DEVELOPMENT OF THE EFFECTIVE STRATEGY USED FOR ESTABLISHMENT OF MITOTICALLY STABLE LINES OF EMBRYONIC STEM CELLS DERIVED FROM BLASTODERM ISOLATED FROM IN VIVO-FERTILIZED EGGS OF DOMESTIC FOWL (GALLUS GALLUS DOMESTICUS)

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Abstract

Pluripotent blastoderm-derived embryonic stem cells (B-ESCs), isolated from domestic fowl (Gallus gallus domesticus) embryos at stage X, provide not only an innovative model for studying avian embryonic development but are also becoming increasingly important for safeguarding populations outside their natural environment and preserving the genetic diversity of poultry species and breeds. The main objective of this study was to develop an efficient protocol for: (1) isolation of chicken blastodermal cells (BCs); (2) establishment of stable embryonic stem cell (ESC) lines from these cells under ex ovo conditions; and (3) characterization of the proteomic profile of B-ESCs based on selected pluripotency biomarkers, namely the transcription factors Oct-3/4 (octamer-binding transcription factor-3/4) and Sox2 (sex-determining region Y (SRY)-box 2). After isolation from fertilized eggs at stage X, BCs were expanded ex ovo to establish stable blastoderm-derived ESC lines. During long-term in vitro culture of B-ESCs comprising at least 15 passages, their adhesive and proliferative capacities were analysed in relation to the culture vessels used. The presence of specific protein markers of pluripotency (Oct-3/4 and Sox2) was detected by Western blotting and immunofluorescence staining. During long-term culture, domestic fowl blastoderm-derived ESCs retained morphology typical of embryonic stem cells. Expression of selected biomarkers of the pluripotent stem-cell phenotype was also confirmed by identifying the proteomic and immunofluorescence profiles of two

representatives of the homeotic transcription factor family (Oct-3/4 and Sox2), containing a highly conserved DNA-binding homeodomain with a characteristic 60-amino-acid helix-turn-helix motif. The results clearly demonstrate that the use of 8-well Ibidi glass microplates coated with the synthetic polymeric substrate poly-L-lysine and polystyrene culture dishes with bottoms coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes) significantly affects the proliferative capacity of domestic fowl B-ESC lines.

Keywords: domestic fowl, blastodermal cells, embryonic stem cells, pluripotency markers, homeotic transcription factors, Oct-3/4, Sox2

Introduction

Research focused on establishing and cryogenically safeguarding cell lines of embryonic origin is gradually becoming, and in the relatively near future may constitute, an integral component of biotechnology-assisted ex situ conservation and genetic rescue of various poultry species and breeds, including the domestic fowl (Gallus gallus domesticus) (Carsience et al., 1993; Blackburn, 2006; Hu et al., 2022). In addition, the development of ex ovo systems that maintain stable cytokinetic conditions and generate a mitogenic and anti-cytostatic microenvironment conducive to stimulation of the proliferative activity of cells of embryonic origin (e.g. blastoderm-derived stem cells or primordial germ cells) provides an alternative to ex vivo research models based on cultures of dermal fibroblast cell lines or mesenchymal stem cell lines derived from tissues and organs of juvenile or sexually mature domesticated birds (Wu et al., 2008; Guan et al., 2010; Na et al., 2010; Bai et al., 2011, 2013; Nandi et al., 2016; Svoradová et al., 2018). Domestic fowl eggs laid approximately 24 hours after fertilization therefore constitute an excellent bioreservoir of blastodermal cell subpopulations (BCs) derived from the embryonic disc (embryodisc). In the poly- and telolecithal eggs of birds, partial discoidal (meroblastic) cleavage at the animal (formative) pole produces embryos at the blastula/discoblastula or gastrula stages. Isolated blastodermal cells are, in turn, an ideal source for establishing mitotically stable, permanent lines of embryonic stem cells (ESCs) of the domestic fowl (Petitte et al., 1990, 2004; Xiong et al., 2020). A measurable benefit of the comprehensive implementation of species-specific reproductive biotechnology and embryonic genetic engineering based on establishment, ex ovo culture and banking of blastoderm-derived ESCs (B-ESCs) is the durable ex situ biotechnological conservation and enhancement of genotypic biodiversity of rare native conservation breeds of selected poultry species, including the domestic fowl (Samiec and Trzcińska, 2022; Sun et al., 2022).

Taking into account all of the above-mentioned agrobiotechnological, biological, animal-science and species-specific ornithological considerations, the overarching objective of the research was to develop and optimize new procedures aimed at creating an in vitro model for:
1) isolation of BCs from fertilized domestic fowl eggs;
2) ex ovo establishment of stable ESC lines from isolated BC subpopulations;
3) expansion in vitro of the established blastoderm-derived ESC (B-ESC) lines;
4) characterization of the proteomic profile of selected pluripotency biomarkers (Oct-3/4, Sox2) in mitotically stable B-ESC lines.

Material and methods

1. Isolation of BCs from embryonic discs of fertilized domestic fowl eggs, establishment of primary cultures and blastoderm-derived ESC (B-ESC) lines, and determination of their cytokinetic parameters depending on the use of different types of culture vessels

In the experiments, fertilized eggs were stored until BC isolation in a room cooled to 17–18°C. Immediately before BC isolation, the eggs were washed with 70% ethanol to maintain aseptic conditions. Embryonic discs were isolated from eggs at stage X of embryonic development using filter-paper rings. Fifteen fertilized eggs were used in each of three experimental replicates. After isolation, the discs were rinsed into sterile 15-mL conical centrifuge tubes using 1×PBS without Ca2+ and Mg2+ ions (CMF-PBS; calcium/magnesium-free phosphate-buffered saline; pH 7.2). Using sterile Pasteur pipettes, the cells were mechanically dispersed to obtain a homogeneous suspension. The resulting cell suspension was then centrifuged for 10 min at a relative centrifugal force of 90×g at room temperature. After removal of the supernatant, the cell pellet was resuspended in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Merck), supplemented with 1.5% fetal bovine serum (FBS; Sigma-Aldrich, Merck), 2.0% (v/v) B-27™ Plus supplement (Thermo Fisher Scientific; a serum-free, chemically defined next-generation supplement containing, among other components, antioxidants and trophic-energy substrates that provides high in vitro survival and supports high proliferative activity, self-renewal and maintenance of the undifferentiated status of embryonic stem cells in monolayer culture), 2.0 µL/mL SCF (Thermo Fisher Scientific; stem cell factor) and a penicillin-streptomycin antibiotic cocktail (Pen-Strep; Sigma-Aldrich, Merck). Enrichment of the expansion medium, designed to stabilize mitotic divisions and maintain the undifferentiated phenotype, stemness and self-renewal of B-ESCs, by simultaneous supplementation with 1.5% FBS (a mitogen-rich, chemically undefined supplement whose concentration was markedly reduced from 10.0% to 1.5%) and 2.0% B-27™ Plus (a chemically defined supplement dedicated to the specific requirements of primary embryonic cells) was dictated by the unique cytokinetic and molecular properties of blastoderm-derived embryonic stem cells and by literature data (Vanhoutteghem et al., 2004; Horiuchi et al., 2006; Zhang et al., 2018). Moreover, appropriate concentration levels and a suitable quantitative ratio of the two supplements (1.5% FBS; 2.0% B-27™ Plus) provided complementary and synergistic effects of these multicomponent, multimitogenic factors on the cytophysiological parameters of B-ESCs and helped stabilize the molecular properties associated with blocking their structural-functional reprogramming and cytodifferentiation under ex ovo conditions (Couteaudier et al., 2015; Zhang et al., 2018; Xiong et al., 2020).

The concentration of seeded cells in 1 mL of suspension was determined using a TC20™ Automated Cell Counter. Blastodermal cells were seeded into culture vessels at a density of 3×10^5 cells/mL, initiating primary B-ESC cultures from which mitotically stable embryonic stem cell (ESC) lines/strains were established by passaging that doubled the cell subpopulations (minimum 15 passages). B-ESC cultures were maintained in an incubator under strictly controlled thermal-atmospheric conditions (38°C, 5% CO2 in air, maximum relative humidity).

To determine optimal culture conditions, different culture vessels and different types of substrates coating the bottom surfaces of the vessels were used. The experimental design therefore comprised two series of experiments. In the first series, cell cultures were established and maintained in: i) SPL Life Sciences Cell Culture Flasks (25 cm²) with a polystyrene substrate (Fig. 1A); ii) Nunc™ EasYFlask™ Cell Culture Flasks (25 cm²) with a Δ-type polystyrene bottom surface that increases cell adherence and electrostatic attraction to the substrate (Fig. 1B); and iii) sterile high-walled 8-well Ibidi microplates with a glass bottom coated with the synthetic polymeric substrate poly-L-lysine (µ-Slide 8 Well high Poly-L-Lysine) (Fig. 1C). In

the second series, cell cultures were established and maintained in 60-mm-diameter polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes) (Fig. 1D). Photomicrographic documentation of cultured B-ESCs was obtained using an inverted microscope (Nikon Ti-U) equipped with a Nikon DS-Fi1c-U3 camera (Tokyo, Japan). After evaluation of cytokinetic parameters (cell adhesion and proliferative growth, as well as the capacity to reach full confluence and contact inhibition), the established blastoderm-derived ESC lines were cryopreserved in containers filled with liquid nitrogen (LN2).

2. Assessment of the proliferative activity of blastoderm-derived ESCs using a nucleotide analogue (BrdU)

To estimate the proliferative potential of the cells, a reagent kit for a colorimetric proliferation assay based on 5-bromo-2′-deoxyuridine (BrdU; Roche, REF 11647229001) was used (Kolb et al., 1999; Liboska et al., 2012). To prepare the test solution, 7.1 µL of BrdU was dissolved in 710 µL of culture medium. Subsequently, 10 µL of this solution was added to each well (except the control) of a 96-well plate containing cells seeded 24 h earlier (10,000 cells/100 µL culture medium/well), and also to the blank, followed by incubation for 2 h. The test solutions were then removed from the wells, the plate was blotted on filter paper and placed in a drying oven (60°C, 1 h). Next, 200 µL of FixDenat fixation/denaturation buffer was added per well and incubated at room temperature for 30 min. During this time, specific anti-BrdU antibodies conjugated with peroxidase (anti-BrdU-POD conjugates) were dissolved in 1.1 mL H2O; 100 µL of this solution was then diluted in 10 mL Antibody Dilution Solution. After 30 min, the solution was removed, the plate was blotted dry, and 100 µL per well of diluted anti-BrdU antibodies conjugated with peroxidase (anti-BrdU-POD) was added and incubated for 90 min at room temperature. During this period, 10 mL of Washing Buffer was diluted with 90 mL H2O. The antibodies were then washed three times with 100 µL per well of diluted Washing Buffer; each time the solution was shaken out and the plate was blotted on filter paper. Subsequently, 100 µL per well of Substrate Solution was added and incubated at room temperature for 10 min. Absorbance was then measured using a Thermo Scientific Varioskan LUX spectrophotometer with SkanIt Software 7.0 RE. Absorbance detection was set at an absorption/excitation wavelength of $\lambda ab/ex = 370$ nm and an emission wavelength of $\lambda em = 492$ nm, and readings were taken 10 and 15 min after addition of Substrate Solution.

2.1. Statistical analysis of experimental results obtained using the BrdU-dependent colorimetric proliferation assay for B-ESCs cultured in vitro

Statistical analysis was performed in Microsoft Excel 365 using the Analysis ToolPak add-in. The absorbance value of the medium (blank) was subtracted from the absorbance values of the technical replicates. Extreme results—one maximum and one minimum value—were removed to reduce the standard deviation. Mean absorbance values at $\lambda ab/ex = 370$ nm and $\lambda em = 492$ nm were then calculated for the technical replicates, followed by mean values for the biological replicates. The resulting absorbance values at the emission wavelength $\lambda em = 492$ nm were subtracted from the absorbance at the absorption/excitation wavelength $\lambda ab/ex = 370$ nm. Standard deviations were calculated. Statistical differences were analysed using Student's t-test, with the significance level set at 0.05 ($\alpha = 0.05$). The measured absorbance values for the control samples (cells + anti-BrdU-POD peroxidase antibody conjugates) were at the same level as those for the blanks (culture medium + BrdU + anti-BrdU-POD peroxidase antibody conjugates), indicating specific activity of the antibodies used.

3. Western blot-based analysis of the B-ESC proteomic profile to confirm stem-cell characteristics and pluripotency based on identification of the expression of the marker proteins Sox2 and Oct-3/4 from the group of homeotic transcription factors

3.1. Isolation of total protein from blastoderm-derived ESCs

To isolate total protein from B-ESCs cultured in μ-Slide 8 Well high Poly-L-Lysine slides and SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes, the culture medium was removed from the vessels. After three washes of the cells with PBS, RIPA buffer (Radioimmunoprecipitation Assay; Thermo Fisher Scientific), intended for cell lysis and total protein extraction, together with protease inhibitors (10 μL/mL lysis buffer; Sigma-Aldrich, Merck), was added to the culture wells. The culture vessels were placed on ice for cytolysis and, after 5 min, adherent cells were mechanically detached from the poly-L-lysine- or collagen-coated substrate using a cell scraper (Biologix Group Limited). The resulting cell suspension was transferred to conical microcentrifuge tubes. The samples were then subjected to ultrasonic disruption (lysis and extraction) and cell homogenization, i.e. sonication, by three 5-s pulses at 50 V. After sonication, the samples were kept on ice for 10 min and then ultracentrifuged at 15,000 rpm for 15 min at +4°C. Supernatants containing the isolated total protein were frozen at –80°C.

3.2. Measurement of total protein concentration by the Lowry method

Total protein concentration in thawed samples was determined using the DC™ Protein Assay Kit II (Bio-Rad Laboratories) according to the Lowry method. The principle of the method involves chemical reduction of copper cations in complex compounds from the Cu2+ oxidation state to Cu+, resulting in a blue colour of the samples. Colour intensity was measured spectrophotometrically at an emission wavelength of 750 nm (λem = 750 nm). A standard curve was prepared from bovine serum albumin solutions (BSA; Sigma-Aldrich, Merck) in RIPA buffer. Standard samples were transferred to 96-well microplates. Absorbance at λem = 750 nm was measured using an Infinite® F50 plate reader (Tecan Group Ltd.). Protein concentration in the test samples was determined from the standard curve.

3.3. Polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

For all samples, a volume containing the equivalent of 30 μg protein was measured. When the sample volume exceeded 20 μL, the samples were concentrated in a cooled vacuum concentrator (LABCONCO) by solvent evaporation. When the sample volume was too small, it was supplemented with an additional volume of RIPA lysis/extraction buffer containing protease inhibitors. To each sample, 19 μL of 2× Laemmli Sample Buffer and 1 μL β-mercaptoethanol (Bio-Rad Laboratories) were added. The samples were then centrifuged. To accelerate denaturation, protein samples were placed in shaking thermoblocks at 250 rpm and 100°C for 5 min. After incubation, the samples containing denatured proteins were cooled to room temperature. Electrophoresis was carried out in a polyacrylamide gel. Electrophoretic separation of the protein mixture was performed for 120–150 min in a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad Laboratories) at a constant voltage of 70 V for the stacking gel and 110 V for the resolving gel.

3.4. Electrotransfer of proteins onto a membrane

After electrophoresis, proteins were transferred from the gel to a polyvinylidene fluoride membrane (PVDF Immobilon®-P; Sigma-Aldrich, Merck). The membrane was activated in methanol for 5 min and then rinsed in distilled water and equilibrated for 3 min in transfer buffer (GTB; Genie Transfer Buffer). Proteins separated in a 10% gel were transferred to the membrane using a semi-dry transfer method. Whatman filter papers pre-soaked in GTB were used to sandwich the gel and membrane. Electrotransfer was carried out for 10–15 min at a constant current of 1300 mA using a Thermo Scientific Pierce Power Blotter (Thermo Fisher Scientific). After transfer, the membranes were washed five times for 3 min on a rocking shaker in TBST (Tris-Buffered Saline with Tween 20; Sigma-Aldrich, Merck).

3.5. Protein detection

The PVDF membrane was incubated in 5% skimmed milk prepared in TBST for 1 h on a rocking shaker at room temperature to block nonspecific antibody-binding sites. After incubation, the membrane was washed five times for 3 min in TBST on a rocking shaker. Each membrane received 5 mL of antibody solution at the specified concentration (Table 1), prepared in TBST containing 1% BSA, and the membranes were incubated overnight at 4°C. After incubation, the membranes were again washed five times for 3 min in TBST. Each membrane was then supplied with 5 mL of horseradish peroxidase-conjugated (HRP) secondary antibody solution (Table 1), prepared in TBST. A further 1-h incubation was performed on a rocking shaker at room temperature. After 1 h, the membrane was washed while shaking three times for 3 min in TBST and three times for 3 min in Tris-buffered saline (TBS).

Table 1. Comprehensive characterization of primary and secondary antibodies used for Western blot (W-B) analysis

Antigen	Primary antibody	Secondary antibody
Sox2 Sex-determining region Y (SRY)-box 2	polyclonal rabbit anti-Sox2 1:500 Abcam	goat anti-rabbit HRP-conjugated 1:2000 Vector Laboratories
Oct-3/4 Octamer-binding transcription factor-3/4	monoclonal rabbit anti-Oct-3/4 1:500 Abcam	goat anti-rabbit HRP-conjugated 1:2000 Vector Laboratories
β-actin	monoclonal mouse anti-β-actin 1:2000 Sigma-Aldrich	goat anti-mouse HRP-conjugated 1:3000 Bio-Rad Laboratories

3.6. Detection of proteins following SDS-PAGE

Using a luminol-based chemiluminescence method (Clarity™ Western ECL Blotting Substrates, Bio-Rad Laboratories), sites on the membrane where antibody-antigen binding had occurred were visualized. First, the membrane was incubated with the reagent for 90 s at room temperature in complete darkness. After incubation, the PVDF membrane was placed in a

ChemiDoc XRS+ system (Bio-Rad Laboratories). Results were generated using ImageLab 4.0 software (Bio-Rad Laboratories).

3.7. Antibody removal ("stripping") with reference to the β-actin loading-control protein

The stripping procedure is used to remove primary and secondary antibodies directed against the proteins under study. It involves eluting membrane-bound antibodies with a low-pH buffer (pH = 2). In this case, β-actin served as the reference protein. β-actin detection was carried out on the same membranes to which the antibodies had previously been applied.

The stripping procedure was initiated by washing the PVDF membranes three times for 10 min in TBS on a rocking shaker. After washing, the membranes were incubated in stripping buffer for 30 min. They were then washed twice for 10 min on a rocking shaker, followed by one 10-min wash in TBST. In the subsequent stripping stages, the membrane was handled in the same way as after electrophoresis: blocking in milk was initiated and, in the final stage, a primary antibody directed against β-actin was added.

3.8. Processing of the results of Western blot-based proteomic assessment of B-ESCs

Western blot analysis of the investigated proteins was repeated twice. Images of each membrane were evaluated using ImageLab 4.0 software (Bio-Rad Laboratories). Band thickness was measured and normalized to the measurement obtained for the reference protein (β-actin).

4. Immunofluorescence analysis of B-ESCs to confirm their stem-cell characteristics and degree of pluripotency

Immunofluorescence staining aimed at detecting stem-cell pluripotency biomarkers, namely the homeotic transcription factors Sox2 (sex-determining region Y (SRY)-box 2) and Oct-3/4 (octamer-binding transcription factor-3/4), was performed using the primary antibodies employed in the Western blot analysis. Details of the primary and secondary antibodies are provided in Table 2. After removal of the culture medium from the wells, the cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min at room temperature. The fixative was then removed and the cells were washed with TBS. After washing, the cell membranes were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Merck) in TBS for 30 s at room temperature. The wells were then emptied and 200 µL of blocking solution was added to each well (details in Table 2). After 40 min of incubation, the blocking solution was removed and primary antibodies were applied. Incubation was carried out for 24 h at +4°C. On the following day, the primary antibodies were removed and the preparations were washed five times for 3 min in TBST on a rocking shaker, after which secondary antibodies were applied (Table 2). Following 1 h of incubation in the dark at room temperature, the preparations were washed again three times for 3 min in TBS on a rocking shaker and mounted in a gel containing a mixture of the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) and Vectashield reagent, which prevents premature fading of fluorescence signals and extends the half-life of excited fluorochrome molecules (DAPI-VECTASHIELD® HardSet™; Vector Laboratories). The preparations were imaged using an OLYMPUS FV1200 FLUOVIEW scanning laser confocal microscope (OLYMPUS, Tokyo, Japan) (imaging parameters in Table 3).

Table 2. Comprehensive characterization of primary and secondary antibodies used for immunofluorescence analysis

Antigen	Blocking solution	Primary antibody	Secondary antibody
Sox2 Sex-determining region Y (SRY)-box 2	5% normal goat serum Sigma-Aldrich	polyclonal rabbit anti-Sox2 1:50 Abcam	goat anti-rabbit Alexa Fluor 488-labelled 1:500 Thermo Fisher Scientific
Oct-3/4 Octamer-binding transcription factor-3/4	5% normal goat serum Sigma-Aldrich	monoclonal rabbit anti-Oct-3/4 1:100 Abcam	goat anti-rabbit Alexa Fluor 488-labelled 1:500 Thermo Fisher Scientific

Table 3. Parameters for imaging cellular structures with an OLYMPUS FV1200 FLUOVIEW confocal microscope

Fluorochrome	Laser (DIODE)	Absorption/excitation wavelength (λab/ex); emission wavelength (λem)
Alexa Fluor 488	473 nm	λab/ex = 473 nm; λem = 520 nm
DAPI	405 nm	λab/ex = 405 nm; λem = 461 nm

Results

After 96 h of culture in SPL Life Sciences polystyrene culture flasks (Fig. 1A) and Nunc™ EasYFlask™ flasks (with a Δ-type surface; Fig. 1B), BCs began to detach from the substrate en masse despite the previously observed progression of adhesion and proliferative growth in vitro. By contrast, after 15 passages of ESC expansion using 8-well Ibidi glass microplates coated with the synthetic polymeric substrate poly-L-lysine (μ-Slide 8 Well high Poly-L-Lysine; Fig. 1C) and plastic culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes; Fig. 1D), total protein was isolated to determine the proteomic profile of selected transcription factors governing pluripotency of the established B-ESC lines by Western blot (W-B) and immunofluorescence analysis of pluripotent-cell marker proteins Oct-3/4 (octamer-binding transcription factor 3/4) and Sox2 (sex-determining region Y (SRY)-box 2) (Figs. 2 and 3).

Effect of the culture substrate on the proliferative activity of domestic fowl blastoderm-derived ESCs

The BrdU assay is a procedure that quantitatively reflects DNA synthesis and thus cell proliferation. 5-Bromo-2′-deoxyuridine is a synthetic analogue of the nucleoside thymidine that is incorporated into DNA in dividing cells during the S phase of the cell cycle. Incorporation of BrdU molecules and their specific binding (addition) to nuclear DNA occurs through repeated substitution reactions in which bromine atoms from BrdU molecules replace methyl groups attached to carbon atoms at position 5 (5-C) of thymidine (Kolb et al., 1999). Incorporated BrdU can be detected using a specific antibody conjugated with the enzyme peroxidase (anti-BrdU-POD conjugate) and its reaction with the added substrate. This reaction enables colorimetric analysis of the proliferative capacity of the cells under study (Liboska et al., 2012).

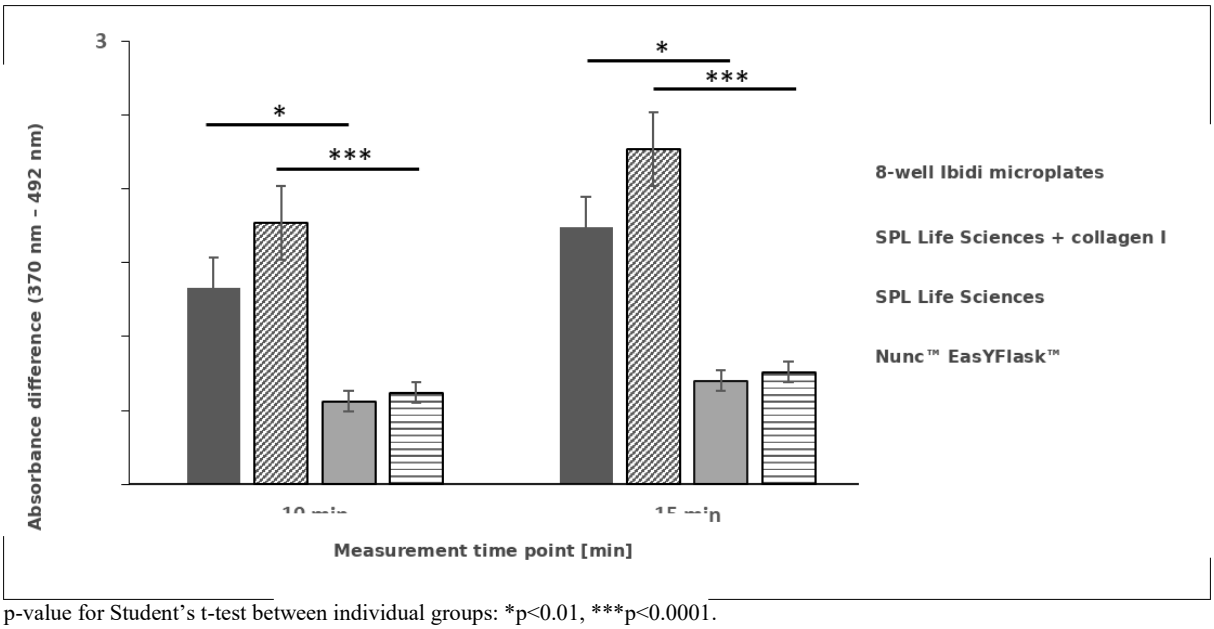
The proliferation-assay results were calculated from absorbance measurements taken 10 and 15 min after addition of Substrate Solution and are presented in Table 4 and Diagram 1. No statistically significant differences were found between cells cultured in SPL Life Sciences Cell Culture Flasks with a polystyrene substrate and cells grown in Nunc™ EasYFlask™ Cell Culture Flasks with a Δ-type polystyrene bottom surface that increases cell adherence and electrostatic attraction to the substrate. Likewise, no statistically significant differences were found between cells grown in high-walled 8-well Ibidi microplates with glass bottoms coated with the synthetic polymeric substrate poly-L-lysine and cells grown in 60-mm-diameter polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes).

The analyses showed a statistically significantly higher level of proliferation in cells cultured using 8-well Ibidi microplates and type I collagen-coated culture dishes than in cells cultured either in SPL Life Sciences Cell Culture Flasks with a polystyrene substrate or in Nunc™ EasYFlask™ Cell Culture Flasks with a Δ-type polystyrene bottom surface.

Table 4. Difference obtained by subtracting the mean absorbance at the emission wavelength λem = 492 nm from the mean absorbance at the absorption/excitation wavelength λab/ex = 370 nm, and Student's t-test analysis of statistical differences between mean values for B-ESCs growing in different culture vessels at 10 and 15 min after addition of Substrate Solution

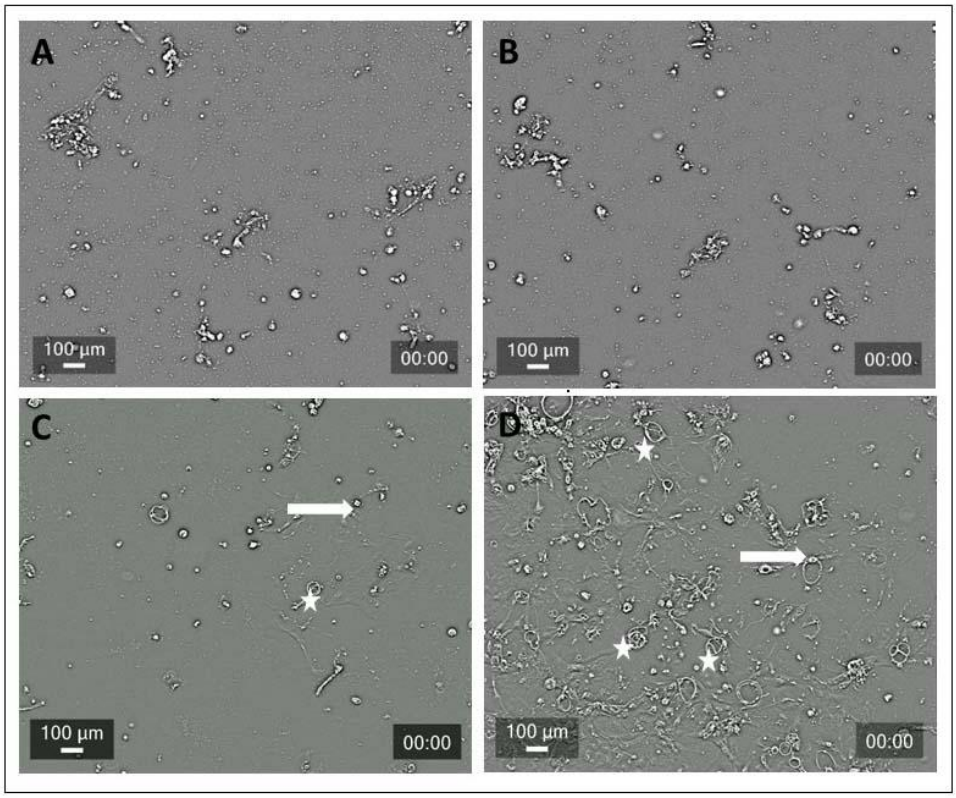
Type of vessels/substrates used for in vitro culture of B-ESCs	**Mean absorbance difference [nm] at 10 min**	**Student's t-test**	**Mean absorbance difference [nm] at 15 min**	**Student's t-test**
8-well high-walled Ibidi glass microplates coated with poly-L-lysine µ-Slide 8 Well high Poly-L-Lysine: 1.5 polymer coverslip	1,33 ± 0,55	0,11	1,74 ± 0,65	0,09
SPL Life Sciences polystyrene culture dishes coated with type I collagen SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes	1,77 ± 0,54		2,27 ± 0,60	
SPL Life Sciences plastic culture flasks with a polystyrene substrate SPL Life Sciences Cell Culture Flasks	0,56 ± 0,30	0,69	0,70 ± 0,35	0,72
Nunc™ EasYFlask™ plastic culture flasks with a Δ-type polystyrene bottom surface Nunc™ EasYFlask™ Cell Culture Flasks	0,62 ± 0,37		0,76 ± 0,44	

Diagram 1. Difference in absorbance for B-ESCs growing in different culture vessels 10 and 15 min after addition of Substrate Solution



p-value for Student's t-test between individual groups: *p<0.01, ***p<0.0001.

Figure 1. Development of an in vitro model for establishing domestic fowl blastoderm-derived embryonic stem cells (B-ESCs) to determine the physicochemical and biological conditions of their isolation, ex ovo adhesion and proliferation

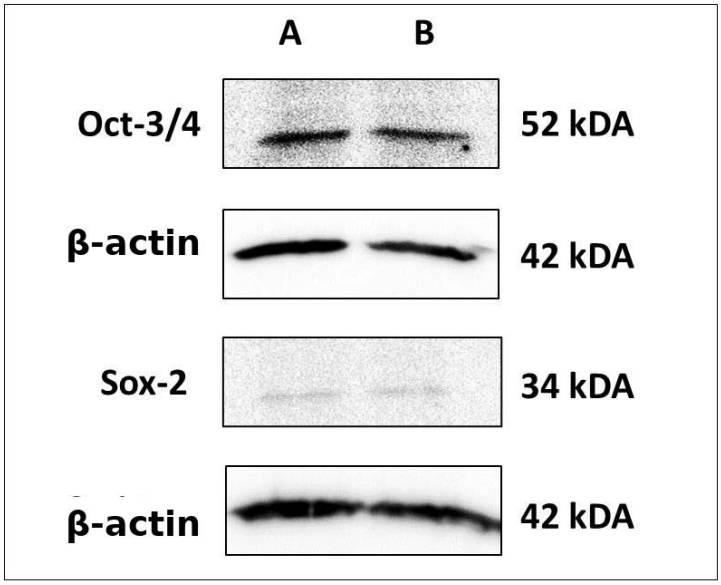


Analysis of proliferative growth and cytological assessment of domestic fowl blastoderm-derived embryonic stem cells (B-ESCs) using a CELLCYTE X scanning microscope (CELLINK Life Science) after 7 days of in vitro culture in: A) SPL Life Sciences Cell Culture Flasks (25 cm²) with a polystyrene substrate; B) Nunc™ EasYFlask™ Cell Culture Flasks (25 cm²) with a Δ-type polystyrene bottom surface; C) 8-well Ibidi glass microplates coated with the synthetic polymeric substrate poly-L-lysine (µ-Slide 8 Well high Poly-L-Lysine: 1.5

polymer coverslip); and D) 60-mm-diameter polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes). In adherent cultures maintained in 60-mm-diameter polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes) (D), B-ESCs proliferating as a monolayer were observed. In cultures maintained both in 8-well Ibidi glass microplates coated with poly-L-lysine (µ-Slide 8 Well high Poly-L-Lysine: 1.5 polymer coverslip) (C) and in SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes (D), B-ESCs showed characteristic large nuclei (asterisks); nucleoli could also be identified in the ultrastructural image of the nuclear nucleoplasm, most often located close to the inner layer of the nuclear envelope (arrows).

To identify the pluripotent character of B-ESCs, expression of selected protein markers of pluripotency (Oct-3/4, Sox-2) was demonstrated in total protein isolated by ultrasonic cell disruption (lysis) from maintenance cultures of cell lines/strains subjected to at least 15 passages (population doublings) (Fig. 2). The presence of both homeotic transcription factors was confirmed, demonstrating the pluripotent nature of the B-ESCs.

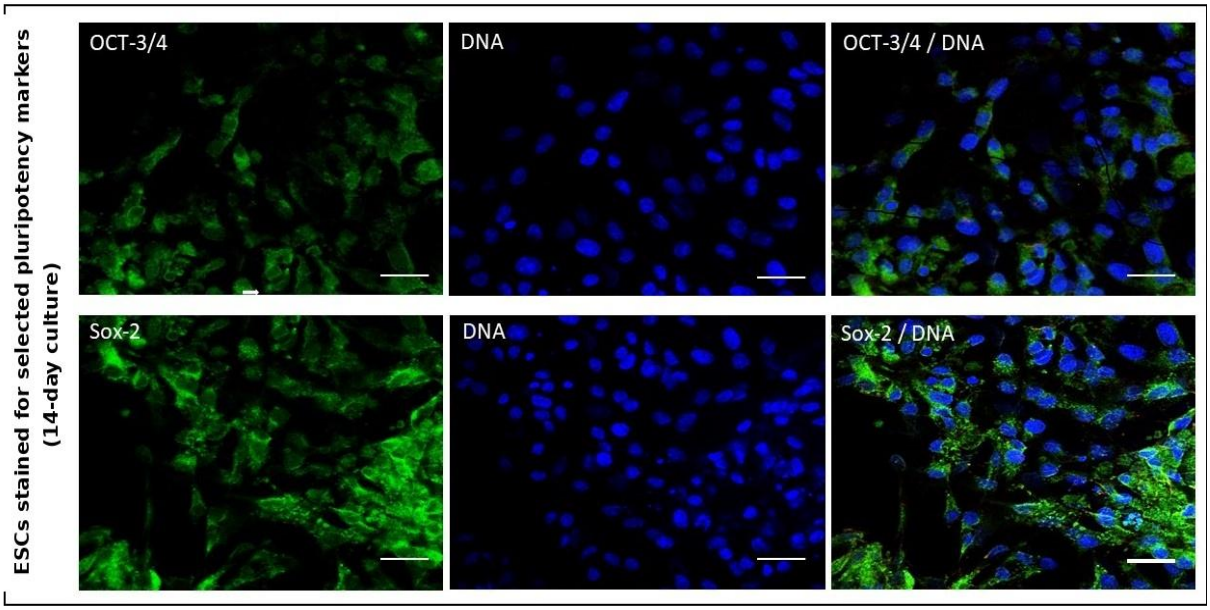
Figure 2. Development of an in vitro model for establishing domestic fowl blastoderm-derived embryonic stem cells (B-ESCs) to determine the proteomic profile of selected transcription factors (Oct-3/4; Sox-2) responsible for stable maintenance of ESC pluripotency ex ovo



Semi-quantitative Western blot (W-B) analysis of the pluripotency biomarkers Oct-3/4 and Sox-2 at the total-protein level, isolated from blastoderm-derived ESCs at approximately passage 15 of in vitro culture using 8-well Ibidi glass microplates coated with poly-L-lysine (µ-Slide 8 Well high Poly-L-Lysine: 1.5 polymer coverslip) (A) and polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes) (B).

To confirm the results obtained by Western blot analysis, after completion of the culture an immunofluorescence assay was performed to identify the intracellular localization of selected pluripotency biomarkers belonging to the homeotic transcription factor group, Oct-3/4 and Sox-2. The results show that after completion of the expansion culture, B-ESCs contain pluripotency marker proteins, providing direct evidence of molecular phenotypic features associated with the pluripotent nature of the stem cells (Fig. 3)

Figure 3. Immunofluorescent localization of selected pluripotency-related protein biomarkers (Oct-3/4 and Sox-2) in ex ovo-expanded blastoderm-derived embryonic stem cells (B-ESCs) originating from mitotically stable lines/strains subjected to at least 15 passages



The photomicrographs show B-ESCs growing as a monolayer. The green signal from Alexa Fluor 488 indicates the presence of the Oct-3/4 marker protein (upper panel) and Sox-2 (lower panel) in these cells. In all images, the blue signal is emitted by the DAPI fluorochrome, which labels DNA in cell nuclei. Scale bar: 100 μm.

Discussion

The research involved effective efforts to develop a new experimental procedure aimed at creating an in vitro model for establishing domestic fowl blastoderm-derived embryonic stem cells (B-ESCs) for the purposes of:

1) determining the physicochemical and biological conditions of isolation, adhesion and ex ovo proliferation of domestic fowl B-ESCs using modern techniques of reproductive biotechnology and experimental and applied embryology within animal assisted-reproduction technologies, embryonic engineering and cell engineering;

2) determining the proteomic profile of selected transcription factors responsible for stable maintenance of pluripotency in domestic fowl B-ESCs (Fig. 2), using modern biotechnology and molecular biology techniques within animal assisted-reproduction technologies, embryonic engineering and cell engineering.

The principal aim of this study was to develop a stable and simplified method for ex ovo culture of domestic fowl embryonic cells displaying ESC stem-cell characteristics (i.e. pluripotent cells). Avian pluripotent stem cells are capable of generating both the germ line and chimeric somatic lineages (Bradley et al., 1984). Until recently, however, avian embryonic cells with pluripotent characteristics could be obtained only in short-term culture (Petitte et al., 2004; Lavial et al., 2009). It is worth noting that remarkable progress has been made in recent years in the culture of avian pluripotent stem cells, enabling establishment of the 9N2-5 cell line (Pain et al., 1996; Acloque et al., 2001). Moreover, strategies for in vitro expansion of avian ESCs have been progressively improved through: 1) the use of culture media enriched with polypeptide growth factors such as human basic fibroblast growth factor (hbFGF), insulin-like growth factor I (IGF1), mouse stem cell factor (mSCF), human interleukin-6 (hIL-6), the soluble fragment of the human interleukin-6 receptor hIL6-sR (Interleukin-6 Receptor Soluble Fragment), and human leukemia inhibitory factor (hLIF) (Park and Han, 2000; Acloque et al., 2001; Choi et al., 2010; Zhang et al., 2018); or 2) co-culture systems using adherent feeder monolayers

formed from immortalized STO mouse fetal fibroblast subpopulations, which can be mitotically inactivated with cytostatic/antimitogenic agents (e.g. mitomycin C; MMC) (Wang et al., 2006; Zhang et al., 2018). STO fibroblast cell lines (S: SIM-derived; T: 6-thioguanine-resistant; O: ouabain-resistant), used to support ESC proliferative activity, are derived from fetuses of the inbred Sandos mouse strain (SIM; Sandos inbred mice) carrying a mutation in the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HPRT), an enzyme catalysing conversion of the purines guanine and hypoxanthine to nucleotides (Saitoh et al., 2012). Consequently, because of the phenotypic lack of activity of this enzyme, immortalized STO fibroblast lines show high resistance to cytostatic compounds such as 6-thioguanine, a major thiopurine metabolite/analogue, and the glycoside ouabain (strophanthin G), an Na+-K+-ATPase inhibitor. STO fibroblasts are simultaneously highly sensitive to HPRT-dependent selection and to the HAT triad comprising hypoxanthine (a nitrogenous base/oxypurine component of the purine nucleoside inosine), aminopterin (4-aminofolic acid, an antimetabolite/folic-acid antagonist; folic acid is an important coenzyme in synthesis of purine and pyrimidine bases and nucleotides forming RNA and DNA) and thymidine (a pyrimidine nucleoside) (Cong et al., 2014). Systems have also been developed for culturing avian ESCs in buffalo rat liver-conditioned medium (BRL-CM) and on feeder monolayers formed from STO mouse fetal fibroblasts (van de Lavoir and Mather-Love, 2006). Today, specifically dedicated cytokines and culture substrates are used to maintain chicken ESCs in an undifferentiated state. Leukemia inhibitory factor (LIF), a member of the interleukin-6 (IL-6) family, is recognized as a highly effective factor whose cytobiochemical properties support maintenance of cells in a cytophysiologically undifferentiated state (Nichols et al., 1990; Horiuchi et al., 2004; Zhang et al., 2018). Exogenous basic fibroblast growth factor (bFGF) has also been shown to prevent differentiation of human embryonic stem cells (Wang et al., 2005). In turn, supplementation with the polypeptide stem cell factor (SCF) promotes maintenance of molecular pluripotency traits in embryonic cells of the domestic fowl (Park and Han, 2000). Although a co-culture system based on an STO mouse fetal fibroblast feeder monolayer and BRL-CM can maintain chicken ESC lines in an undifferentiated state for more than 20 passages, the cell subpopulations are of heterologous (xenogeneic) origin, preparation of the conditioned medium is a complex multistep procedure, and its qualitative and quantitative composition, structural distribution and concentrations of individual polypeptide growth factors generated by cytosecretory and paracrine functions are not precisely defined (van de Lavoir and Mather-Love, 2006). There was therefore a need to develop simplified yet optimal culture conditions capable of maintaining chicken ESC pluripotency during long-term expansion.

Our results suggest that the culture system developed in this study, using 8-well Ibidi glass microplates coated with poly-L-lysine and polystyrene culture dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes), together with culture medium supplemented with SCF and B-27™ Plus (Wartalski et al., 2021), can maintain embryonic cells displaying stem-cell characteristics for more than 15 passages in vitro. The round shape of cell clusters, characteristic of ESCs, was confirmed by morphological observations. The clusters were spherical, although not as round and compact as human, mouse or pig embryonic stem cells. Nor did they have boundaries as distinct as those of mouse embryonic stem cells (mESCs) and human embryonic stem cells (hESCs). Domestic fowl embryonic stem cells exhibit a strongly defined spherical morphology that limits their capacity to adhere tightly to one another. They are also characterized by a spherical nucleus and a low cytoplasm-to-nucleus ratio.

Although some cultured ESCs may display morphology and epitope profiles typical of embryonic stem cells, the most stringent test for confirming developmental potential is demonstration of biomarkers specific to pluripotent ESCs. Interestingly, domestic fowl B-ESCs

cultured for at least 15 passages in the newly developed ex ovo system showed proteomic-level expression of selected markers characteristic of pluripotent stem cells (Oct-3/4 and Sox2).

As a result of this study, mitotically stable embryonic cell lines with morphology and immunophenotypic (epitopic) profiles specific to stem cells, established from the blastoderm of embryonic discs isolated from fertilized chicken eggs at stage X, were able to maintain high proliferative activity during long-term in vitro culture while retaining typical stemness traits, supported by an appropriate culture-vessel substrate and a basal culture medium enriched with only three supplements providing mitogenic and energy-trophic activity (FBS, B-27™ Plus, SCF). The alternative and relatively simple ex ovo model used here enabled maintenance of traits characteristic of pluripotent avian embryonic cells during long-term in vitro culture. These highly encouraging results provide a basis for further refinement and advances in avian B-ESC technology.

Final conclusions

Based on morphological assessment, semi-quantitative Western blot analysis and immunofluorescence analysis, the isolated blastodermal cell fractions from which stable embryonic stem cell lines were established (Figs. 1C and 1D) exhibited phenotypic features of undifferentiated ESCs, including the presence of the transcription factors Oct-3/4 and Sox2 that determine pluripotency (Fig. 2). Accordingly, further experiments aimed at creating in vitro models for establishing mitotically stable ESC lines (Figs. 1C and 1D) from the blastoderm of domestic fowl discoblastulae can successfully use both 8-well Ibidi glass microplates coated with the synthetic polymeric substrate poly-L-lysine (µ-Slide 8 Well high Poly-L-Lysine) and polystyrene culture dishes with bottoms coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes).

Summary: Practical applications of the research results. Future goals and challenges, and prospective directions arising from the broad application potential of the research

The most important advantage and strongest aspect of the research aimed at developing a strategy for establishing stable embryonic stem cell lines (primary embryonic cells) from isolated domestic fowl blastodermal cells is its pioneering and innovative nature. This provides a solid conceptual foundation for implementing and successfully pursuing further, more advanced research and development in: 1) agricultural and reproductive biotechnology; 2) interdisciplinary research at the interface of biomedicine, transgenics, molecular biology and molecular biotechnology; and 3) biotechnology- and embryology-assisted ex situ conservation and restoration of endangered or extinct species and breeds of domesticated and free-living birds (Blackburn, 2006; Wang et al., 2006; Lavial et al., 2009; Woodcock et al., 2019; Soglia et al., 2021; Rieblinger et al., 2021; Sun et al., 2022; Samiec and Trzcińska, 2022).

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DEVELOPMENT OF THE EFFECTIVE STRATEGY USED FOR ESTABLISHMENT OF MITOTICALLY STABLE LINES OF EMBRYONIC STEM CELLS DERIVED FROM BLASTODERM ISOLATED FROM *IN VIVO*-FERTILIZED EGGS OF DOMESTIC FOWL (*GALLUS GALLUS DOMESTICUS*)

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SUMMARY

Pluripotent embryonic stem cell (ESC) lines established from the blastoderm, which had been isolated from embryonic discs of the domestic fowl (*Gallus gallus domesticus*) embryos at the stage X not only serve as an innovative model for studying avian embryonic development but are also gaining increasing importance, both in the context of safeguarding various populations outside their natural environment and in preserving the genetic diversity of poultry species and breeds. The main objective of this study was to develop an efficient protocol for: (1) isolating chicken BCs; (2) generating stable BC-derived ESC lines under *ex ovo* conditions; and (3) determining their pluripotency-related proteomic signatures based on the expression of such biomarkers as Oct-3/4 (octamer-binding transcription factor-3/4, also designated as POU5F1; a member of the family of POU (Pit-Oct-Unc)-domain- and homeodomain-containing transcription factors) and Sox2 (sex-determining region Y (SRY)-box 2; a member of the high mobility group (HMG)-box family of DNA-binding transcription factors). ESCs, which had been established from BCs isolated from fertilized eggs at the stage X, were cultured for a minimum of 15 passages under *ex vivo* conditions. During the *ex-ovo* expansion of blastoderm-derived ESCs, their adhesive and proliferative capabilities were compared depending on the type of vessels utilized for *in vitro* culture. By using Western-blot analysis and immunofluorescence staining, the proteomic landscapes have been examined for the pluripotency-related biomarkers that have been represented by the members of a family of homeobox (Hox) transcription factors (Oct-3/4 and Sox2), which display the presence of homeodomain, i.e., a conserved 60-amino acid helix-turn-helix motif-containing and DNA-binding domain. Throughout the extended *in vitro* culture, chicken B-ESCs have been found to retain the typical morphology of embryonic stem cells. Moreover, the expression of selected pluripotency-related markers of stemness was confirmed at the proteomic levels based on the detecting the presence of such homeotic (homeodomain/homeobox-containing) transcription factors as Oct-3/4 and Sox2. Finally, the use of 8-well Ibidi glass-bottomed microplates coated with synthetic polymeric substrate (poly-*L*-lysine), and cell culture dishes comprised of polystyrene plastic and coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes) exerted a significant impact on the proliferative capabilities of chicken B-ESC lines.

Keywords: domestic fowl, blastodermal cells, embryonic stem cells, pluripotency markers, homeotic (homeobox) transcription factors, Oct-3/4, Sox2