Video Article

Development of a Negative Selectable Marker for *Entamoeba histolytica*

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Abstract

Entamoeba histolytica is the causative agent of amebiasis and infects up to 10% of the world's population. The molecular techniques that have enabled the up- and down-regulation of gene expression rely on the transfection of stably maintained plasmids. While these have increased our understanding of Entamoeba virulence factors, the capacity to integrate exogenous DNA into genome, which would allow reverse genetics experiments, would be a significant advantage in the study of this parasite. The challenges presented by this organism include inability to select for homologous recombination events and difficulty to cure episomal plasmid DNA from transfected trophozoites. The later results in a high background of exogenous DNA, a major problem in the identification of trophozoites in which a bona fide genomic integration event has occurred. We report the development of a negative selection system based upon transgenic expression of a yeast cytosine deaminase and uracil phosphoribosyl transferase chimera (FCU1) and selection with prodrug 5-fluorocytosine (5-FC). The FCU1 enzyme converts non-toxic 5-FC into toxic 5-fluorouracil and 5-fluorouridine-5'-monophosphate. *E. histolytica* lines expressing FCU1 were found to be 30 fold more sensitive to the prodrug compared to the control strain.

Protocol

1. FCU Negative Selection System in *Entamoeba histolytica*

1. The vector control line (HM1/ pKT3M) and the experimental line (HM1/pKT3M-FCU1) were generated by previously described techniques[1,2]. Seed the lines under study from the stock tubes into T-25 flasks and maintain selection by adding appropriate antibiotic (12 µg/ mL G418) in TYI-S-33[3] medium. The flasks should be 70-80% confluent after 16-18 hours of growth at 37°C.
2. Prepare a fresh 50mM stock solution of 5-Fluorocytosine (5-FC, Sigma) in warm TYI-S-33 medium. Vortex rigorously for several minutes to dissolve 5-FC completely. Filter-sterilize the stock solution by passing it through a 0.22 µm filter.
3. Harvest trophozoites by placing the flasks on ice for 3-5 minutes and collecting the cells by centrifuging at 200xg for 5 minutes at room temperature. Resuspend the pellet in fresh TYI medium and count the cells.
4. Design of a typical experiment uses 0.5×10^4 cells per well of a 48 well plate in a final volume of 1 mL; and triplicate repeats for each test condition. In addition, to facilitate fluorescent measurements, a separate plate is used for each strain per time point.
5. Seed 0.5×10^4 cells per well for each test 5-FC concentration, in triplicate. Maintain the antibiotic selection pressure in the TYI medium. Now add the required amount of 5-FC stock solution and trophozoites to each well.
6. Incubate the plates in an anaerobic bag at 37°C.

2. Determination of Selection Efficiency

1. Prepare 2 mg/ mL stock solution of Cell tracker green CMFDA (Invitrogen) in DMSO. To make the working solution dilute the stock 1000-fold in serum-free TYI medium.
2. Remove the medium completely from trophozoite culture plates and replace with 150 µL serum-free TYI containing CMFDA. Continue incubation at 37°C for 1 hr.
3. Remove the dye solution from wells and read the plate in the Spectramax M2 microplate spectrofluorometer. The excitation wavelength should be set at 492 nm and the fluorescence emission read at 517 nm.
4. Compare the fluorescence values for the control cells versus the test transfectants.
5. Continue reading the plates at each time interval. Use appropriate positive (TYI only) and negative (25 µg/ mL hygromycin) control wells in each plate.

3. Representative Results

The *E. histolytica* transfectants showed robust expression of codon optimized recombinant FCU1 (Figure 1).When this protocol is followed correctly it results in selective elimination of *E. histolytica* cells expressing FCU1 in presence of 0.5 mM 5-fluorocytosine. Control cells, in contrast continue to grow normally at up to 5 mM concentration of 5-FC and reach confluence between 72 and 96 hrs (Figure 2-a). The FCU1 carrying cells at 48 hrs are completely lysed when the treated wells are viewed under a microscope. These also show decreased fluorescence when stained with the vital dye CMFDA, which is used in quantitative studies involving large number of samples (figure 2-b). The FCU1/5-FC system is an effective and powerful negative selection tool for *E. histolytica* trophozoites.
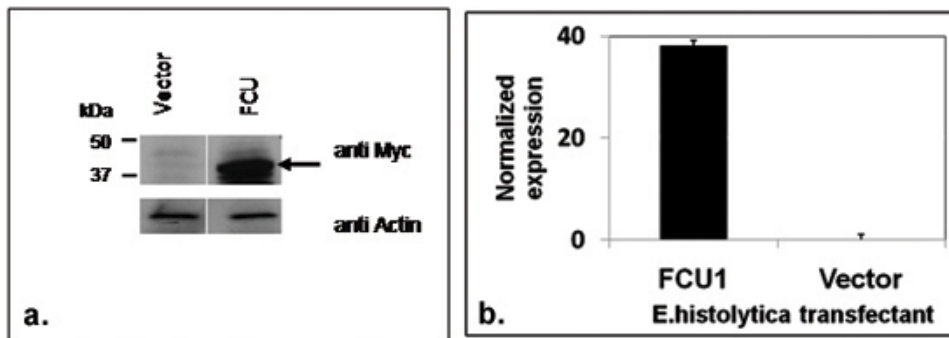
Figure 1. Generation of *E. histolytica* HM1 transfectants expressing negative selectable marker
(**a**) FCU1 recombinant protein was detected on a western blot using anti-c Myc antibody (top panel). As expected, a 42kDa band could be detected (shown by an arrow) in the total lysate from the FCU transfectants but not in the vector alone control transfectants. The bottom panel shows the same blot probed with anti actin antibody as a loading control. (**b**) Results of the quantitative real time PCR showing expression of FCU1 specific mRNA normalized to lgl4 control.
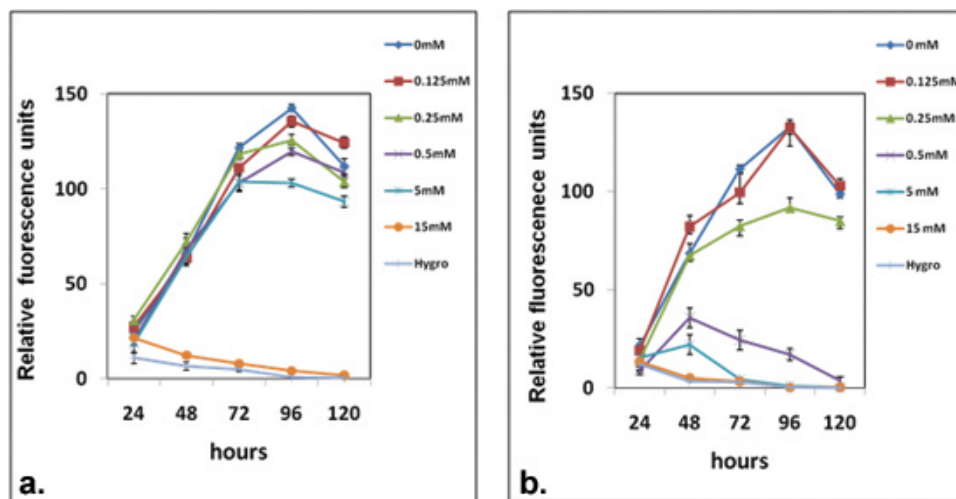

Figure 2. *In vitro* drug sensitivity of *E. histolytica* transfectants expressing negative selectable marker
Survival curves of (**a**) control transfectants and (**b**) FCU1 expressing transfectants cultured in 48-well plate. The cells were grown in presence of increasing concentrations of the prodrug- 5-fluorocytosine; cell survival was quantified by CMFDA staining at each time interval as denoted on the X-axis. The Y-axis represents fluorescence units and is a direct reflection of the surviving cell population. Please note that the FCU1 expressing transfectants showed significant sensitivity at 0.5mM prodrug concentration compared to the control. No cells were observed at 120h time point in these wells as compared to the confluent growth seen in the corresponding control wells under microscope (data not shown). Hygro denotes 25μg/ mL hygromycin used as a negative control.

Discussion

Although there have been substantial advances in Entamoeba molecular biological tools there are no current methods available to delete or disrupt genes[4]. Knockdown of gene specific expression has been achieved by the use of hairpin RNAs[5], small interfering RNA[6] and bacterially expressed dsRNA[7]. However these methods while effective for the respective target genes, have several limitations including use of costly chemicals, continuous selection against antibiotics and the need for constant validation due to the high incidence of reversion occurring over time (Alicia Linford, personal communication)[8].

Any plasmid can replicate in *Entamoeba* as there is apparently not a strict sequence requirement for origins of DNA replication[9], and so once transfected, it usually is difficult to cure a plasmid. This limits the possible use of intact plasmids in recombination and gene knockout experiments. Negative selection markers are key components of gene targeting methods as they can be used to eliminate recombinant subpopulations. We have successfully expressed a codon optimized FCU1 gene in *Entamoeba histolytica* and tested its potential as a negative selectable marker. This gene fusion has been used for suicide gene therapy of human tumor cells and metabolizes the prodrug 5-FC into toxic downstream products resulting in inhibition of RNA and DNA synthesis[10]. FCU1 has been recently used as a negative selectable marker in *Plasmodium*, another protozoan parasite. *Plasmodium berghei* cells expressing FCU1 were found to be almost 1000 fold more sensitive to the prodrug *in vitro* and *in vivo* treatment with 5-FC killed over 99.9% of the infecting recombinant parasites in the erythrocytic-stage mouse model of malaria[11]. *E. histolytica* cells expressing FCU1 showed only a 30-fold increased sensitivity to the prodrug 5-FC in contrast to the sensitivity observed in *P. berghei*. Possible reasons for this could be large pool of nucleotides available in the growth medium competing with the toxic metabolites.
In both *P. berghei* and *P. falciparum* the FCU1 marker has been used in targeted gene disruption. studies[11,12]. We aim to manipulate the Entamoeba genome using homologous recombination. The validation of the FCU1/5-FC negative selection system is a significant step towards this goal.

Disclosures

Acknowledgements

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