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REVIEW ARTICLE

State of the art of nuclear transfer technologies for assisting mammalian reproduction

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Abstract

The transfer of nuclear genomic DNA from a cell to a previously enucleated oocyte or zygote constitutes one of the main tools for studying epigenetic reprogramming, nucleus–cytoplasm compatibility, pluripotency state, and for genetic preservation or edition in animals. More than 50 years ago, the first experiences in nuclear transfer began to reveal that factors stored in the cytoplasm of oocytes could reprogram the nucleus of another cell and support the development of an embryo with new genetic information. Furthermore, when the nuclear donor cell is an oocyte, egg, or a zygote, the implementation of these technologies acquires clinical relevance for patients with repeated failures in ART associated with poor oocyte quality or mitochondrial dysfunctions. This review describes the current state, scope, and future perspectives of nuclear transfer techniques currently available for assisting mammal reproduction.

KEYWORDS
cloning, horse, polar body, pronuclei, spindle

1 | INTRODUCTION

In mammals, the generation of a new life implies the combination of two wholly differentiated cells with a haploid set of chromosomes giving rise to a new cell with diploid content, capable of generating all existing cell types in an adult individual. This feature of totipotency is gradually lost during early preimplantation embryo development, and after that, most cells acquire a unidirectional path of differentiation. Assays performed by Gurdon in 1962 revealed for the first time that the differentiated status of a cell from a vertebrate could be reversed by factors contained into the ooplasm, introducing the concept of nuclear reprogramming (Gurdon, 1962). Subsequently, the development of the somatic cell nuclear transfer (SCNT) technique demonstrated that adult somatic cells from mammals were also reprogrammable by an oocyte (Wilmut et al., 1997). From the birth of Dolly in 1996 to date, at least 25 mammals have been born using this technology (Table 1). Interest in reproductive cloning to artificially generating genetically identical individuals has significantly grown in the last years, mainly in the animal production sector and, especially, in sport horses. Moreover, private companies that offer cloning services of companion animals have also recently emerged. Despite the efforts of several research groups, the commercial interest, and the development of novel molecular analysis tools, SCNT for reproductive purposes is still a low-efficiency technology that frequently leads to alterations during embryo, fetal, placental, and neonatal development.

In the field of human reproductive medicine, and more recently in the bovine and horse industry, technologies such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and preimplantation genetic testing are routinely used in laboratories

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TABLE 1 Mammalian's species born from SCNT or iSCNT from fetal/adult donor cells

N	Year	Donor cell species—cell type	Recipient egg	References
1	1996	Sheep—fibroblast-like cells	Sheep (*Ovis aries*)	Campbell et al. (1996)
	1996	Sheep—mammary gland cells	Sheep	Wilmut et al. (1997)
2	1997	Mouse—cumulus cells	Mouse (*Mus musculus*)	Wakayama et al. (1998)
3	1998	Cow—Fetal fibroblast	Cow (*Bos taurus*)	Cibelli et al. (1998)
	1998	Cow—cumulus cells	Cow	Y. Kato et al. (1998)
4	1999	Goat—fetal transgenic cell line	Goat (*Capra aegagrus hircus*)	Baguisi et al. (1999)
5	1999	Pig—granulosa cells	Pig (*Sus scrofa domesticus*)	Polejaeva et al. (2000)
6	2000	Gaur (*Bos gaurus*)—adult fibroblast	Cow	Lanza et al. (2000)[b]
7	2001	Rabbit—cumulus cells	Rabbit (*Oryctolagus cuniculus*)	Chesné et al. (2002)
8	2001	Cat—cumulus cells	Cat (*Felis catus*)	Shin et al. (2002)
9	2001	Mouflon (*Ovis orientalis musimon*)—granulosa cells	Sheep	Loi et al. (2001)[b]
10	2003	Mule—fetal fibroblast	Horse (*Equus ferus caballus*)	Woods et al. (2003)[b]
11	2003	Horse—adult fibroblast	Horse	Galli et al. (2003)
12	2003	Rat—fetal fibroblast	Rat (*Rattus norvegicus*)	Zhou et al. (2003)
13	2003	Banten (*Bos javanicus*)—adult fibroblast	Cow	Janssen et al. (2004)[b]
14	2003	Deer—fibroblast cells	Deer (*Odocoileus virginiana*)	Westhusin (2003)[c]
15	2003	African wildcat (*Felis silvestris lybica*)—adult fibroblast	Cat (*Felis catus*)	Gómez et al. (2004)[b]
16	2005	Dog—adult fibroblast	Dog (*Canis lupus familiaris*)	B. C. Lee et al. (2005)
17	2005	Ferret—fetal fibroblast	Ferret (*Mustela putorius furo*)	Z. Li et al. (2006)
18	2005	Buffalo—fetal fibroblast	Buffalo (*Bubalus bubalis*)	Shi et al. (2007)
19	2007	Wolf (*Canis lupus*)—adult fibroblast	Dog	Kim et al. (2007)[b]
20	2008	Sand cat (*Felis margarita*)—adult fibroblast	Cat	Gómez et al. (2008)[b]
21	2009	Pyrenean ibex (*Capra pyrenaica pyrenaica*)—adult fibroblast	Goat (*Capra pyrenaica*)	Folch et al. (2009)[a,b]
22	2009	Camel—cumulus cells	Camel (*Camelus bactrianus*)	Wani et al. (2010)
23	2017	Monkey—fetal fibroblast	Monkey (*Macaca fascicularis*)	Z. Liu et al. (2018)
24	2021	Przewalski's horse (*Equus ferus przewalskii*)—adult fibroblast	Horse	Revive and Restore (2020)[b,c]
25	2021	Black-footed ferret (*Mustela nigripes*)—adult fibroblast	Ferret	US Fish and Wildlife Service (2021)[b,c]

[a]The cloned animal died few minutes after birth.
[b]Interspecific somatic cell nuclear transfer (iSCNT).
[c]Milestones not from peer-reviewed articles.

around the world. In general, excluding male factors, the success of ARTs is negatively affected by the number and quality of retrieved oocytes (due to advanced maternal age, breed, ovarian dysfunctions, and other reproductive problems) and the presence of genetic disorders such as mutations of mitochondrial DNA (mtDNA). Therefore, the possibility of recovering the nuclear DNA (nDNA) of an "affected" oocyte (by any of the previously mentioned causes) acquires clinical and productive interest to generate a viable embryo that preserves the female genetic. This can be achieved by transferring the nDNA from the female patient's gamete or zygote to the ooplasm of a healthy donor, known as germline nuclear transfer (NT) technology. Unlike SCNT, the NT technologies involve the replacement of nDNA from oocytes, eggs, or zygotes with a single set of unpaired chromosomes, and the embryos are produced

TABLE 2 Utmost results achieved on NT technologies in humans

Technique	Observations	Results	References
GV-NT	Consenting research patients	One embryo arrested at eight cells and one compacted morula	Takeuchi et al. (2001)
SCNT	Commercially available female dermal fibroblasts of fetal origin and Leigh syndrome patient cells	Blastocyst and embryonic stem cells derivation	Tachibana et al.(2013)
MII-NT	A 36-year-old woman with a history of four pregnancy losses and two decease children from Leigh syndrome	Healthy baby born in 2016	J. Zhang et al. (2017)
PB1-NT	Woman with multiple cycles of severe embryo fragmentation	Nontransferred blastocysts	S. P. Zhang et al. (2017)
PB1-NT	Consenting research patients between 25–31 years old	Nontransferred blastocysts	Ma et al. (2017)
PN-NT	An infertile 34-year-old woman	Healthy baby born in 2017	Manzur (2020). Nadiya Clinic
PB2-NT	37-year-old consenting research patients	Nontransferred blastocysts	Tang et al. (2019)

Abbreviations: GV-NT, germinal vesicle nuclear transfer; MII-NT, metaphase II nuclear transfer; PB1-NT, first polar body nuclear transfer; PB2-NT, second polar body nuclear transfer; PN-NT, pronuclei nuclear transfer; SCNT, somatic cell nuclear transfer.

TABLE 3 Utmost results achieved on germline NT technologies in nonhuman mammals

Technique	Species	Results	Reference
GV-NT	Rabbit	Matured oocytes	G. P. Li et al. (2001)
	Mice	Offspring	Takeuchi et al. (2004)
	Cattle	PA blastocyst	Franciosi et al.(2010)
	Cat	IVF blastocyst	Comizzoli et al. (2011)
	Pig	IVF blastocyst	Dang-Nguyen et al. (2017)
MII-NT	Mice	Offspring	M. K. Wang et al. (2001)
	Nonhuman primate	Offspring	Tachibana et al. (2009)
	Cattle	Offspring	Y. J. Lee et al. (2021)
PB1-NT	Mice	Offspring	Wakayama and Yanagimachi (1998)
	Porcine	PA blastocyst	Lin et al. (2013)
	Nonhuman primate	Offspring	Z. Wang et al. (2021)
PN-NT	Mice	Offspring	McGrath and Solter (1983)
	Pig	Offspring	Prather et al. (1989)
PB2-NT	Mice	Offspring	Wakayama et al. (1997)

Abbreviations: GV-NT, germinal vesicle nuclear transfer; IVF, in vitro fertilization; MII-NT, metaphase II nuclear transfer; PA, parthenogenetic activated; PB1-NT, first polar body nuclear transfer; PB2-NT, second polar body nuclear transfer; PN-NT, pronuclei nuclear transfer.

by fertilization. The technical complexity of NT technologies and its incipient clinical application in humans generate social concern and are currently under the critical eye of the scientific community (Siristatidis et al., 2021). Tables 2 and 3 highlight the utmost results achieved for NT technologies used in humans and mammals, respectively, for reproductive purposes.

2 | REPRODUCTIVE SCNT

The term reproductive cloning usually refers to an artificial form of asexual reproduction that enables the generation of genetically identical organisms and does not involve fertilization. Therefore, cloning is not limited only to SCNT. For example, embryos produced

by separation or bisection of their cells at early stages of development are considered "clones" since they contain the same genetic information and, unlike most embryos produced by SCNT, the same mtDNA. The first evidence of an artificial generation of two genetically identical individuals was reported in the '80s (Sander, 1997). Subsequently, embryo bisection was introduced in veterinary medicine, but the low reproducibility and embryo viability after bisection still impede its commercial use (Rahbaran et al., 2021). If embryo viability is maintained after its division, this technology might potentially increase the final number of embryos and, therefore, the chance of a successful pregnancy (Illmensee et al., 2011). However, a recent study has shown that even though embryo splitting in humans does not induce chromosomal abnormalities, it affects embryo morphokinetics and quality (Omidi et al., 2020). Up to date, there is no country whose regulations allow the division of embryos for clinical purposes, and there are no reports of embryo transfer generated by bisection in humans.

The concept of NT was introduced almost a century ago by Hans Spemann, the now considered "father of cloning" (Spemann, 1921). First births by SCNT using embryo blastomeres as donor cells were reported in several domestic species during the '80s and '90s, such as the sheep (Willadsen, 1986). However, the birth of "Dolly" in 1996 marked a milestone by demonstrating that differentiated adult somatic cells could be used to produce live animals by SCNT (Wilmut et al. 1997).

After removing the meiotic spindle, "enucleation," a diploid somatic cell is injected into the perivitelline space and then fused with the enucleated egg. Both enucleation and cell injection are usually performed under an inverted microscope coupled to a micromanipulation equipment. An alternative protocol includes the direct injection of the donor cell into the ooplasm, but its use is restricted to small cells (Canel et al., 2012; Wakayama et al., 1998). Subsequently, the signal cascade that triggers embryo development is induced by a process known as artificial activation (Figure 1, SCNT), generating an embryo that is cultured in vitro until it is transferred or cryopreserved. The cloning procedure can also be done by removing the zona pelucida of the egg known as zona-free SCNT. This facilitates enucleation and fusion processes but requires a special

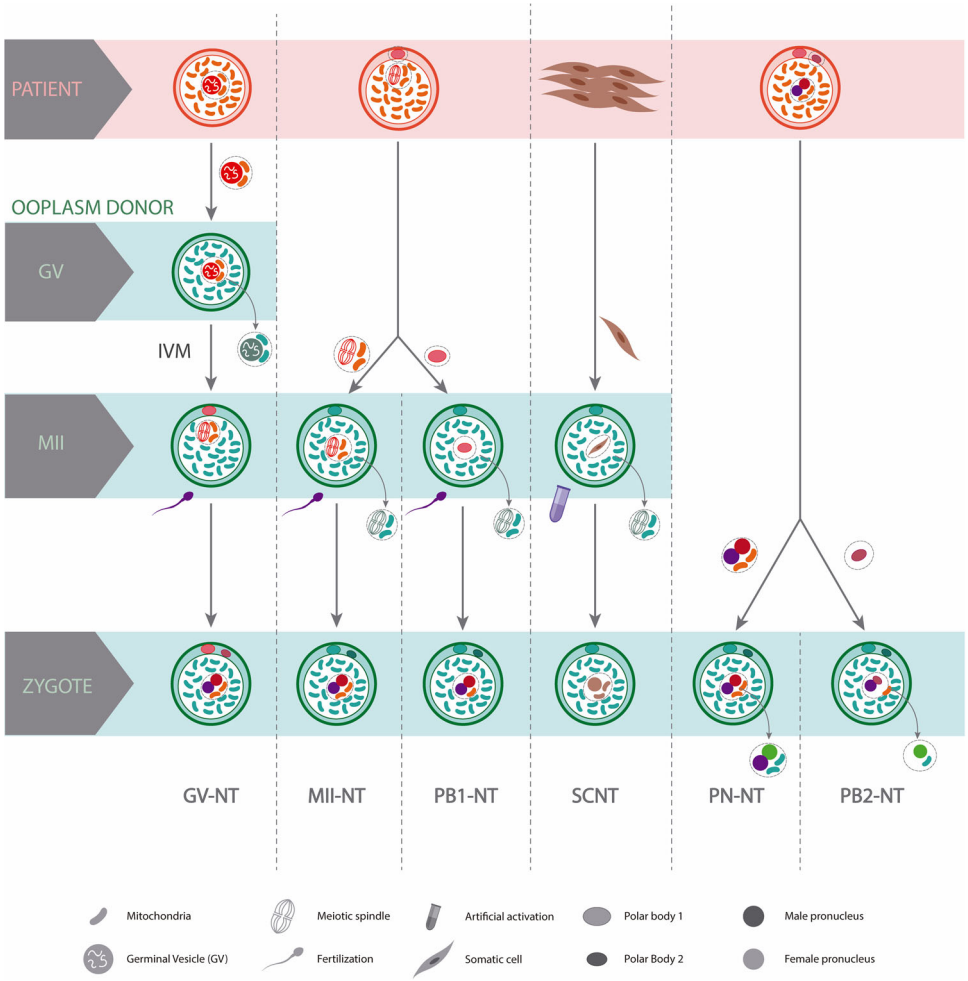

FIGURE 1 Scheme of nuclear transfer technologies with reproductive purposes in mammals. GV-NT, germinal vesicle nuclear transfer; IVM, in vitro maturation; MII-NT, metaphase II or maternal spindle transfer (MST); PB1-NT, first polar body nuclear transfer; PB2-NT, second polar body nuclear transfer; PN-NT, pronuclei nuclear transfer; SCNT, somatic cell nuclear transfer (cloning).

micro-well culture system (Vajta et al., 2000). In addition, zona-free SCNT made it possible to explore some strategies to improve cloning efficiency. Specifically, improvements in embryo developmental competence have been achieved using embryo aggregation. Our research group produced the first equine clone in Latin America in 2010 (Gambini et al., 2012, 2014) and the first equine clone born in Australia (Damasceno Teixeira et al., 2019). Embryo aggregation is currently used to produce equine clone's offspring on a large scale (Gambini & Maserati, 2017) and we estimate that about 1000 cloned horses have been produced worldwide.

SCNT remains challenging, and its inefficiency in producing live offspring limits its application for animal production. Despite this, SCNT is required to preserve valued animal genetics (particularly by the horse breeding industry, Gambini & Maserati, 2017), for conservation of endangered species (briefly described later in this review), for pets genetic preservation (private companies such as ViaGen pets; https://viagenpets.com and Sinogene; https://www.sinogene.org), and to produce genetically modified animals (Galli & Lazzari, 2021). Several modifications to the initial cloning procedure were emerging, as assisted chemical enucleation, new fusion methods, donor cell and recipient oocyte cycle synchronization, new artificial activation methods, use of epigenetic modulating agents, antioxidants, novel culture systems, among others, leading to differences in SCNT protocols among laboratories (Loi et al., 2021).

2.1 | Nuclear reprograming as the main limitation of SCNT

The process of reproductive cloning by SCNT is one of the most complex technologies, requiring a high degree of micromanipulation and the simulation of processes that should naturally occur. Epigenetic reprogramming of the somatic cell during SCNT is often incomplete (Ogura et al., 2021), leading to problems during early embryo development, gestation, placentation, and even during the first weeks after birth. In many cases, the clinical presentations at birth are species-specific and may include higher or lower weight at birth, abnormal size of certain organs, alterations in neuromuscular coordination, respiratory problems, and a weakened immune response, among others.

In recent years, a significant progress on epigenetic events and chromatin architecture has made possible to understand in more detail the molecular mechanisms associated with reprogramming, identifying some “resistant regions.” Some of these regions are associated, for example, with specific epigenetic marks such as the methylation of histone 3 in lysine 9 (H3K9me), considered as one of the main epigenetic barriers of mice cloning (Matoba et al., 2014). To circumvent these limitations, several strategies to improve nuclear reprogramming are now included in cloning protocols, such as correction of the exogenous expression of protamine (Czernik et al., 2016) and Kdm4d, a lysine-specific demethylase, overexpression (Chen et al., 2020; Weng et al., 2020).

In addition to improving nuclear reprogramming, advances in other research areas are still essential to investigate to enhance the development of clones, such as premature activation of oocytes, artificial activation, mito-nuclear communication, cell cycle synchronization and cryopreservation, in vitro culture systems, and the selection and synchronization of the recipients.

2.2 | SCNT for conservation of endangered species

SCNT has a great potential for the maintenance of biodiversity through the recovery of species, to produce embryonic stem cells, and to study the embryo development of endangered species. However, the major limitation is the access to reproductive samples of extinct or endangered species. For this reason, eggs of phylogenetically related species are used as the receptor ooplasm for the somatic cell, a technique known as interspecific cloning or Interspecific (iSCNT).

Although iSCNT is not the primary strategy in endangered species conservation programs, its use could preserve valuable genetics that would otherwise be lost. Attempts to perform iSCNT have been made with eggs from phylogenetically related (intragenus) or distant species in numerous mammalian species with variable results, ending in established pregnancies and births only in a few species (Table 1, iSCNT). For example, it has been possible to cryopreserve epithelial and muscle cells of 15,000 years old tissues from the woolly mammoth (*Mammuthus primigenius*), extinct about 10,000 years ago. This tissue has been proven to form pronuclear-like structures when injected into mice enucleated eggs (H. Kato et al., 2009).

Our research group reported the production of the first zebra (*Equus quagga*) cloned embryos using oocytes from domestic horses with no differences in the expression of trophectoderm or inner cell mass proteins (Gambini, Duque Rodríguez, et al., 2020). Also, in 2020, the partnership of the Frozen Zoo at San Diego Zoo Wildlife Alliance, Revive and Restore, and ViaGen Pets and Equine reported, the birth of the first Przewalski's horse (*Equus ferus przewalskii*) (Revive and Restore, 2020), an endangered species that belongs to the same genus than horses, zebras, and donkeys. Last, in 2021, the birth of a black-footed ferret (*Mustela nigripes*) produced by iSCNT, from cells preserved for 30 years was reported (US Fish and Wildlife Service, 2021). Of note: the two last births mentioned in this section were reported in not peer-reviewed articles. These are clear examples of how iSCNT is currently being used to preserve endangered genetics increasing the genetic variability within animal populations by restoring genomes lost years ago. However, many factors limit the application of iSCNT such as nucleus–mitochondria compatibility (recently reviewed by Mrowiec et al., 2021), the lack of knowledge for optimal in vitro developmental conditions, and the selection of a recipient for to carry on a healthy pregnancy to term.

2.3 | SCNT in nonhuman and human primates

Cloning in nonhuman primates has generated interest due to the phylogenetic relationship with humans. The production of live birth

nonhuman primates clones has been more complex than other mammals and the birth of the first primate clones was achieved only 4 years ago (Z. Liu et al., 2018).

Although the technical feasibility of cloning humans for reproductive purposes exists, its practice is unethical and prohibited in most countries. Nonetheless, some scientists dedicated their efforts to this aim, which has provoked controversy. The first report of human SCNT embryos was in 2001 (Cibelli et al., 2001), and in 2006 the first cloned human embryo was transferred at the four-cell stage, without establishing a gestation (Zavos & Illmensee, 2006).

Efforts to improve SCNT in primates are more focused on therapeutic cloning. That is to say, the production of blastocysts to generate isogenic embryonic stem cell lines to be potentially used in regenerative medicine, precision medicine, development of disease models, and in vitro production of organs. The first report of human SCNT blastocysts was in 2005 with embryonic stem cells used as nuclear donors (Stojkovic et al., 2005). However, the first embryonic stem cells derived from a cloned human embryo were obtained after identifying the main limitations that blocked the development of primate clones: the premature exit from the oocyte arrest in metaphase II and a suboptimal activation (Tachibana et al., 2013). Currently, the rates of blastocysts for human clones are highly variable as in other species, with values around 10% (Chung et al., 2014; J. E. Lee et al., 2020; Tachibana et al., 2013).

3 | NT FOR RESTORING THE DEVELOPMENTAL COMPETENCE OF GAMETES AND ZYGOTES

The reprogramming capacity of the egg revealed by the first NT reports opened the possibility of using donor genomes from oocytes, eggs, or zygotes to rescue infertility. NT could restore failures of embryo development due to maternal cytoplasmic factors by introducing nDNA into a young, healthy, and competent ooplasm. Although most NT technologies reports have been focused on mitochondrial replacement (MR), its application could potentially solve any ooplasmic related infertility in aged patients or with diminished ovarian reserve (Christodoulaki et al., 2021). It is known that aging affects oocyte quality (Duncan et al., 2017) including epigenetic events that could potentially impact later in the offspring (reviewed by Moghadam et al., 2022). Mitochondria are energy-producing organelles that contain their own genome, and unlike nDNA, thousands of copies exist in each cell. The biparental transmission of mtDNA was reported (Luo et al., 2018); however, mammals typically harbor only the maternal mtDNA genotype. In many mitochondrial-related diseases, mutated and normal alleles coexist, and this condition is known as heteroplasmy. NT technologies could restore the homoplasmy condition of the embryo (Jiang & Shen, 2021).

Finally, NT allows the production of "xeno-oocytes" when an ooplasm of a different species from the nDNA donor is used, an exciting tool for revealing species-specific unknown mechanisms. Below, we describe the NT technologies that have been studied, including those with nDNA from oocytes before fertilization (a): germinal vesicle (GV), metaphase II (MII), first polar body (PB1); or after fertilization (b): second polar body (PB2), or pronuclei (PN) (Figure 1). To maximize NT clinical application, it is possible to use cryopreserved nDNA from oocyte/eggs and combine the different NT techniques to produce a final embryo, which is known as "sequential NT." For example, researchers have produced blastocyst by PN-NT after the fertilization of matured oocytes reconstructed by GV-NT (H. Liu et al., 2000).

3.1 | Before fertilization

3.1.1 | GV-NT

Before puberty, mammalian oocytes are arrested in the prophase of meiosis I, also known as the GV stage. Only GV a few oocytes will complete the oocyte maturation process, in which meiosis will restart until reaching the metaphase II stage, acquiring the competence to be fertilized (egg). For GV-NT, the germinal vesicle (or the metaphase I) containing the nDNA of interest is removed and inserted into a healthy oocyte that was previously enucleated. Reconstructed oocytes are then cultured in vitro to induce the maturation process and then fertilized by ICSI to produce an embryo (Figure 1, GV-NT).

In humans, stimulation protocols for oocyte collection for assisted reproductive techology (ART) seek to obtain eggs. In contrast, in vitro maturation protocols have been intensely explored in most domestic animals, allowing the in vitro production of embryos from oocytes to become standard practice. Mouse, rabbit, cat, cow, and pig blastocysts have been produced after GV-NT, achieving births in some of these species (reviewed by Darbandi et al., 2017). In humans, meiosis resumption was observed in vitro after GV-NT, and the development of ICSI embryos up to the morula stage was reported (Takeuchi et al., 2001).

An advantage of GV-NT is that it provides a model for studying cytoplasmic factors involved during the maturation process, and it could rescue oocytes from individuals who do not respond to gonadotropins or fail to resume meiosis. On the other hand, GV-NT is disadvantaged as an alternative for the MR since a relatively high proportion of altered mitochondria remains in the embryo (Yabuuchi et al., 2012). Additionally, GV-NT involves early removal of cumulus cells and in vitro maturation of oocytes, with the associated lower embryo developmental rate, particularly in humans. To date, apart from the species mentioned in Table 3, there are no reports of human or any other mammal's blastocysts produced by GV-NT.

3.1.2 | MII-NT

It consists of removing the meiotic spindle and chromosomes from an affected MII oocyte and inserting it into a healthy MII oocyte, which was previously enucleated (Figure 1, MII-NT). After reconstruction, fertilization is performed, usually by ICSI. This technique is also known as maternal spindle transfer.

This NT technology is considered the safest and most straightforward acquiring the most significant human clinical relevance. However, only murine and Rhesus macaque have been used as animal models, with only one recent study in a large domestic animal model (Y. J. Lee et al., 2021). Thus, additional animal models should be included before spreading the human clinical application of this technology. Although MII-NT emerged as MR therapy, the possibility of providing a healthy cytoplasmic environment to the nDNA of an affected oocyte could offer a solution to other pathologies. For example, it was recently reported that MII-NT could restore developmentally compromised mouse oocytes (Bai et al., 2020; Ogawa et al., 2020; Yamada & Egli, 2017) and failed fertilization after artificially activated assisted ICSI (Tang et al., 2022). The first baby born using this technology was reported by J. Zhang in 2017. It was a case of "Leigh syndrome," a devastating childhood disease associated with mtDNA mutations. Moreover, using a mice model, it was recently shown that MII-NT could overcome the developmental arrest caused by ooplasmic defects (Costa-Borges et al., 2020). Surprisingly, it was reported that a mtDNA drift can occur during in vitro culture of embryonic stem cell lines derived from blastocysts generated by MII-NT (Yamada et al., 2016), and this possibility should be considered for its clinical application. A disadvantage of MII-NT technology is that, as a nuclear membrane does not surround the spindle-chromosome complex, chromosomes may be more readily lost or damaged during micromanipulation (Tachibana et al., 2013), and this could be even more important in aged oocytes with a higher degree of chromosomes alterations.

3.1.3 | PB1-NT

PB1-NT replaces the nDNA of a healthy donor egg with the first polar body (PB1) of the patient's egg (Figure 1, PB1-NT). The polar bodies are formed during oocyte maturation and are dispensable for embryo development. They consist of a small ooplasm with the bivalent chromosomes in the case of PB1 (complementary to those of the MII from the mature oocyte) or the haploid set of chromatids in PB2 (complementary to the content of the female pronucleus, see PB2-NT). Several studies in mice have shown that the DNA of the PB1 can be used to produce viable offspring. However, results obtained in other species have not been so encouraging, except for nonhuman primates (Z. Wang et al., 2021). Porcine embryos produced by PB1-NT and fertilized by IVF or ICSI have failed to develop beyond the eight-cell stage (Lin et al., 2013). In humans, in vitro production of PB1-NT blastocysts followed by ICSI has been achieved. Although the quality of these embryos does not seem to be significantly affected according to genetic, epigenetic, and transcriptional studies, developmental rates are lower (Ma et al., 2017; S. P. Zhang et al., 2017b).

The major advantage of the PB1-NT is that can be combined with MII-NT generating two embryos from a single donor egg. This justifies the efforts of exploring its clinical application in humans and in other species with commercial interest, such as in horses, since the equine industry has a vast interest in obtaining foals from a particular mare. Interestingly, mares selected for assisted reproduction programs are usually aged mares and are an excellent model for investigating human reproductive aging. Since minimum ooplasms is excluded from the PB1 during cytokinesis, the PB1-NT also emerges as a good alternative to prevent the transmission of mtDNA mutation (T. Wang et al., 2014). However, it seems to depend on the species (Z. Wang et al., 2021). Summarizing, some potential advantages of PB1-NT compared to NT-MII are (i) reduced risk of leaving chromosomes behind because of being strongly compacted within CP1, (ii) no need to use cytoskeletal inhibitors during oocyte micromanipulation, (iii) possibility of carrying out PB1-NT and MII-NT together and doubling the chances of success per cycle. However, there are still some limitations and concerns about PB1-NT, mainly related to the genetic, epigenetic, and lifespan quality of the DNA of the PB1, with possible consequences over the offspring and subsequent generations (Wei et al., 2015). It was recently demonstrated that the PB1 could functionally replace MII-NT in generating healthy primate offspring by PB1-NT. The efficiencies of ICSI and pregnancy were comparable to those using nonmanipulated oocytes. Notably, PB1-NT derived offspring showed a <5% mtDNA heteroplasmy level (Z. Wang et al., 2021). The recent scientific advances in this area seem to provide promising scenarios for the clinical application of this NT technology in humans and other mammalian species.

3.2 | After fertilization

3.2.1 | PN-NT

During PN-NT, the pronuclei of a patient's zygote are transferred to a healthy zygote in which pronuclei have been previously removed (Figure 1, PN-NT). Of note, polar bodies are usually removed to avoid their fusion and alter the ploidy of the embryo. In this section, we are also consider the prepronuclear transfer that is performed right after fertilization. PN-NT idea was developed by McGrath and Solter in 1983 and has proven to be relatively efficient in producing offspring in some species. In 1989, the first pig born by PN-NT was reported (Prather et al., 1989), and several years later, some alterations associated with mtDNA were possible to repair in mice. However, variable heteroplasmy levels were observed in adult animals (Sato et al., 2005). The first assay using this technology in humans was reported in 2003 (J. Zhang et al., 2003), resulting in triplets with no births. In 2010, another study demonstrated the possibility of reducing the amount of mtDNA in the patients by this technique, showing its potential to prevent the transmission of diseases associated with mtDNA (Craven et al., 2010). It has also been elucidated that the early stages of PN, around 12 h after fertilization, are more efficient in supporting the development of the reconstructed embryos (Hyslop et al., 2016; Bai et al., 2020). However, a small number of mitochondria from the donor egg is transferred to the recipient ooplasm (Wolf et al., 2017), resulting in variable

heteroplasmy levels. Recently, PN-NT has been effective for improving oocyte developmental competence affected by aging and possibly more efficient than MII-NT (Tang et al., 2020).

Unlike the other NTs, the DNA of the PN is under the replication and telomere elongation processes. Hence, significant epigenetic and chromatin architecture modifications are occurring at this crucial stage of development (Canovas & Ross, 2016; Gambini, Stein, et al., 2020). For this reason, compared to micromanipulation of the egg, micromanipulation of zygotes could be more harmful for the embryo developmental competence. Although survival rates after the micromanipulation of human zygotes are reasonable, the need to "destroy" a zygote to provide the opportunity of another zygote to be reconstructed remains controversial, and it does not occur with NT technologies carried out before fertilization. As a more ethical approach, parthenogenetically activated zygotes could be used, but the negative aspects of artificial activation could impact the development of the future embryo. To date, there are at least 10 births reported by PN-NT in humans, including a patient with a mutation on *TUBB 8* gene (Mazur 2020).

3.2.2 | PB2-NT

PB2 is formed after fertilization and, due to its chromosomal load, it could replace the maternal pronucleus. PB2-NT consists of extracting the second polar body from the patient's zygote and transferring it to a donor zygote, from which the maternal PN has been removed (Figure 1, PB2-NT). Similar to the PB1-NT, this technique rescues nDNA that otherwise will be lost, allowing it to produce an extra embryo.

The first study that successfully demonstrated the ability of the second polar body to replace the female PN was in 1997 (Wakayama et al., 1997) with full-term development of mice embryos. Interestingly, 25 years have passed, and no reports of births in other mammal species have been published. The advantages of PB2-NT are like those of PB2-NT concerning the genetic potential, the number of mitochondria introduced during the procedure, and the possibility of easy micromanipulation without inducing chromosome damage (Tang et al., 2019). Murine blastocyst PB2-NT rates are like those observed for the other NT technologies (Tang et al., 2019; T. Wang et al., 2014). In this species, pronuclei can be easily visualized under a bright light microscope, and the PN size and closeness to the PB2 can reveal its origin. However, in most domestic species, the heterogeneous and lipidic content of the cytoplasm makes it challenging to identify the pronuclei, and their size is not always an indicator of their origin. Therefore, specific markers are required and limit the research and clinical application of this technology in most mammalian species. If a safe protocol to identify the origin of pronuclei is developed, PB2-NT could acquire greater clinical relevance in other species since it could be carried out simultaneously with other NTs, maximizing the production of healthy embryos. Moreover, the PB2 in the mice has a similar pronucleus morphology and chromatin pattern to the FPN (T. Wang et al., 2014), and its viability seems to be greater than that of PB1, persisting during the initial stages of embryo development in several species (Bartholomeusz, 2003). To date, there is only one report of nontransferred blastocysts generated by PB2-NT in humans (Tang et al., 2019). The current state of this technology requires further basic research in various species before its clinical application in humans.

4 | FUTURE PERSPECTIVES

If SCNT achieves repeatability and efficiency, it will become the method of choice to recover valuable genetics for animal production and generate animal models in large mammalian to develop novel therapies for complex diseases. On the other hand, the major drawback for assaying therapeutic cloning in humans is the requirement of good quality oocytes and the ethical and legal restrictions on their use for this purpose. In this sense, the possibility of creating new gametes in vitro from stem cells or the recent reports of blastocyst-like structures generated from human pluripotent stem cells and the derivation of stem cell lines (Yu et al., 2021) is causing great expectations as alternatives to produce isogenic stem cell lines or potentially to produce transferred embryos for animal cloning avoiding SCNT technology.

Although only a few laboratories in the world have proven to perform NT technologies successfully, more than 12 babies have been born using NT, including the recent report of the first baby born in South America by MII-NT (Télam, 2021). The combination of NT technologies could become a routine practice to produce an extra embryo from the same oocyte, not only for humans but for animals with high genetic value, such as horses. Among future alternatives to avoid problems associated with mitochondrial mutations, the possibility of using novel gene-editing tools is emerging (Mok et al., 2020) and it could be combined with NT technologies. Most mitochondrial proteins are encoded by nuclear genes, existing a robust communication network between mitochondria and the nucleus, such that nDNA/mtDNA incompatibility could affect ATP production, thus altering the redox balance (Hill, 2018). Therefore, the possibility of combining different haplotypes of mtDNA with nDNA through NT technologies could allow, for example, the generation of embryos with improved mitochondrial and cellular function by finding the best match for the nucleus of that cell. This could be particularly interesting in sporting horses, where mitochondrial function at the muscular level is at its optimum.

5 | CONCLUSIONS

The transfer of nDNA from a mammal cell to an enucleated oocyte and its subsequent embryo development has been one of the most impactful milestones in modern science. It accelerated the progress of certain research areas such as biomedicine, animal and human reproductive medicine, pluripotency, epigenetics, and stem cells. At the same time, SCNT has had an interesting sociocultural impact

since it became the only available tool to rescue extinct species or companion animals and accelerate genetic progress in breeding livestock species. In combination with gene-editing technologies, SCNT would allow the production of animals with desirable characteristics in less time, such as fast growth, disease resistance, improved meat quality, or sports performance. However, the molecular mechanisms involved in the SCNT process still need a deep understanding.

ARTs are relatively new clinical practices, therefore the safety and potential adverse effects in the short-, medium-, and long-term have yet to be determined and they must always be carried out by experienced operators, under extreme vigilance and considering ethical and legal aspects. The remaining challenges of NT techniques are the levels of mtDNA carryover and the potential reversion of a pathogenic mutation. These technologies also require a higher degree of micromanipulation than the regular procedure performed to generate ICSI embryos which could negatively affect embryo viability (Cheng et al., 2009).

Authors consider that, although clinical NT application in humans has already begun, it is necessary to promote policies to facilitate research in more animal models, including large domestic species, for a deeper understanding of the epigenetic reprogramming events of NT technologies and their long-term effects on the offspring before encouraging its clinical and commercial use.

AUTHOR CONTRIBUTIONS

Andrés Gambini and Olinda Briski designed the idea of the review. Andrés Gambini, Olinda Briski, and Natalia Canel wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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