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Research paper

Set up of an in vitro model to study early host-parasite interactions between newly excysted juveniles of *Fasciola hepatica* and host intestinal cells using a quantitative proteomics approach

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ABSTRACT

Fasciola hepatica is the causative agent of fasciolosis, a parasitic zoonosis of global distribution causing significant economic losses in animal production and a human public health problem in low-income countries. Hosts are infected by ingestion of aquatic plants carrying metacercariae. Once ingested, the juvenile parasites excyst in the small intestine and, after crossing it, they follow a complex migratory route that lead the parasites to their definitive location in the bile ducts. Despite being a critical event in the progression of the infection, the available data on the cross-talk relationships between the parasite and the host at an early stage of the infection are scarce. The objective of the present work is to characterize the proteomic changes occurring in both the parasite and the host, through the development of a novel in vitro model, to shed light on the molecular pathways of communication between the newly excysted juveniles (NEJ) from *F. hepatica* and the host's intestinal epithelium. For this, in vitro excystation of *F. hepatica* metacercariae was carried out and NEJ were obtained. Additionally, optimal conditions of growth and expansion of mouse primary small intestinal epithelial cells (MPSIEC) in culture were fine-tuned. Tegumentary and somatic parasite antigens (NEJ-Teg and NEJ-Som), as well as host cell protein lysate (MPSIEC-Lys) were obtained before and after 24 h co-culture of NEJ with MPSIEC. We used an isobaric tags for relative and absolute quantitation (iTRAQ)-based strategy to detect 191 and 62 up-regulated, and 112 and 57 down-regulated proteins in the NEJ-Teg and NEJ-Som extracts, respectively. Similarly, 87 up-regulated and 73 down-regulated proteins in the MPSIEC-Lys extract were identified. Taking into account the biological processes in which these proteins were involved, interesting mechanisms related to parasite development, invasion and evasion, as well as manipulation of the host intestinal epithelial cell adhesion, immunity and apoptosis pathways, among others, could be inferred, taking place at the host-parasite interface. The further understanding of these processes could constitute promising therapeutic targets in the future against fasciolosis.

1. Introduction

Host invasion processes in fasciolosis start with the excystment of the juvenile forms of the parasite *Fasciola hepatica* in the small intestine. This process occurs after the ingestion of aquatic plants carrying infectious metacercariae by suitable hosts, including ruminants and humans. Following excystment, the newly excysted juveniles (NEJ) cross the intestinal wall in order to follow a complex migratory route leading the parasites to their definitive location in the bile ducts where they become sexually mature (Mas-Coma et al., 2014; Moazeni and Ahmadi, 2016). After that, the chronicity of the infection, as well as the acute form of the disease, typically associated with the liver migratory stages, determine a hepatic pathological process, with an impact on the production of meat, milk and wool, and decreased fertility (Schweizer et al., 2005) in a disease affecting millions of ruminants around the world with annual economic losses of more than 3 billion USD (Toet

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et al., 2014). In addition, fasciolosis is an emerging zoonosis in many areas of Latin America, Africa and Asia, and its epidemiology in humans is linked to a high prevalence in livestock, being children a particularly vulnerable population (Nguyen et al., 2011). Despite its worldwide relevance, the classical vaccination approaches carried out so far, mostly based on the use of parasitic molecules from the adult phase, have not given the expected results (Dominguez et al., 2018); thus, studying the host-parasite relationships at an earlier phase of infection could provide important information to develop new control measures against this liver fluke.

NEJ are capable of crossing the intestinal wall by about 2 h after metacercariae ingestion (Moazeni and Ahmadi, 2016), representing a key process for the parasite life cycle as a "point of no return" in fasciolosis. Importantly, the first contact of the parasite with the host occurs between the cells of the intestinal epithelium and the NEJ, being this a determining phase for the subsequent development of the infection. The intestinal epithelial cells constitute a physical barrier but also interact with the pathogen and with the underlying immune cells, producing immunoeffector molecules involved in the first line of innate immune defense against the parasite (Goto and Ivanov, 2013). The deep characterization of the changes induced in the host cells and in the NEJ upon their interaction is of great importance to define the first events driving the host-parasite relationship inside the vertebrate host. Existing models on the study of these mechanisms, including ex vivo models using NEJ injection into a rat gut segment (van Milligen et al., 1998), as well as in vitro models placing NEJ into compartments containing rat distal jejunal sheets (Garcia-Campos et al., 2016a), are useful in the study of parasitic migration, but do not allow the study of the changes arising both in the parasite and in the host cells before and after their interaction. In vitro models based on cell cultures approaches could represent a better alternative to those already described, since they allow the co-culture of parasites and host cells directly involved in these mechanisms in order to obtain more robust conclusions. These types of in vitro models have been widely used for the study of host-parasite interactions in helminths with intestinal stages by co-culturing intestinal epithelial cells and infective third-stage larvae of *Trichostrongylus colubriformis*, *Ancylostoma ceylanicum* or *Ascaris suum* (Corvan et al., 2015; Feather et al., 2017; Ebner et al., 2018).

The main objective of this work is to develop an in vitro model to shed light on the molecular pathways of communication between *F. hepatica* NEJ and the host's intestinal epithelium. This new model is used to reveal cross-talk interactions between the parasite and its host in the early phase of infection. For this purpose, a high-throughput quantitative proteomics approach using isobaric tags for relative and absolute quantitation (iTRAQ) was performed to detect changes in the proteins expressed by NEJ and by the host intestinal epithelial cells after their in vitro interaction.

2. Material and methods

2.1. In vitro excystation of F. hepatica metacercariae

NEJ were obtained as follows: *F. hepatica* metacercariae (Italian strain) were purchased from Ridgeway Research Ltd. (UK). One thousand metacercariae were excysted in vitro as described previously (Hernández-González et al., 2010). In brief, pure CO_2 was bubbled for 30 s in 10 ml of cold distilled water. Sodium dithionite to a final concentration of 0.02 M was added to the water, and the tube closed and incubated at 37 °C until a fine cloudy precipitate was produced. Metacercariae were then added to the tube and further incubated at 37 °C for 1 h. After incubation, parasites were washed twice by sedimentation with warm distilled water, and 5 ml of Hank's balanced salt solution (Sigma, USA) plus 10 % (v/v) rabbit bile and 30 mM HEPES (Sigma) pH 7.4 were added to the dry metacercariae. Parasites were then incubated for 4 h at 37 °C. Emerging active parasites were collected with a 20 µl pipette, washed twice in cold PBS and immediately subjected to protein extraction (see Section 2.3) or used for the in vitro interaction with host cells (see Section 2.2).

2.2. Growth and expansion of a mouse intestinal epithelial cell line in culture, and cell culture stimulation with NEJ

Mouse small intestinal epithelial cells (C57BL/6 MPSIEC) from Cell Biologics (ref. C57-6051) were grown in epithelial cell growth medium (Innoprot). MPSIEC plates were precoated with an attachment factor solution (0.2 % gelatin). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5 % CO_2 and 95 % air. Medium was changed every 3 days. Expansion was done by trypsinizing the cells (Trypsin/EDTA, Innoprot) and replating them when the proliferating cells reached a sufficient density. Passaging was done at ratios of 1:3. Cell counts were performed using the equipment Countess® Automated Cell Counter (Invitrogen) following the manufacturer's instructions. After cultures were fine-tuned, the optimum stimulation conditions were selected, which resulted in the use of 60 mm plates in passage five with a confluence of 100 %. Confluent cells were treated with 200 NEJ per plate for 24 h in triplicate. Parasite and cell culture viability were monitored by taking images under an inverted microscope. Cytotoxicity of co-culture was ruled out after calculation of the amount of lactate dehydrogenase (LDH) released to the cell media as measured with the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific) following the manufacturer's protocol. Then, NEJ were collected with a 20 µl pipette and cells were separately collected by detachment with a cell scraper, and centrifuged at 15,000 *g* for 10 min at 4 °C, both washed twice in cold PBS and immediately subjected to protein extraction (see Section 2.3). Untreated cells, as well as NEJ before cell co-culture were used as controls (Fig. 1).

2.3. Protein extraction

Both NEJ before and after cell co-culture were used for tegument protein isolation as previously described (Garcia-Campos et al., 2016b). In brief, parasites were washed three times in PBS followed by incubation for 30 min at room temperature with 1 ml of 1 % Nonidet P40 (NP40) in PBS. Then, NEJ were centrifuged at 300*g* for 5 min and the supernatant collected. NP40 was removed from the supernatant with 0.3 g of Bio beads (Bio-Rad) according to the manufacturer's recommendations. The pellet obtained after tegument removal, containing denuded NEJ, was suspended in RIPA buffer (Sigma-Aldrich) and used for somatic proteins isolation. After incubation, both samples were centrifuged at 1000 *g* for 5 min and the supernatants resulting from this process, named NEJ-Teg and NEJ-Som, respectively, were collected. On the other hand, treated and non-treated MPSIEC were lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10 % glycerol, 1 % Igepal CA-630, aprotinin, pepstanin, pepstatin, and leupeptin at 1 µg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). The supernatant including soluble proteins was collected and the resulting extract was named MPSIEC-Lys. All biological triplicate extracts were mixed and labelled with the number 1, if they corresponded with controls, or with the number 2, if they had been obtained after cell culture stimulation with NEJ (Fig. 1). A cocktail of protease inhibitors was added to all samples following the methodology described by Maizels et al. (1991) and protein contents was determined by DC protein assay commercial kit (Bio-Rad). Finally, all samples were aliquoted and stored at −80 °C until use.

2.4. In-gel digestion and iTRAQ labelling

Protein extracts (10 µg for *F. hepatica* extracts; 40 µg for mouse cell extracts), were suspended up to 50 µl in sample buffer, and then applied onto 1.2-cm wide wells of a conventional SDS-PAGE gel (1 mm-thick, 4 % stacking, and 10 % resolving). The run was stopped as soon as the

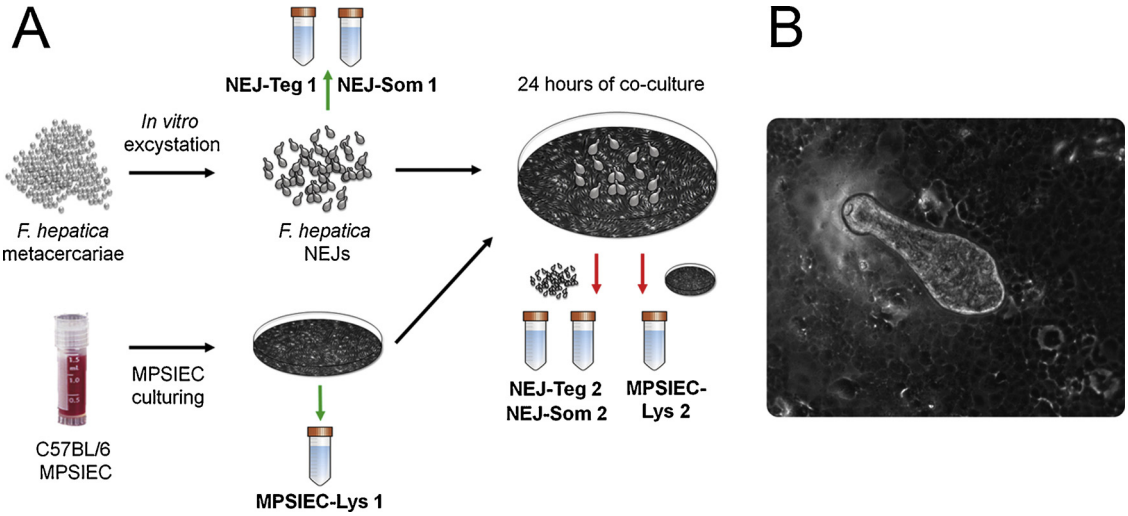

Fig. 1. Experimental design and in vitro model. *F. hepatica* juveniles after in vitro excystation and mouse intestinal epithelial cells were co-cultured for 24 h. Parasitic tegument and somatic extracts, as well as host cell lysates were obtained before and after co-culturing (A). Representative image of MPSIEC stimulated with the NEJ (100x) (B). Tegument extract from newly excysted juveniles, NEJ-Teg; somatic extract from newly excysted juveniles, NEJ-Som; mouse primary small intestine epithelial cell lysate; MPSIEC-Lys. In all extracts number 1 represents before co-culturing and number 2 represents after co-culturing.

front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie blue staining, excised, cut into cubes (2 × 2 mm), and placed into 0.5 ml microcentrifuge tubes. The gel pieces were destained in acetonitrile:water (ACN:H_2O, 1:1), reduced with 10 mM DTT for 1 h at 56 °C and alkylated with 50 mM iodoacetamide for 1 h at room temperature in darkness. Samples were finally digested with sequencing grade trypsin (Promega, Madison, WI) as described by Shevchenko et al. (2001), supernatants dried down, desalted using OMIX Pipette tips C18 (Agilent Technologies) and dried down in a SpeedVac. The resultant peptide mixture was labeled using the iTRAQ reagent 8plex Multi-plex kit (Applied Biosystems) as follows: reagent 113 for NEJ-Teg 1, 114 for NEJ-Teg 2, 115 for NEJ-Som 1, 116 for NEJ-Som 2, 119 for MPSIEC-Lys 1 and 121 for MPSIEC-Lys 2 (Köcher et al., 2009). Briefly, peptides were dissolved in 0.5 M triethylammonium bicarbonate (TEAB), adjusted to pH 8. For labeling, each iTRAQ reagent was dissolved in 50 μL of isopropanol, added to the respective peptide mixture and incubated at room temperature for two hours. Labelling was stopped by the addition of 0.1 % formic acid. Whole supernatants were dried down and the six samples (4 samples from *F. hepatica* and 2 samples from mouse cells) were respectively mixed to obtain two labeled mixtures. Mixtures were desalted and fractionated using a Pierce High pH Reversed-Phase Peptide Fractionation Kit.

2.5. Reverse phase-liquid chromatography RP-LC–MS/MS analysis

Each labeled mixture were dried, resuspended in 10 μl of 0.1 % formic acid and analyzed by RP-LC–MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). Peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Proxeon), and then separated using a 0.075 mm x 250 mm C18 RP column (Proxeon) operating at 0.3 μl/min. Peptides were eluted using a 240-min dual gradient from 5 to 25 % solvent B in 180 min followed by gradient from 25 to 40 % solvent B over 240 min (Solvent A: 0.1 % formic acid in water, solvent B: 0.1 % formic acid, 80 % acetonitrile in water). ESI ionization was performed using a Nano-bore emitters Stainless Steel ID 30 μm (Proxeon) interface (Alonso et al., 2015). The instrument method consisted of a data-dependent top-20 experiment with an Orbitrap MS1 scan at a resolution ($m/\Delta m$) of 30,000 followed by either twenty high energy collision dissociation (HCD) MS/MS mass-analyzed in the Orbitrap at 7500 ($\Delta m/m$) resolution. MS2 experiments were performed using HCD to generate high resolution and high mass accuracy MS2 spectra. The minimum MS signal for triggering MS/MS was set to 500. The lock mass option was enabled for both MS and MS/MS mode and the polydimethylcyclosiloxane ions (protonated (Si(CH3)2O))6; m/z 445.120025) were used for internal recalibration of the mass spectra. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan) using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 40 % for HCD fragmentation, and dynamic exclusion applied during 30 s periods. Precursors of unknown or +1 charge state were rejected.

2.6. Database searching and bioinformatic analysis

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1) and validated with X! Tandem (The GPM, thegpm.org; version CYCLONE 2010.12.01.1) against a database comprising the *F. hepatica* predicted proteome [WBPS13 database, corresponding to Bioproject PRJEB25283 (https://parasite.wormbase.org)] (Cwiklinski et al., 2015) appended to the *Mus musculus* proteome (Uniprot) and the cRAP database (https://www.thegpm.org/crap/). Parameters for Mascot and X! Tandem searches included a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 50 ppm and fixed modifications of O^{18+} of pyrrolysine and iTRAQ8plex labelling of lysine and the n-terminus. In X! Tandem searches variable modifications specified were Glu- > pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, Gln- > pyro-Glu of the n-terminus, oxidation of methionine, methylthio of cysteine and iTRAQ8plex labelling of tyrosine. In Mascot searches variable modifications specified were oxidation of methionine, and iTRAQ8plex labelling of tyrosine. Scaffold Q+ (version Scaffold_4.2.1) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at greater than 95 % and 99 % probability, respectively, as specified by the Peptide Prophet algorithm (Keller et al., 2002), and contained at least two identified peptides (Nesvizhskii et al., 2003). Proteins containing similar peptides that could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony. A false discovery rate (FDR) of < 0.1 % was calculated using protein identifications validated by the Scaffold Q + program. Scaffold Q + was used to quantify the isobaric tag peptide and protein identifications. Channels were corrected in all

samples according to the algorithm described in i-Tracker (Shadforth et al., 2005) and acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run, and intensities for each peptide identification were normalized within the assigned proteins. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data. Blast2GO (Conesa et al., 2005) was used to classify proteins according to gene ontology (GO) categories.

3. Results

3.1. Protein identification and quantification

An MS/MS approach using isobaric labelling was followed to identify and quantify the proteins present in the samples. A total of 67,966 and 33,764 spectra were obtained in both 4-plex labelled experiments (*F. hepatica*, 113–116, and *M. musculus*, 119–121, respectively), representing 3299 and 2822 unique proteins containing two or more unique peptides identified using an FDR < 0.1 % (Supplementary Files 1–2). Mouse proteins were also identified in NEJ-Teg 2 and NEJ-Som 2 samples and, similarly, *F. hepatica* proteins were identified in the MPSIEC-Lys 2 sample (Supplementary Files 1–2). The quantification analysis was performed using Scaffold Q+, and only proteins with a $\log_2$ fold-change > 0.6 or < −0.6 (for up-regulated and down-regulated proteins, respectively) were taken into consideration for further analysis. The expression level of 303 proteins in the tegument of *F. hepatica* NEJ was differentially regulated by the incubation of the parasites with mouse intestinal epithelial cells (191 up-regulated and 112 down-regulated; Fig. 2A; Supplementary File 3). Furthermore, the levels of 119 proteins were regulated in the somatic extract of *F. hepatica* NEJ after incubation with mouse small intestinal epithelial cells (62 up-regulated and 57 down-regulated; Fig. 2A; Supplementary File 4). Similarly, the expression levels of 87 proteins were up-regulated and 73 were proteins down-regulated in the protein lysate of mouse intestinal epithelial cells after incubation with *F. hepatica* NEJ (Fig. 2B; Supplementary File 5).

3.2. Incubation with mouse epithelial cells induces a protein composition shift in F. hepatica NEJ

Interestingly, up-regulated proteins from the tegument of *F. hepatica* NEJ after incubation with mouse cells were implicated in different biological processes such as phosphorylation, transport and proteolysis (Fig. 3). Among these proteins we positively identified several kinases (tyrosine kinase, PEPCK and serine/threonine kinase among others), ubiquitin-like proteins, legumain-like proteins and several cathepsin L enzymes (CL1 and CL1D). Conversely, up-regulated proteins from the somatic extracts of the parasite were implicated in several metabolic processes such as primary metabolic process, nitrogen compound metabolic process and cellular nitrogen compound metabolic process, as well as in other biological processes such as protein phosphorylation (i.e. tyrosine-protein kinase PR2) and cytoskeleton organization (i.e.

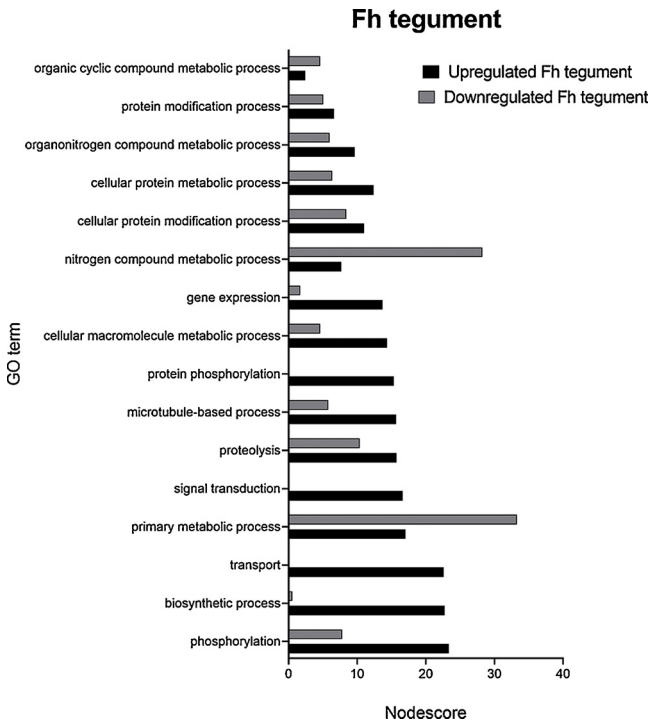

Fig. 3. Bar graph representing the top 10 biological processes from up- and down-regulated proteins in the tegument from *F. hepatica* NEJ after incubation with mouse intestinal epithelial cells. Y-axis represents the nodescore from Blast2GO.

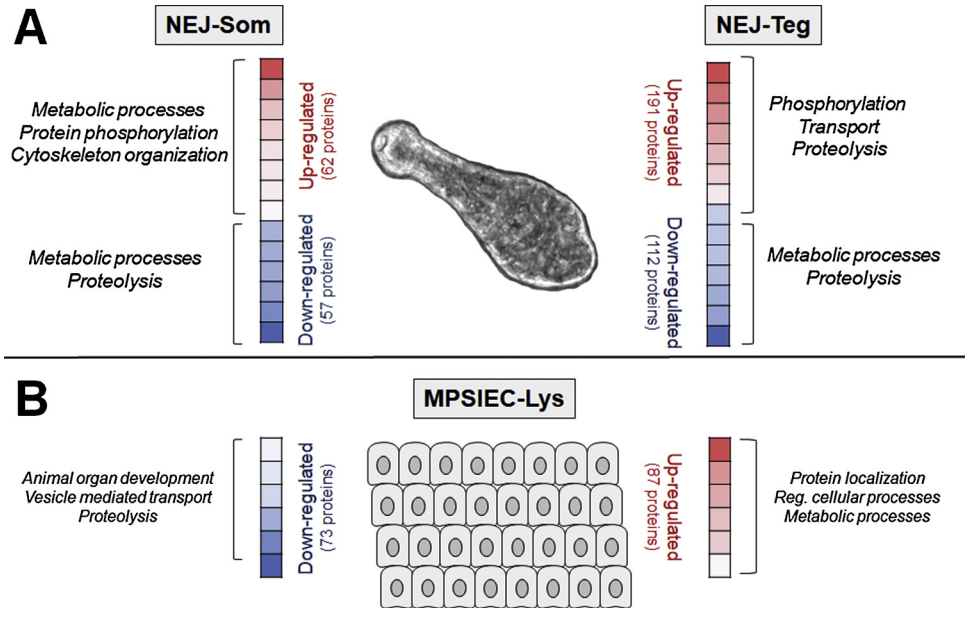

Fig. 2. Diagram showing the number of up- and down-regulated proteins and main associated biological processes from the somatic and tegumental extracts of *F. hepatica* NEJ (A) and mouse intestinal epithelial cells lysate extract (B) after experimental incubation. Gene ontology biological processes and the corresponding gene ontology identifiers are given in Supplementary File 6.

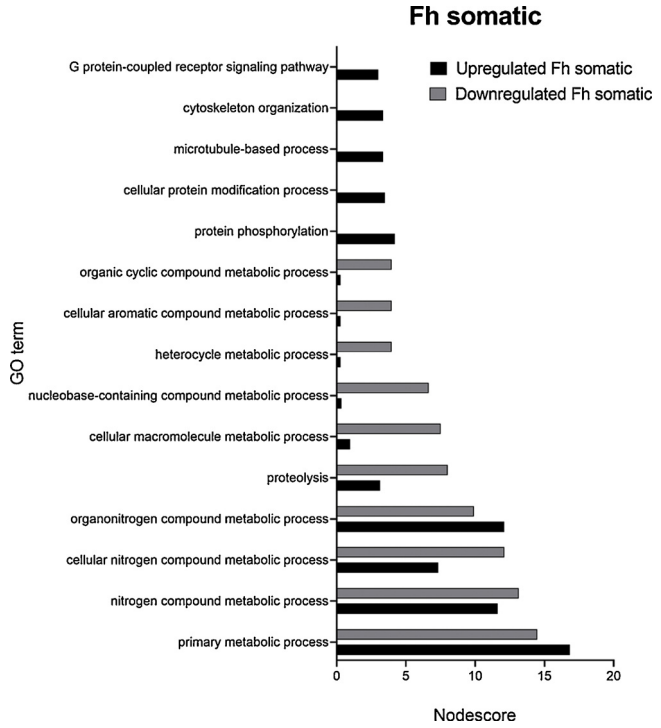

Fig. 4. Bar graph representing the top 10 biological processes from up- and down-regulated proteins in the somatic extracts from *F. hepatica* NEJ after incubation with mouse intestinal epithelial cells. Y-axis represents the nodescore from Blast2GO.

tubulin alpha chain and serine/threonine-protein kinase MARK2) among others (Fig. 4). Down-regulated proteins from both *F. hepatica* extracts (tegumental and somatic proteins) are heavily implicated in several metabolic processes (i.e. nitrogen compound metabolic processes and primary metabolic process) and other biological processes such as proteolysis (Figs. 3 and 4). Interestingly, among these proteins we found several cathepsin L (CL1D, pro-CL3, CL3 and CL4), as well as cathepsin B (CB) and other proteolytic enzymes such as a papain family cystein protease in the tegument and a serine protease (mastin precursor) in the somatic extract.

3.3. Mouse epithelial cells show specific proteomic adaptation after incubation with F. hepatica NEJ

Changes in the proteomic composition of mouse epithelial cells were detected after incubation with *F. hepatica* NEJ. The most important changes in up-regulated proteins from MPSIEC-Lys 2 at the biological level (based on the frequency and the nodescore value provided by Blast2GO) were related to protein localization, regulation of cellular processes and organic cyclic compound metabolic processes and nucleobase-containing compound metabolic processes (Fig. 5A). Among the proteins implicated in these processes, we found plastin-1, clathrin light chain and syntaxin-6 (protein localization) as well as inositol-1-monophosphatase, phosphoinositide phospholipase C and phosphodiesterase (regulation of cellular processes). Down-regulated proteins from MPSIEC-Lys 2 were mainly implicated in tissue development and regeneration such as animal organ development (i.e. tropomyosin alpha-3 chain and myosin 3), cell adhesion (i.e. polycistin-1 and stabilin 1), and other processes (i.e. annexin 5, nucleoside diphosphate kinase A and superoxide dismutase) as well as in protein metabolism such as proteolysis and vesicle mediated transport (Fig. 5B).

4. Discussion

It is suggested that the lack of knowledge regarding the underlying biological, biochemical and immunological components from the host-parasite interface represents one of the main reasons that could explain that currently not many targets have been successfully developed into vaccines against parasites (Stutzer et al., 2018). Therefore, understanding how parasites infect and migrate within their host tissues is of paramount importance to discover new interventions that strategically block host-parasite interactions (Cwiklinski et al., 2019). In the case of the study of fasciolosis caused by *F. hepatica*, these strategies should be ideally focused on the deep characterization of the first hours of cross-talk between the parasite and its host, e.g., in the changes induced in the NEJ and in the host intestinal epithelial cells upon their interaction, since this step is critical to define the first events driving the host-parasite relationship inside the vertebrate host. The aim of the present work is to characterize, starting from developing a novel in vitro model, the proteomic changes taking place during the early phase of fasciolosis between the NEJ and the host intestinal epithelial cells.

Regarding the parasite, we decided to work separately with tegument and somatic extracts, although it is expected to be difficult to obtain a complete separation of both antigenic compartments. Nevertheless, we obtained a tegumental enriched fraction as it has been postulated by other authors working with similar methodology (Garcia-Campos et al., 2016b). This extract also comprised excretory-secretory proteins, presumably regurgitated from the fluke gut, so it could be considered that it is a mixture of excretory/secretory and membrane-associated proteins (Morphew et al., 2013; Toet et al., 2014). The tegument of *Fasciola*, like that of other parasitic trematodes, is a biologically active and metabolically complex matrix continuously exposed to host tissues (Ravidà et al., 2016). It is a very dynamic structure, since changes in both its morphology and its composition from its juvenile to its adult form have been described, revealing the response of the migrating parasite to the changing demands of the host environment (Bennett and Threadgold, 1975). In our study, we found a large reorganization of the NEJ-Teg extract after 24 h of contact with the MPSIEC, which resulted in 303 differentially regulated proteins. In this regard, tegument turnover has been postulated as a successful strategy that ensures survival of platyhelminth worms helping them in reducing antigenicity of their outer surface (Skelly and Alan Wilson, 2006; Mulvenna et al., 2010). Observing the biological processes in which these proteins might be involved, we found 14 and 10 up-regulated proteins related to transport and signal transduction functions, respectively, in the NEJTeg 2 extract. This would be in agreement with the sophisticated communication mechanisms required by parasites with the aim of enhancing their own survival and transmission when facing a physical and molecular barrier from the host (Coakley et al., 2016; Roditi, 2016). These mechanisms should include not only the down-regulation of the host immune responses at the intestinal level, but also the expression of mediators and transporters allowing parasites to adapt and survive in a hostile environment, as can be inferred from the up-regulated proteins identified in this study. These included, among others, proteins related to innate immunity (leucine-rich repeat-containing protein), vesicle exocytosis (calcium-dependent secretion activator), pH homeostasis (voltage-gated hydrogen channel) and receptors for host molecules (cholecystokinin receptor). Interestingly, cholecystokinin is a hormone with important functions in pancreatic secretion, gallbladder function, intestinal motility and intestinal inflammation (Chandra and Liddle, 2007). Its synthesis and secretion is carried out by enteroendocrine cells in the duodenum and jejunum (Brubaker, 2012), the portion of the intestine in which NEJ should cross the intestinal barrier (Mas-Coma et al., 2014).

Analysis of the somatic proteome of the NEJ allowed us to identify 119 differentially regulated parasitic proteins after incubation with MPSIEC. In this case, many of the modified biological processes identified resulted in altered structural functions, such as cytoskeleton

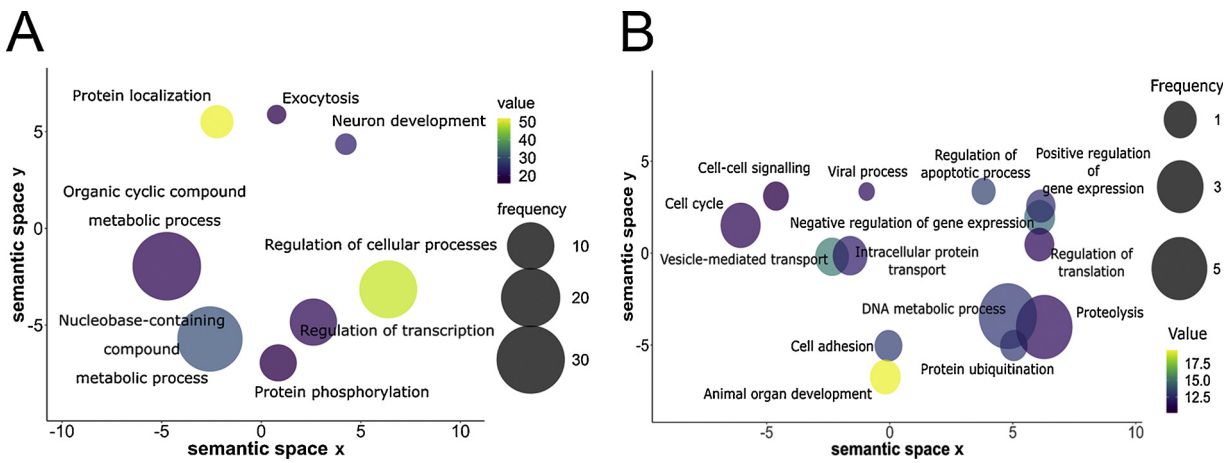

Fig. 5. Biological processes of up-regulated (A) and down-regulated (B) mouse primary small intestinal epithelial cells lysate (MPSIEC-Lys) proteins ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database.

organization or microtubule-based processes. In this sense, large-scale transcriptional changes have been described within the tubulin family as *F. hepatica* developed and migrated from the gut, across the peritoneum (Ryan et al., 2008; Cwiklinski et al., 2015). Tubulins are known targets of triclabendazole (TCBZ), a derivative of benzimidazoles, which are nitrogen containing heterocyclic compounds (Asif, 2017). Although the nature of the interaction of *F. hepatica* with TCBZ remains undefined and resistance has been described increasingly since the mid-1990s, it is considered the drug of choice to control fasciolosis (Kelley et al., 2016). Despite the fact that the actual biochemical mechanism of TCBZ resistance remains unclear, changes in the target molecule or the ability of parasites to metabolize the drug have been pointed out in this phenomenon (Brennan et al., 2007; Kelley et al., 2016). Intriguingly, we have found a down-regulation activity of nitrogen compound metabolic processes in both antigenic compartments (NEJTeg 2 and NEJSom 2). This might be related to the parasitic secretion of molecules with capacity to metabolize benzimidazoles, as well as with the ability to evade the action of the reactive nitrogen species. The production of these nitrogen intermediates by immune cells has been identified as a mechanism of cell-mediated cytotoxicity to NEJ (Piedrafita et al., 2001). Accordingly, NEJ would require metabolize host nitrogen compounds as a detoxification mechanisms in the early phase of the infection as have been previously studied in bacteria (Poole, 2005).

Finally, we could identify a representative number of differentially regulated proteins belonging to two important functional groups in both extracts NEJSom and NEJTeg: phosphorylation and proteolysis. Protein phosphorylation regulates many cellular processes by the action of protein kinases, in a mechanism that implies the transfer of phosphate groups from ATP to specific substrates (Manning et al., 2002). Their prominent roles in controlling parasite development and differentiation have been widely demonstrated in the study of schistosomes (Grevelding et al., 2018). In this species of trematodes, translocation of different groups of protein kinases from cytosol to membrane fractions has been related to transformation processes, as well as with motility and survival mechanisms of juvenile schistosomula (Wiest et al., 1992; Ressurreição et al., 2016; Grevelding et al., 2018). In the latter, the participation of host dopamine activating protein kinases in the early-stage schistosomules was demonstrated (Hirst et al., 2016). Interestingly, the up-regulated expression of a dopamine D2 receptor in the NEJTeg2 extract was shown in our study. In this sense, a dopamine D2 receptor with several potential phosphorylation sites for protein kinase has been related to parasite motility in *Schistosoma mansoni* (Taman and Ribeiro, 2009). We found in our study 13 and 3 up-regulated proteins related to phosphorylation processes, mainly kinases, in the tegument and somatic extracts from NEJ after contact with MPSIEC, respectively. Phosphorylation over-expression has not been described in the study carried out by Cwiklinski et al. (2018), in which the authors analysed, among other aspects, the proteomic changes in the NEJ after 24 h of incubation in axenic culture medium (RPMI 1640 medium). This suggests that the up-regulated expression of protein kinases found in our study, which could be of importance in the development and differentiation of NEJ once they cross the intestine, would be induced only after the contact with the intestinal barrier of the host.

Proteolysis, as a result of parasitic secretion of molecules with protease activity, is considered as a key mechanism in order to ensure the invasion and survival of trematodes. Their pivotal role in a large number of host-parasite interactions supposes that some of these proteases have been considered promising targets for the development of novel chemotherapeutic drugs and vaccines against a number of trematodiases, including fasciolosis (Kasný et al., 2009). Within this type of molecules the cathepsin-like cysteine peptidases represent not only the most numerous group (> 80 % of the total protein secreted by adult flukes), but also the most studied for its important functions in virulence, infection, tissue migration and modulation of host innate and adaptive immune responses (Cwiklinski et al., 2019). Immunolocalisation studies of *F. hepatica* NEJ carried out by Cwiklinski et al. (2018) showed that cathepsin cysteine peptidases are localised within the somatic extracts of the parasite, specifically to the gastrodermis of the bifurcated gut, which would act as a reservoir of the proteolytic capacity of the parasite. Our results show a down-regulation of this type of proteins in both, somatic and tegumental parasitic extracts after the contact with MPSIEC. Therefore, we can hypothesize that interaction with host intestinal epithelial cells could cause the secretion of some parasitic cathepsins.

In addition, the expression associated with *Fasciola* development of these proteases has been correlated with the passage of the parasite through host tissues (Stack et al., 2011). Consequently, when the immature flukes enter the liver and feed on/migrate through the tissue, the secretion of FhCL3 and FhCB drops, while the percentage of other cathepsin L clade members (mainly FhCL1) within the secretome of the parasite become increases (Robinson et al., 2008). Interestingly, the results obtained in our study showed this shift in the regulation of cathepsin expression after the contact of the NEJ with MPSIEC (See Fig. 6). We observed a predominance of the cathepsin isotypes B, L3 and L4 in the NEJ-Teg 1 extract, which is substituted by an up-regulation of cathepsins L1 and L1D after co-culturing with MPSIEC [FhCL1_1 and FhCL_4 according to the terminology described by Cwiklinski et al. (2019)]. These results could suggest the importance of FhCB, FhCL3 and FhCL4 in the early stages of the parasite, playing important roles in excystment or gut penetration, as well as of the CL1

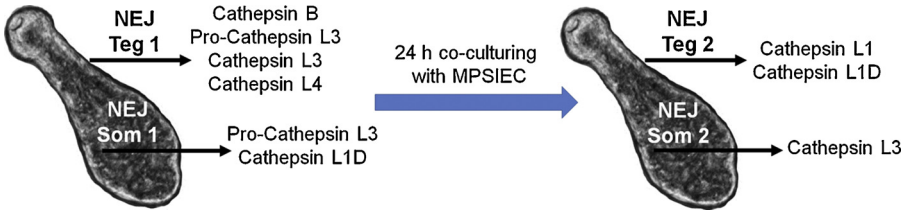

Fig. 6. Protein profile of the up-regulated cathepsin cysteine peptidases in the tegument and somatic extracts of the NEJ before and after co-culturing with the MPSIEC. Tegument extract from newly excysted juveniles, NEJ-Teg; somatic extract from newly excysted juveniles, NEJ-Som. In all extracts number 1 represents before co-culturing and number 2 represents after co-culturing.

clade in later stages (e.g. immature migratory flukes), as it has been reviewed by Cwiklinski et al. (2019). In their proteomic studies carried out with NEJ without the involvement of the host intestinal epithelial cells, Cwiklinski et al. (2018) found that FhCL3 proteases comprise the major components of the NEJ somatic proteins (including tegumental fraction), after 24 h of incubation in culture medium. This suggests that the interaction between the NEJ and the MPSIEC, and not only time after excystment, could be responsible for the triggering of the expression of a different protease profile in the parasite. This new cathepsin repertoire, including components typically associated with the adult stage of the parasite could be part of the secretome that the parasite requires for its proteolytic activity during its developmental/migratory phase, as well as to evade the host's defensive mechanisms in an early stage of the infection by the replacement of its cathepsin profile.

Secondly, we obtained MPSIEC lysates before and after stimulation with NEJ in order to identify the proteomic changes induced in the host intestinal epithelial cells upon contact with the parasite. We found 160 differentially regulated proteins from the MPSIEC cultures, representing approximately half of proteins whose expression had changed in the parasite's tegument extract. This is in line with a similar study carried out with the same cell type stimulated with larvae L3 of *Ascaris suum* in which authors are surprised to find such a low magnitude of response (indicated by fold-change values) and the lack of conclusive activation signatures (Ebner et al., 2018).

Among other processes, we found 43 and 21 up-regulated proteins in stimulated MPSIEC related to regulation of cellular processes and protein localization, respectively. Interestingly, we identified seven proteins belonging to the family of Rab proteins, within both groups. Rab family of proteins comprise Ras-like small GTPases with important roles in vesicle trafficking processes (Barr and Lambright, 2010). Furthermore, recently, their ability to regulate some processes related to innate immunity and inflammation by controlling the formation, transport and fusion of intracellular organelles has been described (Prashar et al., 2017). Similarly, we found an up-regulated ficolin-1 in the MPSIEC-Lys 2. Ficolins are innate pattern recognition receptors playing important roles within the innate immune response to numerous pathogens, including some parasites, via the recognition of pathogen-associated molecular patterns (PAMPs) (Cestari et al., 2013; Ren et al., 2014; Bidula et al., 2019). Taking into account that intestinal epithelial cells are considered as a first line of defense connecting pathogens and underlying immune cells in order to maintain intestinal homeostasis and promote host defense (Allaire et al., 2018), these up-regulated proteins suggest possible signaling mechanisms, which could be used by the host to trigger activation of the innate immune system through activity of the Rab pathway or ficolins, among others, as a response to parasite stimuli.

On the other hand, and interestingly, we found a decrease in the expression of the positive regulation of host immune system in the MPSIEC-Lys 2, including down-regulation of three proteins related to ubiquitination. Since surfaces of the mammalian intestine interact directly with the external environment, intestinal epithelial cells have evolved a number of strategies for controlling the invasion of infectious agents. In this regard, innate immunity is a highly effective first line of defence against pathogens (Hooper, 2015). The ubiquitin system have increasingly received attention for its important role in responding to pathogen infection as a part of the host innate immune system, as well as its modulation by pathogenic antigens has recently pointed out in some bacteria responsible for intestinal diseases, such as *Escherichia coli*, *Salmonella* and *Shigella* (Bhoj and Chen, 2009; Zhou and Zhu, 2015; Li et al., 2016). Regarding *Fasciola* juveniles, it has been described that as soon as NEJ arrive at the intestinal wall, they secrete immunomodulatory molecules that influence the host innate response (Dalton et al., 2013). Consequently, the observed down-regulation of protein ubiquitination might be an immune evasion strategy carried out by NEJ in the early stage of infection.

Finally, parasite effects on the host intestinal barrier morphology could be related to 7 and 8 down-regulated proteins identified in the MPSIEC after contact with NEJ linked with cell adhesion and the regulation of the apoptotic processes, respectively. Cell-cell interactions, as part of cell adhesion mechanisms in intestinal epithelial cells are critical to maintain gastrointestinal homeostasis, especially during pathological conditions (Efstathiou and Pignatelli, 1998). Furthermore, disruption or reduced expression of cell adhesion proteins and loss of epithelial barrier function represents one of the main strategies used by intestinal parasites to invade host tissues (Di Genova and Tonelli, 2016). Our data showed a down-regulated expression of some cell adhesion proteins, such as polycystin 1, stabilin 1 and protein S100-A10 in MPSIEC-Lys 2. Among them, polycystin 1 has found to be part of a complex with the essential epithelial cell adhesion molecules E-cadherin and catenins (Huan and van Adelsberg, 1999). Intriguingly, parasite E-cadherin and catenin, as well as mouse polycystin 1 were up-regulated in NEJ-Teg extract after the contact with MPSIEC, suggesting a potential parasitic adhesion mechanism to the host intestinal barrier prior invasion. Regarding apoptotic processes, we found, among others, a down-regulated expression of host superoxide dismutase, a potent antioxidant that protects against oxidation-induced apoptosis (Briehl et al., 1997). Similarly, the induction of intestinal epithelial cell apoptosis by intestinal parasites such as *Giardia lamblia*, *Cryptosporidium sp.*, and *Entamoeba histolytica* have been showed as a mechanism contributing to tissue invasion (Di Genova and Tonelli, 2016).

In conclusion, “omics” technologies are helping advance our understanding of the host-parasite relationships in the study of fasciolosis (Cwiklinski and Dalton, 2018). Our data are in accordance with these studies showing the adaptation of NEJ to the host environment and the capacity for rapid evolution in terms of the number of differentially regulated proteins identified in the parasite extracts after the contact with the host epithelial barrier. The analysis of the biological processes in which these proteins are involved show different strategies allowing the parasite to develop and differentiate, some of them only triggered after communicating with its host, for invading its tissues or evading its defence mechanisms. In addition, the inclusion of the MPSIEC in our in vitro model allow us to know how the parasite stimuli could influence on the host intestinal epithelium functions, that are important for parasite invasion and survival, such as the modulation of the innate immune system, the disruption of cell-cell interactions or the regulation of apoptosis. Future studies aimed at unravelling these cross-talk relationships between the NEJ of *F. hepatica* and the host intestinal barrier could define new pharmacological or vaccine targets for the early elimination of fasciolosis, before the adult worms are established in its definitive location.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2020.109028.

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