Evidence for asymmetric cell fate and hypoxia induced differentiation in the facultative pathogen protist *Colpoda cucullus*

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**Abstract**

Accumulating evidence shows that the evolutionary origin of the asymmetric cell fate and stemness begin in the last eukaryotic ancestor (LECA). The mechanisms of asymmetric division and stemness are conserved in descendants and pathogen protists as *Entamoeba invadens* and *Giardia lamblia*. It is to be expected that mechanisms for asymmetric cell fate are also conserved in free-living protists and occasionally facultative pathogens. For a better understanding of the ancestral mechanisms involved and their evolution in the protists, we looked for similar mechanisms of cell cycle progression, growth and differentiation in the facultative pathogen protists *Colpoda cucullus*. We compare what is known about *Colpoda cucullus* to our findings in *E. invadens*.

**Keywords**: *Colpoda cucullus*, asymmetric division, non identical daughter cells, hypoxia, terminal differentiation, hypoxia induced encystment

**Introduction**

In recent papers [1,2] and comments posted at ResearchGate and Academia.edu (see www.researchgate.net/profile/Vladimir_Niculescu/publications and http://uni-tuebingen.academia.edu/VladimirNiculescu/Papers) it was shown that the evolutionary origin of the asymmetric cell fate and stemness begin in the last eukaryotic common ancestor (LECA). The discovery of self-renewing stem cell lines in single celled eukaryotes and cell differentiation mechanisms inherited from the common ancestor re-opens the discussion concerning the basal mechanisms of differentiation. Mechanisms of cell differentiation similar to those of *E. invadens* were probably conserved in many protists that likely have hidden stem cell lineages in their life cycle. Encystment occurs as terminal differentiation step in prominent commensals and pathogens such as *Entamoeba*, *Naegleria*, *Acanthamoeba*, *Iodamoeba*, *Giardia*, *Leishmania* and *Balantidium* and also in many free-living protists (Niculescu VF 2014. The multilined cell system of *Giardia lamblia* in the light of the modern protist stem cell biology).

Recent years *Colpoda cucullus* has been intensively studied by a Japanese research group [4-8]. In the present paper we compare what is known about *Colpoda cucullus* cell differentiation to our findings in *E. invadens* [1,2] and *Giardia* (Niculescu VF 2014. The multilined cell system of *Giardia lamblia* in the light of the modern protist stem cell biology).

**Materials and methods**

The Japanese researchers cultured *Colpoda* in a nutrient poor culture medium consisting only of 0.05% w/v dried wheat leaves infusion [4-8]. Low-density cultures (LD cultures) were inoculated with $10^3$ vegetative cells/ml. Non-proliferating *Aerobacter aerogenes* cells [4-6] and *Klebsiella pneumoniae* [7,8] were added to the culture medium as a food source. Both *A. aerogenes* and *K. pneumoniae* are oxygen consuming bacteria (OCB) [9,10]. In order to induce encystment, authors suspend cells at a high cell density in Tris-HCl/CaCl$_2$ encystment medium. Bacteria and polystyren latex particles were used to suppress encystment. Unfortunately, the authors failed to report the amounts of bacteria added in culture, thus neglecting to consider the effects of hypoxia and different pO$_2$ values on *Colpoda* growth and behaviour.
Results and discussion

Encystment

It was reported that ≤10^3 OCB/ml did not suppress Ca^{2+} mediated encystment allowing ~90% Colpoda cells to encyst, while 10^4 OCB/ml reduced encystment to ~62% [4] and 10^5 OCB/ml completely repressed cyst formation, independent of whether the bacteria are living or have been boiled. Similar inhibitory effects have been shown using polystyrene latex particles (3x10^3 PLP/ml). The results suggest that signaling mechanisms for pino- and phagocytosis are retained in mitotically repressed trophozoites, and therefore neither non nutrient media nor Ca^{2+} ions are the true encystment inducers, capable to convert vegetative cells into ITD cysts (ITD, induced terminal differentiation).

It could be that the true inducer is a shift in hypoxia, similar to the hypoxic/hypopsmotic pressure in OCB sediments which shift E. invadens to ITD. Evidence for this assertion is the “overpopulation” (OP) mediated encystment observed in encystment assays inoculated with high Colpoda cell density [6,8]. The authors differentiate between “induced” (IND) and “not induced” (NI) encystment occuring in 1mM Tris-HCl pH 7.2 with or without Ca^{2+} ions, and “spontaneous” (S) encystment occuring in culture medium (0.05% w/v infusion of dried wheat leaves). For “induced” assays they suspended high density Colpoda populations (3x10^4 cells/ml) in 1mM Tris-HCl pH 7.2/0.1mM CaCl_2 encystment medium referred to as HD/Ca^{2+}(IND). For “not-induced” assays authors transferred low density populations of 1-2x10^3 cells/ml in encystment medium without Ca^{2+} ions and bacteria referred to as LD/Tris (NI) [7] and [8]. It is not inducement by Ca^{2+} ions, but rather hypoxic changes that occur during and after cell transfer (t0-t6) which in fact induce encystment. HD populations themselves consume appreciable amounts of dissolved oxygen from the encystment medium, changing the pO2 value. Hypoxic changes following oxygen consumption stops the vegetative life cycle of Colpoda, initiating terminal differentiation (encystment). Additional amounts of bacteria ≥10^4 OCB/ml [4] increased oxygen consumption in HD/Ca^{2+}(IND) assays. Increased hypoxia suppresses encystment of HD populations. When 10^4 OCB/ml was used, 62% of the cells encyst, 38% cells are refractory. In assays without bacteria all cells encyst.

Asymmetric cell fate, population heterogeneity and “spontaneous” encystment

Colpoda populations are heterogenous and the daughter cells non-identical.

Growth in LD cultures is not logarithmical. The culture conditions chosen by authors [8] suppressed encystment during the growth phase (t0-t24). In the early growth phase (t0-t12) only few cells divide. In the main growth phase (t12-t24) the population doubled and the progeny enter in a stationary state. Stationary cells begin to encyst spontaneously (S encystment) but not because they are starved. 50% of the cells remain vegetative at least 14-15 hr after the end of the growth phase (t24-t38), others even longer.

Population heterogeneity is seen in [8]. In both 24 h and 36 h hold samples two distinct cell fractions were observed: (i) one of about 60% cells capable to encyst but only in HD/Ca^{2+}(IND) assays vs. (ii) a second cell fraction of about 32% capable to encyst both in HD/Ca^{2+}(IND) and LD/Tris (NI) assays. This is the clear indication that Colpoda divide producing two non identical daughter cells, namely a cycling D1 cell and a mitotic repressed D2 cells. During the growth phase the population performed in fact two asymmetric divisions [8]. At t24 it contains a ~30% cycling cell fraction (D12, second generation of D1cells) arrested in the G1 phase and a ~60% of quiescent D21-2 cells (two generations of D2 cells) arrested in G0. In HD/ Ca^{2+}(IND) assays, hypoxia due to oxygen consumption by the Colpoda cells (3x10^4 cells/ml), converted D2(G0) cells to form cysts. D1 (G1) cells encysting both in HD/Ca^{2+}(IND) and LD/Tris (NI) assays were induced mostly by oxidative stress when cells were harvested from the more hypoxic culture medium.

Encystment in culture [8] occurred similarly. Increasing hypoxia due to consumption of oxygen by rapidly growing cells (t0-t24) represses encystment, while decreasing hypoxia due to reduced oxygen consumption during the stationary phase induces encystment (t24-t48). It is evident that one of the two cell fractions (D1 cells) begins to differentiate earlier in LD/Tris (NI) and the other fraction (D2 cells) later [7]. There is a difference in onset of differentiation of about 5 hr. In Entamoeba invadens mitotic arrested D2 cells are sub-tetraploid (<4C) and need more time for additional endocycles, before encystment. Colpoda cysts are tetraploid and D2/G0 cells of Colpoda could also be sub-tetraploid.

Polystyrene latex particles (1.1µm) and the bacterial dyalized product MWCO: 10.000 seem to repress preferentially the 33% D1 cell fraction in HD/Ca^{2+}(IND) assays [4] while the 62% D2 cell fraction is refractory. The results reflect differences in the endocytotic potency of both fractions. However, small particles such as bacteria (living or killed 10^{7}-10^{8} cells/ml), molecular bacterial products and dyalized MWCO: 1000 stop D2 (G0) encystment also. It seems that mitotic arrested D2 (G0) cells may return to a vegetative life cycle if they feed rapidly by pinocytosis or on small opsono-phagocytic particles as bacteria. PLP lacking adequate surface receptors and high diluted MWCO: 10.000 are inadequate feed for quiescent cells (mitotic repressed D2/G0 cells) and are not capable of stopping the induction of encystment.

Conclusions

The current data suggests that cell proliferation in Colpoda occurs by asymmetric cell division. Analyzing the recent data from Japanese research groups [4-8] in the light of results acquired from our experiments using E. invadens and in consideraton of the recurring ratio of about 30:60 (1:2) seen in the most assays with Colpoda suggests the following hypothesis: Colpoda cucullus is another example of a single-celled eukaryote having molecular mechanisms for asymmetric
cell division, inherited from the common ancestor. Similar with E. invadens and Giardia (Niculescu VF 2014. The multilined cell system of Giardia lamblia in the light of the modern protist stem cell biology) Colpoda has a self renewing cell line (SRL) consisting of cycling cells, constant in number (D1 fraction). SR cells proliferate by asymmetric division giving rise to repeated generations of mitotic arrested cells (D2 fraction). Proliferation and terminal differentiation (encystment) are hypoxia-dependant. Colpoda cells and oxygen consuming bacteria used as nutrients (OCB) consume dissolved oxygen in culture, reducing pO2 levels. Critical ranges of hypoxia stop proliferation. Oxygen consumption decreases by reduced metabolic activity of stationary phase cells at a hypoxic range that induce Colpoda cells for terminal differentiation and ITD cysts. All cells encyst in non-nutrient media when hypoxia reached permissive levels, D1 cells earlier and D2 cells later. The delay is probably caused by additional rounds of DNA synthesis (endocycles) needed before cells begin cyst differentiation. Ca2+ ions are not the true inducer but HD/Ca2+(IND)- conditions may favored D2 cell encystment respectively endocycling.

In conditions where there are sufficient nutrients encystment is arrested. Colpoda cells (D1 and D2) retain endocytotic activity and re-enter the vegetative cell cycle to replenish the SRL line. In conditions of limiting food resources, the population splits: the minor D1 cell fraction continues cell cycle progression while the major D2 cells terminally differentiate to cysts. There are no differences between “spontaneous” encystment observed in culture and the other forms of cyst differentiation called by the authors as IND-, NI- and overpopulation mediated encystment. All encystment processes described [4-8] occurred in conditions of hypoxia and are probably induced by an favorable pO2 value achieved by oxygen consumption (ITD encystment), either by oxygen consuming bacteria (OCB) or be Colpoda themselves. As we found in Entamoeba [1] “spontaneous” encystment is a form of autonomous encystment by cyclic terminal differentiation (ATD encystment) that occurs successively in media rich in nutrients (cyclic encystment). D2 cells produced in culture by the most oxidative line MOL lineage (s-SRL line) encyst autonomously to ATD cysts. ITD encystment is a non-autonomous differentiation process occurring in hypoxic/hypoo- 

smotic respectively non-nutrient media. All daughter cells may be induced if they are in the early G1/ante-RP stage (D1 cells) or in G0 state (D2 cells).

A full understanding of the differentiation biology of Colpoda requires accurate measurment of pO2 to clarify hypoxia’s role in triggering terminal differentiation in culture and encystment medium. More experiments are necessary in culture, additionally varying the amounts of OCB. Hypoxic studies will clarify whether Colpoda has a single-lined or a multi-lined cell system, similar with those of Entamoeba and Giardia and if there is a second cell line capable of autonomous terminal differentiation (ATD) and whether ATD cysts exist in the system. In Entamoeba we found a most oxidative cell line MOL capable of autonomous differentiation and cysntment and an MHL line, capable of induced differentiation (Niculescu VF, Extrinsic and intrinsic signaling control cell fate specification in the stem cell system of Entamoeba, Academia.edu February, 17, 2014), similar with those SRL observed in Colpoda.

However, it is unknown at what point in the cell cycle do D1 cells become mitotically determined and refractory for differentiation (point of no return). In Entamoeba and Giardia we found postmitotical daughter cells in state of double potency being capable to continue a vegetative life cycle or to exit the vegetative life cycle if cells are induced for ITD encystment. The state of double potency ends in normal cultures at the point of cell fate bifurcation, where young cells become mitotically determined (D1 cells) lacking the capacity for ITD encystment (Niculescu VF 2014. The multilined cell system of Giardia lamblia in the light of the modern protist stem cell biology).

Competing interests
The author declares that he has no competing interests.

Acknowledgement
The author expresses his gratitude to Dr. Dennis Thomas (english speaker) for reading of the manuscript and excellent support.

Publication history
EIC: Todd R. Callaway, Food and Feed Safety Research Unit, USA. Received: 19-May-2014 Final Revised: 18-Jul-2014 Accepted: 24-Jul-2014 Published: 02-Aug-2014

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Citation:
Niculescu VF. Evidence for asymmetric cell fate and hypoxia induced differentiation in the facultative pathogen protist Colpoda cucullus. Microbiol Discov. 2014; 2:3.
http://dx.doi.org/10.7243/2052-6180-2-3