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Viable pluripotent chick blastodermal cells can be maintained long term in an alkaline defined medium

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Abstract Most chicken embryonic cell culture methods call for neutral pH media of different natures, with disregard of the peculiar electrochemical environment in which avian embryos develop, with a 4 pH unit gradient across the thin blastoderm and the vitelline membrane. We report results of a culture system in alkaline media (pH >9) with atmospheric conditions. Blastodermal and blood cells, maintained for 8 wk with minor differentiation in the absence of the standard growth factors, developed a thick, mucoid-like matrix in which a large proportion of the cell mass grew embedded, with no direct contact to cultureware. After up to 8 wk, blastoderm explants and dissociated blastodermal cells, cultured in either M199 or Dulbecco's modified Eagle's medium (DMEM) in the absence of supplemental CO_2, expressed several pluripotency markers (SSEA1, VASA) and embryoid bodies were formed. The assayed conditions impose an undoubted electrolyte stress on the cells which, notwithstanding, maintained their viability and remained undifferentiated. We hypothesize that a rise in pH and the activation of active cation exchanger like Na^+/H^+ antiporter could mediate the observed differentiation arrest.

Keywords Blastodermal cell culture · CO_2-free media · Chicken

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Introduction

Chick embryos are an important model for developmental biology research and a promising transgenesis platform for the production of a vast range of proteins (Han 2009). To that end, the possibility of culturing blastodermal and primordial germ cells that can be transfected, selected for transgene integration, and later propagated through germline chimeras has been sought by many researchers. A successful in vitro culture for these cells will also be of interest for germplasm preservation programs based on germline technologies where primordial germ cells of endangered breeds and species can be cryopreserved and later propagated through germline chimeras (Petitte 2006).

At oviposition or stage X (Eyal-Giladi and Kochav 1976), avian embryos already contain 40–60,000 blastodermal cells (BDCs), and it is around this time that their commitment to form different tissues is established. A small set of cells (less than 100), the primordial germ cells (PGCs), are clearly committed to form the germline, and there might be others which, at this stage, retain their pluripotency, that is, their ability to contribute to any somatic or germinal tissue (Pettite et al. 1990; Etches et al. 1997; Carsience et al. 1993; Thoraval et al. 1994; Kagami et al. 1997). Their propagation in vitro has been subject of numerous investigations (Etches et al. 1997; Pain et al. 1996). However, after a short time in culture, BDCs may rapidly differentiate loosing their potency. PGCs can also be isolated and cultured from embryonic blood at a further stage of development, HH14-16 (Hamburger and Hamilton 1951), when these cells migrate from the extra-embryonic germinal crescent to the primitive gonads, but their propagation in vitro entails similar problems (Naito et al. 1994; Tajima et al. 1993; van de Lavoir et al. 2006; Yasuda et al. 1992).

A number of different media supplementation strategies have been proposed for BDC culture whereby sera, various growth factors, hormones, or co-culture with other cell strains

facilitate cell multiplication without significant differentiation. This is a difficult compromise since high rates of cell division are usually associated to differentiation and the exact components halting differentiation are often unknown and, in some cases, variation from lot to lot is not predictable. At any rate, most research has been conducted with standard temperature (37–40°C), pH (7.0–7.4), and atmosphere conditions (5% CO_2) (Etches et al. 1997; van de Lavoir et al. 2006a; van de Lavoir et al. 2006b). The last two conditions do not mimic the environment where the early chick embryo develops: pH of egg albumen at the beginning of incubation remains substantially alkaline for several days (Reijrink et al. 2010), and CO_2 concentration in the air cell of freshly laid eggs is below 0.10% (Keener et al. 2000) due to a rapid loss of CO_2 immediately after laying. There is only one study investigating the effects of a media pH increase (from 7.2 to 8.0) which reported an enhanced short-term survivability of PGC cultures derived from the germinal crescent (Kuwana et al. 1996).

The aim of the present study was to assess the feasibility of BDC and PCG culture under alkaline pH in the absence of supplemental CO_2 or growth factors, at a very early stage of embryo development in which cell pluripotency allows for further propagation or for manipulation.

Materials and Methods

Egg source Freshly laid chicken eggs were obtained from Cobb SA (Alcalá de Henares, Madrid, Spain). Housing and management of the laying hens (*Gallus gallus*, Linn) comply with welfare and sanitary EU standards.

Albumen pH In order to mimic albumen's pH during the first hours of embryonic development, the pH of the albumen of unincubated fertilized eggs was monitored after oviposition and every 2 h for the first 8 h of incubation and twice a day thereafter for 8 d. Two sets of fertilized eggs were placed in an incubator (38°C; 65% RH; automatic turning, 90° at 1 cycle/h) and removed at pre-established intervals for albumen pH measurement. The eggs were cracked open, the albumen was poured in a clean beaker, and the pH was measured with a Crison GLP21 pH meter. For each set and sampling time, two or three eggs were used.

Culture of BDCs and PGCs BDCs from 30 stage X embryos were plated on both Nunc (15–18 and 35 mm, cat no. 143982 and 150318) and Falcon (35 mm cat no. 353801) culture dishes, either as explanted whole embryos or after dispersing cells with accutase (PAA, Dartmouth, MA, L11-007) according to the manufacturer's instructions for cell detachment from cultureware, and maintained in plain M199 (Sigma M4530) or Dulbecco's modified Eagle's medium (DMEM) (Sigma D5546) media with antibiotic/anti-fungic (Sigma A5955), at 37°C and 40–50% relative humidity, in atmospheric conditions with no CO_2 addition. The media were completely replaced every 3 d for 2 wk and every 10 d thereafter. The media recovered from each well were filtered and frozen.

After 1 mo in culture, blastodermal-like cells (BDLCs) derived from explanted blastoderms were subcultured by gently detaching them from approximately one fourth of the well surface and transferring them to a new well where they were maintained in culture for an additional month. The remaining BDLCs, which over this first month in culture had colonized the plate off the embryo, were not detached but left in culture on the same original well. Wells seeded with accutase-dispersed BDCs were also maintained for 2 mo with no subculturing, having their media refreshed on days 3, 6, 9, 12, 15, 25, 35, 45, and 55.

Bloodborne PGCs were cultured from approximately 2–4 μl of whole blood taken from the dorsal aorta of 20 HH14-15 chicken embryos. Blood from a single embryo was seeded on each well and kept in culture in the conditions described above. Frequency of media changes was as described for BDC cultures.

Immunocytochemistry of cultured BDCs and PGCs After stopping the cultures, pluripotency of cultured cells was assessed by an alkaline phosphatase (AP) immunocytochemical detection system using anti-SSEA-1, anti-dVASA (DSHB; Ames, IA), and anti-cVASA (kindly provided by Dr B Pain; Lyon, France). Briefly, BDLCs and blood cell cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h at 4°C. To minimize non-specific binding, the fixed cells were treated for 3 h with 3% BSA and 0.1% Triton X-100 in PBS before immunostaining. The optimal concentration of each antibody was selected based on the results of preliminary experiments (1/300–1/500 for anti-SSEA1, anti-dVASA, and anti-cVASA antibodies). Cells were incubated for 24–48 h with the primary antibodies at 4°C and subsequently reacted for 24 h each with biotinylated anti-mouse (Sigma B0529, for monoclonal first antibody) or anti-rabbit (Sigma B5283, for polyclonal: anti-c-VASA) immunoglobulins, followed by AP-conjugated streptavidin (Sigma E2636). Fast Red was used as substrate chromogen. To avoid interference by the potential endogenous activity of AP, the cells were treated with 0.02 M levamisole or heat inactivated at 70°C for 30 min.

Antibodies against neural (anti-NeuN monoclonal (Millipore, Ontario, Canada), anti-tubulin IIIβ monoclonal (Millipore), anti-tubulin III TUJ 1 polyclonal (Covance, Princeton, NJ), anti-L1-CAM monoclonal, anti-NgCAM polyclonal) oligodendrocyte and astrocyte (anti-O4 monoclonal, anti-glial fibrillary acidic protein (GFAP) polyclonal (Dako, Carpinteria, CA)) markers, along with anti-mouse

Alexa 488 or anti-rabbit Alexa 568 as secondary antibodies (2 h at room temperature) were also used to identify these cell types. Cells were observed under an inverted fluorescence microscope (Nikon, Eclipse TE300; Tokyo, Japan, fitted with a H910104A, TE-FM Epi-Fluorescence Lamp).

In some wells, toluidine blue was used as a counterstain for immunocytochemistry assays and to reveal the presence of glycosaminoglycans/proteoglycans. Briefly, paraformaldehyde-fixed cells were permeabilized using 1% DMSO and 0.1% Triton X-100 in PBS (four 5-min washes). After washing with PBS, toluidine blue was added at 0.1% for 3 min.

Trypan blue and fluorescence Yolk granules present in various types of blastoderm cells, including PGCs, were labeled with trypan blue. Once the bottom of the wells was covered by attached blastoderm cells, 4 μl of trypan blue 0.4% was added and incubated overnight on the aforesaid culture conditions. Binding of trypan blue to yolk confers red fluorescence to the cells (Callebaut 1983, 1984 and 2005) which can be observed under inverted fluorescence microscope (Nikon, Eclipse TE300).

Results

Albumen pH The pH of albumen from fertilized eggs reached maximum values slightly above 9.50. For the first 3 d of incubation, pH ranged between 9.0 and 9.5 and remained above 8 by day 7 (Fig. 1).

Culture and immunocytochemistry of cultured BDLCs and PGCs Cultures of stage X BDC, either as whole embryos or as dispersed cells, were successfully maintained for at least 2 mo with M199 medium. They reached more than 90% confluence after 24 h in culture, with the cell layer covered by a thick, mucoid-like coat. Best results were obtained with M199xFalcon or Nunc (6×4, 15 mm) plates, intermediate results with M199xNunc (35 mm), very poor with DMEMxFalcon, and unsuccessful cultures with DMEM and Nunc (35 mm).

Trypan blue produced a scattered stain on whole explants with the corresponding sprinkled blue spots among the cells that, off the explants, had colonized the wells. In plates seeded with accutase-dispersed embryos, trypan blue stained a variety of cell types, with different morphologies, evenly distributed throughout the plates (Fig. 2*a*), among them duplets (Fig. 2*a'*), putative endophyll cells (Fig. 2*b*, *c*, *c'*), and putative PGCs (Fig. 2*d*, *e*, *e'*).

Between 24 and 72 h, round openings appeared in most plates, surrounded by a cell dense rim which gradually evolved to form craters (Fig. 3*a–d*). Some formed by rapid accumulation of liquid beneath the cell layer and the development of a round button-like embossment which eventually detached from the cell layer leaving the nude plate surface visible. Others appeared in regions where cells had previously formed hilly cumuli. In many instances, the circumferences were grouped (Fig. 3*e*). Also, in reseeded plates from BDC, almost perfect circles were formed, delimited by a single-cell palisade that remained without appreciable changes until the cultures were stopped. In trypan blue-treated wells, the cells forming these rims and craters showed blue-stained, fluorescent intracellular granules (Fig. 4*b*, *b'*). However, no such formations were found when accutase was used to disaggregate stage X embryos or when cultured on DMEM.

Cell aggregates resembling endophyll or polyinvaginated cells—defined as clumps of cells of 30–50 μm with strong carbohydrate component in their surface (Bellairs and Osmond 2005)—were observed after the second day of culture in plates growing explanted whole embryos (Fig. 2*b*, *c*, *c'*) which showed red fluorescence (Fig. 2*c'*). Cells reseeded after 1 mo in culture continued their growth on the new plates. Alike newly isolated BDCs, a thick mucoid matrix was formed with some cells attached to the dish surface and some embedded in the mucoid matrix, although only 50–70% of the plate surface was covered by cells, compared to >90% after the initial seeding. Among cells attaching to the plate after reseeding, a high proportion showed the characteristic bluish color and red fluorescence of trypan blue (Fig. 2*e*, *e'*).

Blood cells from HH14-15 embryos ($n=20$) were successfully cultured, with most of the erythrocytes in suspension disappearing after 10 d. In all wells, embryoid bodies (one to three per well) as well as SSEA1-, dVASA-, and cVASA-positive cells (48±24.2 cells per field when examined with ×40 magnification) were observed (Fig. 5*b–d*). Duplets of cells undergoing mitosis could be found in both blood (Fig. 5*a*) and BDLC cultures (Fig. 2*a'*).

Neuron-like cells, with a morphology resembling different types of nervous cells, developed in some plates. Figure 6 shows fields with neuron-like cells of different shapes and sizes, with axons of differing thickness and degree of ramification, as well as putative sensory cells. On the latter, toluidine blue stain revealed thick ciliary expansions on soma and axons, the latter ending in a well-defined growth cone (Fig. 7). Most of these neuron-like cells showed fluorescence when immunoassayed for NeuN, indicating a clear neural origin (Fig. 6*a'*, *b'*). However, axons were not clearly stained with either neuron-specific (tubulin IIIβ, L1CAM, and NgCAM) or glial cell-specific (GFAP and O4) antibodies.

Discussion

The unusual culture conditions assayed in this work succeeded in maintaining a mixed population of BDCs, including putative PGCs, for several weeks. Two important features differed from previously reported culture conditions: (1)

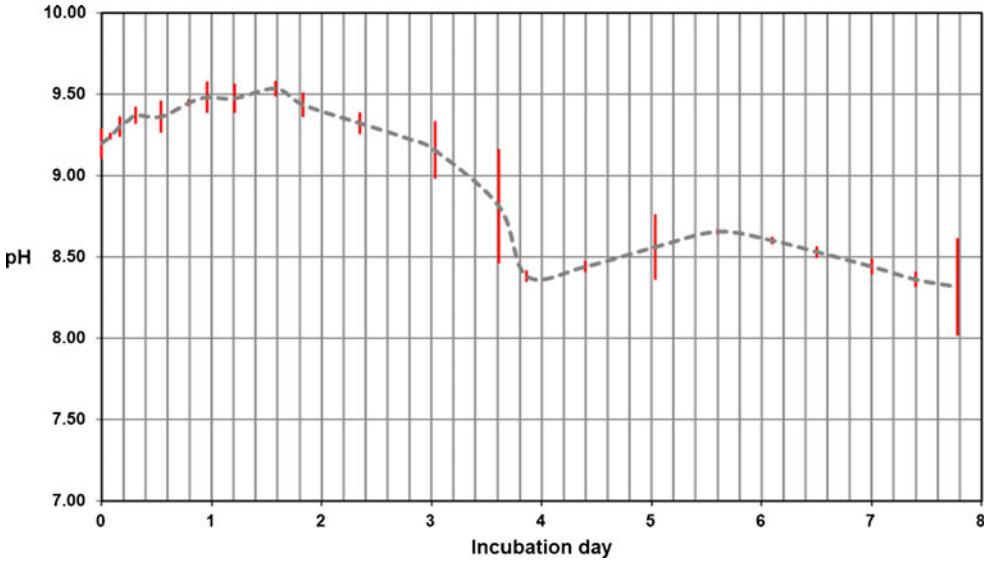

Figure 1. The pH of albumen of fertilized eggs was measured every 2 h for the first 8 h of incubation and every 12 h thereafter. pH peaked at 9.5 during the second day of incubation and remained above 8 by day 7.

The only nourishment provided was medium M199 or DMEM, with no serum, growth factors, or interleukins added, and (2) no CO_2 was supplied. After the first 7 d in culture, the cell number and their morphological appearance remained with no substantial change for the rest of the time.

Proliferation of avian stem cells in culture with no significant differentiation has been achieved only when a variety of growth factors, stem factors, and leukemia inhibitory factors are supplemented to the media. Etches et al. (1997) cultured chick BDCs in M199, as in the

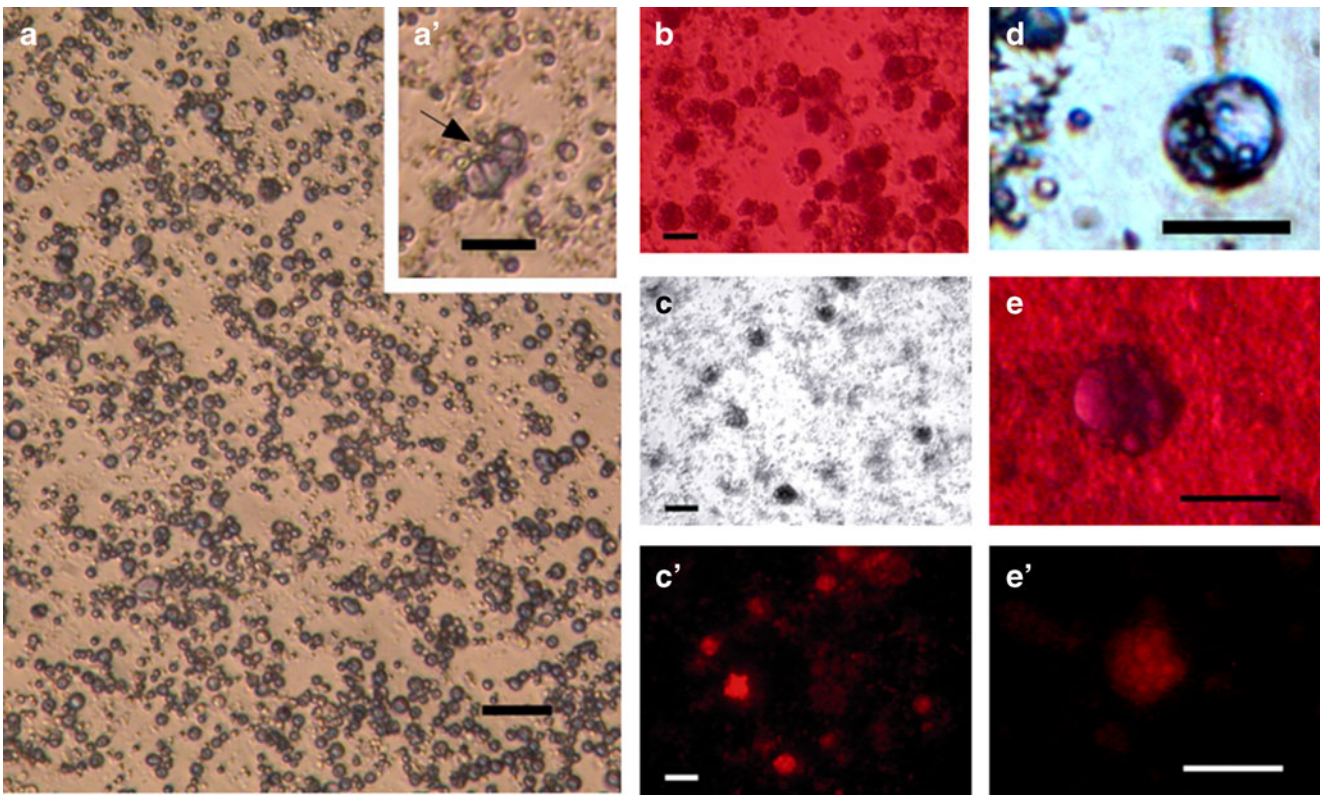

Figure 2. Cell suspensions obtained by accutase digestion of stage X chick blastoderms were cultured in M199 media, in the absence of supplemental CO_2 or specific growth factors, and stained with trypan blue. *a* Cells 72 h after accutase digestion and overnight trypan blue incubation (*bar* 100 μm). *a'* Recently divided duplets among cells in *a* (*arrow*; *bar* 50 μm). *b*, *c*, *c'* Putative endophyll cells (*c'* red fluorescence in *c*; *bar* 50 μm). *d*, *e*, *e'* Putative PGC (*e'* red fluorescence in *e*; *bar* 50 μm).

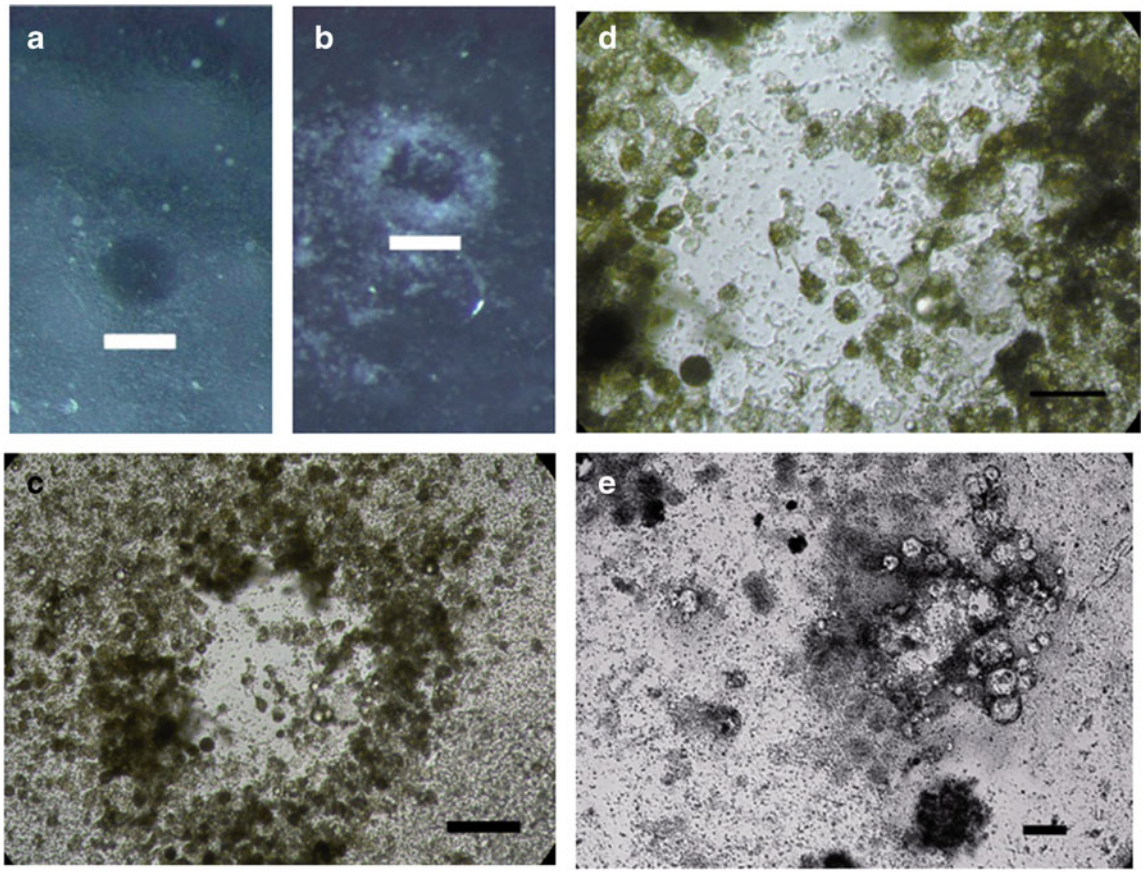

Figure 3. After reaching confluence, chick blastodermal-like cells cultured in M199, in the absence of supplemental CO_2 or growth factors, a variable number of circular openings which evolved to form a sort of craters developed. (*a*) Stereomicroscope image of a round, flat opening. (*b*) A crater formed by a thick rim of cells around a nearly round central area where cells had disappeared (*bar* 250 μm). (*c*, *d*) Bright field of *b*, where the engrossed rim can be appreciated (*bar* 200 and 100 μm, respectively). (*e*) Aggregate of circular structures similar to that shown in *b* (*bar* 200 μm).

present study, but with the addition of fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), stem cell factor (SCF), and insulin-like growth factor-1 (IGF-1). Although successful PGC cultures have been also reported on feeder-free systems, the need for additional growth factors remains (i.e., bFGF FGF2, insulin, and activin A) (Choi et al. 2010; Whyte et al. 2015). By contrast, our culture conditions allowed the maintenance of BDCs on a nearly quiescent state of development, an important feature as these cells might retain the ability to contribute to somatic tissues and to the germline of chimeras for different purposes. A large proportion of cells in our cultures grew embedded in the mucoid matrix, with no direct contact with the plasticware, a known key feature halting differentiation in cell cultures (van de Lavoir and Mather-Love 2006).

The registered albumen pH values in the incubated eggs are in full agreement with previous reports that, upon incubation, egg white pH rises sharply above 9.0 for the first 2 d and looses alkalinity progressively to almost neutrality by day 14 (Dawes 1975). More recently, Kuwana et al. (1996) report albumen pH values between 9.0 and 9.7 during the three initial days of embryo development while values remained greater than 8.0 up to day 6. Despite being known for a long time (Romanoff and Romanoff 1929; Sharp and Powell 1931), this feature of the early embryo environment has not been implemented in in vitro systems for BDC culture. In our experiments, the used media without supplemental CO_2 maintained the BDLCs in alkaline environment. Its pH drifted to a mean value of 9.6, close to albumen's pH in fertilized eggs at least during the first 3 d of incubation.

It is accepted that, despite this remarkable alkalinity of the albumen, pH in the subgerminal fluid is closer to neutrality (7.4) while that of yolk is clearly acidic (6.5). The embryo is supposed to maintain this strong pH gradient across the thin blastoderm and vitelline membrane by continuously pumping H^+ out of the albumen into the subgerminal fluid (Stern 1991). However, mean pH of the interstitial fluid of early chick embryos is 8.3 according to the only available report (Gillespie and McHanwell 1987). The culture conditions used in our experiments might not reproduce the complex electrochemical environment of the avian embryo, but they impose an

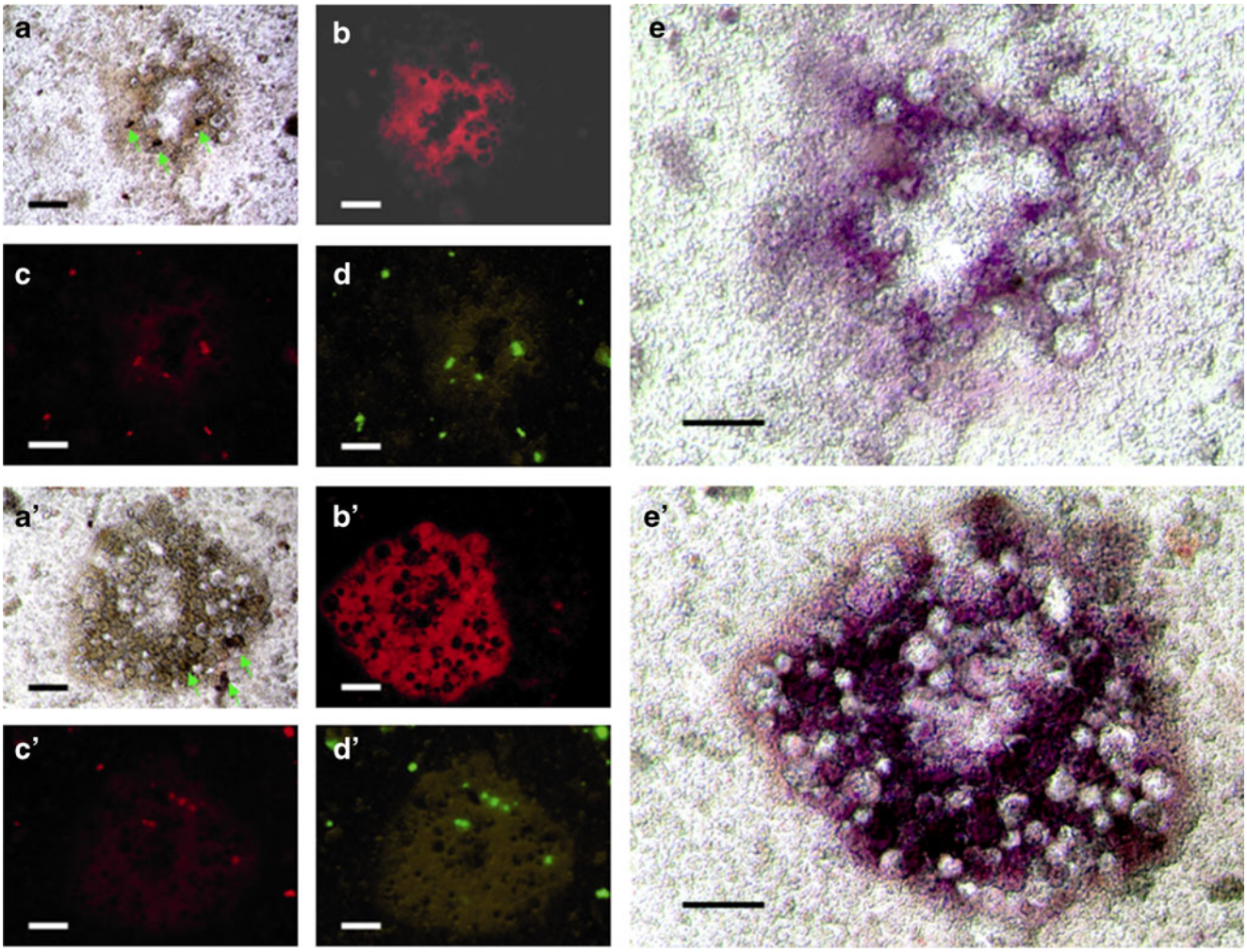

Figure 4. SSEA1-positive cells (*a*, *a'*, *green arrows*) on aggregates of circles and craters formed on chick blastodermal-like cultures where cells that had taken up trypan blue showed a characteristic red fluorescence (*b*, *b'*). NgCAM+ (*c*, *c'* Alexa 568, red fluorescence) and Olig O4+ (*d*, *d'* Alexa 488, green fluorescence) cells were also present in these formations. *e*, *e'* Toluidine blue stain (*bar* 200 μm).

electrochemical stress of a similar nature upon the cultured cells. It is possible that an increased Na/H antiporter activity had an anti-differentiation effect in our cultures, since it has been attributed a role in cell response to apoptosis suppression factors (Rajotte et al. 1991) and in cancer development (Stock et al. 2012).

Kuwana et al. (1996) reported a transitory favorable effect of alkaline media on PGC survival (increasing viability from 5.75% at neutral pH to 95% at pH 8) and succeeded in long-term culture of cells from chick embryos at different stages (from BDCs—stage 1—up to gonadal cells from hatching males). However, no further development of their strategy has been explored since. In our work, a higher alkalinity of media, more closely resembling the basic environment of the early developing embryo, resulted in cells maintaining pluripotency markers at least after 2 mo in culture, while their number and appearance barely changed. Avian embryos are adapted to stand diapause, a temporary suspension of active development after oviposition despite adequate temperature, water potential, or gaseous environment (Ewert 1993), through a variety of mechanisms. The stimuli which trigger and terminate diapause in the avian embryo are not well understood, but electrochemical environment could be an important factor on it. It is possible that under alkaline conditions in our cultures, growth of certain cell populations is delayed while others multiply.

The viability of our cultures is indicated by two main observations. After reseeding, the cells attached to the plate and they developed the same thick mucoid matrix seen initially after plating. Duplets of cells that had recently undergone mitosis were observed after initial plating and after reseeding.

We applied trypan blue to our cultures trying to reproduce, after Callebaut (1983; 1984), the selective labeling of certain cell lineages carrying γ and δ ooplasm granules in their cytoplasm. He identified that these two components of the oolem were very selectively taken up by cells in the endophyll and in Rauber's sickle, with PGCs being an important fraction of them. In our experiment, we hypothesized that these cells,

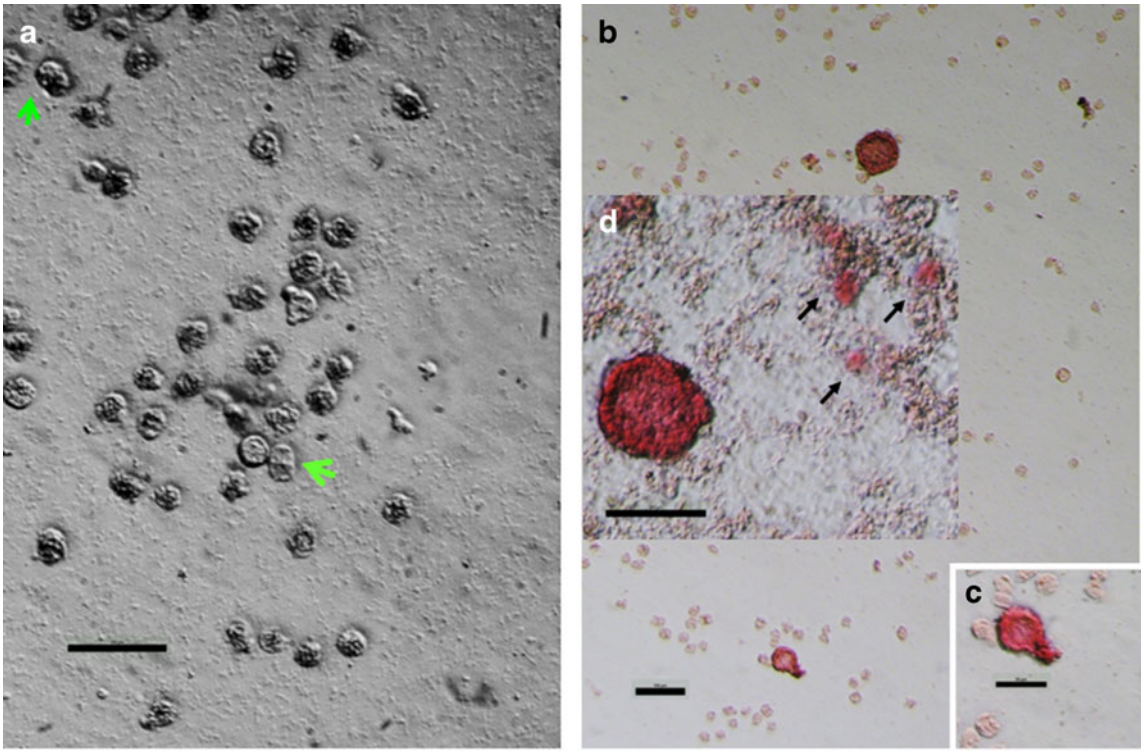

Figure 5. Blood from HH14-15 chick embryos was cultured in M199 in the absence of supplemental CO_2 or specific growth factors. (*a*) After 1 wk in culture, cell divisions were observed in non-erythrocyte cells (duplets, *green arrows*; *bar* 100 μm). (*b–d*) After 10 d, embryoid bodies could be observed and characterized as SSEA+ (*b bar* 100 μm; *c bar* 50 μm) and dVASA+ (*d bar* 100 μm). Isolated dVASA+ cells were also observed (*d*, *black arrows*).

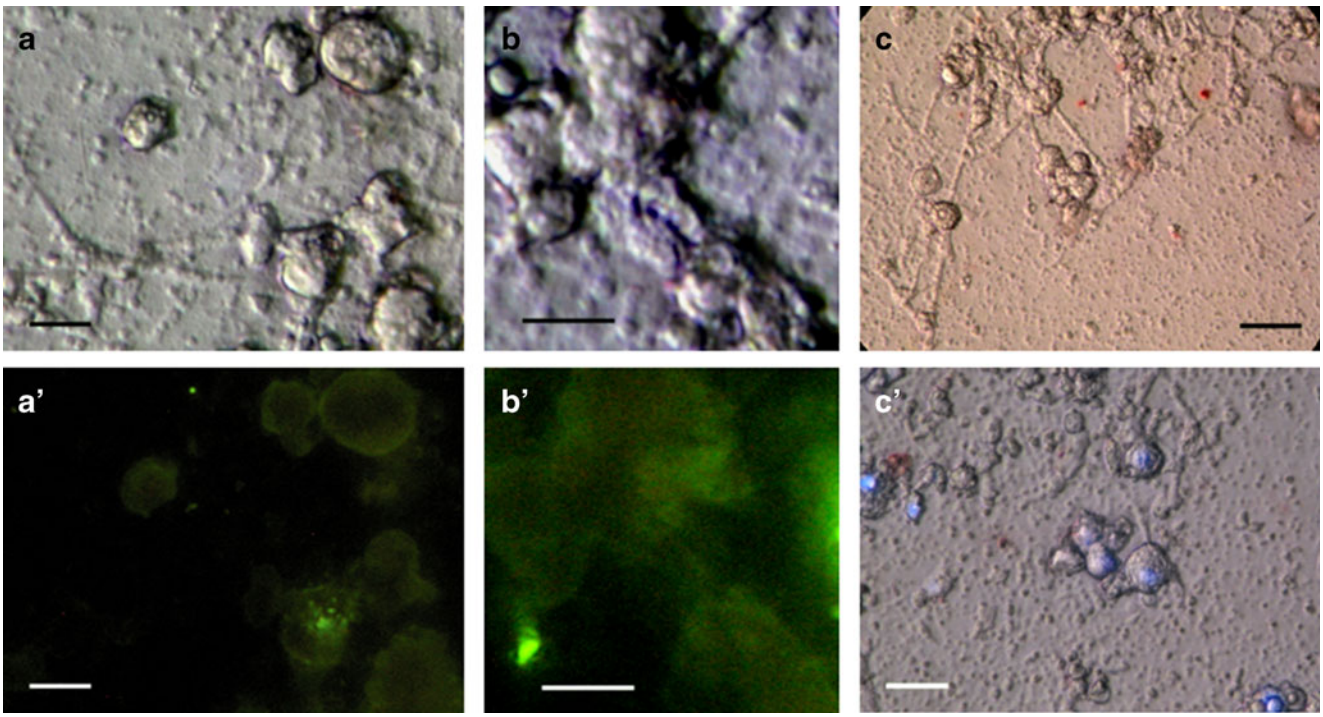

Figure 6. Neuron-like cells were observed in chick blastodermal-like cell cultures. Among them, anti-NeuN+ sensory-like cells (*a*, *b* bright field; *a'*, *b'* green fluorescence in the nuclei of a big and a small cell) and sensory-like cells negative for β tubulin III (*c* bright field; *c'* DAPI on nuclei but no tubulin III labeling). *Bars* 100 μm.

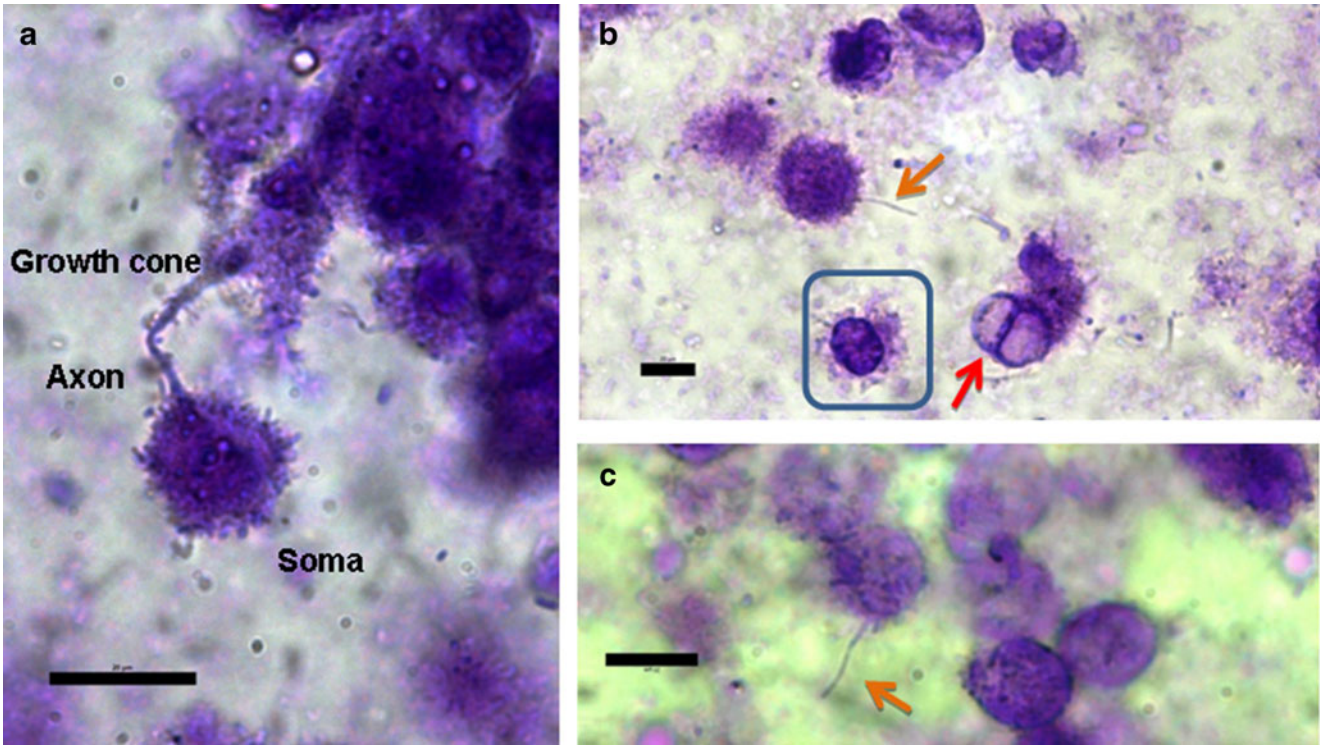

Figure 7. Toluidine blue stain of sensory-like cells developed in chick blastodermal-like cell culture in M199 in the absence of supplemental CO_2 or specific growth factors; bright field showing axons (*orange arrows*, *b*, *c*), duets (*red arrow*, *b*) and ciliary projections (*blue box*, *b*). *Bars* 20 μm.

containing intracytoplasmic materials with a strong affinity for trypan blue, would retain the label for a long time and would therefore be easily traced in culture. Our labeling procedure was probably too long and yielded a very large number of labeled cells; probably, 20–40% of all cells showed a blue stain. A minor fraction had dark blue, vacuole-like structures with a distinct red fluorescence. Among the latter are, most likely, Callebout's labeled cells, but they are only a small subset of all cells labeled.

Several remarkable features were observed in our cultures. The circular openings and craters, developed in all plates with BDLCs, started to show up the day after the seeding and were clearly shaped by day 7. This type of formations resembling blood islets was surprisingly regular in shape and, to our knowledge, has not been described before. Those initially appearing as round embossments could probably form as a result of the activation of the systems for water transport into the subgerminal cavity, associated with the maintenance of pH and ion gradients in the developing embryo (Stern 1991). In those BDCs treated with trypan blue, the blue color was also evident in the cells forming circumferences which conferred them a red fluorescence. The possible angioblastic nature of these structures could not be tested, as no good markers are available for chick angioblasts, but their shape and timing support the hypothesis of a vascular or endothelial nature. Additional support is the finding that these were intensely stained with toluidine blue (Fig. 4*e*, *e'*), known to bind proteoglycans in vascular connective tissue (Domenga et al. 2004).

Intermingled with these cells, a few SSEA-1-positive cells could also be evidenced. The possible co-localization of putative PGCs and angioblasts, or other endothelium-forming cells, supports the idea of a common precursor for all of them. Based on his studies on the origin of PGCs and fate of endophyll cells, Dubois (1967, 1969) hypothesized that precursors that he called "presumptive PGCs" divide giving rise to one PGC and one somatic cell which, in our opinion, could have a vascular fate. This is also in agreement with recent findings (Zhou et al. 2009) that PGCs are the common ancestry of angioblasts and hematopoietic cells. This strategy of PGCs being present where and when blood islets and vessels originate would be the best guaranty for their bloodborne migration to the primitive gonads. Finally, specific labeling for neural markers (NgCAM and O4) was also demonstrated on these formations, indicating that they are not just isolated foci of abnormal cell growth, but highly organized structures developed by the interaction of different cell types.

When blood cells from stage HH14-15 embryos (the period when PGCs migrate from the cephalic region to gonadal ridge) were cultured under these conditions for, at least, 1 mo, putative PGCs and structures resembling "embryoid bodies" (EBs) were observed, both expressing SSEA1 and cVASA, which supports their pluripotency. These hotchpotches are commonly obtained by culturing stem cells in hanging drops, a system allowing the aggregation of ESCs. Other studies have developed EBs using embryonic germ cells (EGCs) from gonads of Beijing Fatty Chicken (Wang

et al. 2010) and duck (Guan et al. 2010) grown in suspension with co-culture system, serum, and wells covered with gelatin. By contrast, our system provides not only a simplified procedure for EB development and culture, but also a strong indication that our culture conditions are appropriate for stem cell handling.

Different cell lineages have been reported to derive from BDC cultures, among them hematopoietic, neuronal, and muscular cells. With the starting explants and the culture conditions used in our trials, neuron-like cells were observed. NeuN was shown in the nucleus of most small neuron-like cells and few large ones (Fig. 6). This, along with their characteristic morphology (Fig. 7), supports the conclusion that these are neural cells.

The fact that axon projections were not stained for any of the most representative markers such as tubulin IIIβ, L1CAM, NgCAM, GFAP, and O4 is not surprising, since the expression of these antigens in chick embryonic nerve cells begins when organogenesis is already initiated, no earlier than day 4. Our neuron-like cells, morphologically coincident with cells reported by other studies, could be very early, barely differentiated nerve cells. This is consistent with the overall slow differentiation of our cultures. Finally, toluidine blue stain revealed a typical sensory cell morphology, particularly resembling retinal ganglion cells, although other cell types such as photoreceptors and terminal neurons unable to regenerate and cannot be ruled out.

In summary, the assayed alkaline, CO_2 un-supplemented, defined culture media were capable of maintaining chick embryo blastodermal and blood cultures for 2 mo. The fact that pluripotent cells as well as EBs were maintained for this length of time suggests that our conditions were able to slow down/contain differentiation. However, complex structures, which might be associated to early angiogenesis, and numerous neuron-like cells were also present that could not be fully characterized. Additional research is also needed to verify the viability of these cells after freezing/thawing, a key feature for further applications of this culture strategy.

In conclusion, culture conditions mimicking the extreme alkaline environment when early avian embryos develop succeeded in maintaining a population of undifferentiated embryonic cells even in the absence of the various growth factors and differentiation inhibitors commonly used for their expansion in vitro. The probable activation of Na/H transport systems might be one of the reasons why differentiation was, to a significant degree, halted.

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