

DIRECTIONS AND POSSIBILITIES OF USING BIOLOGICAL MATERIAL DERIVED FROM FREE-LIVING ANIMALS

Monika Trzcińska*, Marcin Samiec*

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

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

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



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

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

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Abstract

Considering the phylogenetic development of vertebrates (mammals, birds, fish), many taxa representing the phylum of chordates (Chordata) and the subphylum of vertebrates (Vertebrata) currently have the status of endangered or extremely endangered species or are extinct in the wild. Therefore, ex vivo preservation and biological and molecular characterization of somatic and stem cell lines isolated from various tissue explants originating from free-living animals will enable: 1) the development of ex vivo research models based on cell culture engineering and cytological toxicology, and 2) the use of the collected biological material in assisted reproductive technologies (ARTs), applicable to the programs intended for the ex situ conservation and restoration of endangered vertebrate representatives. Taking into account the above considerations, the aim of this work is to present the possibilities of multifaceted evaluation of in vitro cell culture models of various wildlife species (including wild boars, roe deer, red deer, red foxes, raccoon dogs, wildfowl and fresh-water fish) for the purposes of: 1) identifying and exploring the cytological, biochemical, molecular, epigenetic, transcriptomic and proteomic factors and mechanisms; and 2) determining the effects of a broad spectrum of trophic and mitogenic supplements and environmental toxins on intracellular processes, with particular emphasis on cells and tissues of the reproductive system. The source of tissues and organs indispensable for establishment of somatic and stem cell lines can be male and female specimens at different age, being representatives of the selected vertebrate classes and derived from native subpopulations of a variety of wildlife species: 1) mammals – wild boars, roe deer, red deer, raccoon dogs, red foxes and others; and 2) birds – pheasants, partridges and others; as well as 3) fresh-water fish (herbivorous and carnivorous). Based on the established cell lines, in vitro research models focused on cell and tissue engineering and reproductive biotechnology and toxicology can be elaborated. For this reason, mitotically stable somatic cell lines and multipotent stem cell lines of various origins can be subjected to: 1) structural-functional

assessment mediated by a broad panel of methods in such research fields as molecular biology, transcriptomics, proteomics, epigenomics, immunocytochemistry, among others, and 2) biophysical and biochemical functional tests using nanotechnology and molecular toxicology techniques. Summing up, the in vitro cell culture engineering models in wildlife species, which will have been developed within the framework of the implementation of this research work profile, can be extrapolated to other mammalian species, including humans and farmed livestock species, especially those of their representatives that have a close degree of phylogenetic relatedness with the corresponding free-living species. a detailed comparative characterization of cell culture models between wildlife and livestock species will be also helpful in this regard.

Keywords: *wildlife species, ex vivo research models, adult somatic and stem cells, reproductive biotechnology and toxicology*

Introduction

At the present stage of biological evolution, many taxonomic representatives of vertebrates (mammals, birds and fish) are classified as endangered or critically endangered species, or as species extinct in the wild. The main causes of the drastic decline or extinction of so many mammalian, avian and fish species in their natural habitats appear to include:

- 1) exploitative anthropogenic activity resulting in a high degree of human interference with the habitats of individual species/subspecies (Comizzoli and Holt, 2019; Perry and Mitchell, 2022);
- 2) agricultural and industrial degradation of biotopes inhabited by free-living species;
 - a) eutrophication of inland waters and marine water bodies caused by excessive emissions of carbon dioxide and other greenhouse gases, global warming of climate zones and increasing temperatures of surface waters;
 - b) pollution within aquatic ecosystems and groundwater, as well as in crop and forage plants and in free-living and domesticated herbivorous, carnivorous and omnivorous animals, caused by bioaccumulation and the lack of biodegradation of cytotoxic compounds, e.g. toxins synthesized by toxin-producing cyanobacteria, i.e. cyanotoxins (Thawabteh et al., 2023; Shi et al., 2023; Kurbatova et al., 2023; Roy et al., 2023);
- 3) poaching (Bolton et al., 2022; Hildebrandt et al., 2021);
- 4) degradation of threatened natural niches inhabited by individual vertebrate species/subspecies as a consequence of climate change and natural disasters (Roy et al., 2023; Moreira-González et al., 2023);
- 5) epidemic and pandemic transmission of pathogens causing intra- and interspecific infectious diseases in animals (Caroli and Pizzi, 2012; Bolton et al., 2022).

Practical rationale for research using cells, tissues and organs isolated from wildlife species in various areas of reproductive biotechnology and toxicology, as well as in broadly interdisciplinary studies

The rapidly shrinking habitats of many endangered vertebrate species/subspecies, resulting from the expansion of urbanized and agricultural ecosystems, are a major cause of reduced genetic drift among wild populations/subpopulations of a given vertebrate species. This, in turn, contributes to a reduction in intra- and interpopulation genotypic variation among many endangered vertebrates. A rapid decrease in the frequency and distribution range of threatened vertebrate taxonomic units may also lead to loss of biological equilibrium or progressive decline in biodiversity as a consequence of complete extinction of a given species (Comizzoli and Holt, 2019; Thongphakdee et al., 2020; Jacques et al., 2023).

Therefore, the development and optimization of methods for *ex situ* preservation of biological material originating from free-living animals creates opportunities to develop new *in vitro* models of cell and tissue engineering for the purposes of:

- 1) conservation of species diversity;
- 2) preservation and stabilization of ecosystem balance between biocenosis and biotope;
- 3) reduction in the population size of highly invasive species with strong adaptive capabilities in anthropogenic and urbanized ecosystems (wild boars, red foxes and raccoon dogs) (Woelders et al., 2012; Ryder and Onuma, 2018; Gavin-Plagne et al., 2020; Praxedes et al., 2021; Soglia et al., 2021).

Detailed specification of research directions using biological material obtained from selected animal species inhabiting natural ecosystems in Poland

In this context, the activities of the Department of Reproductive Biotechnology of the National Research Institute of Animal Production, aimed at *ex vivo* preservation and structural-functional characterization of proliferatively active somatic and stem cell lines isolated from various tissue explants originating from free-living animals (Table 1), constitute an attractive research profile. This research panel will enable the development of experimental models based on various *in vitro* culture and toxicology systems. Such *ex vivo* cellular models can be successfully extrapolated to experimental human cell models and to various domesticated vertebrate species, including livestock. These models will be used, among other purposes, to investigate the effects of environmental toxins and biotoxic compounds synthesized by toxin-producing cyanobacteria on cell lines established from biopsies isolated from various tissues and organs, including tissues and organs of the reproductive system. Such models will also facilitate intracellular delivery pathways for exogenous compounds with cell-supporting properties through experimentally designed carrier nanoparticles (Kikuchi et al., 2016; Chen et al., 2018; Borges et al., 2020; Son et al., 2021; Dua et al., 2021; Elyasi Gorji et al., 2021).

Taking the above into consideration, the establishment of *ex vivo* research models for identification and exploration of the molecular network of intracellular factors and processes is fully justified because somatic and stem cells of various origins (including those from reproductive organs and tissues of both sexes) constitute sources of nuclear donor cells for somatic cloning of various free-living and farmed animal species (Li et al., 2013; Xu et al., 2019; Jeong et al., 2020; Hildebrandt et al., 2021; Nguyen et al., 2021; Comizzoli et al., 2022). In turn, intra- and interspecific somatic cloning may provide an effective research tool for *ex situ* conservation and biotechnological rescue of endangered or extinct vertebrate species (Magalhães et al., 2020; Wang et al., 2020; Nguyen et al., 2021; Thongphakdee et al., 2020; Hildebrandt et al., 2021; Samiec and Skrzyszowska, 2021). Also important are biotechnology-assisted methods for restoring endangered or extinct vertebrates, aimed at reintroduction and recovery of species/sub-species biodiversity in many natural ecosystem niches, including those under strict human protection, such as nature reserves (León-Quinto et al., 2014; Comizzoli, 2015; De Oliveira Silva et al., 2021; Sun et al., 2022; Hu et al., 2022).

Table 1. Biorepositories of the National Research Institute of Animal Production containing *ex vivo*-preserved stem and somatic cell lines derived from tissue biopsies obtained from selected free-living mammalian species

Species	Origin of adult stem or somatic cells	Method of <i>ex vivo</i> preservation	Number of cell lines	Number of female donors	Number of male donors
Wild boar	AT-MSCs	Cryopreservation	6	-	4
	ES-FCs		8	-	6
	EC-CTs		9	-	7
Common raccoon dog	AT-MSCs		4	4	-
	ES-FCs		5	4	-
	EC-CTs		4	4	-
European red fox	AT-MSCs		6	1	2
	ES-FCs		3	2	1
	EC-CTs		6	2	3
European roe deer	AT-MSCs		6	-	2
	ES-FCs		5	-	3
	EC-CTs		6	-	3

AT-MSCs – adipose tissue-derived mesenchymal stem cells; ES-FCs – ear skin-derived fibroblast cells; EC-CTs – ear cartilage-derived chondrocytes.

In this respect, comprehensive evaluation of *in vitro* cellular models of various wildlife species (including representatives occurring in Poland, such as wild boars, roe deer, red deer, red foxes, raccoon dogs, game birds and freshwater fish) is important for identifying and understanding molecular, epigenetic, transcriptomic and proteomic factors and mechanisms, as well as for investigating the effects of various trophic and mitogenic supplements and environmental toxins on intracellular processes (Thomas et al., 2022; Yan et al., 2022; Afshar et al., 2023; Lautaoja et al., 2023). In the latter case, it is also extremely useful to determine the rate of uptake and facilitate intracellular transport of exogenous cytotoxic compounds by means of carrier nanoparticles, as well as to assess the kinetics of formation and secretion of exosomes (extracellular microvesicles) together with characterization of their biochemical composition (quantitative distribution of phospholipids, glycolipids, proteins and miRNA molecules as modulators/regulators of epigenetic information) (Zhang et al., 2015; Yoo et al., 2015; Rincon-Benavides et al., 2023; Williams et al., 2023). This will make it possible to establish research models involving cell cultures derived from wildlife species, including freshwater fish inhabiting inland waters polluted as a consequence of eutrophication caused by cascading effects of endotoxins (microcystins and nodularins) synthesized by toxin-producing cyanobacteria (*Microcystis aeruginosa*, *Nodularia spumigena*) (Yu et al., 2023; Kurbatova et al., 2023; Roy et al., 2023).

Investigating the mechanisms of extracellular and intracellular toxicity caused by environmental toxins using *in vitro* models is particularly important in the context of bioaccumulation of these cytotoxic compounds in organisms of various aquatic and terrestrial vertebrates, including humans and farm animals (Nowruzi et al., 2023; Schwark et al., 2023; Shi et al., 2023). A useful research tool for recognizing different molecular pathways of cytotoxin uptake, as well as the effects of extracellular supplements or carrier nanoparticles coated with extracellular supplements, environmental toxins or cyanotoxins on somatic and stem cells expanded *ex vivo*, is to combine characterization of RNA and miRNA transcriptomic profiles and proteomic profiles (using RT-qPCR and Western blot analyses) with immunolocalization of proteins or glycoproteins in specific intracellular regions and organelles, including the membrane skeleton

and cytoskeleton, as well as in exosomes formed and externalized by cells (Han et al., 2016; Yoo et al., 2017; Samiec et al., 2022; Yan et al., 2022; Yu et al., 2023).

Moreover, the *in vitro* cellular models of wildlife species developed within this research direction can be extrapolated to other mammalian species, including humans and domesticated and farmed animals, especially representatives that are phylogenetically closely related to the corresponding free-living species. Detailed comparative characterization of such cellular models at the cytophysiological and molecular levels will be helpful in this regard.

Summary, final conclusions and future research objectives

Developing effective methods for preserving cells and tissues originating from free-living animals, including different species inhabiting ecosystems in Poland, as well as developing *in vitro* models of cell and tissue engineering using biotechnology, toxicology and nanotechnology, constitutes an innovative direction of research and development. These approaches are particularly important for conserving species diversity, maintaining biotope and biocenotic balance, and for activities aimed at reducing populations of highly invasive species with strong adaptive capabilities in anthropogenic and urbanized ecosystems (wild boars, red foxes and raccoon dogs).

The establishment of *ex vivo* research models for identification and exploration of the molecular network of intracellular factors and processes is justified because somatic and stem cells of various origins (including those derived from reproductive tissues and organs of both sexes) constitute sources of nuclear donor cells for somatic cloning of various free-living and farmed animal species. For this reason, *ex vivo* preservation and structural-functional characterization of somatic and stem cell lines isolated from various tissue explants originating from wildlife species represent an attractive research direction that enables the development of experimental models involving cell culture engineering and molecular toxicology of wild vertebrate species (mammals, birds and fish). Such *ex vivo* cellular models can be successfully extrapolated to experimental and preclinical cellular models in humans and other vertebrate species, including farm animals, particularly those taxonomically closely related to their counterparts inhabiting natural ecosystems and not subjected to anthropogenic pressure.

In this context, a *sine qua non* condition for future experimental and applied work within the adopted research panel appears to be comprehensive evaluation of *in vitro* cellular models of various wildlife species (including representatives occurring in Poland, such as wild boars, roe deer, red deer, red foxes, raccoon dogs, game birds and freshwater fish) in terms of identifying and understanding molecular, epigenetic, transcriptomic and proteomic factors and mechanisms, as well as investigating the effects of various trophic and mitogenic supplements and environmental toxins on intracellular processes, with particular emphasis on cells and tissues of the reproductive system.

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Monika Trzcińska, Marcin Samiec

SUMMARY

Considering the phylogenetic development of vertebrates (mammals, birds, fish), many taxa representing the phylum of chordates (*Chordata*) and the subphylum of vertebrates (*Vertebrata*) currently have the status of endangered or extremely endangered species or are extinct in the wild. Therefore, *ex vivo* preservation and biological and molecular characterization of somatic and stem cell lines isolated from various tissue explants originating from free-living animals will enable: 1) the development of *ex vivo* research models based on cell culture engineering and cytological toxicology, and 2) the use of the collected biological material in assisted reproductive technologies (ARTs), applicable to the programs intended for the *ex situ* conservation and restoration of endangered vertebrate representatives. Taking into account the above considerations, the aim of this work is to present the possibilities of multifaceted evaluation of *in vitro* cell culture models of various wildlife species (including wild boars, roe deer, red deer, red foxes, raccoon dogs, wildfowl and fresh-water fish) for the purposes of: 1) identifying and exploring the cytological, biochemical, molecular, epigenetic, transcriptomic and proteomic factors and mechanisms; and 2) determining the effects of a broad spectrum of trophic and mitogenic supplements and environmental toxins on intracellular processes, with particular emphasis on cells and tissues of the reproductive system. The source of tissues and organs indispensable for establishment of somatic and stem cell lines can be male and female specimens at different age, being representatives of the selected vertebrate classes and derived from native subpopulations of a variety of wildlife species: 1) mammals – wild boars, roe deer, red deer, raccoon dogs, red foxes and others; and 2) birds – pheasants, partridges and others; as well as 3) fresh-water fish (herbivorous and carnivorous). Based on the established cell lines, *in vitro* research models focused on cell and tissue engineering and reproductive biotechnology and toxicology can be elaborated. For this reason, mitotically stable somatic cell lines and multipotent stem cell lines of various origins can be subjected to: 1) structural-functional assessment mediated by a broad panel of methods in such research fields as molecular biology, transcriptomics, proteomics, epigenomics, immunocytochemistry, among others, and 2) biophysical and biochemical functional tests using nanotechnology and molecular toxicology techniques. Summing up, the *in vitro* cell culture engineering models in wildlife species, which will

have been developed within the framework of the implementation of this research work profile, can be extrapolated to other mammalian species, including humans and farmed livestock species, especially those of their representatives that have a close degree of phylogenetic relatedness with the corresponding free-living species. a detailed comparative characterization of cell culture models between wildlife and livestock species will be also helpful in this regard.

Keywords: wildlife species, *ex vivo* research models, adult somatic and stem cells, reproductive biotechnology and toxicology