Stem cell proliferation driven by opposite sex excretory-secretory products (ESPs) in *Schistosoma mansoni*

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Introduction

Schistosome parasites infect ~240 million people globally, mainly in Africa[1,2]. Adult male and female schistosomes must physically pair and remain paired to complete/sustain maturation and enable egg production; this must be facilitated by molecular signalling between the sexes[3,4].

Previously we demonstrated that the exchange of male or female excretory-secretory products (ESPs) between groups of opposite-sex adult worms activated two protein kinases: p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK)[5]. Treatment with SB203580 and U0126 attenuated the ESP-mediated phosphorylation (activation) of p38 MAPK and ERK, respectively[5].

The **aim of this research** was to investigate whether the ESPs could mediate downstream somatic/germinal stem cell proliferation in the opposite sex parasites and if ERK or p38 MAPK signalling pathways underly these processes. Furthermore, to reveal, by LC-MS/MS, the nature of the molecule(s) responsible for the induction of signalling. Understanding these processes has implications for novel approaches to schistosomiasis control.

Methods

Effect of ESPs and inhibitors on stem cell proliferation

- Adult male and female worms were cultured for 6 days in RPMI with antibiotics (Fig 1a) and ESPs were exchanged, and EdU (20 µM) added for 24 h.
- Next, worms were fixed, dehydrated and stored. Rehydration was followed by proteinase K treatment, incubation in Alexa Fluor 488, and mounting in SlowFade Gold/DAPI.
- In parallel, worms were pre-incubated with inhibitors before ESP exposure. CLSM imaging was performed, and EdU+ cells were counted in tegument, testes, ovaries, and vitellaria.

Proteomics of total male/female ESPs and extracellular vesicle (EV)-depleted ESP fraction

- Adult male and female ESPs were collected, concentrated, subject to protein estimation, and run onto SDS-PAGE gels. Gel areas containing proteins were excised and sent for LC-MS/MS analysis.
- EV-depleted ESP fractions were prepared using previously validated methods[6], separating the filtrate containing soluble ESPs from remaining fraction containing EVs (>100,000 Da); these were sent for LC-MS/MS as above.

Results 1: Exposure of adult worms to opposite-sex ESPs stimulates p38 MAPK- and ERK-dependent stem cell proliferation in vital organs

Given the importance of stem cells to schistosome development and reproduction, we hypothesized that ESPs from one sex could affect stem cell proliferation in the other. To test this, adult worms were chased with EdU on day 0 to establish baseline proliferation, and after 6 days in single-sex culture, with or without exposure to opposite sex ESPs (Fig 1a).

- After 6 days in single sex culture, the number of EdU+ cells in males and females plummeted (Fig 1b).
- Strikingly, when males were exposed to female worm ESPs, the number of EdU+ cells increased in the testicular lobes (25-fold increase) and sub-tegument (70-fold) (Fig. 1b; p≤ 0.001).
- Incubation of these worms with SB203580 or U0126 for 1 h before exposure to female ESPs and EdU chase, abolished the increase in proliferating cells (p ≤ 0.001; Fig. 1b).
- Similar effects were observed in female worms exposed to male ESPs, with ESP-mediated increases in EdU+ cell number observed in ovaries (10-fold), vitellaria (60-fold) and sub-tegument (5-fold).
- This increase was attenuated when female worms were pre-incubated with SB203580 or U0126 prior to exposure to male ESPs (p ≤ 0.001; Fig. 1b).

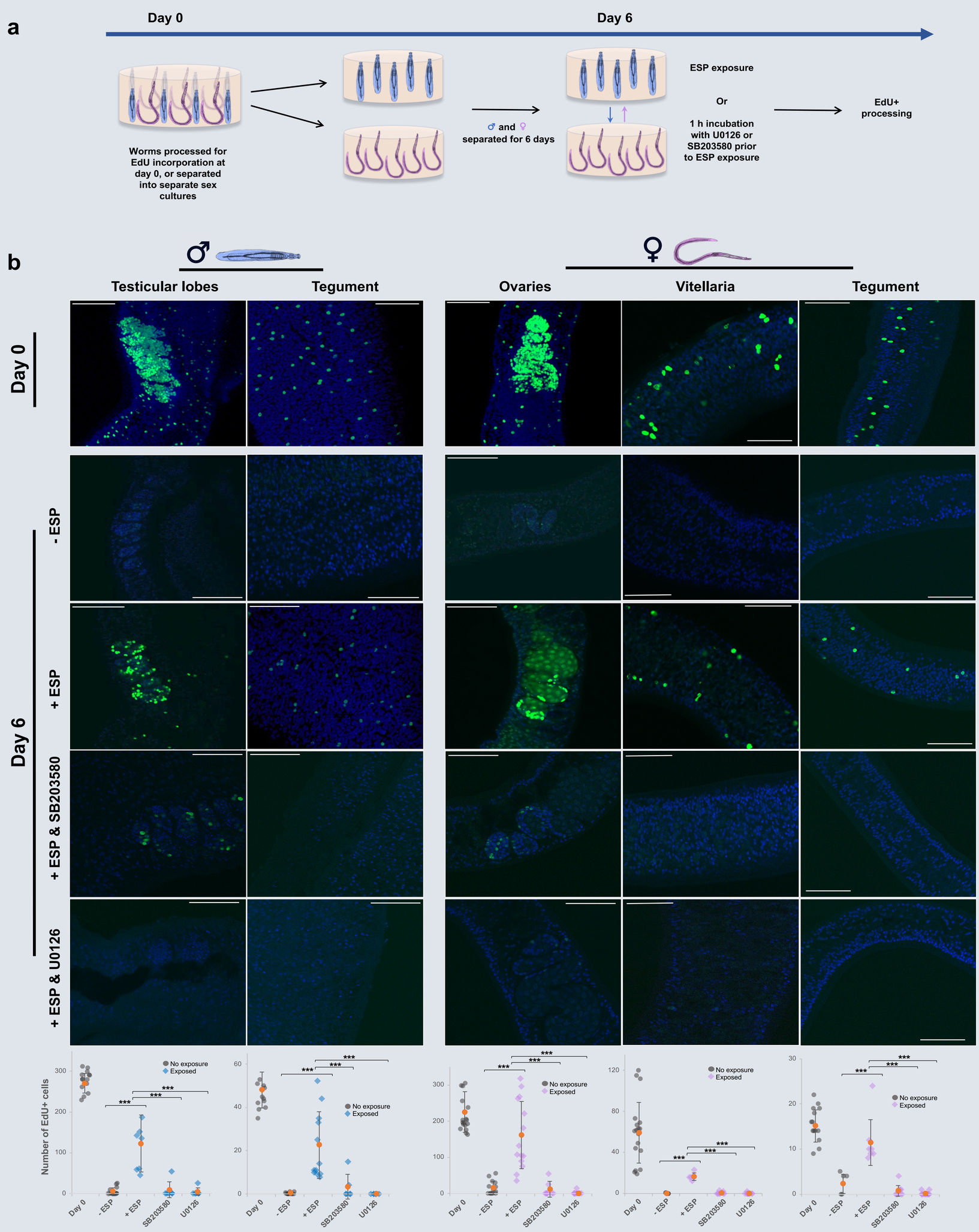

Fig 1a *Cartoon illustrating the experimental workflow.* ***b*** *Adult male or female worms were either chased with EdU on day 0 for 24 h or separated into groups of five males or females for 6 days prior to being exposed to opposite sex ESPs (or not, control) for 24 h, either with or without 10 µM SB203580 or 10 µM U0126, and chased simultaneously with EdU for 24 h. Worms were processed for EdU detection, stained with DAPI and images captured by CLSM. Representative images show maximum projections (50, 25, and 20 z-sections for the testes and ovaries, vitellaria, and teguments, respectively). Scale bar = 50 µm. Graphs show EdU⁺ cell counts (from individual sections of an entire z-stack) from at least three independent assays for male testicular lobes and tegument, and female ovaries, vitellaria and tegument with the mean (±S.D.) also shown; ***p ≤ 0.001 (ANOVA).*

Results 2: Proteomic analysis of total ESPs and EV-depleted E SPs

- 705 proteins identified: 583 in ESPs from both sexes, 74 and 48 exclusively from males and females, respectively (Fig 2a). ~17% of proteins were 'uncharacterized'.
- 362 proteins identified in the EV-depleted ESP fractions: 286 from both sexes, 62 and 14 exclusively from males and females, respectively (Fig. 2b).
- Comparative analysis of total ESPs vs EV-depleted ESPs revealed 6 male and 2 female uncharacterised proteins were unique to EV-depleted ESP fractions. 49 proteins present in EV-depleted fraction but not in total ESPs.

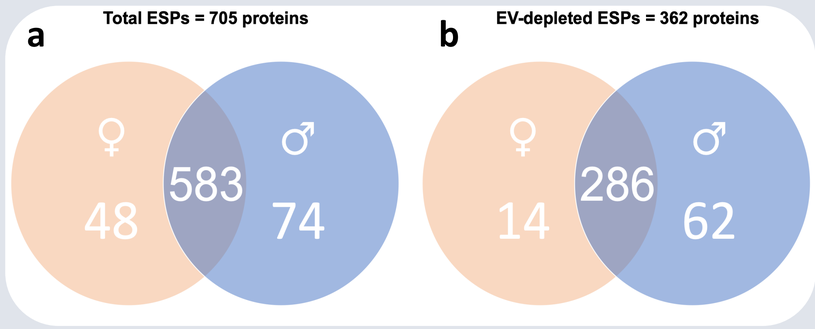

Fig 2. *LC-MS/MS analysis of total ESPs and EV-depleted ESPs of male and female* S. mansoni *identified a total of 705 and 362 proteins, respectively.* ***a.*** *ESPs were collected from 240 worms of each sex, concentrated, and processed for LC-MS/MS; 583 proteins were identified in both males and females, 74 proteins were unique to males and 48 to females.* ***b.*** *Concentrated ESPs from 240 worms of each sex were depleted of EVs and processed for LC-MS/MS analysis; 286 proteins found in both males and females, 62 exclusively in the males and 14 in females.*

Discussion

We demonstrate that adult male and female schistosome ESPs induce proliferation of somatic and germinal stem cells in opposite-sex worms, and this effect is ERK- and p38 MAPK-dependent. This finding, which supports the notion that schistosomes may communicate remotely to benefit their development, reproduction and possibly survival, provides a new (non-contact) paradigm for how schistosomes may interact. Experiments using human serum were not practical due to the signal-based interference that would occur from components such as growth factors; thus, it remains uncertain if the observed ESP-mediated responses occur *in vivo*.

Detailed proteomic analyses of adult male and female total ESP and EV-depleted ESP fractions discovered differences in the proteins present between the sexes, but the number of different proteins observed between the sexes made interpretation in relation to the stem cell findings challenging. Despite not being able to identify the molecules responsible for the ESP-mediated responses in the opposite sex worms, the proteomic data is valuable for further studies.

We anticipate that this and our previous research[5], which includes novel findings on *S. mansoni* ESPs, cell signalling, and stem cell proliferation, will stimulate future research and discussion into male-female schistosome interactions and their implications for schistosome control.

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