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The many problems of somatic cell nuclear transfer in reproductive cloning of mammals

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ABSTRACT

In 1996, when Dolly the sheep was born, a new, utopian era was expected to begin. Science fiction and popular culture instantly threatened the public with shortly upcoming human clones, portraying it as a very easy and instant procedure. Practice has proven otherwise; it exposed how little is known about the early development of mammals and epigenetic reprogramming. Unfortunately, somatic cell nuclear transfer success rate in mammals has not changed much since its very beginning. It is not uncommon that hundreds of oocytes need to be reconstructed to obtain a single live birth. In this review we provide a brief summary of the progress and problems of the field; beginning with selection of the donor cells and their susceptibility to different methods of epigenetic reprogramming; methods of the later gene activation, placental abnormalities, and their possible causes; to health issues that such offspring is prone to.

1. Introduction

Even though cloning became a matter of public discussion only in the late twentieth century, the first attempts were undertaken ca. 1892; when Driesch separated blastomeres of a sea urchin embryo and demonstrated that two separate organisms were formed [1]. After 60 years Robert Briggs and Thomas J. King performed the first nucleus transfer, from blastula cells into enucleated frog eggs [2], but cloning mammals hadn't been made possible for next 45 years. In 1996, over a hundred years of research resulted in the first successful attempt to clone a mammal from an adult somatic cell [3]. Not only the science world saw it as a breakthrough; popular media sought to portray the apparently and shortly upcoming human cloning as the biggest threat to society, resulting in titles such as “Greetings from Frankenstein: Is the Cloned Human on the Way?” and continued to portray scientists as the insane [4]. In reality, reproductive cloning is performed by a transfer of a somatic cell nucleus into an enucleated oocyte and results in a single cell, analogous to a fertilized oocyte, usually referred to as a reconstructed oocyte. Despite the fears of uncontrolled and easy cloning, practice shows that quarter a century later little is understood in the matter and many more years of innovative research are required for cloning to be as successful as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

This year Dolly the sheep would have been 26 years old. While many more species were reported to be cloned, overall success rate from the creation to a viable and healthy offspring remains at a similar, low level. In 2002, Y. Tsunoda and Y. Kato have published a commentary, summarizing the live birth ratio as low as 0.5%–18% in sheep, cattle, pigs and mice [5]. A review from 2007 estimated it as low as 1–5% [6]. A nation-wide survey in Japan, that covered 9 years of Somatic Cell Nuclear Transfer (SCNT) efforts (1998–2007) showed no improvement in development and survival until 6 months of cloned calves (on average 4.3% success rate, with 7.5% in 1998 and 2.8% in 2007) [7]. Similar statement was made as of 2020 [8]. Nonetheless, today reproductive cloning serves as a tool of creation of champion polo horses, farm animals of exceptional genetic makeup and as a way of bringing back beloved pet companions.

Dolly was the only viable infant of 277 couplets created with mammary epithelium cells [3]. In 1998, the ‘Honolulu technique’ was developed [9], resulting in the first successful murine cloning.

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There, in mice, the cumulus cells showed to be the most suitable nuclei donors and the only ones able to produce offspring. Out of 4 experiment series, the highest birth rate was reported to reach 2.8%. The perhaps unexpected difficulties caused a 5 year gap between the first mouse and rat cloned [10]. In rats, specifically, oocytes activate spontaneously within an hour after being removed from the oviducts. The original protocol, allowing for the first successful rat nuclear transfer followed by live births, involved collecting the oocytes in the presence a proteasomal inhibitor (MG132). It reversibly inhibited the oocyte from entering anaphase II, thus preventing precocious activation. Starting with 129 reconstructed and transferred embryos, three pups were delivered alive and two of them survived (which marks 1,5%) and later also produced offspring [11]. It is highly desired to create rat clones populations, as they make a useful model for medical research. As new lines of inbred animals are created and other become excinct, to achieve a 98.6% homozygous, isogenic population, over twenty consecutive generations of brother x sister or parent x offspring crossing must occur [12,13]. This process could perhaps be replaced by somatic cell-cloned animals.

The first cloned pet animal, “CC”, simultaneously the first ever cloned cat, was particularly famous for a different coat color than the nuclear donor, as it is influenced by developmental factors, other than genetic [14]. She was also the first clone to reproduct, with three out of four kittens viable. The offspring and the mother herself were reported to live in good health, and CC died in 2020 at 18 years old [15]. Many difficulties had to be overcome to clone the first dog as well, and the specimen was born in 2005. 1095 embryos were transferred to 123 bitches and three pregnancies were confirmed, with “Snuppy” being the only viable offspring [16]. Later, in 2011, Jung et al. reported their effort to clone pekinese dogs, where the reconstructed oocytes were activated using 4 different techniques (needle and chamber fusion methods, both divided in groups using low and high voltage). 180 embryos were transferred, and out of 3 fetuses only one pup was born alive (0.5%) [17]. Despite inaccesibility to canine oocytes, relatively rare ovulation and very low *in vitro* maturation efficacy of canine oocytes [18], a resounding success was achieved and canine alongside feline cloning is commercially available as of 2022 at a cost of ca. $50 000 and $35 000, respectively [19]. Recently an attempt to clone Macaque Monkeys was reported [20]. With an optimized protocol, out of 301 reconstructed embryos, four live births were obtained and only two of them survived. This makes 1,3% birth rate, which is also rather similar to the results from 20 years prior.

The first ever cloned calves were obtained from only ten embryo transfers, and of eight live births four survived [21]. The authors speculated that no abnormalities were observed post-mortem, and the demise was due to environmental causes, including pneumonia following a heatstroke, drawing in superfluous amniotic fluid, and one as a consequence of dystocia. Despite high success ratio of the initial study, a 2001 report on cloning efficiency, which included 13 citations of adult SCNT in cattle, showed that most of them didn't achieve 2% live births compared to the number of reconstructed embryos [22]. In 2007, 49.1% of 160 laboratories worldwide were reported to clone cattle [23]. As this practice became widely available among breeders and due to the consumers' concerns, Food and Drug Administration (FDA) offers Consumer Health Information. It addresses, among others, safety of consumption of meat derived from cloned specimens and the fact that the cloned animals are meant for breeding, and not direct consumption, as the cost of obtaining such animals is also very high [24]. SCNT remains the main method, when it comes to obtaining gene edited livestock [25].

Prometea, the first equine clone, was born in 2003. She was the only viable foal obtained from 841 reconstructed zygotes [26]. In 2008, a review of SCNT progress in horses was released [27]. Authors cited five studies (all of them published from 2003 to 2007) in which a total of 3577 equine oocyte reconstructions were performed and only 15 foals were born. Highest success rate was achieved when donor cells were treated with roscovitine, which is thought to synchronize the treated donor cells in G0/G1. Several protocols of activation were additionally applied in these groups, including ionomycin and/or sperm extract [28]. Commercial equine cloning is also performed (ex. Viagen, Sinogene), but international studbooks registering racing thoroughbreds refuse to recognize clones [29]. Argentine Polo Pony clones are accepted in competitions. It is claimed that Crestview, a commercial laboratory that offers such service, cloned over 200 horses before 2016 [30] with a 30–40% pregnancy rate and 10% of the pregnancies end up with a live birth rate [31].

Despite extreme difficulties, SCNT is often seen as a hope to restore extinct species or help preserve the endangered. In popular media, Mammoth “de-extinction” is brought up every few years as some small progress is made in the matter [32]. Pyrean Ibex was successfully cloned, using tissue from the last known specimen, but the infant died shortly after birth due to left lung atelectasis. An additional lobe was also found occupying majority of its thoracic cavity, with no connection to the tracheobronchial tree [33]. Similarly, the first cloned gaur died within the first 48 h after birth [34]. Other endangered species, including Grey Wolves [35], Black Footed Ferret [36], Przewalski's Horse [37], were successfully cloned. To the authors' best knowledge, as of 2022, SCNT is not considered a major strategy in preserving any endangered species.

In Table 1, we summarized selected publications, focused on species and corresponding success rate which illustrate two and a half decades of advances in the field. They are extremely valuable, especially in the cases where the species are endangered by extinction.

2. Protocol issues

The procedure of cloning by SCNT begins with the nucleus donor cell and the reciepient ooplast. A brief summary of the process is illustrated in Fig. 1. While the process can be presented generally, it needs to be recognized that in practice, there is high species-specificity involved.

It is common for the rather older literature to suggest that achieving G0 phase is necessary for the nucleus donor cell before it is fused together with the ooplast [38], which can be obtained through serum starvation. More modern evidence shows that non-starved G1 cells serve the same purpose with success [39,40] and in some cases, are equally successful to G0 cells [41]. From this perspective cumulus cells might provide a great facillitation, as 80% of them prove to be arrested in G0/G1 stage naturally and are very suitable as nucleus donors due to the fact that no *in vitro* culture is required [42,43]. On the other side of the fusion, to obtain the ooplast, the oocyte's meiotic spindle complex is removed, requiring a proper visualization technique. For example, bovine oocytes can be treated with UV and fluorochrome bisbenzimide (Hoechst 33342) staining, to which they are relatively resistant [44]. Primate embryos only show comparable blastocyst formation rate (15–16%) when Oosight imaging system is introduced, which does not involve the staining. A speculation was raised by the authors, that Hoechst 33342 might activate primate oocytes prematurely or cause degradation of maturation factors, which could be later linked to epigenetic reprogramming failure [45]. Later, the positive impact of replacing staining with Oosight imaging system in bovine embryos was also demonstrated [46]. In mice specifically, the spindle complex can be made visible using Nomarski or Hoffman optics, also without staining, in 37 °C [47].

Table 1
Summary of the species and corresponding success rate.

Year	Species	Results	Other comments
1997 [3]	Sheep	Dolly the sheep was born; the only viable offspring of 277 attempts	The first mammal cloned from adult somatic cells
1998 [9]	Mouse	Four experiments, highest birth rate 2.8%	First mice cloned
2003 [10]	Rat	3 pups born of 129 reconstructed embryos	First rats cloned, later reproduced naturally
2002 [14]	Cat	One viable offspring of 82 transferred embryos, total 188 reconstructed oocytes	The first cat cloned, the first pet cloned, later produced healthy offspring. She died at the age of 18 due to kidney failure [130]
2005 [16]	Dog	Three pregnancies obtained from 1095 embryos transferred; one viable offspring	Snuppy the dog was later cloned as well [131]
2011 [17]	Dog	One viable offspring of 180 transferred embryos	Four different activation techniques were tested in this experiment
2007 [35]	Wolf	251 reconstructed embryos, 2 live births	Endangered species; Grey Wolf somatic cells nuclei were transferred to canine oocytes
2003 [26]	Horse	Only one viable foal obtained from 841 embryos	First cloned horse
2016 [109]	Horse	16 blastocysts resulted in 11 pregnancies, 3 foals delivered viable	All of the placentas showed abnormalities
2020 [127]	Rabbit	61 blastocysts formed from 165 reconstructed oocytes	This result was achieved using electrofusion and melatonin used as a protective agent against electrofusion damage
2020 [36]	Black footed ferret	1 live birth	Donor cells cryopreserved over 30 years prior to the procedure.
2021 [128]	Dromedary Camel	1033 reconstructed oocytes, 28 births, 19 calves survived	No significant differences in effectiveness regarding the breed of the oocyte donor
2020 [37]	Przewalski's horse	One foal born to a domestic horse dame	Cells cryopreserved in 1980 were used in this procedure
2019 [100]	Crab eating macaque	5 live births out of 325 embryos transferred	Involved (CRISPR)/Cas9 editing

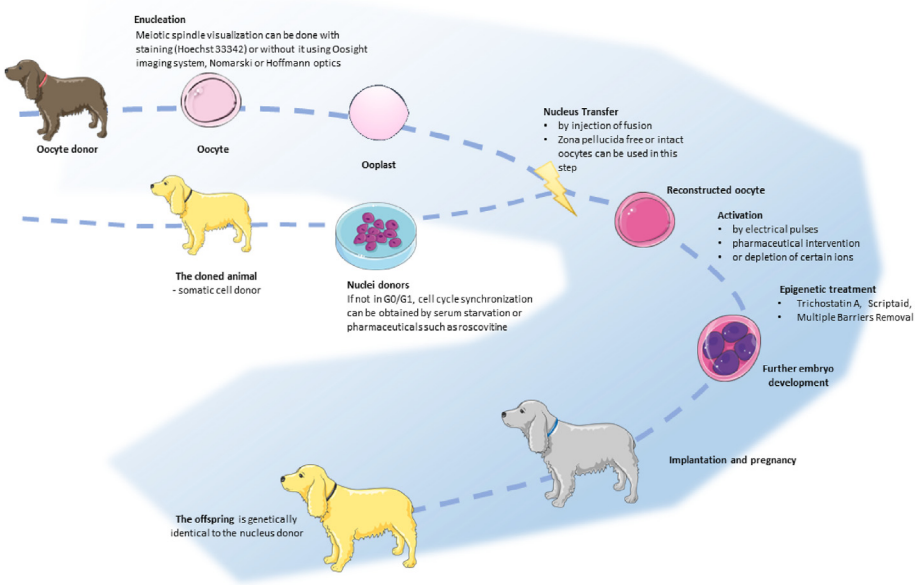

Fig. 1. A general illustration of the SCNT protocol.

There is an ongoing debate, whether oocytes stripped of Zona Pellucida are better candidates for this process, as SCNT using Zona Pellucida-Free (ZF) oocytes tends to be simpler and quicker [48]. It seems to have no affect on bovine [49,50], equine [26,51] and murine embryo development [52]. However, it does not apply to any species given - in cats, removing zona pellucida later leads to complete impairment and failure of such embryos' implantation [53]. ZF embryos require individual, longer lasting culture, i.e., until the blastocyst develops. Additionally, treating the cells with phytohemagglutinin further increases the chances of both cells' fusion [54]. Zona-enclosed embryos, which are harder to obtain, can be transferred at cleavage [54]. Regarding the difficulties with ZF oocytes in cats, specific gene expression abnormalities were identified during the *in vitro* culture of such: SOX2 and NANOG - considered as markers of pluripotency expression - were under-expressed and BAX, apoptosis marker, proved overexpressed [53]. To authors' best knowledge, there are no successful cases reported of ZF feline cloning.

The metaphase II stage arrested, and enucleated oocytes become the recipients of the donor nucleus, whether it is injected or the cells are fused [55]. Until their activation, the development is managed by factors which were already present in the recipient oocyte, such as Maturation/Meiosis/Mitosis-Promoting Factor (MPF) [56]. Physiologically, sperm cells introduce Phospholipase C Zeta 1 (PLCZ1, absent in somatic cells), which, through calcium oscillation and MPF breakdown, induces the meiotic arrest release and further development [57]. Alike fertilized oocytes, the reconstructed oocytes need to be activated to enter the division cycle.

This process must be recreated artificially and so far multiple activation methods have been investigated.

The protocol which resulted in Dolly's birth consisted of electrical pulses between 34th and 36th hour after the ovulation was induced in donor ewes [3]. In the aforementioned Honolulu technique, best results were achieved when the oocytes were activated one to 3 h after the nucleus transfer, in calcium-free, 10 mM Sr^{2+} and 5 μg ml^{-1} cytochalasin B environment [9]. Other reports describe PLCZ cRNA injection, which results in repetitive fertilization-like Ca^{++} rises in bovine reconstructed oocytes [58]. This method was tested alongside with two more common protocols involving ionomycin/6-dimethylaminopurine (DMAP) and ionomycin/cycloheximide (CHX). In this study, PLCZ showed lower, but comparable efficiency (27.7% embryos developed to blastocyst stage vs. 35.9% for ionomycin/CHX). Another study describes a comparison of three activation methods in porcine embryos: electrical fusion/activation, electrical fusion/activation combined with MG132, and electrical fusion in low Ca^{2++} followed by chemical activation with thimerosal/dithiothreitol. The second treatment was the most effective, but only 2 piglets (1.9% of embryos transferred) were delivered [59]. It was also proposed to activate the reconstructed oocyte by reducing zinc ions concentration within the cell. As a result, even in the absence of cellular Ca^{2+} changes, the oocyte can be activated, when Zn^{2+} is depleted with heavy metal chelators in the intercellular space [60].

In 2019 another study was published, which focused on creating a faster but equally reliable porcine protocol. Four experiments took place and were compared to a control group, which was treated with the routine protocol for the laboratory (ionomycin exposure for 5 min followed by $SrCl_2$ and CHX for 4 h, total 245min). It was concluded that similar percentage of embryos which developed to the blastocyst stage was achieved with all methods, including a 20 min procedure, where oocytes were exposed to ionomycin for 5 min plus 15 min of TPEN (N,N,N0,N0-Tetrakis(2-pyridylmethyl) ethylenediamine) treatment [61]. Unfortunately, time-saving procedures might not be as beneficial after all, as a 2019 meta-analysis reveals that delaying the activation by over an hour significantly increases the blastocyst formation rate [62].

3. Epigenetics

It has been clear for decades that the biggest challenge regarding the cloned embryo development lays in its epigenetic reprogramming; physically, among many, resulting in hardships regarding the implantation, placenta development and function, but also abnormal offspring syndrome [63], later obesity, immunodeficiency, respiratory defects and early death [64]. It has been concluded that this epigenetics issue is vastly responsible for the low birth rate [65,66]. It is critical for the cell nucleus to return to totipotency through undifferentiation. If incomplete before genome activation, the failed patterns will be copied and expressed, resulting in fetal abnormalities and death [67,68]. Dysregulations at the time of genome activation have been studied and multiple aberrations have been proven. As many as 259 differently expressed mRNAs have been reported in a study only at the one cell stage of an SCNT embryo, compared to *in vivo* fertilized embryos, making ca. 1.6% of all detected transcripts (137 of higher, 122 of lower expression) [69].

As mentioned, no epigenetic manipulations were applied in the murine protocol [9]. In case of Dolly, the cell cultures were serum-starved only with a suggestion, that such quiescence could possibly help the reprogramming [3]. Both cases show that the oocyte itself has the ability to reprogram the transferred nucleus. To increase the birth rate pharmaceutical intervention can be applied. Among many, DNA methylation, histone acetylation and methylation were studied as potential molecular targets with a variety of results. Trichostatin A (TSA) is a drug of Histone Deacetylase Inhibitor (HDACi) class [70]. Its action, through interfering with enzymatic removal of acetyl groups from histones, assures the DNA accessibility for the transcription factors. In case of somatic cell nucleus remodeling, the addition of TSA is expected to support unfolding of the nucleosome so it can resemble the totipotent pattern. In 2006, two groups, independently, have established the most appropriate protocol for murine SCNT using TSA and fivefold increase in the embryo's development was reported [71,72]. Another report delivered data of enhanced blastocyst development rate in monkey (4%–18%) [73]. Embryos reconstructed using adult bone marrow Mesenchymal Stem Cells (MSC)and treated with TSA prove the success rate to grow exponentially, with 65,2% of electrofused embryos reaching the blastocyst stage, of higher cytological and molecular quality compared to 45,5% in those untreated [74]. Nonetheless, questions were raised about TSA's toxicity and teratogenicity [75]. In a recent meta-analysis of its effectiveness on pig SCNT blastocysts yield, an important conclusion was drawn, that TSA improves the embryo development when the nuclear donor cell is of fetal and not adult source. Furthermore, even though the embryonic development is usually increased, births are reduced [76]. In rabbits, a study was performed where out of six recipients two became pregnant with reconstructed embryos treated with TSA [77]. Out of eleven pups, seven were born alive but all died within an hour to 19 h. The authors recommend further investigation on long-term effects of such treatment, but don't associate the mortality with it as early deaths are common in cloned animals. Another substance of the same class, Scriptaid, has been shown to provide similar therapeutic effects [78] with less toxicity [79] but a different study showed improved blastocyst formation in bovine embryos only when treated with TSA and not Scriptaid [80]. A 2022 meta-analysis on Scriptaid's potency to increase the number of blastocyst cells or the cleavage rate in porcine embryos derived from SCNT concluded that only the former might be true [81].

Other epigenetic treatment candidates researched include Chetomin, a fungal secondary metabolite (which supposably inhibits the trimethylation on histone 3 lysine 9 (H3K9 me3)) and caffeine (as a protein phosphatase inhibitor) [82]. In the cited article authors did not observe any improvement in bovine and equine embryo development compared to TSA, even after a 48 h exposure to Chetomin. Caffeine, on the other hand, was shown to improve the developmental capability of porcine embryos [83] and primate embryos [84]. Another H3K9 methyltransferase inhibitor, Chaetocin, was studied but opposite to expectations, it impaired the ovine embryo development [85]. More recently, a different, promising approach on epigenetics was proposed: Mutliple Barriers Removal [86]. The following was proposed: Histone 3 lysine 9 trimethylation, the first barrier, can be removed by injecting a demethylase (Kdm4d) mRNA into reconstructed embryos 5 h post-activation. Similarly can be done with Kdm5b mRNA injection into the enucleated oocyte. Combining both increased the rate of murine cloned embryos' development into live animals to 11% [87]. Also knock-out of H3K9me3 transferases in the donor cells prior to SCNT brought improvement of almost 50% in blastocyst formation compared to 6,7% in control group [88]. Authors point out remethylation to be another barrier, which could be overcome by injecting siRNAs of methyltransferases Dnmt3a and Dnmt3b into the enucleated oocytes. Finally, the same authors reported that 92.3% of enucleated oocytes that underwent Kdm4b+5b mRNA and siDnmt3a+3b co-injection reached the blastocyst stage. So far no data is available whether any viable offspring was ever obtained using this method. Unfortunately, promising embryonic development during the *in vitro* culture might have nothing to do with survival until birth and later viability of such newborn. In

epigenetics, it is perfectly illustrated when donor cells of different origin are used: equine adult skin fibroblasts show similar blastocyst development rate, and even birth rates to equine MSC, but foals created with MSC are not burdened with malformations or diseases and present higher viability [89]. This might be due to the fact that MSC show lower activity of Histone Deacetylases and DNA methyltransferases (DNMTs) [74], which is possibly the reason for the susceptibility to epigenetic reprogramming. Using MSC as the nucleus donors also seems to improve the success rate of the procedure in equines – in a study where MSC (along with adult fibroblasts) were compared as donor cells against a control group of Artificial Insemination (AI) embryos. A total of 594 MSC embryo-transfers resulted in 37 pregnancies and 20 viable foals (3,4% success rate, compared to 1,9% for adult fibroblasts and 71,6% for embryo transfer) [89]. Good developmental perspectives have been shown in porcine [90–92], caprine [93] and bovine embryos [94] cloned with MSC.

4. Abnormal placental development

Although the placenta developed de novo many times throughout the evolutionary history [95], it is usually associated with Placentalia, a subdivision of the class Mammalia. In the live bearing, the placenta allows for the embryo to implant in the uterine wall; this process, if imperfect, may result in multiple pathologies. Generally, they are inevitable in SCNT pregnancies. Due to this reason, the majority of them are lost during the first trimester [96] or peri-implantation [97]. In bovine embryos, a reduced number of placentomes, hydroallantois and general enlargement are observed [98,99]. In 2006 genetic aberrations in the developing placenta have been proven. Authors confirmed that certain genes responsible for the trophoblast proliferation, differentiation and function were expressed differently to the control group (embryos derived from AI and IVF, leaning towards high proliferation genes expression and low or absent function-associated ones both pre- and post-implantation. At day 17 the SCNT-derived trophoblast showed no signs of interferon-tau production, which is the pregnancy recognition signal in cattle [100]. Another study identified 123 differentially expressed circRNAs, which were found related to 60 host genes and 32 miRNAs [101]. Especially the miRNA abnormality is to be linked to the problematic epigenetics of SCNT. In the cited study, transcriptomes of the mRNA, lncRNA and miRNA of placental cotyledon tissue at day 180 after gestation were sequenced and 362 mRNAs, 1272 lncRNAs and nine miRNAs were found to differ in expression [102]. Thus, the problematic placental development is most likely emergent and not fundamental.

Placentas and fetal membranes of SCNT animals have also been examined by ultrasound imaging and histologically which revealed even more abnormalities [103]. Amniotic membranes, placentomes, umbilical cords, and fetal fluid were studied by ultrasound and compared to an AI control group. It disclosed focal edema, larger placentomes and increased umbilical cord diameter with hyper-echodensity areas and spikes around it. Histological findings included degenerated inflammatory cells, chorioallantoic membrane edema, and decreased epithelial thickness. Clinical observations prior to the study fall into place with it, as placentome hypertrophy, enlarged umbilical vessels and placental edema were reported [104,105].

In mice, placental hyperplasia and expanded spongiotrophoblast layer develop [106] with poor proliferation rates of the trophoblast cells followed by rapid growth in later stages [107]. Recently, causes of this enlargement were studied [108]. In natural conception in mice, genes Sfmbt2, Gab1, and Slc38a4 are paternally expressed, but in SCNT placentas, they are expressed biallelically. At first, a maternal knockout of previously recognized enlargement-related genes (Sfmbt2, Gab1, Slc38a4) was performed with no improvement in placental quality but correcting the expression of clustered miRNAs within the Sfmbt2 gene the physiological placental phenotype was restored. The pups grew into normal, fertile adults. Although after miRNA knockout the birth rate increased more than twice (6.7% vs 3% in the wild SCNT clones) the numbers were considered statistically insignificant. Therefore, even though the abnormal placental development can at least be partially prevented, more underlying issues are probably to be identified.

In equines the placenta faces developmental issues as well. A detailed study of equine SCNT pregnancies was published in 2016 [109]. Nuclei obtained from fibroblasts of a 29-years old mare were transferred. Sixteen blastocysts underwent an embryo transfer, resulting in eleven pregnancies, during which the placentas were monitored for abnormalities. Three foals were delivered viable. Pathologies were found in all assessed placentas; even in the three successful pregnancies. In these, placental edema, engorged allantoic vessels and a large umbilical vessel were found. Two mares were diagnosed with bacterial placentitis and treated with trimethoprim sulfamethoxazole, pentoxifylline and flunixin meglumine. In the five mares that experienced abortion the placentas were found heavier than normal, up to 35% of the fetal weight. All of them showed some form of placentitis. Villous hypoplasia, atrophy or necrosis, vasculopathy of allantoic and umbilical vessels, hemorrhages and placental mineralization were reported as well. Additionally, four of five umbilical cords were longer than regular and edema, hemorrhage and mural thickening of the umbilical vessels were observed. There were four cases of large allantoic vesicles. Severe placental separation was diagnosed in some as well. Two more mares were found to have had bacterial placentitis only after the abortion, despite no ultrasonographic findings. The viable foals experienced hypoxic-ischemic encephalopathy, pneumonia, omphalophlebitis and omphaloarteritis. Two foals had umbilical hernias blood clots in the urinary bladders. Incomplete calcification of carpal bones and multiple rib fractures were found in one foal. Two foals were discharged on day 12, the third one suffered hemothorax due to a fractured rib causing puncture of the pericardium and was euthanized. These findings are mostly consistent with previous research [110,111], but this particular study was the first one to report placentitis in SCNT pregnancies.

5. Other findings

Even though the majority of cloned animals live in good health, it is estimated that nearly one in three dies within the first 6 months of life [112]. Large/Abnormal Offspring Syndrome (LOS), respiratory failure, abnormal kidney development, cardiovascular and liver pathologies are often reported [22,112,113]. LOS is widely associated with cloned and IVF offspring. By definition, it results in a phenotype consisting of excessive overall fetus size, muscular deformities, abnormal and asynchronous organ growth combined with placental dysfunctions. Its occurrence is unpredictable [114], but so far has not been observed in horses [27], when overgrowth is expected, parturition induction can be performed a week before the due date [115]. Abnormal fetal development is linked to obesity later in life in animals [116] and adult murine clones struggle with increased body mass [117].

Il-Hwa Hong et al. [118] reported necropsy findings in 12 stillborn/deceased SCNT canine neonates. Significant increase in muscle mass and macroglossia were commonly observed. The tongue size caused airway obstruction in many cases. Moreover, anterior abdominal wall defects, edema and atelectasis of the lung tissue,

increased heart and liver size were present in almost all of the dogs and one case of failed cerebrum medulla development was found. Interestingly, this particular neonate presented normal muscle mass and tongue size. These abnormalities raised questions on myostatin expression in such specimens, as myostatin-deficient animals share such phenotype. The team found reduced expression in myostatin in their tongues and muscle and provided some interesting discussion. They explain that myostatin is one of the TGF-β family members, which is regulated by IGFs during cell cycle and its differentiation. Another study showed variable, abnormally high IGF2R, IGF1R mRNA expression in adult SCNT produced goats [119], otherwise healthy. IGF2 mRNA expression also varies in bovine clones [120].

Aging in animals cloned from adult somatic cells is researched due to justified concerns of the cloned offspring to be at the biological age of the nucleus donor at birth. Among the most concerning are probably the epigenetic dysregulations, problematic in these specimen as discussed above. Telomere length also rose questions, as they were expected to be shorter, proportionally to the age of the donor of the nucleus. Dolly's telomeres were confirmed to be 20% shorter to the control group [121], and it seems to be a tendency in cloned sheep, according to a 2017 review [122]. Surprisingly, it also reported telomere length in cloned cattle to be appropriate for their age or even elongated, but overall, in other species it was often found reduced compared to natural offspring of the same age. Recent study in Dromedary Camels showed that telomere lengths in clones was adequate to the control group [123]. Interestingly, TSA was proved to facilitate telomere elongation in cloned pigs [124].

The aforementioned abnormal placental development might be linked to cardiovascular issues in the early life, according to Batchelder et al. [125]. The study suggests that anomalous placental membranes, causing abnormal circulatory pressure, interfere with the fetal heart's development. This thesis was supported by echocardiographic findings (initially decreased aortic diameters and increased myocardial thicknesses) in seven cloned calves, which showed that during the first 30 *ex utero* life, they made adjustments and by the end of the study the differences to the control group resolved. Other authors also agree that although SCNT animals vary from non-SCNT control offspring in the first two months of life, it is no longer the case after this period is over [112]. In another report, seven of ten cloned piglets were similarly evaluated between 40 and 65 day of life [126]. Decrease in fraction ejection, mitrial insufficiency and decreased cardiac output was observed in some. Authors also included necropsy findings in five piglets who died during the study and in four of them, cardiovascular deviations were observed, including an enlarged heart with dilated, thin-walled ventricles with myocardial necrosis, myocardial degeneration and excess pericardial fluid and congestive heart failure. The surviving five piglets showed no symptoms of cardiac disease.

General examination, hormonal and hematologic assessments have been performed in cattle cloned from somatic cells [127]. Fetuses obtained before term showed variable organ sizes and deformities, including abnormally large kidney, autolysis of both kidneys in a stillborn and large fatty liver. Neonates had higher average birth weight compared to the AI and IVP control groups. Rectal temperature of clones was found to be higher until 50 days of age, including random 24–36 h peaks up to 41 °C with no other clinical signs observed, non-reactive to any treatment applied (NSAIDs, wet towels and ventilation). T_4 plasma levels were examined in speculation, whether it could be the reason for the fever, but the answer was negative. RBC parameters were found to be consistent within all groups. The neutrophil:lymphocyte ratio, reflecting cortisol levels at birth, was higher in clones. Despite this, later ACTH stimulation tests found no significant differences. Regarding the growth hormone, normal level was found, as well as in insulin and glucose responses. To the authors' best knowledge, these macroscopic findings are not found to originate within any specific developmental deviation.

6. Suggestions for further research

In a short summary, the low efficiency reflects how complicated SCNT and further embryo development is. Some recommendations and assumptions can be made with a precaution, that even though sharing similar problems and results, probably no two studies can be directly compared. Different methods and approaches are researched in order to further rectify the whole procedure as such. A faster and simplified SCNT would limit both oocyte's and donor cells' exposure to external factors and manipulations and require shorter training of the staff with similar success rates. Here, an inevitable conclusion has to be stated - that more successful cloning will require deeper understanding of oocyte's physiology, not only as a mammal oocyte, but also the species specificity and uniqueness. Especially the relationship between nucleus-donor cell type and introduced pharmaceuticals need further studies; which might be very complicated due to multiple possibilities and combinations. Perhaps the future of SCNT lies in the Mutliple Barrier Removal technique, as it potentially withdraws the need of using any classical pharmaceutical treatment, but on the other hand, may require more injections *per se*. Yet again, blastocyst formation or cleavage rate, often presented as determinants of a successful study, may not have anything to do with the embryo's pregnancy survival and the newborn's viability. While many studies are promising, their actual value can only be verified by obtaining healthy offspring.

Aside from discussed technical difficulties, ethics of cloning, widely discussed in the popular media, cannot be forgotten. So far no successful human reproductive cloning attempt was documented, not only due of the legal regulations, but also due to lack of reasonable cause for such; when it comes to infertility, multiple methods other than SCNT can be used. In animal cloning, doubts could be raised especially in case of commercial dog cloning. World famous Snuppy, the first dog cloned by SCNT, out of 1000 embryos transferred into 123 surrogates, was the only pup surviving until adulthood [16]. To our knowledge, such businesses do not publicly share information on the surrogates' living conditions, especially those, who experience abortion, nor about the dogs that do not get adopted due to lack of predictability regarding the live births count.

Despite multiple issues, of which some we briefly described, progress continuously takes place. In the end, SCNT may not be as easy as portrayed quarter a century ago, but each and every advance may be a giant leap for the science of medicine.

Author contributions

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Declaration of competing interest

None.

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