

BIOENGINEERING BASED ON EMBRYONIC STEM CELLS OF SELECTED SPECIES OF BREEDING POULTRY – SCIENTIFIC, RESEARCH AND APPLICATION POTENTIAL

Marcin Samiec*, Monika Trzcńska*

National Research Institute of Animal Production, Department of Reproductive Biotechnology and Cryoconservation, 32-083 Balice near Kraków, Poland

E-mail: marcin.samiec@iz.edu.pl, E-mail: monika.trzcinska@iz.edu.pl

* Marcin Samiec and Monika Trzcńska contributed equally to the preparation of this scientific article (equal authorship contribution).



Marcin Samiec ID: <https://orcid.org/0000-0002-4060-1893>

Monika Trzcńska ID: <https://orcid.org/0000-0001-9758-1304>

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Abstract

At the current stage of research, it is necessary both to accelerate work on the development of “game changer” research strategies enabling a significant improvement in the efficiency of generating blastoderm-derived embryonic stem cells (B-ESCs) of the domestic chicken that are stable in terms of proliferative potential and pluripotency-related traits, and to optimize previously developed methods for establishing and expanding B-ESCs, which in many cases remain more or less suboptimal and insufficiently reproducible in terms of the efficiency achieved. It is also necessary to note the lack of progress in developing procedures enabling efficient and sustained ex ovo cytodifferentiation of domestic chicken B-ESCs, as well as the absence of any methods for extra-ovum establishment, expansion, pluripotency stabilization or differentiation of B-ESCs from other avian species, including farmed poultry species other than the domestic chicken. This paper presents activities aimed at developing entirely new research procedures focused on creating innovative in vitro models for establishing blastoderm-derived embryonic stem cells (B-ESCs) of selected farmed poultry species (chicken, duck and goose) for the following purposes:

- 1) determining the physicochemical and biological conditions of isolation, adhesion, proliferation, extracellular stabilization of phenotypic pluripotency traits, and systems for ex ovo differentiation (cytodifferentiation) of B-ESCs, or pathways of extracorporeal neoplastic transformation (carcinogenesis) of B-ESCs or their differentiated derivatives (somatic cells of mesodermal, endodermal or ectodermal origin), using modern techniques of reproductive and molecular biology and biotechnology as well as experimental and applied embryology within assisted animal reproduction technologies, embryo engineering and cell engineering;*
- 2) determining a broad spectrum of predictive molecular parameters, including the detection of transcriptomic, proteomic and exosomal biomarkers as well as protein immunofluorescence indicators that determine:*

- a) stable maintenance of pluripotency and stemness by undifferentiated B-ESCs;
- b) preservation of traits responsible for permanent (irreversible) cytodifferentiation of B-ESCs toward partially/lowly or terminally/highly specialized somatic cells of mesodermal, endodermal and/or ectodermal origin;
- c) stabilization of conditions conducive to oncogenic (neoplastic) transformation toward poorly differentiated/malignant or highly differentiated/non-malignant tumor cells, thereby enabling identification of endogenous molecular factors responsible for initiation and progression of tumorigenesis and metastasis in the innovative biomedical in vitro models of human carcinogenesis presented in this paper and based on ex ovo-expanded B-ESC lines of domestic chicken, domestic duck and domestic goose.

In summary, the principal advantage and strongest feature of the research profile presented in this article, aimed at developing strategies for the establishment, differentiation or in vitro neoplastic transformation of stable embryonic stem-cell lines derived from blastodermal cells of selected taxonomic representatives of farmed birds (chicken, duck and goose), is its pioneering and innovative character. This provides a solid programmatic basis for the implementation and successful completion of further, more advanced research and development work in: 1) agricultural and reproductive biotechnology; 2) interdisciplinary research at the interface of biomedicine, transgenics, molecular biology and biotechnology; and 3) biotechnologically and embryologically assisted ex situ conservation and restoration of endangered or extinct species and breeds of farmed and wild birds.

Keywords: farmed birds, landfowl, waterfowl, blastoderm-derived embryonic stem cells (B-ESCs), cell culture engineering, agricultural and reproductive biotechnology, experimental and applied embryology

Introduction

Research aimed at establishing and cryogenically preserving embryonic-origin cell lines is gradually becoming, and may in the relatively near future constitute, an inherent component of biotechnology-assisted ex situ conservation and genetic rescue of various species and breeds of farmed birds, including the domestic chicken (*Gallus gallus domesticus*), domestic duck (*Anas platyrhynchos forma domestica* and *Cairina moschata f. domestica*) and domestic goose (*Anser domesticus*) (Carscience et al., 1993; Blackburn, 2006; Hu et al., 2022). Moreover, the development of ex ovo systems that maintain stable cytokinetic conditions and generate a mitogenic and anti-cytostatic microenvironment conducive to stimulation of proliferative activity and preservation of pluripotency in embryonic cells (e.g. blastoderm-derived stem cells or primordial germ cells) represents an entirely alternative direction to ex vivo research models based on the culture and differentiation of fibroblast cell lines from the integumentary system 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). Therefore, eggs of selected farmed poultry species (chicken, duck and goose) laid approximately 24 hours after fertilization constitute an excellent biological reservoir of blastodermal cell (BC) subpopulations derived from the embryonic disc. In the poly- and telolecithal eggs of birds, partial discoidal (meroblastic) cleavage at the animal pole gives rise to embryos at the blastula/discoblastula or gastrula stages. Isolated blastodermal cells are, in turn, an ideal source from which mitotically stable permanent embryonic stem-cell lines, i.e. primary embryonic stem cells (ESCs), can be established in chickens, ducks and geese (Petitte et al., 1990, 2004; Xiong et al., 2020). A tangible benefit of the comprehensive implementation of species-specific reproductive biotechnology and genetic embryo engineering

based on various systems for establishment, collection, extracorporeal culture and ex ovo differentiation (cytodifferentiation) of blastoderm-derived ESCs (B-ESCs) is long-term biotechnological ex situ protection and an increase in genotypic biodiversity of rare native conservation breeds of selected farmed bird species, including domestic chicken, domestic duck and domestic goose (Samiec and Trzcińska, 2022; Sun et al., 2022).

Taking into account all of the above agri-biotechnological, biological, animal-breeding and species-specific ornithological considerations, the overarching objective of this paper is to present and characterize the advantages of research procedures designed to create in vitro models for:

- 1) isolation of BCs from fertilized eggs of domestic chicken, domestic duck and domestic goose;
- 2) ex ovo establishment of stable ESC lines from isolated BC subpopulations;
- 3) in vitro culture supporting self-renewal and maintenance of the molecular features of pluripotency in established blastoderm-derived ESC lines (B-ESCs);
- 4) ex ovo cytodifferentiation of established, mitotically stable pluripotent B-ESC lines into partially and/or terminally differentiated somatic cells of different/diversified histological origin, in other words cells derived from different germ layers (mesoderm, endoderm and/or ectoderm);
- 5) determination of quantitative and qualitative molecular biomarkers (transcriptomic and proteomic indicators, exosomal microRNA signatures and protein immunofluorescence profiles with identification of intracellular immunolocalization) that determine:
 - a) stabilization of pluripotency-related traits and stemness of individual B-ESC lines; and/or
 - b) permanent (irreversible) induction, i.e. initiation, progression and termination, of B-ESC cytodifferentiation into partially or terminally specialized somatic cells of different histological origin; and/or
 - c) effective oncogenic/neoplastic transformation toward poorly differentiated (often malignant) or highly differentiated (generally non-malignant) tumor cells, thereby enabling detection of predictive biomarkers of tumorigenesis and metastasis in innovative biomedical ex vivo models of human carcinogenesis developed within the present research task and based on ex ovo-established B-ESCs of farmed birds;
- 6) estimation of variability in molecular parameters of pluripotent B-ESCs and the mesodermal, endodermal and/or ectodermal differentiated cell lines derived from them, depending on the effects of a broad spectrum of ectopic epigenetic and/or excitotoxic and/or trophic-energy and/or mitogenic (pro-cytokinetic) and/or antimitogenic (cytostatic) and/or carcinogenic and/or neurodegenerative and/or cytoprotective and/or antioxidant factors.

Current state of research and challenges in the bioengineering of embryonic stem-cell cultures in farmed birds

Pluripotent avian stem cells are capable of generating both germline and chimeric somatic lineages (Bradley et al., 1984). However, to date, avian embryonic cells displaying pluripotency-related characteristics have generally been obtained only under short-term culture conditions (Petitte et al., 2004; Lavial et al., 2009). It should be emphasized that over a long period of approximately 30 years, successive progress in developing methods for culture of pluripotent stem cells has been achieved only in the domestic chicken (*Gallus gallus domesticus*), and even this progress remains highly insufficient. One of the first achievements was the establishment of the 9N2-5 cell line (Pain et al., 1996; Acloque et al., 2001).

Nevertheless, in view of the above, it is essential both to accelerate work on “game changer” research strategies that can significantly improve the efficiency of obtaining domestic chicken B-ESCs stable in terms of proliferative potential and pluripotency-related traits, and to

optimize previously developed methods for establishing and expanding B-ESCs, which in many cases are more or less suboptimal and insufficiently reproducible in terms of the efficiency achieved. There has also been no meaningful progress in developing procedures that enable efficient and permanent ex ovo cytodifferentiation of domestic chicken B-ESCs, nor are there any methods for extra-ovum establishment, expansion, pluripotency stabilization or differentiation of B-ESCs from other avian species, including farmed poultry species other than the domestic chicken. Moreover, strategies for in vitro expansion of avian ESCs have been gradually improved by: 1) using 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, and human leukemia inhibitory factor (hLIF) (Park and Han, 2000; Acloque et al., 2001; Choi et al., 2010; Zhang et al., 2018); or 2) using co-culture systems with adherent feeder monolayers formed from immortalized STO mouse fetal fibroblasts, often subjected to mitotic inactivation with cytostatics/antimitogens (e.g. mitomycin C; MMC) (Wang et al., 2006; Zhang et al., 2018). It should be stressed that STO fibroblast cell lines (S: SIM-derived; T: 6-thioguanine-resistant; O: ouabain-resistant), used to support ESC proliferative activity, originate from fetuses of the inbred Sandos mouse strain (SIM) carrying a mutation in the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HPRT), which catalyzes conversion of guanine and hypoxanthine to nucleotides (Saitoh et al., 2012). Consequently, immortalized STO fibroblast lines, owing to the phenotypic absence of this enzyme activity, display high resistance to cytostatics such as 6-thioguanine and the glycoside ouabain (strophanthin G), an Na⁺/K⁺-dependent ATPase inhibitor. At the same time, STO fibroblasts are highly sensitive to HPRT-dependent selection and to the HAT triad: hypoxanthine, aminopterin (4-aminofolic acid, an antimetabolite/folic-acid antagonist) and thymidine (Cong et al., 2014).

Avian ESC culture systems have also been developed using buffalo rat liver-conditioned medium (BRL-CM) and feeder monolayers formed from mouse STO fetal fibroblasts (van de Lavoie and Mather-Love, 2006). Currently, 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 considered a highly effective factor with cytobiochemical properties that support maintenance of cells in a physiologically undifferentiated state (Nichols et al., 1990; Horiuchi et al., 2004; Zhang et al., 2018). It has also been demonstrated that exogenous basic fibroblast growth factor (bFGF) can prevent differentiation of human embryonic stem cells (Wang et al., 2005). In turn, supplementation with the polypeptide stem cell factor (SCF) promotes maintenance of the molecular features of pluripotency in embryonic-origin cells of the domestic chicken (Park and Han, 2000). Although the co-culture system based on a feeder monolayer of mouse STO fetal fibroblasts and BRL-CM allows chicken ESC lines to remain undifferentiated for more than 20 passages, the cellular subpopulations are heterogeneous (xenogeneic), preparation of the conditioned medium is a complicated multistep procedure, and its qualitative and quantitative composition, structural distribution and concentrations of individual polypeptide growth factors resulting from cytotsecretory and paracrine functions are not precisely defined (van de Lavoie and Mather-Love, 2006). This created a need to develop simplified yet optimal culture conditions for maintaining chicken ESC pluripotency during long-term expansion.

The results of our preliminary studies suggest that ex ovo culture systems for domestic chicken and duck B-ESCs based on such culture vessels as 8-well Ibidi glass microplates coated with poly-L-lysine and polystyrene dishes coated with type I collagen (SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes), together with culture medium supplemented with mitogenic and energy-trophic agents such as SCF, B-27™ Plus and FBS, provide an optimal extra-ovum microenvironment conducive to maintaining the morphological and ultrastructural phenotypic features of chicken and duck embryonic cells with stem-cell attributes for more than 15

passages in vitro (Samiec et al., 2023). The rounded shape of cell clusters characteristic of B-ESCs was confirmed by morphological observations. The clusters were spherical, but not as rounded and compact as human, mouse or pig embryonic cells. B-ESCs of both farmed poultry species also lacked the sharply defined boundaries typical of mouse embryonic stem cells (mESCs) and human embryonic stem cells (hESCs). Domestic chicken and domestic duck embryonic stem cells exhibit a strongly pronounced spherical morphology, which determines their limited ability to adhere tightly to one another. They are also characterized by a spherical nucleus and a low cytoplasm-to-nucleus ratio (Samiec et al., 2023). As part of this research panel, biorepositories dedicated to B-ESCs of both poultry species were established at the Department of Reproductive Biotechnology and Cryoconservation of IZ-PIB. Their specifications are presented in Table 1.

Table 1. Biorepositories comprising ex ovo-preserved embryonic stem cells isolated from in vivo-fertilized eggs of farmed birds

Species	Stem-cell origin	Preservation method	Number of cell lines	Number of fertilized eggs
Domestic chicken (Gallus gallus domesticus)	B-ESCs	Cryopreservation	18	90
Domestic duck (Anas platyrhynchos forma domestica; Cairina moschata f. domestica)			28	140

B-ESCs – blastoderm-derived embryonic stem cells.

Target directions for the potential application of bioengineering of embryonic stem cells derived from the blastoderm of farmed bird eggs

Research on cell-culture engineering of blastoderm-derived embryonic stem cells (B-ESCs) has broad application potential that may be reflected in the development of entirely new research procedures aimed at creating innovative in vitro models for establishing B-ESCs of selected farmed poultry species (chicken, duck and goose) for:

1) determining the physicochemical and biological conditions of isolation, adhesion, proliferation, extracellular stabilization of phenotypic pluripotency traits and systems for ex ovo differentiation (cytodifferentiation) of B-ESCs, or pathways of extracorporeal neoplastic transformation (carcinogenesis) of B-ESCs or their differentiated derivatives (somatic cells of mesodermal, endodermal or ectodermal origin), using modern techniques of reproductive and molecular biology and biotechnology as well as experimental and applied embryology within assisted animal reproduction technologies, embryo engineering and cell engineering;

2) determining a broad spectrum of predictive molecular parameters, including detection of transcriptomic, proteomic and exosomal biomarkers and protein immunofluorescence indicators that determine:

- a) stable maintenance of pluripotency and stemness by undifferentiated B-ESCs;
- b) preservation of traits responsible for permanent (irreversible) cytodifferentiation of B-ESCs toward partially/lowly or terminally/highly specialized somatic cells of mesodermal, endodermal and/or ectodermal origin;
- c) stabilization of conditions conducive to oncogenic (neoplastic) transformation toward poorly differentiated/malignant or highly differentiated/non-malignant tumor cells, thereby enabling identification of endogenous molecular factors responsible for initiation and progression of tumorigenesis and metastasis in the entirely novel biomedical in vitro models of human carcinogenesis developed within the present research task and based on ex ovo-expanded B-ESC lines of domestic chicken and/or domestic duck and/or domestic goose.

In summary, the fundamental scientific and research challenge for future studies in ornithological experimental and applied embryology and in the reproductive biotechnology of farmed landfowl and waterfowl will be to develop efficient systems for establishment and proliferative culture that guarantee stabilization of phenotypic pluripotency traits in embryonic stem cells of selected farmed bird species (chicken, duck and goose) under ex ovo conditions. The innovative research and development direction of this research profile should be emphasized because, to the best of our knowledge, the following studies have not yet been undertaken:

- 1) studies aimed at creating ex ovo models for differentiation of domestic chicken B-ESCs toward highly specialized somatic cells of mesodermal, endodermal and/or ectodermal origin;
- 2) studies aimed at creating ex vivo models for establishment and expansion of undifferentiated pluripotent B-ESCs in domesticated taxonomic representatives of waterfowl (ducks and geese);
- and 3) studies aimed at phylogenetic comparative characterization of molecular attributes of extra-ovum pathways responsible for maintaining phenotypic pluripotency traits and for extra-ovum induction of pro-mesodermal, pro-endodermal or pro-ectodermal cytodifferentiation of primary embryonic cells among different avian species, including domesticated taxa represented by domestic chicken, domestic duck and domestic goose.

Finally, the most important advantage and strongest feature of the research issues presented in this article, which are aimed at developing strategies for establishment, differentiation or in vitro neoplastic transformation of stable embryonic stem-cell lines (primary embryonic cells) derived from blastodermal cells of selected taxonomic representatives of farmed birds (chicken, duck and goose), is their pioneering and innovative character. This provides a solid programmatic basis for the implementation and successful completion of further, more advanced research and development work in: 1) agricultural and reproductive biotechnology; 2) interdisciplinary research at the interface of biomedicine, transgenics, molecular biology and biotechnology; and 3) biotechnologically and embryologically assisted ex situ conservation and restoration of endangered or extinct species and breeds of farmed and wild birds (Blackburn, 2006; Wang et al., 2006; Laval et al., 2009; Woodcock et al., 2019; Soglia et al., 2021; Rieblinger et al., 2021; Sun et al., 2022; Samiec and Trzcińska, 2022; Samiec et al., 2023).

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BIOENGINEERING BASED ON EMBRYONIC STEM CELLS OF SELECTED SPECIES OF BREEDING POULTRY – SCIENTIFIC, RESEARCH AND APPLICATION POTENTIAL

Marcin Samiec, Monika Trzcińska

SUMMARY

At the current stage of investigations, it is indispensable both to accelerate the elaboration of “game changer” research strategies, enabling a significant improvement in the efficiency of generating stable – in the context of proliferative potential and pluripotency-related attributes – blastoderm-derived embryonic stem cells (B-ESCs) of the domestic fowl, and to optimize previously developed methods intended for establishment and *in vitro* expansion of B-ESCs (in many cases more or less suboptimal), and insufficiently replicable in terms of the efficiency obtained. It is also necessary to note the lack of any progress in the development of procedures allowing for efficient and sustainable *ex ovo* cytodifferentiation of chicken B-ESCs, as well as the non-existence of any methods for extra-ovum derivation, multiplication, pluripotent stabilization or differentiation of B-ESCs in other avian species, including breeding poultry species other than the domestic chicken. Within the framework of the present work, efforts will be unveiled to develop completely new research procedures aimed at creating innovative *in vitro* models for establishing B-ESCs of selected species of breeding poultry (domestic chicken, duck, goose) for the purposes of:

1) recognizing the physico-chemical and biological conditions of isolation, adhesion, proliferation, extracellular stabilization of phenotypic traits of pluripotency and systems applied to *ex ovo* cytodifferentiation of B-ESCs or pathways of extracorporeal neoplastic transformation (carcinogenesis) of B-ESCs or their differentiated derivatives (somatic cells of meso-, endo- or ectodermal origins) – by using modern techniques of not only reproductive and molecular biology/biotechnology, but also experimental and applied embryology, within the framework of assisted animal reproductive technologies, and embryonic cell engineering;

2) unravelling a broad spectrum of predictive molecular parameters, including the detection of transcriptomic, proteomic, exosomal biomarkers, and protein immunofluorescence indicators that determine:

(a) either the stable maintenance of pluripotency and stemness by undifferentiated B-ESCs;

(b) or the preservation of the attributes responsible for the permanent (irreversible) cytodifferentiation of B-ESCs into partially/lowly or terminally/highly specialized somatic cells of meso-, endo- and/or ectodermal provenance;

(c) or perpetuation of conditions conducive to oncogenic (neoplastic) transformation toward low-differentiated/malignant or highly-differentiated/non-malignant tumor cells, which gives the possibility to identify endogenous molecular factors responsible for the initiation and progression of tumorigenesis and metastasis in the novel *in vitro* biomedical models of human carcinogenesis deciphered within the framework of this research work, based on the *ex ovo* expanded B-ESC lines of domestic chicken, domestic duck and domestic goose.

To sum up, the most important advantage and the strongest point of the research profile presented in this article targeted at the elaboration of strategic approaches to establishment, differentiation, or *in vitro* neoplastic transformation of mitotically stable B-ESC lines in the selected taxonomic representatives of farmed birds (chicken, duck, goose) is its pioneering and innovative character, which provides a solid program basis for the implementation and success of the assumptions of further, more advanced research and development work in the field of: 1) agricultural and reproductive biotechnology, and 2) interdisciplinary research at the interface of

biomedicine, transgenics, molecular biology and biotechnology, as well as 3) biotechnologically- and embryologically-assisted *ex situ* conservation and restoration of disappearing or extinct species and breeds of domesticated and wild birds.

Keywords: breeding birds, landfowl, waterfowl, blastoderm-derived embryonic stem cells (B-ESCs), cell culture engineering, agricultural and reproductive biotechnology, experimental and applied embryology