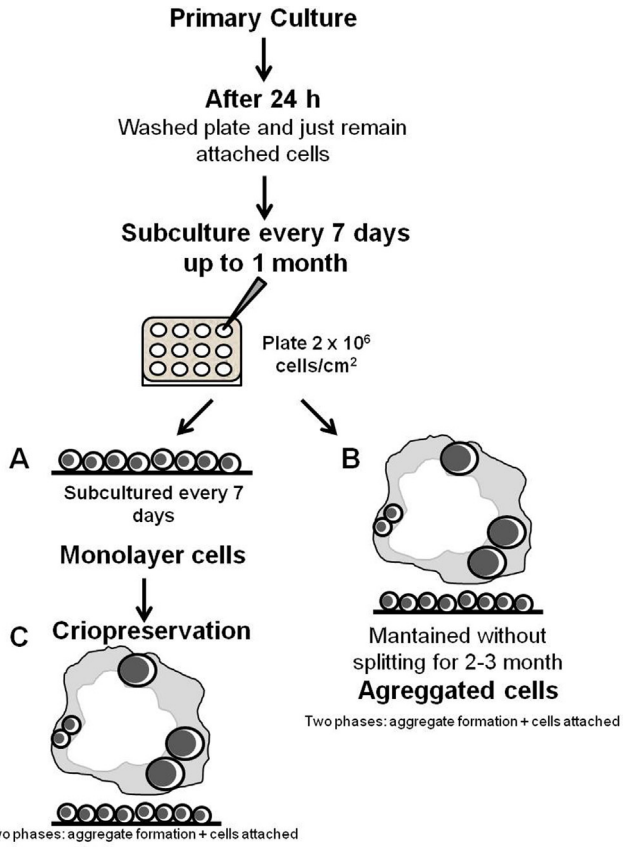

Fig. 1. Flow chart summarizing the in vitro germinal cell culture systems for *Echinococcus granulosus*.

2.2. Chromosome analysis

Karyotypic analysis was performed on *E. granulosus* germinal cells at passage 20 using a conventional method described in Ye et al. (2006). In brief, the cells were dosed with colchicine 10 μg ml^{-1} for 12 h in plastic petri dishes and harvested by centrifugation (600 × *g*, 5 min). Single cells were suspended in hypotonic solution of 0.075 M KCl for 30 min, and fixed two times in 3:1 metanol–acetic acid fixative solution, 15 min for each time. Slides were prepared using the conventional drop-splash technique (Freshney, 2005) and then air dried. Chromosomes were stained with 5% Giemsa (Sigma, USA) for 20 min. Finally, chromosomes were observed microscopically and one hundred photographed cells at metaphase were counted.

2.3. Fluorescence microscopy

In toto immunohistochemistry was carried out as described Fairweather et al. (1988). Monolayer and aggregates cells were fixed with 4% (w/v) paraformaldehyde for 4 h at 4 °C and washed with 0.3% (v/v) PBS solution plus 0.2% (w/v) Triton X-100, sodium azide and 0.1% bovine serum albumin (BSA). Then, they were incubated for 12 h at 4 °C with anti-human alpha-tubulin as primary antibody (1:1000 dilution, Santa Cruz, USA -sc5286-), and washed with PBS at 4 °C. Finally, cells were incubated with goat anti-mouse IgG conjugated with FITC for 2 h at 4 °C, washed and counterstained with 2 μg ml^{-1} propidium iodide (Molecular Probes P-3566, to observe all cell nuclei in optimal contrast conditions) and they

were observed with an inverted confocal laser scanning microscope (Nikon C1 Confocal Microscope). The images were collected every 5 s in sections of 30 nm after excitation with 493 nm light and a collection emission signal above 630 nm. Images were processed using Corel Draw and Corel Photopaint.

2.4. Electron microscopy

For scanning electron microscopy (SEM), 4 months old cellular aggregates were harvested and fixed with 2.5% (v/v) glutaraldehyde in 0.1% (v/v) sodium cacodylate buffer for 48 h at 4 °C, and were washed several times in cacodylate buffer. The specimens were then dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%) and finally immersed in hexamethyl-disilazane for 18 h. They were sputter-coated with gold and inspected on a JEOL JSM-6460 LV scanning electron microscope at 15 kV.

2.5. Cryopreservation and thawing of cells

For freezing cells, *E. granulosus* monolayer cells were grown to late log phase, harvested by centrifugation and high cell density suspension ($2–4 \times 10^6$ cells ml^{-1}) and suspended in culture medium with 5% dimethyl sulphoxide (DMSO) and 25 or 50% FBS. Cells were cooled at 1 °C per min to −80 °C. Following storage at liquid nitrogen, the seed stocks after 1, 6 and 12 months were thawed quickly (1–2 min in sterile water at 37 °C in a beaker) and transferred immediately in pre-warmed medium for the best recovery (Freshney, 2005). Preserved cells were diluted slowly to prevent osmotic damage by DMSO (10 ml over about 2 min added dropwise at the start diluting the cells and cryoprotectant), centrifuged for 2 min at $100 \times g$, discarded the supernatant, and resuspended at a high cell density in fresh medium for culture. Viability, proportion attached versus those still floating and proliferation were evaluated. During the first 24 h after thawing, cells were cultured in medium supplemented with antibiotics and 25% FBS and then transferred to normal supplemented medium as described above.

2.6. Determination of infectivity to mice

Cells at passage 48 (one year of maintenance in vitro) were washed with PBS, harvested with trypsin, re-suspended in fresh media, homogenized and counted using the Neubauer chamber.

Animal procedures and management protocols were carried out in accordance with the 2011 revised form of The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. Four female CF1 mice (body weight 25 ± 5 g) were intraperitoneally injected with 1.5×10^6 cells. Food and water were provided ad libitum. Groups of 2 mice were killed at 6 and 12 months after injection, and the presence of cysts in the peritoneal cavity was analyzed.

3. Results

After four subcultures (every 7 days up to 1 month, Fig. 1A), *E. granulosus* germinal cells were able to divide, maintaining a high proliferation rate and low saturation density. Culture cells grew for al least 24 months (beyond 100 passages) and showed no signs of reduced proliferation capacity. The monolayer cells appeared homogeneous in size (approximately 4 μm) with the presence of small nuclei (Fig. 2A–C). Additionally, confocal microscopy by propidium iodide stain and α-tubulin immunodetection confirmed the presence of small cells (Fig. 2D and E).

To determine the chromosome number of *E. granulosus* germinal cells, karyotype analysis was performed. The metaphase of *E. granulosus* germinal cells is shown in Fig. 2F. Results showed that the number of chromosomes ranged from 18 to 72, but that there were mainly 36 chromosomes, which occupied 32% of the metaphase cells counted at passage 20 (Fig. 2F). In addition an important degree of heteroploidy was observed (44% polyploidy and 43% aneuploidy), suggesting the occurrence of transformation during culture.

After 1 month, some culture replicates were left without splitting and two different cell populations were observed. Cells attached to the bottom of the flask and other cells grew in suspension, clumping together and forming loose aggregates, and, after 2–3 months in culture without splitting, became larger and denser (Fig. 1B). Microscopic analysis of aggregates showed a tridimensional organization consisting of cell clusters with a central cavity surrounded by different cell types immersed in an abundant extracellular matrix (Fig. 3A and B). By confocal microscopy, two different cell subpopulations based on nucleus size were recognized: cells with large and small nucleus (Fig. 3C and D). Studies by SEM showed an irregular surface with the presence of a large number of *E. granulosus* germinal cells and cavities (Fig. 3E and F).

E. granulosus germinal cells were successfully cryopreserved (Fig. 1C). Cells at passages 24 and 48, corresponding to 6 and 12 months respectively, were used. It was possible to recover 80% of germinal cells after thawing. Cells grew to confluence after 3 weeks (Fig. 4A) and recovered the aggregation capacity (Fig. 4B). No differences were found when 25 and 50% of FBS was used, regardless of whether the cultures were cryopreserved for 1, 6 or 12 months.

To investigate whether cells proliferate in the peritoneal cavity of mice as cysts, $1.5 \times l0^6$ cells were injected into CF1 female mice. Six and twelve months after inoculation, mice were killed and their peritoneal content examined. After 6 months, no cysts were recovered, whereas after 12 months, a mean of three cyst-like structures per mouse were found in the liver or in the surrounding area. Histopathological studies showed an abnormal growing parasite tissue (data not shown).

4. Discussion

During the last decades, due to the interest in fundamental molecular and cellular aspects concerning development and host–parasite interplay, several attempts have been made to culture parasitic helminths in vitro. Nevertheless, only a few comparable culture techniques have been reported (Toledo et al., 1997; Cousteau and Yoshino, 2000; Coyne and Brake, 2001). Particularly for *Echinococcus* spp., the impossibility to find suitable growth conditions to encourage parasite culture free of host contamination made the first attempts unsuccessful (Fiori et al., 1988; Furuya, 1991). However, Yamashita et al. (1997) and Spiliotis et al. (2008) obtained a long-term in vitro culture system for *E. multilocularis* cells, and we developed *E. granulosus* primary cell cultures from the germinative layer (Albani et al., 2010).

In the present study, we achieved a cell line from *E. granulosus* germinal layer. The established culture had the potential to be subcultured for at least 24 months, cells showed no contact inhibition and were successfully reestablished after cryopreservation. *E. granulosus* germinal cell monolayer was able to maintain a continuous growth with a high proliferation rate in 199 medium supplemented with hydatic fluid, reducing agents and under atmospheric oxygen conditions. The attached cells were frozen and intraperitoneally injected in CF1 mice developing abnormal structures of parasite tissue. Cells were subcultured at least 100 times without changes in cell viability, morphology or proliferation rate. Cells divided showing an extended life span, which is a feature of cell lines.

Using *E. granulosus* cells derived from protoscoleces, Smyth (1962) reported a karyotype of $2n = 18$, which was confirmed for *E. multilocularis* by Sakamoto et al. (1967) and Lukashenko (1964)

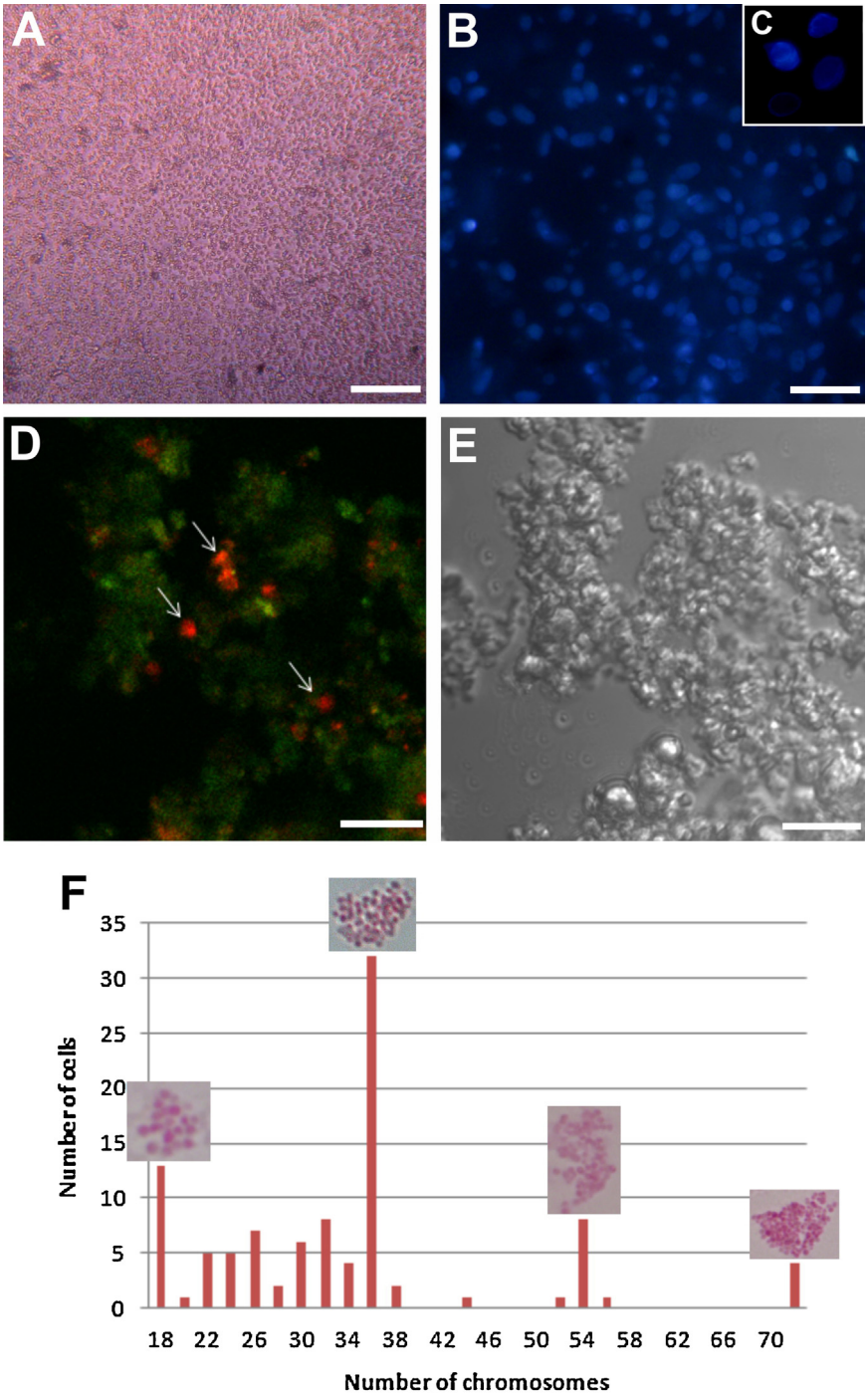

Fig. 2. Characterization of *E. granulosus* cells growing as a monolayer. (A) Light microscopy of cells growing indefinitely after several subcultures. (B) DAPI staining showing total nuclei. (C) High magnification image showing the morphology of nuclei. (D and E) Fluorescence and merge confocal microscopy of cells attached to the substrate incubated with propidium iodide (red nucleus) and α-tubulin FITC-conjugated antibody (green cytoplasm). Arrows point to small cells. (F) Chromosome analysis of *E. granulosus* cells at passage 20, showing the most representative chromosome metaphases and chromosome number distribution in 100 metaphases. Scale bar: (A) 40 μm, inset 10 μm; (B and C) 15 μm.

using germinal cells and scoleces, respectively. In contrast, our results showed that the number of chromosomes in the attached *E. granulosus* germinal cells varied from 18 to 72 and that the cells with 18 ($2n$) or 36 ($4n$) chromosomes were clearly predominant. These results evidence the presence of polyploidy, as described previously for flatworms (Otto and Whitton, 2000). Moreover, the subcultured cells presented an important degree of aneuploidy, which is a typical feature of continuous cell lines, and their chromosome number was often between diploid and tetraploid values. The aneuploidy observed could also explain the lack of contact inhibition and reduced anchorage dependence. We were not able to draw further conclusions related to the karyotype due to the difficulties related to the small size of the cells. However, our results are consistent with those reported by Tsai et al. (2013). The authors revealed the presence of tetraploidy in protoscoleces and trisomy of chromosome 9 in protoscoleces and metacestodes.

Although various larval stages of nematode species and *E. multilocularis* have been successfully frozen (Eckert, 1988),

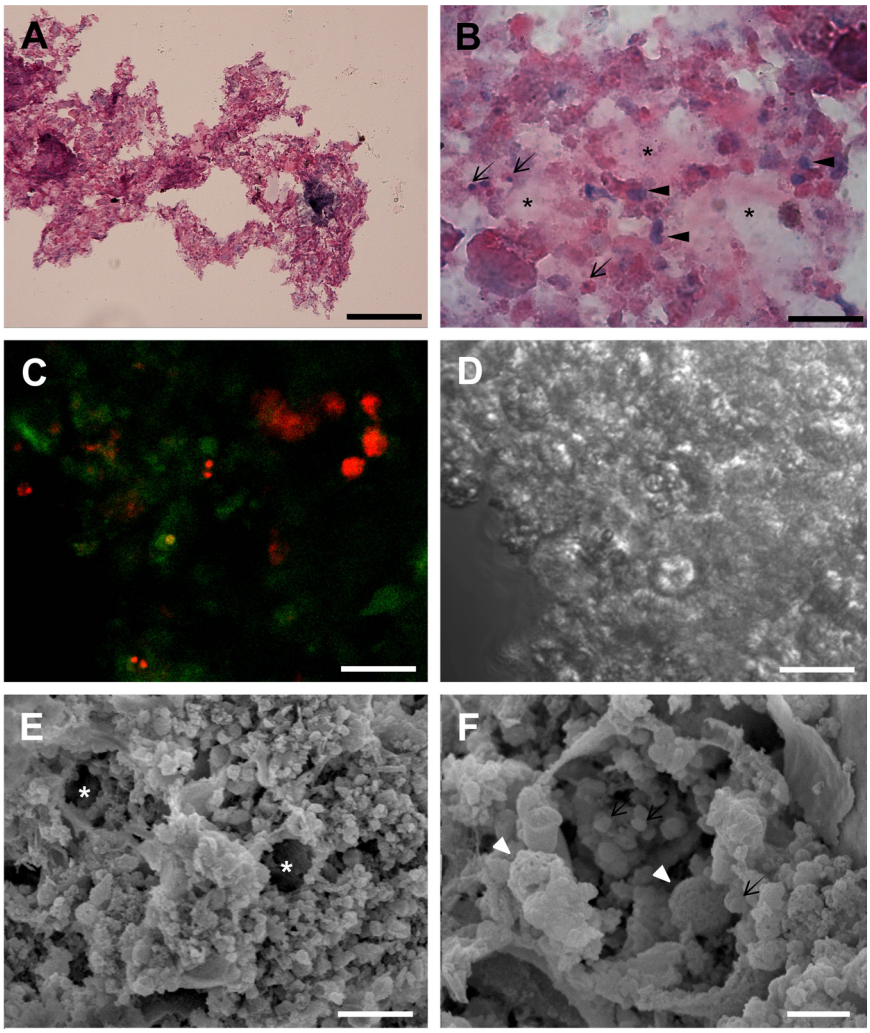

Fig. 3. Cell aggregate morphology of *E. granulosus* germinal cells. (A and B) Hematoxylin–eosin staining revealing the presence of two cell nucleus sizes. (C and D) Fluorescence and light field confocal microscopy showing two cell nucleus size subpopulations. (E and F) Scanning electron microscopy showing numerous germinal cells. Arrows, small nuclei; head arrows, large nuclei; asterisks, central cavities. Scale bar: (A) 200 μm, (B) 20 μm, (C and D) 15 μm, (E) 50 μm, (F) 10 μm.

cryopreservation of helminths is still limited (James, 1985). In this study, reestablishment of the cell culture after cold storage in liquid nitrogen and a high survival rate at high concentration of FBS independently of time of cryopreservation used were successfully achieved. These culture cells provide important substrates for biological assays of vaccine potency and efficacy, drug screening, and genetic manipulations of the *E. granulosus* larval stage.

E. granulosus germinal cells were able to grow both attached to the substrate and in suspension, forming aggregates. Aggregates appeared as loose structures that became denser and more complex after 2–4 weeks in culture. Using optical, SEM and confocal microscopy, we showed the presence of two different cell subpopulations, differing in nucleus size. The three-dimensional organization of the aggregates suggests the existence of some

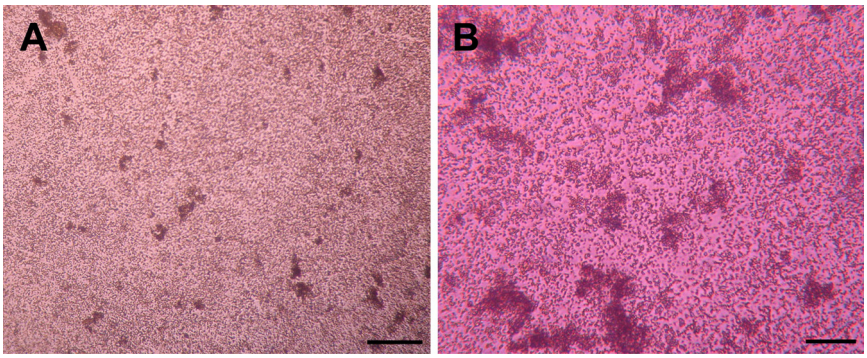

Fig. 4. Light microscopy of *E. granulosus* cell culture after cryopreservation. (A) Confluent cultures after 3 weeks of culture post-thawing. (B) Cells clustered together forming small aggregates. Scale bar: (A and B) 60 μm.

kind of functional compartmentalization and that they are similar to human embryonic stem cell aggregates (Freshney et al., 2007; Labarge et al., 2007). Brehm and Spiliotis (2008) suggested that the cell arrangement around a cavity in *E. multilocularis* could represent a primitive lacuna in the process of cyst development. Spiliotis et al. (2008) reported that *E. multilocularis* primary cells are capable of fully regenerating infective metacestode vesicles when cultured with rat hepatocytes in a trans-well system. These authors also mentioned that these conditions lead to rapid proliferation of the primary cells and to the formation of cell aggregates which develop central cavities and give rise to young metacestode vesicles after about 4–5 weeks of co-culture. Comparatively, our results showed a slower development of the aggregates. Thus, we think that the co-culture in a trans-well system would be a good option for future attempts to stimulate the regeneration processes and, particularly, vesicle formation. Further studies, including the use of defined growth factor additives, should be carried out to promote the differentiation toward different cell types.

The availability of a cell line from *E. granulosus* germinal layer provides a very important tool for practical purposes including the screening of new drugs for the treatment of cystic echinococcosis.

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