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The evolutionary history of eukaryotes: how the ancestral proto-lineage conserved in hypoxic eukaryotes led to protist pathogenicity

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Abstract

The aim and the background of this paper was to investigate how ancient primitive eukaryotes evolved to the successful parasites they are today. In preparing this work, the most significant literature of the last years has been studied. We expand it by the results of our own research.

Results and conclusion: Anaerobic single-celled eukaryotes, such as *Entamoeba invadens* and *Giardia lamblia*, became successful invasive pathogens by using mechanisms inherited from the common ancestor (LECA). As described in previous papers pathogen protists have a surprising stem cell network (ancient protolineage) controlled by intrinsic and extrinsic mechanisms of cell conversion and differentiation inherited from the ancestor. Mechanisms leading to pathogenicity were not acquired after the organisms became parasitic, they were present before the single-celled organisms switched to parasitism. Only organisms possessing an extended ancestral stem cell system capable of switching between most oxidative (MO) and most hypoxic (MH) niches by changing metabolic pathways and antigenicity could develop into successful invasive pathogens like Entamoeba or Giardia. Related organisms not conserving all ancestral features evolved to become luminal commensals or free-living protists.

Keywords: *Entamoeba invadens*, stem cells, LECA, anaerobic metabolism, hypoxic and oxidative niches, commensalisms, parasitism

Introduction

Until recently, the evolutionary origin of stemness was largely unknown [1]. The central question has remained the same: did stem cells originate as single-celled organisms or not? [2]. It was Ernst Haeckel, 145 years ago, who first considered single-celled organisms as the phylogenetic ancestors of multi-celled organisms [3]. However, until recently protist researchers could not develop adequate model systems to elucidate the evolutionary stem cell history and the correlations between life cycle and the multi-cellularity of single-celled organisms went unrecognized.

A few years ago the earliest stem cell lineages were considered those that occurred in basal Metazoans such as Spongia and Cnidaria (Hydra). The lack of available data for protists led developmental biologists to hypothesize that multi-cellularity originates from a number of multifunctional cell types existing only in metazoan ancestors [4,5]. As a result, some authors considered populations of protists to be homogenous communities, where each individual was self-sustaining and all cells are identical [6]. Thus, the appearance of stem cells was considered as a significant developmental event that happened exclusively in basal metazoans.

The discovery of self-renewing stem cell lines in protist pathogens such as *E. invadens* [7-11] re-opens the discussion on the evolutionary origin of stemness (Niculescu, Researchgate Feb. 17, 2014) and helps us better understand mechanisms leading to the parasitic way of life. Only a few years ago our knowledge of multicellularity in protist pathogens such as *Entamoeba* and *Giardia* was still rudimentary. Switching from vegetative forms (trophozoites) to a dormant/resistant form (cyst) and vice versa was considered the only differentiation events occurring in protists. "Spontaneous" encystment observed in cultures rich of nutrients remained unexplained, as was why an organism such as *Entamoeba* has two kinds of trophozoites (magna and minuta). Similarly, VSP switching in *Giardia* was considered more a mutational antigenic advantage rather than

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a feature of differentiation.

In recent years, progress has been made in deciphering the stem cell biology of *Entamoeba*. Some years ago [8,9] we observed two different intra-specific self renewing stem cell lines (SRL) in [9] amoebic long term cultures (LT) grown in sediments with oxygen consuming bacteria (OCB niche). One is a more oxidative TD6^{LT} line that resides in most oxidative MO niches and the other is a more hypoxic line QD24^{LT} line residing in most hypoxic MH niches. In LT cultures TD6^{LT} proliferate only in the early growth phase as a non-continuous stem cell line, while QD24^{LT} is a continuous cell line, proliferating indefinitely in cultures and subcultures.

Materials and methods

Materials and methods were described in a previous paper [9]. Amoebae were cultured in hypoxic sediments with 5-10 mg metabolically repressed bacteria (OCB associates) and strong hypoxic sediments with 15 mg OCB. For terminal differentiation T stem cells were induced in strong hypoxic OCB sediments and hypoosmotic nutrient-free medium (AaEM).

Results

Hypoxia is the controlling factor for stemness and lineage development in *E. invadens*. It controls asymmetric and symmetric cell fate, cell line conversion and cell cycle length [8]. More oxic and light hypoxic conditions promote *E. invadens* stem cells to fast cycling while high and strict hypoxia leads stem cells to slow cycling respectively symmetric proliferation and cell cycle arrest in G2/M. In the Aa (Sm) cul tures of *E. invadens* hypoxia can be adjusted by the varying amount of OCB. The biochemical oxygen demand (BOD) of the microorganisms determines the pO_2 value in the niche. Amoebic phagocytosis reduces the number of OCB and indirectly controls hypoxia dynamics. The OCB sediments mimic in cultures the different stem cell niches for *E. invadens* present in the host.

In the present paper we hypothetized that the MH/MO stem cell lineage of *E. invadens* (**Table 1**) conserved an early eukaryotic stem cell protolineage developed by LECA and present our results, sustaining this hypothesis.

The primary stem cell lines p-SRL

Searching in primary cultures for lineage development and cell line hierarchy [8] we recently found the primary self renewing stem cell line (p-SRL) generated by the eight metacystic amoebulae (A2 cells) resulting from the totipotent innercyst cell. A2 amoebulae convert by intrinsic mechanisms (A/P conversion) into cycling primary stem cells (CSP) that form the p-SRL line. The primary stem cell line produced by asymmetrical division new cycling stem cells (CSP) and mitotic arrested sister cells (MAP) that are in fact reserve stem cells (RSC). Depending on passage conditions, oxidative stress and hypoxia in the new OCB niche, P cells convert in subcultures either to a secondary s-SRL stem cell line by P/S conversion (more oxidative conversion) or, to a tertiary t-SRL stem cell line by a more hypoxic P/T conversion.

The subsidiary stem cell lines s-SRL and t-SRL

The secondary s-SRL line is a MO stem cell line that proliferates only in low hypoxic niches. *In vitro* it grows only in the more oxidative growth phase of the culture (t0-t28/t30) and proliferates in 5-6 hrs by AGT5-6. In contrast to the s-SRL, the tertiary t-SRL line is a ubiquitous stem cell line proliferating in culture in all oxidative and hypoxic conditions (t0 t128). The t-SRL line cycled by AGT6 (fast cycling), AGT11-12 and AGT24 (slow cycling). Oxidative stress changed and CST proliferation from fast cycling to slow cycling and vice versa. The t-SRL is the only continuous stem cell line of *E. invadens* growing indefinitely in culture and hosts.

Terminal differentiation (encystment)

Mitotically arrested T cells (MAT) are reversible differentiated cells capable of cell cycle re-entry and cell line replenishing. When induced, they are capable of terminal differentiation, producing ITD cysts, while mitotically arrested S cells (MAS, precysts) encyst by autonomous terminal differentiation-without any other inducement-giving rise to ATD cysts by cyclic encystment in culture (CE). The ATD cysts were known previously as "spontaneously" occurring cysts.

Strict hypoxia and symmetric cell fate

In strict hypoxic niches, (**Table 2**) the cycling T cells (CST) switch to symmetric cell fate giving rise to with switched all cells to asymmetric cell fate cells. Both daughter cells proliferate by extreme slow cycling (AGT≥48). A slight oxygen increase during symmetric cell proliferation stopped cells at a G2/M checkpoint for cell fate (bifurcation point). At this checkpoint cells stop proliferation and wait for further decisions. When hypoxia increases again, cells continue strict hypoxic proliferation by symmetric division. Increasing pO_2 values and hypoxia decrease switched all cells to asymmetric cell fate and asymmetric division.

Disscussion

The ancient hypoxic-oxidative protolineage of *E. invadens* is a bridge between the ancestral stem cell system developed by the last eukaryotic common ancestor LECA and the highly evolved stem cell systems of metazoans, mammalians and humans. It has conserved intrinsic and extrinsic mechanisms of cell differentiation, inherited from the ancestor. The discovery of stem cell lines and stem cell lineages in a single-celled organism like *E. invadens* is surprising and showed that asymmetric cell fate and stemness appeared much earlier in the evolutionary history of eukaryotes than anyone has imagined. It is reasonable to assume that the last common eukaryotic ancestor (LECA) was therefore a more complex ancestor than previously thought. 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.

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Table 1. The stem cell system of *E. invadens*.

Cell type	Cell line	Conversion capacity	Cycling stem cells (D1)	Cell cycle duration (AGT in hrs)	Mitotic arrested cells (D2)	Terminal differentiated cells
P	p-SRL	P/S, PT	CSP	AGT/6-24	MAP (RSC)	--
T	t-SRL		CST	AGT/6-24	MAT	ITD cysts
S	s-SRL	S/T	CSS	AGT5-6	MAS (precyst)	ATD cysts (CE cysts)

Primary, secondary and tertiary stem cells (P, S, T); D1, D2, asymmetric daughter cells; D1 cells: self-renewing, cyclic stem cells (CSP, CSS, CST); p-SRL, s-SRL, t-SRL, self renewing stem cell lines; D2 cells: mitotic arrested, reversible differentiated daughter stem cells, as reserve stem cells (MAP and MAT, as RSC); ATD cysts, autonomous differentiated cysts; CE, cyclic encystment; ITD, hypoxic induced cysts; AGT, average generation time.

Table 2. The ancient stem cell protolineage of the hypoxic eukaryote *E. invadens*.

pO_2 value of the niche (in %)*	Hypoxic/oxidative range	Asymmetric/symmetric cell fate (A/S)	Stem cell lines (protolineage)	AGT (in hrs)	Proliferation
0.1-1.0	strict hypoxic (SH)	S	identical cells	AGT≥48	G2/M arrest
1.0 - 2.0	high hypoxic (HH)	A	t-SRL	AGT24	slow cycling
2.0 - 5.0	moderate hypoxic/ mild hypoxic (MH)	A	t-SRL	AGT11-12	--
		--	p-SRL	AGT<15	--
>5.0 (≤ 8.1 mg/l)**	low hypoxic (LH); light oxidative (LO)	A	s-SRL	AGT6	fast cycling

*pO_2 values according to Carreau et al., 2011; **oxygen concentration (dissolved oxygen) in 26°C warm water (http://water.epa.gov/type/rsl/monitoring/vms52.cfm).

Encystment occurs as the terminal differentiation step in prominent commensals and pathogens such as *Entamoeba, Naegleria, Acanthamoeba, Iodamoeba, Giardia, Leishmania* and *Balantidium* and also in free living exotics as *Blephyrisma*, *Pleurotrichia, Uroleptus, Didinium, Opistonecta* and *Colpoda* [12]. Some of them encyst "spontaneously" that means autonomous independent of whether nutritive reserves are abundant or not. *Colpoda* even produces two different types of cysts. One is the *reproductive*, independent cyst, produced under favorable conditions forming by successive divisions two, four or eight daughter cells. The second type is a *protective* cyst formed when food is lacking [13]. Asymmetric division and differentiation events occurring in the facultative pathogen ciliate *Colpoda cucullus* show convergence with the mechanisms of cell differentiation observed in the pathogen protist *E. invadens* [14]. *Colpoda's* two types of reproductive and protective cysts are further evidence that mechanisms of autonomous terminal differentiation (ATD) are ancestral and conserved in free-living and facultative pathogens also.

LECA and the hypoxic-oxidative protolineage

The above data suggest that the basic mechanisms for stem cell differentiation were evolved by LECA. According to Morrison et al., [15], there are basic common properties of stem cells which extend across many species and common molecular mechanisms shared by all stem cells. About 2000 MY ago during the early Paleoproterozoic age, the oxygenation began with cyanobacteria, that produced oxygen by anaerobic photosynthesis. By the end of Paleoproterozoic age LECA completes its basal development (1750-1600 MYA) [16] O_2 produced by cyanobacteria was absorbed in oceans and seabed rocks to iron-oxides. During the Meso-and Neo-proterozoic ages O_2 is released from the oceans but it absorbed by land surfaces. The free gas accumulation in the atmosphere began towards the end of proterozoic, as O_2 sinks filled (850 MYA, see "The great oxygenation event", Wikipedia). With the beginning of the late Neoproterozoic diversification, photosynthesis occurred oxygenically with a variety of eukaryotes producing oxygen as a major by product. Although eukaryotic branching began early in Proterozoic age, the explosion in the number and distribution of abundant marine eukaryotes began about 800 MYA [17]. Researchers believe that most of the organic matter (photosynthetic organisms, cyanobacteria relatives, algae and marine eukaryotes) remained suspended in the *upper oxygenated ocean* providing the mid-neoproterozoic plankton and the marine ecosystem. The organic material was exported into *anoxic sub-surface layers* and finally remineralized via anaerobic fermentation by sulfate- and iron reducing bacteria in the depth. Organic matter and O_2 export lead to *partially oxygenated sub-surface waters* and new ecological niches.

It may be, that in stratified nutrient-rich water columns moving between anoxic to progressively oxygenated niches rich in nutrients, LECA adopted an adaptative strategy using O_2 to optimize its metabolic pathways. The molecular machinery present in LECA at the time of the great Cambrian explosion

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is however largely unknown [18], but our experience with *E. invadens* [8,9] leads to the assumption that by switching back and forth between high, middle and low hypoxic sub-surface layers rich on nutrients, LECA needed alternative mechanisms for utilizing available resources and thus ensuring its survival. It converts between symmetric and asymmetric cell fate and differentiated cell lines for accelerated cell cycle, and autonomous encystment. At the end of this evolutionary period, LECA developed the first hypoxic-oxidative protolineage linking together the more oxidative neo-proterozoic mechanisms for stemness, asymmetric cell division and cell differentiation with the paleo-proliferation patterns of the symmetric cell division, identical daughter cells and slow cycling. It improved proliferation to fast cycling using the oxygen as a catalyst for its anaerobe metabolism.

Thus, at the begining of the Cambrian LECA was a "*complete" eukaryote* [19] possessing a modern nucleus and associated features such nuclear pore complexes, linear chromosomes and centromeres, nucleolus, endoplasmic reticulum, Golgi apparatus, mitochondria of alpha-proteobacteria origin and flagella, a complex cytoskeletal network based on actin and microtubulin, a complete endocytosis vesicle system, a modern cell cycle [20], stemness and sexuality: it underwent mitosis and meiosis. All hypotheses from the origin of eukaryotes believe that early eukaryotic lineages contributed to the modern eukaryotic genome either by vertical endosymbiotic gene transfer (EGT) or by diverse forms of horizontal gene transfer (HGT, LGT). Researchers agreed that the eukaryotic genome is a mosaic of archaea-related, bacteria-related and eukaryotic-specific genes. Genes from bacteria were transferred in the eukaryotic stem branch by EGT and HGT [20,34].

Fossils of the late Mesoproterozoic and early Neoproterozoic showed that most of the cytological and molecular complexities of eukaryotes evolved very early in the Proterozoic, but environmental conditions delayed their diversification within clades until oxygen and predation pressure increased significantly [21]. Javaux [21] makes a very complex presentation based on mesoproterozoic eukaryote fossils. She differentiates between early low-level characteristics and the high-level characteristics of the extant phagotropic heterotrophs and facultative aerobes at the time of the eukaryotic explosion. 1500-1300 MYA early mesoproterozoic fossils showed marked cytological, genetical and ecological complexities and attest to the evolution of multicellularity and cell differentiation in relation to environmental heterogeneity. LECA follows the vertical diversification of marine ecological niches (water column gradients), rather then simply horizontal distribution.

Paleoproterozoic and mesoproterozoic eukaryotes dividing by binary fission, had vegetative and resting stages (cysts) with more or less sophisticated excystment structures. First cyst structures were found 1800 MYA and were considered as a response to starvation. LECA molecular machinery evolved im Meso- and Neo-proterozoic from simple proliferation and differentiation mechanisms (binary fission, induced encystment) to more complex molecular mechanisms for asymmetric cell fate, multicellularity, stemness and vegetative differentiation. This evolution followed the progressive oxygenation and nutrient enrichment of ocean sub-surface layers. The new ecological niches from the mid-neoproterozoic lead LECA to stem cells, autonomous encystment and organized protolineages.

Commensalism and parasitism

Between branching and developing or adapting to the parasitic way of life bacterivorous Amoebozoa such as *Entamoeba* and heterotrophic Excavata such as *Giardia* occupied niches as commensals in association with oxygen consuming bacteria (OCB niches) [8,9]. While existing as *commensals,* representatives of Amoebozoa and Excavata underwent str-uctural and genomic developmental loss, reducing their no longer needed mitochondria to mitosomes [22]. In contrast to Lwoff's popular doctrine of regressive evolution, that states mitochondria and flagella loss, are a consequence of parasitism [23], it is today clear that the reductive development occurred much earlier in the evolutionary history, before the commensals switched to parasites [24]. Thus, more than 1000 free-living protists are amitochondriate [25], without passing through parasitic conversion.

The transition from the commensal to the parasitic way of life, succeeded only in luminal protists that could overexpress exoenzymes. Only protists with complex stem cell lineages and differentiation mechanisms as *E. invadens* and *E. histolytica* become pathogens, while commensals as *E. coli*, *E. dispar* and *E.moshkowskii* remain apathogenic. There are good reasons to expect that the pathogenic stem cell line is the t-SRL, a cell line living indefinitely in a wide scale of oxic and hypoxic niches. It is most capable of tissue invasion and pathogenicity. In contrast, the most oxic s-SRL stem cell line that encysts autonomously, is the non-continuous apathogenic stem cell line of *E. invadens*.

Cytopathic effectors causing host cell damage were reported by Mirelman [26]. Comparing cystein proteinase enzymes from non-pathogenic (*E. dispar, E. coli*) and pathogenic strains (*E. histolytica, E. invadens*) Seghal [27] concluded as early as 1996 *"that pathogenesis is determined more by quantitative levels of key molecules than by total absence of these in non-pathogenic species"*. In recent years new insights into tissue invasion mechanisms identified genes differentially- expressed in virulent and avirulent phenotypes [28-30]. Regulatory mec-hanisms for enzyme over-expression and down regulation are expected to occur in stem cell line differentiation.

Harboring in hypoxic and oxidative niches

Similar to their branching relatives, amitochondrial pathogens such as *Entamoeba* and *Giardia* are *hypoxic eukaryotes* capable of leaving and surviving in a wide scale of environments of different pO_2 levels, from strict hypoxic to light oxidative. The alternativ terms "*anaerobe"* or "*micro-aerophile*" are less suitable.

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These terms suggest leaving in anoxic niches (anaerobe) or in environments of moderate oxygen content (micro-aerophile). "*Hypoxic*" means a wide spread of pO_2 ranges below 5%. *Entamoeba* live and proliferates in luminal microenvironments and tissue niches varying their oxygen content (pO_2 between <1% and ≥5%). pO_2 is a key indicator of the physiological state of the host tissues, resulting from the balance between oxygen delivery and oxygen consumption. *E. histolytica* moves from luminal compartments and OCB niches into more oxidative intestinal tissues (4.4% to 7.6%±0.3% O_2)* and via blood transfer (5.3% to 13.2% O_2)* into liver (5.4%±0.7% O_2)* and muscle (3.8%±0.2% O_2)* [**31**]. Similarly, *E. invadens* invades in reptilia any part of the bowel and tissues, passages portal vessels and embolizes to liver (abcesses and tissue necrosis) and also in lung, heart, kidneys, brain and muscles, where tissue physioxia declines by oxygen deprivation, as a consequence of inflammation and pathogenic events.

mean values of pO_2

According to Carreau et al., 2011 [**32**] the terms currently used to distinguish between the tissue oxygenation levels were "physiological normoxia" or "organ physioxia", meaning 3% to10% pO_2 levels. Hypoxia spans the lower pO_2 ranges (1%-5% pO_2). Any researcher [**31**] can differentiate between mild and moderate hypoxia (2%-5%) and strict hypoxic condition (≤1%). Mammalian cells in cultures were commonly grown at higher oxygenation than organ physioxia (11%-19.5% pO_2). After exposure to hypoxia mammalian cells respond by producing hypoxia-inducible factors (HIF-α and HIF-1α) that control cell production and *cellular glucose uptake* [**33**]. In mammalian stem cells hypoxia is considered as favoring the stem cell state, as seen in multiple stem cell lineages residing in strict hypoxic niches at notorious pO_2 values between 0.1% and 1.0%. A characteristic of strong hypoxic niches (pO_2≤1%) is that stem cell proliferation decreases to slow-cycling or cell cycle arrest, due to regulation by HIFs. Higher oxygen tensions (2% to 5% O_2) do not affect the proliferation of human embryonic stem cells (ESC).

Conclusions

Undoubtedly, the pathogenetic success of *Entamoebae* originates mainly from the widely conserved oxidative-hypoxic protolineage and the multliple cell types specialized for an effective invasive infection that ends in the case of *E. invadens* by host death. Despite the limited life span of the non-continuous primary p-SRL, mitotic arrested P cells (MAP) survive for longer periods of time even in oxydative niches repressing cell cycle re-entry. When permissive environmental conditions are encountered, MAP cells restore secondary and tertiary stem cell lines that start new invasive waves. The most oxic s-SRL remains luminal and give rise to ATD cysts, as long as this non-continuous stem cell line proliferates and survives. Increased hypoxia converts S cells (CSS) into a continuous tertiary stem cell line t-SRL capable of tissue invasion in all oxygenic and less oxygenic tissues. CST cells produce permanently mitotic arrested MAT cells capable of phagocytosis. MAT cells are capable of re-entering the cell cycle until the host is killed. In hypoxic non-nutritive conditions MAT cells encyst to ITD cysts. In strong hypoxic niches CST cells switch to fast cycling and symmetric division giving rise to identical daughter cells.

The ancient oxidative-hypoxic protolineage inherited from LECA assures a successful parasitic life style in *E. invadens*, in that the continuous t-SRL stem cell line ensures invasiveness and pathogenicity and the non-continuous most oxic s-SRL cell line ensures rapid cyst formation. Mechanisms of hypoxic induced cell conversion and autonomous cell differentiation in nutrient-rich environments are inherited from the common ancestor (LECA). Parts of the oxidative-hypoxic protolineage and their mechanisms for asymmetric cell fate seemed to be conserved in other free-living and opportunistic pathogenic protists such as *Colpoda* [**14**] and others.

Competing interests

The author declares that he has no competing interests.

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