GENERATION OF MONOGENETIC CATTLE BY DIFFERENT TECHNIQUES OF EMBRYONIC CELL AND SOMATIC CELL CLONING – THEIR APPLICATION TO BIOTECHNOLOGICAL, AGRICULTURAL, NUTRITIONAL, BIOMEDICAL AND TRANSGENIC RESEARCH – A REVIEW*

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Abstract

The development of effective approaches for not only the *in vitro* maturation (IVM) of heifer/cow oocytes and their extracorporeal fertilization (IVF) but also the non-surgical collection and transfer of bovine embryos has given rise to optimizing comprehensive *in vitro* embryo production (IVP) technology and improving other assisted reproductive technologies (ARTs), such as cattle cloning by embryo bisection, embryonic cell nuclear transfer (ECNT) and somatic cell nuclear transfer (SCNT). The primary goal of the present paper is to demonstrate the progress and achievements in the strategies utilized for embryonic cell cloning and somatic cell cloning in cattle. Moreover, the current article is focused on recognizing and identifying the suitability and reliability of bovine cloning techniques for nutritional biotechnology, agri-food and biopharmaceutical industry, biomedical and transgenic research and for the genetic rescue of endangered or extinct breeds and species of domesticated or wild-living artiodactyl mammals (even-toed ungulates) originating from the family *Bovidae*.

Key words: cattle, cloning, embryo bisection, embryonic cell nuclear transfer, somatic cell nuclear transfer, nutritional biotechnology, agri-food and biopharmaceutical industry, biomedical and transgenic research, endangered and extinct breed or species

The development of the methods used for the *in vitro* maturation (IVM) and fertilization (IVF) of bovine oocytes and of the techniques for non-surgical collection and transfer of bovine embryos has been instrumental in the elaboration of not only *in vitro* embryo production (IVP) technology in cattle but also other assisted

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reproductive technologies (ARTs) based on the use of modern embryonic genome engineering techniques, such as embryonic cell cloning and somatic cell cloning.

This paper aimed to demonstrate the achievements of assisted reproduction in cattle using embryonic cell and somatic cell cloning. Another objective is to present the potential application of bovine cloning in food biotechnology, agri-food industry, pharmacology, and biomedicine and in the genetic conservation of near-threatened, vulnerable, endangered, critically imperilled, vanishing or extinct breeds and species of domesticated and free-living even-toed mammals (*Artiodactyla*) stemming from the family *Bovidae*.

Cloning of cattle by embryo bisection

Microsurgical embryo bisection is a way to double the number of embryos and thus a relatively simple method for cloning mammalian embryos, resulting in genetically identical pairs of individuals, i.e., monogenetic twins. Embryos at all cleavage stages can be subjected to bisection, even those in which intercellular connections, the gap junctions, had not yet formed between dividing blastomeres. The simplest example of this type of bisection is the isolation of blastomeres from very early-stage (2- to 4-cell) embryos that have been deprived of their zonae pellucidae. Most often, however, bisection is performed at advanced stages of preimplantation development, such as late morula or blastocyst (Ozil, 1983; Velásquez et al., 2017; Casser et al., 2019). At these stages of embryogenesis, embryos exhibit complete compaction, with the formation of gap junctions between blastomeres, and as a result, the embryos do not break apart into several pieces when they are cut microsurgically. The bisected embryo “halves” of similar size retain their postimplantation developmental potential after transfer into the reproductive tracts of hormonally synchronized recipient females. To date, many pairs of monogenetic offspring have been produced in cattle following non-surgical intrauterine transfer of embryo “halves” obtained by bisection of compact morulae or blastocysts. In Poland, the first genetically identical calves were generated in 1988 at the Department of Animal Reproduction Physiology (currently the Department of Reproductive Biotechnology and Cryoconservation) of the National Research Institute of Animal Production in Balice (Skrzyszowska et al., 1988). Correct bisection ensures the correct composition of both “halves”, not only in terms of cell number but also with regard to the ratio of inner cell mass (ICM) to trophectoderm cells, comparable to that of the whole embryo (Figure 1).

Bisection is a relatively simple and efficient method for doubling the number of embryos and producing monogenetic twins (efficiency of 25–30%). However, this technique is rather invasive because during the microsurgical embryo splitting at the compact morula or blastocyst stage, some of the embryonic cells are destroyed and eliminated. Particularly severe may be cell losses within the inner cell mass, which have a significant role in reducing the developmental potential of the obtained blastocyst “halves” (Skrzyszowska and Smorąg, 1989; Casser et al., 2019). Little information is known about how the bisection procedure affects the transcriptional activity of the genes in embryo “halves”. A study performed by Velásquez et al. (2017) with elongated blastocysts obtained nine days after transfer of the “halves” and whole bovine blastocysts shows that bisection impacts the transcriptomes of these blastocysts,

mainly by downregulating key genes involved in the 1) remodelling and degradation of the extracellular matrix, 2) control of cell growth, 3) intracellular detoxification of products of oxidative stress, and 4) transport of metabolites (catabolism products of sugars, nucleotides and purine ribonucleotides, proteins, monoamines, lipids, and fatty acids) between individual intracellular compartments and organelles.

Figure 1. Schematic representation of bisecting an embryo at the blastocyst stage. The upper part of Figure 1 depicts a blastocyst bisection that has been correctly accomplished. The median vertical (sagittal) plane of the microsurgical division runs longitudinally through the middle of the spherical figure of the embryo (the vertical line of the lengthwise cutting of the embryo into two symmetrical “halves” is achieved with a microblade, perpendicular to the horizontal plane running across the embryo). The symmetrical division plane is oriented such that the bisected embryo “halves” are equivalent and contain two equally divided parts of the embryo, which are composed of both the ICM/embryoblast cell line (marked in bright red) and the trophoblast/trophectoderm cell line (highlighted in bright green). In the case of incorrect blastocyst bisection, the schematic representation of which is shown in the lower part of Figure 1, the lateral vertical plane of the microsurgical division of the spherical figure of the embryo runs oblique (vertical line of the oblique cutting of the embryo into two asymmetrical “halves” is performed with a microblade, in the plane perpendicular to the horizontal plane running across the embryo). The asymmetrical division plane is oriented such that the bisected embryo “halves” are non-equivalent and contain two unequally divided parts of the embryo, which are composed of both the inner cell mass cell line and the trophoblast cell line. One embryo “half” contains most of the embryoblast cells, and the other “half” most of the trophoblast ring cells of the whole blastocyst prior to the bisection procedure

Our studies that were performed to improve embryo splitting efficiency have given rise to the development and optimization of an innovative technology for the production of monogenetic twins on the basis of a modified approach for blastocyst bisection (Skrzyszowska et al., 1997, 1999; Figure 2). The optimized original bisection method considerably reduces cell losses compared to the losses that occur during standard embryo bisection, as it limits these blastomere losses to several cells. This novel method consists of provoking a specific method of blastocyst hatching from the zona pellucida that had been perforated by microsurgical puncture, incision, treatment with acidic Tyrode’s solution (pH 2), or laser-mediated zona drill-

ing. Under *in vitro* culture conditions, approximately 50% of perforated blastocysts expand through a slit in the zona pellucida, reaching, at a certain stage of hatching, a state in which part of the blastocyst outside the zona pellucida is of similar size to the embryo part inside the zona pellucida. The so-called hatching embryo resembles a figure eight. At this stage of hatching, both parts of the embryo are only connected by a thin cellular bridge. Cutting this “linker” blastomere bridge with a glass microneedle or metal blade involves a small degree of invasiveness, and the procedure only causes damage to several cells (Figure 2). After transfer into the uterus of recipient heifers or cows, the blastocyst “halves” that have been created by modified embryo bisection maintain their competences to develop *in vivo* to term (Figure 3).

Figure 2. Schematic representation of the modified approach for bisecting embryos at the blastocyst stage. In the original modification of the bisection method, which has been elaborated in the Department of Animal Reproduction Physiology (currently: Department of Reproductive Biotechnology and Cryoconservation) of the National Research Institute of Animal Production in Poland, microsurgical, chemical- or laser-assisted perforation of the blastocyst zona pellucida is performed below the spatial location of the inner cell mass (marked in bright red). A schematic representation of this perforation is presented in the upper part of Figure 2. Perforation of the zona pellucida (performed, e.g., by a metal microblade) induces a specific manner of blastocyst hatching, which is shown in the lower part of Figure 2. The hatching embryo has a “figure eight” shape. The two parts of the hatching blastocyst (one part inside and the other part outside the zona pellucida) contain evenly distributed subpopulations of the bright red-highlighted inner cell mass cell line and of the bright green-highlighted trophectoderm cell line and are only interconnected by a thin cellular (blastomere) bridge. A small incision is made with a glass microneedle through the cellular bridge formed by induced blastocyst hatching, which divides the blastocyst into two autonomous “halves” – one surrounded by the zona pellucida and another depleted of the zona pellucida. The vertical plane of the microsurgical embryo splitting is confined to the blastomere bridge, which had previously served as the only intraembryonic connection or specific intercellular link between both parts of the hatching blastocyst, thus minimizing the proportions of cell loss during the embryo bisection procedure

The benefits of monogenetic twin production in cattle (Figure 3) are quantifiable both practically and cognitively, not only in regard to embryological experiments

aimed at evaluating early embryonic development, embryo mortality, and peri-implantation endocrine signalling between the surrogate recipient and the embryo but also from the aspect of cytogenetic and physiological studies, in particular those concerned with nutrition. During the bisection procedure, it is also possible to collect cells for molecular analysis aimed at embryo sexing, which may also have application value, for example in contributing to farm animal breeding progress (Lopes et al., 2001). A limitation of this method is that only clones consisting of a pair of genetically identical individuals (i.e., monogenetic twins) can be produced.

The incorporation of an efficient embryo bisection method into the scheme/programme of multiple ovulation and embryo transfer (MOET) could expand the scope of this programme and contribute to accelerating breeding progress in cattle.

Figure 3. Pair of monogenetic calves born after the transfer of blastocyst “halves” into the uterine horn of a recipient cow. The blastocyst “halves” were generated using an original technique for modified embryo bisection that had been developed and optimized at the Department of Animal Reproduction Physiology (currently: Department of Reproductive Biotechnology and Cryoconservation) of the National Research Institute of Animal Production in Poland

Cloning of cattle by embryonic or somatic cell nuclear transfer

More numerous clones are potentially obtainable by embryonic cell nuclear transfer (ECNT) or somatic cell nuclear transfer (SCNT) into the cytoplasm of an enucleated recipient cell. Enucleated recipient cells for nuclear donor embryonic or somatic cells are *in vivo*- or *in vitro*-matured metaphase II-stage oocytes whose genetic material (meiotic chromosomes in the form of metaphase plate configured by a karyokinetic spindle) has been microsurgically removed.

Cloning as a method of asexual reproduction offers the opportunity to produce and/or multiply monogenetic and single-sex offspring of high genetic and breeding value that are genotypically and phenotypically identical to the progenitor donor of the transcriptional and mitochondrial apparatus of the somatic cell only with regard to genomic DNA. Specimens produced by embryonic cell or somatic cell cloning differ in phenotypic traits, determined by random segregation of maternal (oocyte-inherited) as well as embryonic cell- or somatic cell-inherited (somatogenic) mitochondrial genome (mtDNA) as a result of extranuclear inheritance of genetic material (Samiec, 2005 a, b; Samiec and Skrzyszowska, 2018 a, b).

One of the most spectacular achievements in bovine somatic cell cloning was the resurrection of a champion bull, which died of senility at the age of 13.5 years in 1993 (Hoshino et al., 2009). This bull belonged to the Wagyu population of cattle endemic to Japan, and their genotype is associated with the highly heritable trait of meat marbling. Therefore, fattening bulls and heifers of the beef Wagyu breed are genetically predisposed to deposition of intermuscular and intramuscular fat and to the production of juicy and tender meat with relatively high contents of mono- and polyunsaturated fatty acids in intramuscular fat lipids. An identical genetic copy (clone) of the Wagyu beef bull was produced by the SCNT technique using clonal cell lines and primary cultures of tissue samples that had been isolated from the bull's scrotal organs (testes, epididymides and spermatic cords), frozen at -80°C for ten years, and stored in liquid nitrogen for the next three years (without cryoprotectant). More specifically, the nuclear donors in the cloning procedure were *in vitro*-cultured fibroblast-like (fibroblastoid) or epithelial-like (epithelioid) cells isolated from long-cryopreserved pieces of the caput epididymis and spermatic cords of a dead progenitor bull (Hoshino et al., 2009). Special consideration should also be given to the cloning of the last surviving Enderby Island cow (a dairy cattle breed endemic to New Zealand's Enderby Island), where the nuclear donors were *in vitro*-cultured mural granulosa cells isolated from ovarian follicles. In turn, recipient cytoplasts for follicular cell nuclei, isolated from the ovaries of the last surviving Enderby Island cow, were derived from enucleated oocytes, which reached meiotic maturity under the *in vitro* culture of oocytes recovered postmortem from the ovaries of cows of various breeds. This achievement confirms that intraspecies cloning could be an effective solution for the restoration and reintroduction of endangered conservation breeds of cattle (Wells et al., 1998).

Factors determining the efficiency of somatic cell cloning and the health status of cloned cattle

One of the main constraints on somatic cell cloning efficiency in cattle is abnormal epigenetic adaptation of transferred somatic cell nuclei to the biochemical environment of the oocyte cytoplasm, i.e., incomplete or abnormal remodelling/reprogramming of their transcriptional activity in the cytoplasm of enucleated oocytes, and later in the blastomeres of developing bovine cloned embryos (Zhang et al., 2009, 2019 a; Niemann, 2016; Samiec and Skrzyszowska, 2018 a, b). Deviations from the normal progress of the epigenetic reprogramming of the transcriptional activity of the somatic cell-derived nuclear genome in cloned embryos are the reason for placentation abnormalities and numerous anatomo-histological defects in the placenta of cloned fetuses in cattle (Hiendleder et al., 2004; Su et al., 2011; Chavatte-Palmer et al., 2002, 2012; Biase et al., 2016). Because errors occurring in the transcriptional reprogramming of somatic cell-derived nuclear genome also provoke an increased immune response from the immune system of recipient surrogates, major histocompatibility complex antigens of the cloned embryos/fetuses are identified by the intrauterine environment of the female recipient as allograft antigens (in contrast to embryos and resultant conceptuses developed from *in vivo*-fertilized zygotes, which are treated by the mother's immune system as semi-allografts). Other

consequences of the abnormal remodelling/reprogramming of somatic cell nuclei include the high mortality of cloned embryos and fetuses during the peri-implantation period and perinatal period of ontogenesis, respectively, as well as high pregnancy loss (i.e., resorption of cloned fetuses) during the first trimester of pregnancy (Hill et al., 2002). Attention should also be given to the malformations of cloned conceptuses and offspring arising from lethal or sublethal anatomic and histopathological defects in different internal organs (i.e., lungs, heart, liver, kidneys), the frequently diagnosed large offspring syndrome (LOS) in cloned calves, and the incidence of large placenta syndrome (LPS; placental hyperplasia and hypertrophy or placentomegaly) in bovine cloned fetuses (Farin et al., 2006; Zhang et al., 2009; Su et al., 2011; Chavatte-Palmer et al., 2012; Smith et al., 2012; Biase et al., 2016).

When compared to other assisted reproductive technologies in cattle, the somatic cell cloning efficiency in this mammalian species, expressed as the percentage of offspring born per number of reconstructed oocytes, remains low at 5–15%. Nevertheless, the biotechnological potential of somatic cell cloning in cattle and other livestock species is far ahead of our understanding of the biological determinants, particularly the molecular and epigenetic aspects, of this method (Samiec and Skrzyszowska, 2018 a, b). However, the biological foundations that have been laid for embryonic genome engineering, especially over the last 20 years, made possible the development of an innovative technology of *in vitro* bovine embryo production by the somatic cell cloning procedure, which may meet the conditions for its use for laboratory purposes or, in some cases, for limited practical applications (Loi et al., 2016; Niemann, 2016). A wider practical application of the assisted reproductive technology would only be possible after the somatic cell cloning efficiency in cattle matches the efficiency of *in vitro* fertilization (30–40%) or the efficiency of artificial insemination (60–80%) as part of the MOET programme conducted in this mammalian species. However, due to the relatively high (30–40%) incidence of lethal or sublethal malformations or anatomic-histological defects in cloned fetuses and offspring, commercial-scale somatic cell cloning of cattle is not feasible, at least at this stage of research advancement around the world, including in Poland.

Milk and meat productivity in cloned cattle

Due to rigorous natural selection, calves with congenital defects are eliminated, while surviving cloned animals display no clinical changes/anomalies in adulthood. Watanabe and Nagai (2011) reported that cloned animals (dairy and beef cattle breeds) have a health status equivalent to that of non-cloned animals. Moreover, both groups of animals were found to be substantially equivalent in terms of milk and meat production (Watanabe and Nagai, 2011). The parameters tested for milk production included the following: 1) milk yield, 2) lactation curve, 3) overall milk composition, 4) total concentration, and structural and functional distribution/characterization of lipids, 5) content and percentage of different groups and types of fatty acids, 6) milk solids-non-fat (MSNF), 7) total concentration, and structural and functional distribution/characterization of milk proteins, 8) lactose concentration, and 9) somatic cell count and density. Very small (non-significant) differences have been established in the biochemical and physical properties of milk between cloned

and non-cloned animals. Statistically significant differences were only noted for the composition of milk fatty acids (Heyman et al., 2007). It is noteworthy that the whole lactation milk yield of the cows depends on both their genetic profile and their living environment. The impact of environmental factors during the period between birth and lactation can best be judged in cloned specimens, which by design have an identical genetic profile. Differences in whole lactation milk yield between cloned and non-cloned animals can then be clearly identified. Cloned cows differ very slightly from non-cloned cows in milk yield. These differences may be partly attributable to the more frequent incidence of subclinical mastitis. A study by Montazer-Torbati et al. (2016) demonstrated that milk production does not differ significantly between cloned and non-cloned animals, but the protein and fat levels observed in milk were less varied in cloned individuals. It was further shown that during the first lactation cycle, the milk lipid content in cloned cows was lower. On day 67 of lactation, milk fat content and milk protein concentration were also lower in cloned cows. The same authors suggest that the lower levels of these biochemical parameters may be correlated to the higher apoptotic index of mammary cells.

In turn, beef cattle breeds were evaluated for production parameters, such as: 1) meat yield based on fattening performance and body weight gain, 2) dressing percentage, or the ratio of carcass weight to live weight based on percentage of half-carcass and different carcass cuts, 3) physico-chemical properties of meat, 4) content and structural and functional distribution/characterization of amino acids, and 5) fatty acid profile. Considering the parameters mentioned above, only slight differences were found between cloned animals and their non-cloned counterparts (Heyman et al., 2007; Watanabe and Nagai, 2009, 2011). A statistically significant level of between-group differences only occurred for the fatty acid profile of skeletal muscle tissue (Yang et al., 2007; Heyman et al., 2007).

Somatic cell cloning as a suitable and reliable tool for production and multiplication of transgenic cattle

The application value of somatic cell cloning technology in cattle is related to the possibility to generate and multiply genotypically and phenotypically identical transgenic animals, which have transformed nuclear genomes and are valuable for the expression products of modified genes (Samiec and Skrzyszowska, 2011). It is worth noting the extremely promising findings of Zhang et al. (2019 b). For the cattle cloning procedure, these investigators used as nuclear donors transgenic somatic cells, the genomes of which were injected with lysine-rich cDNA constructs containing sequences of three genes coding for structural and functional milk proteins (ligation of exon segments of β -casein, α S2-casein and lactoferrin genes). It was observed that the lysine-rich *pBC1-LR-NEO^r* fusion transgene, whose tissue-specific expression was controlled by the β -casein gene promoter, not only was successfully integrated into the genomic DNA of bovine cells, but above all exhibited intense transcriptional activity towards the mammary cells. The lysine-rich recombinant fusion polypeptide, containing the combined amino acyl sequences of the three structural and functional proteins – β -casein, α S2-casein and lactoferrin – was highly detectable in the milk of three transgenic cows. In turn, the concentrations of polylysine polypeptides and

proteins remained at significantly higher levels in the milk from genetically modified cows compared to the milk samples from non-transgenic animals. Transgenic cows that provide lysine-rich polypeptides in milk could be used to produce, on a commercial scale, dairy products enriched with proteins containing many residues of this essential amino acid. The universal importance of lysine for the proper progression of many physiological processes (including lipid metabolism or lactation), as well as its fundamental role in mammalian organisms as an essential amino acid that builds polypeptide chains in the molecules of many proteins (including blood plasma proteins, structural proteins of the skin, bones and tendons, and hormone-, enzyme- and antibody-forming proteins), cannot be overestimated.

The use of the SCNT technique in cloning beef cattle breeds could be an efficient method to produce and replicate the populations of transgenic fattening bulls and heifers, the dressing percentage of which is characterized by higher parameters as a result of genetic modification aimed at inactivation of the myostatin-coding gene (Proudfoot et al., 2015) or post-transcriptional (pre-translational) suppression of mRNA molecules coded by the myostatin gene alleles (Tessanne et al., 2012). Myostatin is a hormonal protein, specific for striated and smooth muscle tissue, that, in a paracrine manner, inhibits gain (hypertrophy and hyperplasia) of skeletal and smooth muscles. The presence of a single gene-targeted (knocked-out) allele of the myostatin gene (Proudfoot et al., 2015) or the presence of a post-transcriptionally (pre-translationally) silenced mRNA, synthesized through the expression of a single non-targeted (wild-type) allele of the myostatin gene (Tessanne et al., 2012), as confirmed using molecular biology techniques in heterozygous transgenic specimens produced by somatic cell cloning, appear to lead to a considerable increase in the meatiness of bulls and cows for slaughter, resulting from the hypertrophy and hyperplasia of not only striated muscle tissue but also smooth muscle tissue. However, the kinetics of skeletal and smooth muscle hypertrophy and hyperplasia can be accelerated even more in cloned fattening bulls and heifers that are homozygous transgenic animals, which are characterized by the presence of two knocked-out (i.e., doubly gene-targeted) alleles of the myostatin gene (Proudfoot et al., 2015) or the presence of two post-transcriptionally silenced copies of the mRNA molecules encoded by both transcriptionally active alleles of the myostatin gene (Tessanne et al., 2012). In turn, the quality and taste properties of beef can be enhanced through the production of transgenic clonal cattle with overexpression of genes coding for adipocyte-type fatty acid-binding protein (A-FABP). Somatic cell-cloned transgenic beef calves with the confirmed presence of additional *A-FABP* gene copies are characterized by the capacity for increased accumulation of intramuscular fat with a relatively high concentration of unsaturated fatty acids in skeletal muscles, which is responsible for increased delicacy, juiciness and tenderness of beef (Guo et al., 2017).

The attractiveness of replicating transgenic heifers and bulls through somatic cell cloning is determined by applicability of the hormonal or enzymatic expression product of the modified gene. It is precisely this application potential that decides the scale and scope of the research. Although the first animal cloned was a sheep, research focused on somatic cell cloning of cattle was much wider in scope. Trans-

genic nuclear transfer-derived cows can become live bioreactors for producing humanized or easy-to-digest dietetic milk (Yang et al., 2011; Lu et al., 2016; Wang et al., 2008, 2017) or milk containing human recombinant therapeutic proteins (e.g., biopharmaceuticals or nutraceuticals). The latter may find clinical application in the treatment of patients with genetically determined diseases (Jang et al., 2006; Salamone et al., 2006; Monzani et al., 2013; Luo et al., 2015). It is also worth pointing out that transgenic cloned cows that produce milk containing immunoenzymatic proteins of bacterial or human origin (lysostaphin or lysozyme) have clinically proven resistance to bacterial mastitis (Liu et al., 2013, 2014).

Application potential of somatic cell cloning in cattle

It seems that after making the transition from basic to applied research, the techniques for intra- and interspecies somatic cell cloning of cattle, due to their application potential in the development of science and economy in Poland through their use in agriculture and fields of interdisciplinary research, could contribute to: 1) conservation of genetic resources and establishment of the genetic reserves of near-threatened, vulnerable or endangered native breeds of cattle (e.g., Polish Red, Whitebacked); 2) restoration and multiplication of the subpopulations of disappearing and rare conservative breeds of Polish Red and Whitebacked cattle to maintain biodiversity and to increase the levels of intra-population and inter-specimen genetic variation; and 3) revival and reintroduction into the wild of extinct, free-living species of the family *Bovidae*, which are the ancestors of some primitive breeds of domestic cattle (*Bos taurus*), such as the aurochs (*Bos primigenius*), the extinction of which was recorded in Poland in 1627. It should be noted that in Poland under the patronage of the Polish Foundation for Recreating the Aurochs, chaired by Professor Mirosław Ryba, and as part of a multi-faceted interdisciplinary project coordinated by Professor Ryszard Słomski from the Department of Biochemistry and Biotechnology, Poznań University of Life Sciences and by the Institute of Human Genetics of the Polish Academy of Sciences in Poznań and carried out in collaboration with Professor Zdzisław Smorąg from the Department of Reproductive Biotechnology and Cryoconservation of the National Research Institute of Animal Production in Balice, and with Professor Jacek A. Modliński from the Department of Experimental Embryology of the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences in Jastrzębiec research has been performed on a laboratory scale to partly or completely reconstruct the nuclear and/or mitochondrial genomes and to form a bank of molecules of the reconstructed mitochondrial genomes (mtDNA) of the aurochs, which were isolated from pedicle (horn primordial), bone and tooth tissues, derived from preserved museum exhibits of the horns and neuro- and viscerocranium of this species (Zeyland et al., 2013). Moreover, practically applied research into the cloning of cattle could serve to achieve other tangible benefits, including: 4) the improvement of the breeding (genetic) and productive value of different cattle breeds, such as increasing their milk yield, meat yield and reproductive ability (prolificacy and fertility); and 5) implementation (translation) of basic research into interdisciplinary sciences for the generation of animal biotechnological (transgenic) products for the biopharmaceutical, nutraceutical and food technology industries (e.g., bovine

mammary glands as bioreactors for producing humanized milk or milk containing human recombinant therapeutic proteins).

In general, when compared to conventional breeding and natural reproduction of cattle, the ontogenesis of cloned fetuses and offspring is characterized by a high incidence of spontaneous abortions among recipient females (surrogate heifers/cows) of nuclear-transferred embryos, a high proportion of premature births (including stillbirths of cloned conceptuses) in foster mothers, and high rates of periparturient and neonatal mortality in cloned calves (Farin et al., 2006; Watanabe and Nagai, 2011). A nationwide survey in Japan (Watanabe and Nagai, 2009) estimated that one-third of delivered nuclear transfer-derived calves died during the periparturient period due to stillbirth (abortions) and neonatal death due to the incidence of congenital malformations. These malformations are most often the result of epigenetic errors, which are already detected at the stages of embryonic (Yamanaka et al., 2011), foetal (Liu et al., 2008 a, b; Niemann et al., 2008) and neonatal development (Watanabe, 2013). Meanwhile, the frequency of death losses in cloned cattle due to factors contributing to the incidence of various diseases – among animals surviving more than 200 days after birth – appears to be identical to that observed in conventionally bred cattle (Watanabe and Nagai, 2009). It should also be noted that among the progeny of cloned cattle (obtained through natural mating or assisted reproduction, i.e., artificial insemination), the incidence of death loss throughout their lifetime remained at the same level as for conventionally bred animals (Watanabe and Nagai, 2009).

It has also been shown that the offspring of cloned cattle exhibits a proper epigenetic status as measured by the quantitative methylation profile of genomic DNA cytosine residues; moreover, their status is comparable to the status of cattle produced by conventional assisted reproduction. Yamanaka et al. (2011) provided evidence that the DNA methylation profile in blastocyst cells developing from zygotes generated by either artificial insemination of cloned females using the semen of cloned or non-cloned bulls or artificial insemination of non-cloned females using the semen of cloned bulls remained at the same level as the DNA methylation profile in blastocyst cells developing from zygotes generated by artificial insemination of non-cloned females using the semen of non-cloned bulls. It has also been confirmed that the DNA methylation profile in blastocysts developing from *in vivo*-fertilized oocytes generated by artificial insemination of non-cloned and cloned heifers/cows was considerably higher compared to the group of cloned embryos generated by SCNT, in which the DNA methylation profile persisted at a similar level to the profile observed when analysing the somatic cells used as nuclear donors in SCNT procedures (Yamanaka et al., 2011). In turn, Long et al. (2014) have noticed that the offspring of cloned animals display no anatomo- and histopathological changes characteristic of their parents. Nonetheless, the low efficiency of the somatic cell cloning technique (measured not only by birth rates of healthy nuclear transfer-derived offspring but also by the rates of periparturient/perinatal and postparturient/neonatal survival) is due to the high embryonic, foetal and neonatal mortality rates. These last mentioned factors are underpinned by abnormal or incomplete reprogramming of the epigenetic memory of somatic cell nuclei in the cytoplasm of reconstructed and artificially activated oocytes and in the blastomeres of nuclear-transferred embryos, resulting in abnormal

transcriptional activity of the nuclear and mitochondrial genomes at the embryonic, foetal and postnatal stages of ontogenesis.

Prospects for the use of cloned cattle as food source – state of the art and future targets

Thus far, technologies developed and optimized to efficiently or repeatedly generate and multiply somatic cell-cloned cattle have not been focused on the use of nuclear transfer-derived animals for food production, although there are no objectively justified grounds for the suspension or withdrawal of the somatic cell cloning technique from the production of livestock for consumption. This also concerns the breeding of SCNT-derived specimens and their progeny, the import of cloned animals and/or nuclear-transferred embryos, and meat and milk products originating from cloned animals. Based on the available research, in 2008–2009, the US Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), and the Japanese Food Safety Commission (FSC) concluded that food products from the meat and milk of cloned animals are equivalent to corresponding products made from the meat and milk of animals generated by conventional assisted reproductive techniques such as artificial insemination (AI) and embryo transfer (ET). Despite these assurances, the commercial use of these products in the agri-food industry and in commercial trading is prohibited in many European Union countries, not only due to the lack of social acceptance for food products of such origin but also because of the low efficiency of SCNT technology in farm livestock species. Considering the above, it must be stressed that for commercial purposes, it is necessary to disseminate information about the low risk of using modern assisted reproductive technologies (ARTs) and to carry out further research aimed at the considerable improvement of cattle cloning efficiency.

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