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Cloning in action: can embryo splitting, induced pluripotency and somatic cell nuclear transfer contribute to endangered species conservation?

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ABSTRACT

The term 'cloning' refers to the production of genetically identical individuals but has meant different things throughout the history of science: a natural means of reproduction in bacteria, a routine procedure in horticulture, and an ever-evolving gamut of molecular technologies in vertebrates. Mammalian cloning can be achieved through embryo splitting, somatic cell nuclear transfer, and most recently, by the use of induced pluripotent stem cells. Several emerging biotechnologies also facilitate the propagation of genomes from one generation to the next whilst bypassing the conventional reproductive processes. In this review, we examine the state of the art of available cloning technologies and their progress in species other than humans and rodent models, in order to provide a critical overview of their readiness and relevance for application in endangered animal conservation.

Key words: cloning, conservation, endangered, pluripotency, somatic cell nuclear transfer, gamete, oocyte, egg, spermatozoa, sperm, iPSC, SCNT.

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I. INTRODUCTION

Our planet is in the midst of an extinction crisis; about a quarter of the world's mammal species are currently threatened with extinction. Preserving threatened species is not simply a matter of numbers, but requires a stable population of individuals with sufficient genetic diversity such that the species can resist inbreeding depression and poor health. Most current management programs define a sustainable population as one in which 90% of the genetic diversity present in the founder base (preferably ≥20 individuals) can be maintained for a period of at least 100 years (Lees & Wilcken, 2009; Lacy, 2013). In many cases the global population of a species is already below this threshold; for example, the northern white rhino *Ceratotherium simum leucophaeus*. Furthermore, individuals with valuable genetic profiles may die before reproducing or having their gametes preserved. It is in these situations that cloning techniques may be useful. The smaller a population, the greater is the value in accessing a single founder animal with an unrelated genetic profile. That is, where only a few individuals are left, introducing even a single animal into a breeding program can significantly boost the genetic diversity and therefore the general health and future prospects of the whole population. Thus, in general, it is likely that the goal of cloning in a conservation context would be to restore the genetic contribution of a specific individual, rather than to generate large numbers of animals. It may also provide a means to re-establish a founder population in cases where no live individuals of a given species remain.

The term clone derives from the Greek *klōn*, meaning 'twig', and was introduced into the English language in 1903 to describe the practice of propagating plants *via* cuttings, thus generating genetic copies of the original plant (Steensma, 2017). Expansion of the term to include reference to animals was first suggested in 1912 by George H. Shull: 'I believe that no violence will be done by extending this term [clone] to include animals which are similarly propagated by any asexual method, and I suggest the general adoption of the word "clone" for all groups of individuals having identical genotypic character, and arising by asexual reproduction of any sort, including apogamy (i.e. so-called "parthenogenesis," unaccompanied by a reduction division)' (Shull, 1912, p. 28). Yet it was not until J.B.S. Haldane's infamous speech at the *Ciba Foundation Symposium on Man and his Future* in 1963, in which he pondered the possibility of cloning humans of superior ability or talent (Haldane, 1963), that cloning appears to have really permeated the lexicon. Although Haldane stated that he took this possibility seriously, it was nonetheless cast in a light of science fiction, alongside a reference to Huxley's *Brave New World* and with a proposed goal of generating 'asexual progeny of people with very rare capacities' in order to 'raise the possibilities of human achievement dramatically' (Haldane, 1963, cited in Wolstenholme, 2009, p. 353). Thus, at that time, mammalian cloning was largely a concept of science fiction or at best, a long-term speculative projection: his speech was entitled '*Biological Possibilities for the Human Species in the Next Ten Thousand Years*'. Haldane's speech, and contemporaneous works of science fiction, would go on to frame the entire scientific field in the public eye for decades to come, associating cloning with mass propagation of genetic copies of superior individuals, despite such a goal never being at the centre of the scientific aims of the pioneers of embryo splitting, nuclear transfer, nor induced pluripotent stem cell (iPSC) technologies.

Dolly the sheep made headlines as the first cloned mammal in 1996, although she was in fact the first mammal to be cloned from an adult somatic cell. Prior to this, mammals had been cloned from embryonic cells on multiple occasions during the 1980s and 1990s, while frogs had been cloned by nuclear transfer as early as 1952 (Briggs & King, 1952). Nevertheless, it is the procedure that resulted in Dolly – somatic cell nuclear transfer – that seemed to consolidate the meaning of 'cloning' in the colloquial vocabulary. In this review, we highlight that cloning simply refers to the generation of genetically identical individuals, and draw attention to multiple alternative approaches to this task. Even when we ignore the cloning of plants and self-replicating bacteria, limiting ourselves to vertebrates and specifically to mammals, somatic cell nuclear transfer is just one of several possibilities. Broadly speaking, the three biological approaches to mammalian cloning are embryo splitting, nuclear transfer, and cell reprogramming, in addition to instances of 'natural cloning' as in the case of monozygotic twinning. There are also several emerging laboratory techniques that result in the propagation of genotypes to the next generation of offspring while bypassing conventional fertilisation, and should, therefore, be included in a discussion of cloning and associated techniques. A timeline of key events in the scientific history of cloning and associated biotechnologies is presented in Fig. 1. This review aims to inform the reader of the wide range of techniques presently capable of generating cloned mammals, along with their historical context, current state of the art and potential role in the conservation of endangered species. The major pertinent biological issues and applications associated with each strategy are also discussed.

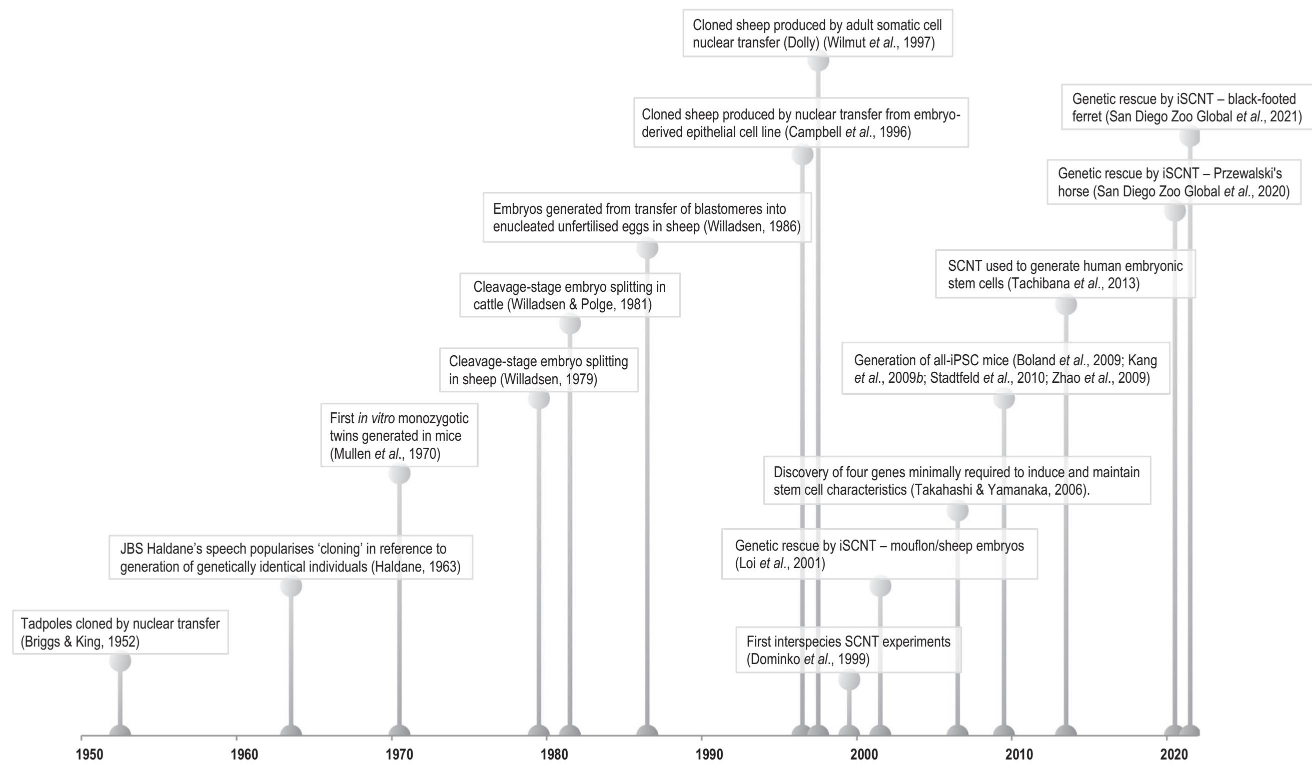

Fig. 1. A timeline of key scientific events in cloning by monozygotic twinning, somatic cell nuclear transfer and stem cell technologies. iSCNT, interspecies somatic cell nuclear transfer; SCNT, somatic cell nuclear transfer.

II. MONOZYGOTIC TWINNING AND EMBRYO SPLITTING

(1) Historical and biological context

Identical (monozygotic, MZ) twins are produced post-fertilisation when a single embryo splits into two parts, and each clump of cells continues to develop as a separate entity. As both originate from the same embryo, they are genetically identical and thus clones. MZ twinning is reasonably widespread across mammalian taxa, but its frequency within species varies widely. Generally MZ twinning is very rare, with the notable exception of the nine-banded armadillo (*Dasypus novemcinctus*), in whom every litter comprises four identical quadruplets (Prodöhl *et al.*, 1996). In the human population the frequency of MZ pregnancies historically and across different geographic locations has remained largely consistent (at around 0.4% of all pregnancies), but there are exceptions and indeed some populations exhibit particularly high rates. For example, a relatively isolated population in the Brazilian region of Rio Grande do Sul has consistently shown higher rates of MZ twinning than the general population (proportion of all twin pregnancies: 42–47% *versus* ~ 30%) (Matte *et al.*, 1996; Mardini *et al.*, 2017), leading to investigation of the factors underlying this propensity. Temporal and geographic analyses have pointed to a genetic component (Bortolus, Zanardo & Trevisanuto, 2001; Mardini *et al.*, 2017), and in some instances specific gene mutations have been associated with increased rates of MZ twinning within a geographically distinct population or a family line (Mardini *et al.*, 2017; Liu *et al.*, 2018*b*). An apparent X-linked pattern of inheritance has been reported and the candidate variants appear to be enriched for genes involved in tight-junction signalling (Liu *et al.*, 2018*b*), tentatively implicating this as the biological mechanism underlying MZ twinning, although a causal relationship is yet to be confirmed and the process remains largely mysterious.

Another distinct human population displaying higher rates of MZ twinning comprises couples using assisted reproductive techniques (ARTs) to conceive. In spontaneous pregnancies, MZ twinning occurs in about 0.4% of pregnancies while in ART pregnancies this rate is up to 4.9% (MacKenna *et al.*, 2020). While the common practice of transferring multiple embryos to increase pregnancy chances is widely known to increase the incidence of dizygotic twins in ART pregnancies, a definitive mechanism to explain the association between ART and MZ twinning remains elusive. Several associations have been drawn in attempts to tease out the cause of MZ twinning in ARTs; prolonged *in vitro* culture (Liu *et al.*, 2018a) and embryo biopsy (Kamath, Antonisamy & Sunkara, 2020; Sellers *et al.*, 2020), as well as maternal age (Liu & Shi, 2021), have been put forward as risk factors. Curiously, MZ twinning appears to be more likely when transferring blastocysts *versus* cleavage-stage embryos (MacKenna *et al.*, 2020). Disruption of communication between blastomeres, that is, a 'loose' configuration of the inner cell mass (ICM), has been tentatively proposed as an underlying mechanism (Otsuki *et al.*, 2016).

Mimicking the MZ twinning process *in vitro* is the simplest form of animal cloning, and thus the first instances of artificial mammalian cloning involved embryo splitting by physically separating the blastomeres of the cleavage-stage embryo. Mullen, Whitten & Carter (1970) were the first to generate MZ twin embryos in this way in mice. In laboratory animal species, such embryos have contributed to basic research seeking to investigate regulatory processes during development. In domestic species, this technique had the additional appeal of potentially improving the efficiency with which genetically valuable animals could be produced, with scope to extend this application to conservation of rare and endangered animals in *ex situ* conservation programs. Willadsen (1979) succeeded in early experiments to split cleavage-stage embryos in sheep, and subsequently in cattle (Willadsen & Polge, 1981), further demonstrating the capacity to produce triplets and even quadruplets *via* this technique.

Producing multiple genetically identical individuals at the same time can be useful in multiple livestock-breeding, research and conservation settings. Thus, embryo splitting is used in the cattle-breeding industry, assisting production efficiency and reducing the time required for sire evaluation by several years (Hashiyada, 2017). However, the advent of other advanced reproductive techniques, including efficient *in vitro* production of embryos and somatic cell nuclear transfer, have nudged embryo-splitting techniques out of the research spotlight and provided more appealing ways and often less-cumbersome means to improve animal production. Nevertheless, investigation into the factors responsible for stimulating MZ twinning, whether related to *in vitro* conditions, genetically controlled factors in the *in vivo* fertilisation environment, or programming/tight junction mechanisms within the embryo itself, is of great interest and may reveal new avenues for the efficient production of livestock and endangered mammals. The fact that species range in their incidence of spontaneous twinning from never to always (as in the case of the nine-banded armadillo) is of interest because it points to genetic and structural interspecies differences in the mechanisms governing early embryo development, totipotency and the potential of the zygote to survive embryo splitting and other interventions.

(2) *In vitro* twinning: the process

There are two laboratory techniques that can be used to achieve *in vitro* MZ twinning (Fig. 2), depending on the developmental stage of an embryo: blastomere separation/biopsy (using cleavage-stage embryos) or bisection (using morulae or blastocysts).

Blastomere separation involves gentle dissociation of the individual cells of the developing embryo and their transfer to a new zona pellucida. The zona pellucida can be removed from the embryo by enzymatic digestion and blastomeres separated by pipetting, or a micromanipulator can be used to remove individual cells. Separated blastomeres, for example in pairs, are then inserted into surrogate empty zonae pellucidae and the reconstructed embryos allowed to resume development *in vitro*. Embryo bisection is a relatively simple procedure; a microblade is used to cut downwards vertically from the top, using an inverted microscope and a micromanipulator fitted with a metal blade. It is essential that bisection is carried out through the midline, splitting the morula or both the ICM and the trophoblast of blastocysts into two equal parts. The demi-embryos are then cultured for a few hours *in vitro* to enable them to 'round up' into a blastocyst like structure before transfer to a surrogate dam as per routine embryo transfer. An important distinction between the two techniques is that blastomere separation relies on totipotency of individual cells, while embryo bisection requires the contribution of distinct (pluripotent but not totipotent) cell lineages that have already established in the morula and blastocyst stages.

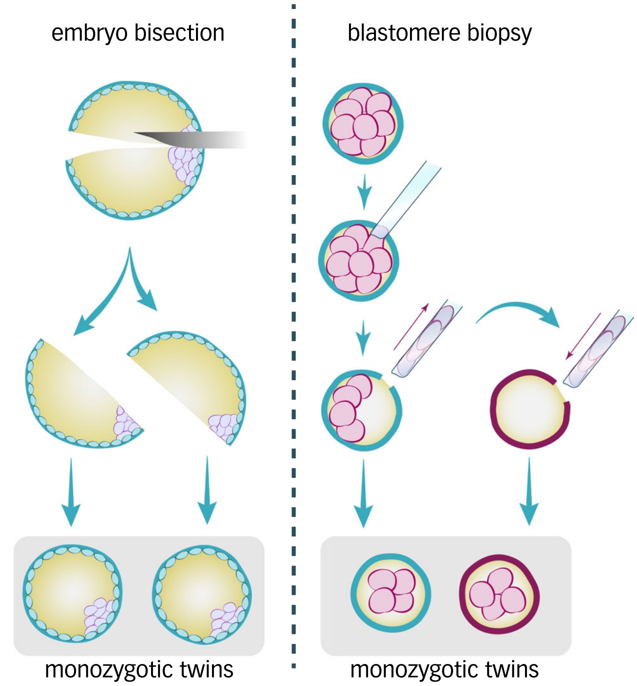

Fig. 2. Schematic illustration of *in vitro* monozygotic twinning by embryo bisection (left) or blastomere biopsy (right).

(3) State of the art of *in vitro* monozygotic twinning

Early methods of generating twins *via* embryo splitting were labour-intensive as they preceded the development of other required ART techniques that significantly simplified the process. For example, *in vitro* production of embryos had not yet been established for cattle, with the first *in vitro* fertilisation (IVF) calf born in 1981 (Brackett *et al.*, 1982). Being able to produce embryos *in vitro*, rather than having to recover each experimental embryo surgically or *post-mortem* would substantially simplify the process and increase the efficiency with which embryo splitting could be studied and applied. In the same vein, since adequate embryo culture conditions had not yet been developed, early experiments with blastomere separation involved the transfer of resultant embryos into agar capsules and transplantation into sheep oviducts – a significantly more cumbersome and disruptive process than the optimised cell culture conditions available (and routinely used in livestock ART) today. Thus, the applied use of embryo splitting was limited for some years as the evolution of ARTs in the livestock industries had to reach a point where MZ twinning could be supported with a number of adjunct strategies. The rapid improvements seen in superovulation/ovum pick up (OPU) and *in vitro* embryo production (IVP) efficiency for cattle and other species in recent years have therefore allowed the greatest progress for *in vitro* MZ twinning and facilitated the uptake of this technique.

In cattle, blastomere separation presently appears to produce MZ twins more efficiently than embryo bisection, but the choice of technique depends on a variety of factors, availability of equipment, and relevant expertise, particularly as the operator's level of experience in the specific technique used significantly impacts success rate (Hashiyada, 2017).

Cloning *via* blastomere separation also requires a supply of surrogate zonae pellucidae into which the blastomeres are transferred. Harvesting these zonae is a labour-intensive step, which significantly complicates the process in practical terms. However, some efforts have focused on developing alternative approaches that negate the need to generate empty zonae. Microencapsulation techniques to create artificial zonae have been attempted in the mouse, consisting of sodium alginate, calcium alginate, poly-L-lysine or agarose (Krentz *et al.*, 1993; Elsheikh *et al.*, 1997; Cosby & Dukelow, 1990; Nagatomo *et al.*, 2017) while other approaches have involved culturing embryos without a zona at all, using culture dish microwells as an alternative (Tagawa *et al.*, 2008). Further developments of zona alternatives using novel biomaterial approaches may significantly improve the efficiency of twinning by blastomere separation. Likewise, future improvements in embryo freezing/vitrification will likely expand the utilisation of this technique by providing additional flexibility with regard to timing of transfer of the embryos to their surrogate dams (Juanpanich *et al.*, 2018).

In summary, the techniques described herein represent a method of 'cloning' animals that closely mimics the spontaneous (albeit poorly understood) *in vivo* process of MZ twinning. Both the natural and artificial versions of this process can provide useful insights into the development of genetically identical individuals. For many years any differences between MZ twins within a pair were assumed to be entirely the products of differences in their environment. Twin cohort studies have been indispensable in medical research as they provide what was assumed to be an identical genetic background (Craig *et al.*, 2020). However, observations of twin discordance for various traits suggested that in fact there could be genuine genetic and epigenetic differences within twin pairs, and have piqued research interest in recent years, in synergy with powerful and sensitive genomic sequencing technologies (Jonsson *et al.*, 2021). In contrast, research activity seeking to enhance livestock reproduction efficiency with the aid of embryo splitting or blastomere separation appears to have plateaued, perhaps outshined by the emergence of newer reproductive technologies (see Sections III–V) that provide greater opportunities for concurrent genetic manipulation, along with improvements in other aspects of livestock ART efficiency.

(4) Scope and limitations for application of *in vitro* twinning in endangered animal conservation

In vitro MZ twinning techniques have the potential to increase the number of *in vitro* embryos produced. Therefore, in any species where IVP is feasible, whether by IVF, intracytoplasmic sperm injection (ICSI) or nuclear transfer, these techniques could be deployed as adjuncts to increase the likelihood of a successful breeding outcome (i.e. term pregnancy and live birth). This potential comes with caveats; for example, the assumption that embryo survival is compromised by extrinsic factors and not genetic aberrations or aneuploidy intrinsic to the original embryo itself, as duplicating a non-viable embryo would not be beneficial. If effective methods to bypass the use of additional zonae pellucidae are refined, twinning by blastomere separation may be particularly useful where oocyte availability for IVP is limited, as is the case for many endangered species and in particular for species in which oocyte availability is limited by ovarian physiology, as is the case in equids (Alvarenga, Carmo & Landim-Alvarenga, 2008).

Embryo splitting and blastomere separation were instrumental in the journey towards artificial cloning and in our understanding of totipotency in cells. Although conceptually relatively simple techniques, they do require specific expertise and equipment (e.g. use of a micromanipulator); provided these are available, in theory the methods can be applied to most mammalian species, although the efficiency will vary and the stage of embryo development at which twinning is likely to be successful would need to be determined on a species-by-species basis. As is evident from examining the history of livestock MZ twin generation, the success of this technique is intricately tied to adjunct methods in ART, including the ability to obtain and mature oocytes, and the capacity to generate embryos *in vitro*, optimal culture conditions, transfer of embryos to surrogate uteri and successful implantation. This can pose a significant obstacle for applications in endangered species as many of these are poorly studied (Comizzoli & Holt, 2019) and generating viable embryos in culture tends to require species-specific conditions (Herrick, 2019). On the other hand, generating additional embryos *via* embryo splitting/blastomere separation can expedite these investigations by providing greater numbers of embryos to work with, and can be particularly useful in species where superovulation is not developed or not effective. Another exciting prospect is the ability to expand embryo numbers further *via* serial embryo splitting, as has been demonstrated in mouse embryos (Illmensee, Kaskar & Zavos, 2006), although this has not been successfully replicated in any other species to date.

A broader limitation is that the individual being 'cloned' by these methods is itself a newly developed embryo and as such, the genotype and phenotype have not yet been proven, while other techniques (see Sections III–V) can be applied to clone donors of known performance or health. In the field of animal conservation, however, there can be great value in having multiple individuals with the same genetic identity to assist in establishing insurance populations, populations in different geographic regions, or increasing the chances of achieving breeding outcomes within the shortest possible timeframe. In instances where IVP of embryos is possible for a given species, *in vitro* twinning could thus be useful, although it is important to note that this remains untested in species beyond domestic livestock and would likely face obstacles pertaining to interspecies differences. Furthermore, these embryos need to be transferred to a surrogate for gestation and the success of this also depends on a number of factors and on thorough knowledge of a given species' reproductive physiology, particularly the implantation receptivity window – often a major limitation in rare and endangered species. Ultimately, generating more embryos for transfer is advantageous as inevitably some transfers will fail. In some instances this will be a result of intrinsic embryo factors and these cannot be compensated for by additional embryos of the same genotype. Others may fail due to the uterine environment, surrogate nutrition or pathology, oestrus asynchrony, or other, as yet unknown extrinsic or surrogate factors, and it is in these instances that duplicate embryos offer a viable insurance policy for increasing the chance of a successful outcome post-transfer. A particular advantage is the similarity of *in vitro* twinning techniques to the natural process of MZ twinning, and this may bypass some of the risks and uncertainties associated with cell reprogramming and epigenetic inputs of somatic cell nuclear transfer (SCNT) and iPSC technologies. This endows the former with a favourable safety profile and optimal health of the offspring generated. In this light, perhaps embryo splitting and blastomere separation should be revisited as a relatively simple but potent conservation tool.

Being a long-established and relatively safe technique, embryo splitting has been suggested for human IVF practice as a means to generate greater numbers of embryos per oocyte, potentially enabling infertile couples to have more embryos transferred or cryopreserved (Wood, 2001). This may be particularly helpful in cases where low numbers of oocytes were obtained or where there is a need to minimise the number of ovarian stimulation cycles. The approach has yet to be incorporated into clinical practice but similar logic can be applied to species of conservation interest – specifically to those in which *in vitro* production of embryos has been identified as a goal. Oocytes are, by their nature, a scarce resource and require huge labour inputs to obtain from live animals; oocyte aspiration is conducted transvaginally or transrectally, in some cases under general anaesthesia, and poses risks to the animal each time it is performed. Superovulation protocols are species-specific and can take many years to develop (e.g. Palomino *et al*., 2016). Furthermore, superovulation protocols leading up to the aspiration include multiple injections and therefore multiple (potentially stressful) handling events. Hence any approach that helps to increase the numbers of embryos generated per procedure, in a cost-effective way and without detracting from animal welfare, should be welcomed in the conservation arena. Early embryo development is a relatively conserved process in comparison to the diverse physiology of ovulation, ovarian anatomy, and responses to hormonal stimulation, meaning the techniques of embryo splitting and/or blastomere biopsy can be practiced on other species to develop competence, and should be largely transferable between species. We therefore argue that a place for *in vitro* twinning techniques in mammalian conservation has been somewhat overlooked in recent years but should be reconsidered as a worthwhile adjunct to *in vitro* production of embryos.

III. NUCLEAR TRANSFER

(1) Historical and biological context

A long-standing paradigm of biology had been that the nuclei of somatic cells are differentiated irreversibly, and the DNA contained within is modified permanently such that it cannot dictate totipotent development. *In vitro* twinning experiments involving blastomere separation clearly reveal that there is a period in early embryos where individual cells retain their pluripotency. A key question that arose from this research was: at which point does this ability disappear irrevocably, or does it disappear at all? As multiple approaches to the generation of MZ twins *in vitro* were developed, the toolbox for exploring the limits of developmental plasticity was expanded. Blastomere-separation experiments such as those carried out by Willadsen (1986) were in effect also the first domestic animal mammalian nuclear transfer experiments, as blastomeres from 8- and 16-cell sheep embryos were inserted into enucleated unfertilised eggs and fused using Sendai virus or electrofusion. The resulting embryos, comprising genetic material from one animal and oocyte machinery from another, successfully developed to term following transfer to recipient ewes and gave rise to live lambs. Similar experiments followed for cattle and similarly generated live offspring (Willadsen & Polge, 1981). The techniques were refined, incorporating isolation of the blastomere nucleus, and expanded to progressively later stages of donor embryos, testing the limits of the donor nucleus' ability to dictate the development of an entire organism.

In the late 1980s and early 1990s, growing interest in biopharmaceuticals inspired attempts to genetically modify animals to secrete biomedically useful compounds. Researchers sought a method that would permit alteration of the animal's genome at the time of embryo production, to ensure maximal integration of the modified genes. Producing embryos by nuclear transfer provided a promising platform for this, and a major breakthrough was achieved by the Roslin Institute (UK) team when they successfully transferred nuclei from an embryo-derived epithelial cell line into enucleated oocytes to generate cloned sheep – the first ever nuclear transfer offspring to develop to term from a differentiated cell (Campbell *et al.*, 1996). Interestingly, cloning itself was not the end-goal of this research program, and it was on the same trajectory that this research group, later that year, achieved the birth of Dolly: a lamb cloned using the nuclear material from a mammary gland cell of an adult sheep (Wilmut *et al.*, 1997). The achievement provided long-awaited concrete proof that cell differentiation was reversible and did not involve the permanent modification of genetic material required for development. Dolly survived to adulthood, suffered no morphological abnormality, and was able to reproduce successfully. This work proved reproducible and results were soon replicated across numerous laboratories and a variety of livestock and laboratory species (reviewed by Keefer, 2015).

(2) SCNT: the process

Cloning by nuclear transfer comprises preparation of enucleated oocytes (cytoplasts), extraction of DNA from a donor cell and transfer into the cytoplast, fusion and activation to kick-start the embryo development process (Fig. 3). In addition, it is necessary to synchronise the cell cycle of the donor cells to ensure nuclear compatibility once the DNA is transferred, and steps to 'reset' the epigenetic status of the donor nucleus are also proving beneficial to the success rate of SCNT.

Donor oocytes are sourced from recently deceased animals (e.g. slaughterhouse samples), surgically removed ovaries, or *via* aspiration of oocytes by OPU. Donor DNA can be obtained from any cell type and is typically sourced from fibroblasts or other somatic cells. *In vitro* culture of the donor cells allows the alignment of cell cycle phase as well as the potential to introduce any desired genetic modifications to the donor genome. The nucleus is extracted from the donor cell and injected into the enucleated oocyte, followed by a fusion step. In a normally fertilised oocyte, the sperm entering the oocyte would induce the release of intracellular calcium in a series of pulses in a process known as activation, however, as this step is bypassed in SCNT, an *in vitro* alternative that mimics activation is required to initiate development of the reconstructed embryo. A range of methods have been used for this step, including exposure to calcium ionophore, ethanol, strontium, or pulses of alternating current (Akagi, Matsukawa & Takahashi, 2014; Patel *et al.*, 2014).

Following nuclear transfer, embryos are typically cultured *in vitro* until the blastocyst stage and in live animal production settings are then transferred to surrogate uteri as per routine embryo transfer. As for all applications of embryo transfer, this requires adequate knowledge and synchronisation of the surrogate animal's oestrus cycle to match the developmental stage of the cloned embryo.

(3) State of the art of SCNT

The creation of Dolly, a clone of an adult somatic cell, demonstrated that cell differentiation was a reversible process and opened up a huge field of research. In contrast to the lay media's focus on creating large numbers of genetically superior individuals, the real appeal of SCNT in agricultural bioscience was the scope for genetic alteration of animals of known phenotype at the point of nuclear manipulation. A series of firsts followed, with SCNT individuals generated across different species, using different donor cell types and modifications of the original technique. Along with these scenarios came a variety of challenges. The first adult SCNT mice were cloned using cumulus cells as sources of donor nuclei (Wakayama *et al.*, 1998). In the same year, eight calves were derived from differentiated cells of a single adult cow, five from cumulus cells and three from oviductal cells. All calves were visibly normal, but four died at, or soon after, birth from presumed environmental causes since *post-mortem* analysis revealed no abnormalities (Kato *et al.*, 1998). Subsequent attempts revealed that a proportion of SCNT calves developed to an abnormally large size *in utero* (Behboodi *et al.*, 1995); this was also observed in sheep but not goats or any other species. The phenomenon also occurred in non-SCNT embryos produced *in vitro* and was eventually linked to aberrations in growth factor receptors incurred during embryo culture in high serum conditions (Young *et al.*, 2001). However, this issue has not been resolved completely and continues to pose a barrier to the wider application of SCNT in cattle and sheep (Galli & Lazzari, 2021). The mechanisms driving the incidence of large offspring syndrome in SCNT foetuses remain under investigation but are generally attributed to incomplete reprogramming of the donor nucleus' epigenetic landscape.

The first attempts at SCNT in pigs proved even more challenging. The low embryo survival rates combined with the pig's unique physiology, whereby a minimum of four healthy embryos is required to attain successful maternal recognition of pregnancy (Polge, Rowson & Chang, 1966), meant that several species-specific adjustments were required at multiple steps of the process. The first successful pig SCNT employed a double nuclear transfer method, where somatic cell (granulosa) donor nuclei were first transferred to enucleated oocytes, activated, and the resulting pronucleus transferred to an enucleated zygote (Polejaeva *et al.*, 2000). Notably all five of the resulting piglets survived and were healthy, in contrast to many of their contemporaneously produced single nuclear transfer counterparts in other species.

Successful SCNT has now been attained in the vast majority of domestic species, in addition to rhesus monkeys *Macaca mulatta* (Meng *et al.*, 1997) and even humans, to the extent permitted by regulation (Tachibana *et al.*, 2013). The procedure has acquired notable success in the horse. Following the first adult SCNT-cloned equine offspring in 2003 (Galli *et al.*, 2003), where 841 successfully fused reconstructed embryos culminated in a single pregnancy carried by the original nuclear donor, the technique went on to produce

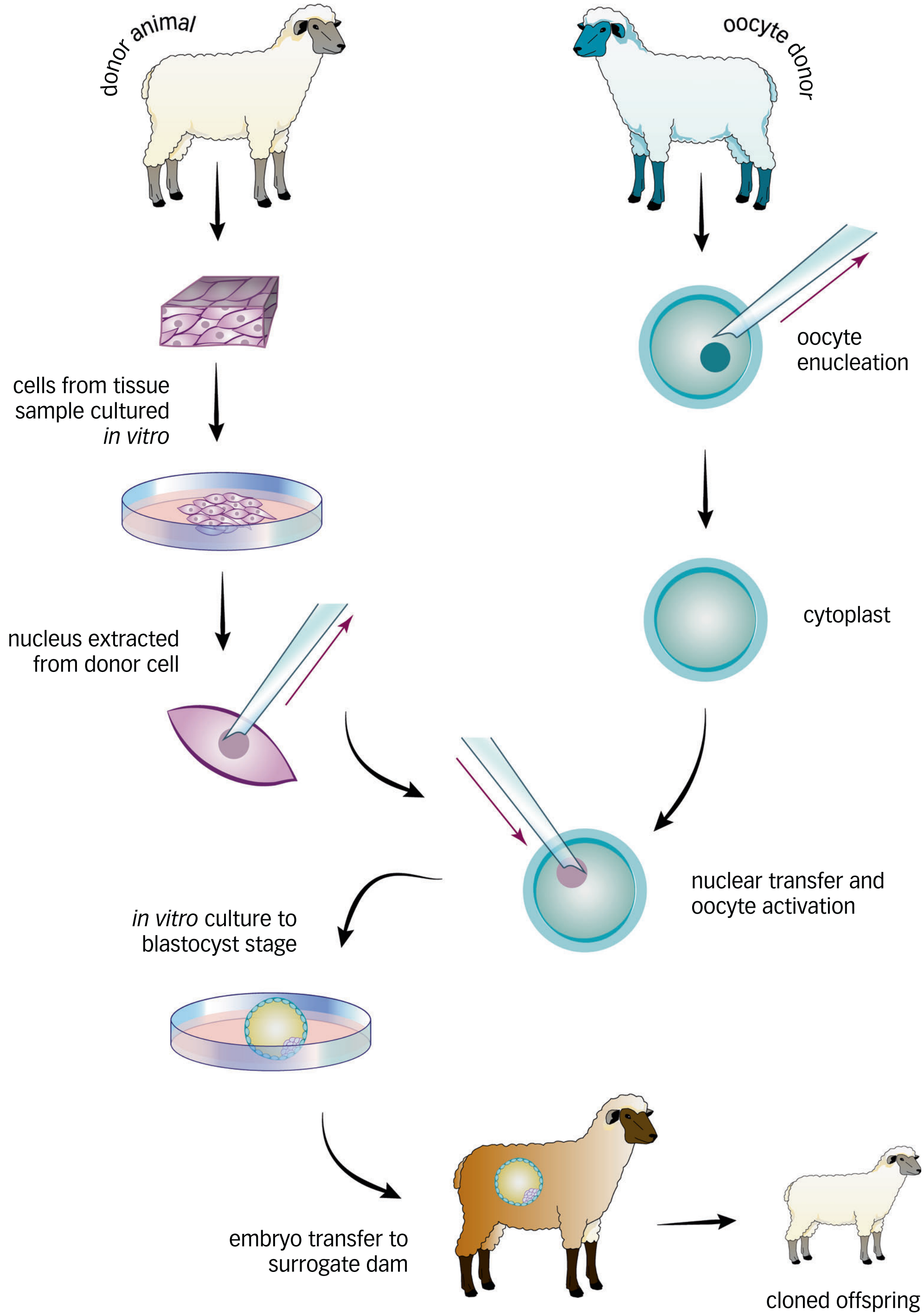

Fig. 3. Schematic illustration of somatic cell nuclear transfer.

many healthy foals without many of the complications experienced in other domestic species. SCNT-cloned foals are now produced routinely in multiple centres around the world, and the technology has found particular use in propagation of high-performing sport horses. A prominent example is polo, where entire teams of genetically identical horses have successfully competed at the highest level of the sport. Such active utilisation of SCNT is even more remarkable in this species as many aspects of ART and IVP in the horse have been poorly studied or are notoriously challenging, including very limited ability to superovulate mares and obtain oocytes, poor rates of *in vitro* maturation, and a persistent inability to generate embryos *via* IVF (necessitating the use of ICSI instead) (Leemans *et al.*, 2016).

Commercial support for the technology buoyed substantial improvements in the efficiency and health of equine SCNT-produced offspring (Olivera *et al.*, 2016). Its success has now extended to endangered equid species: the birth of the first Przewalski's horse (*Equus ferus przewalskii*) foal was announced in 2020, allowing the restoration of biobanked genetics from an individual that died 40 years prior, which would otherwise be eliminated from the species' gene pool (San Diego Zoo Global, Revive & Restore & ViaGen Equine, 2020).

Since its advent, the SCNT procedure has evolved substantially and many of the initial procedural hurdles have been overcome, such as spindle visualisation (which used to require damaging ultraviolet light), and premature activation. Cell cycle synchronisation of the donor cell nucleus continues to pose a challenge as the chemicals typically used to achieve this can have toxic effects and compromise nuclear stability in subsequent development. Oocyte activation, encompassing a series of events that would naturally be triggered by the spermatozoon at fertilisation to initiate reprogramming and metabolic activity in the oocyte cytoplasm, is also a key step that has seen numerous options arise over recent years. Mimicking the action of the sperm in launching this process has proved challenging; remarkably we still do not know exactly how the sperm triggers oocyte activation and thus finding an *in vitro* substitute for this process has proved difficult. Multiple options for oocyte activation are available and it is highly species dependent. Calcium oscillations, inhibition of kinase activity, and electrical pulses have all been used to aid activation, but no optimal protocol exists that is definitively first choice for all species.

A variety of somatic cell types have been used as SCNT donors and the choice of cell type affects the efficiency of the procedure as well as the health of the resultant offspring. Use of cumulus cells as nuclear donors appears to facilitate the highest cloning efficiency with the lowest number of abnormalities in cloned animals (Forsberg *et al.*, 2002; Kato, Tani & Tsunoda, 2000; Tian *et al.*, 2003; Wakayama *et al.*, 1998). Another distinct advantage is that the vast majority of cumulus cells are arrested at the G0/G1 phase of the cell cycle which is consistent with the oocyte, thus minimising the need for potentially damaging interventions to synchronise the cell cycle phase (Kishigami *et al.*, 2006). The use of pluripotent cell types might be expected to yield the best outcomes but this is not always the case; while a range of undifferentiated donor cell types have been used for SCNT with success [e.g. neural, mesenchymal and keratinocyte stem cells (Mizutani *et al.*, 2006; Inoue *et al.*, 2007; Li *et al.*, 2007)], there have also been reports concluding that differentiated donor cells are more efficient than adult stem cells for cloning by SCNT (Sung *et al.*, 2006).

Adjustments to the above factors have improved SCNT over recent years but efficiency rates remain low; generally below 10% of reconstructed embryos go on to develop to term in all species. Presently, the main underlying cause of this low efficiency is considered to be incomplete epigenetic reprogramming of the donor nucleus by the oocyte. Somatic cell differentiation brings with it a plethora of epigenetic modifications, including DNA methylation, histone modification and microRNA (miRNA)-mediated regulation, that control the expression of genes and contribute to both the phenotype and the unique epigenetic memory of each cell. The cloning of Dolly proved that the epigenetic slate *can* be wiped clean, at least sufficiently so to allow a 'brand new' embryo to develop, but it is clear that this process happens successfully in only a small proportion of the reconstructed embryos; we do not fully understand how this epigenetic memory is erased or why this process does not happen correctly in the vast majority of SCNT attempts. Incomplete epigenetic reprogramming results in gene expression aberrations (Humpherys *et al.*, 2002; Wang *et al.*, 2020), which are in turn deemed responsible for cloned animals exhibiting shorter lifespans (Ogonuki *et al.*, 2002) and conditions such as respiratory failure, hepatic failure, abnormal kidney development, liver steatosis, and large offspring syndrome.

The past two decades have seen research targeted toward deciphering the epigenetic changes required and developing strategies to augment reprogramming as part of the SCNT process. Several strategies have proved effective and these include strategic approaches focused on the key categories of epigenetic changes: DNA methylation (by using DNA-demethylating agents or knocking down genes for DNA methyltransferases, the enzymes that drive DNA methylation), histone modification (e.g. modifying histone marks using histone deacetylase inhibitors to increase histone acetylation and open up the chromatin structure), and X-chromosome inactivation [e.g. by deletion or knockdown of X-inactive specific transcript (Xist) to restore X-linked gene expression patterns]. All these treatments have, to some extent, been able to improve the developmental ability of cloned embryos (reviewed in Wang *et al.*, 2020). Approaches involving non-coding RNA species represent an emerging field; for example long non-coding RNAs (lncRNAs) appear to participate in many of the abovementioned epigenetic modification processes and can regulate the developmental competence of cloned embryos (Holoch & Moazed, 2015; Wu *et al.*, 2018). While early evidence suggests that non-coding RNAs are key mechanisms in nuclear reprogramming, practical strategies to improve SCNT success based on these findings are yet to be developed.

In addition to nuclear reprogramming, the roles of mitochondria and mitochondrial DNA (mtDNA) are receiving increasing research attention and emerging as important factors in the success of SCNT. Early embryos rely on the mitochondria inherited from the oocyte, and as such, reconstructed SCNT embryos contain mostly mitochondria and mtDNA inherited from the enucleated donor oocyte, and in some instances a small amount from the nuclear donor. Highly coordinated interactions between the nuclear and mitochondrial genomes are central to normal cell function and early embryo development, and the mismatch between these genomes, the quality of the mtDNA (e.g. accumulated mtDNA mutations) and the degree of mixing between the two mtDNA populations (mtDNA heteroplasmy) can negatively impact these processes (Srirattana & St John, 2019). A combination of nuclear and mitochondrial genes control mitochondrial function. Particularly in interspecies SCNT (iSCNT), nucleo-mitochondrial incompatibilities pose substantial challenges (Mrowiec, Bugno-Poniewierska & Młodawska, 2021). Suboptimal mitochondrial function has been suggested to contribute to the low rates of SCNT embryo development, and is assumed to be the consequence of incomplete nuclear reprogramming resulting in aberrant expression of mtDNA transcription factors (Czernik *et al.*, 2019). On the other hand, novel findings implicate the mitochondria themselves in nuclear reprogramming and further highlight the importance of mitochondrial health in SCNT success (Zhong *et al.*, 2019; Bae *et al.*, 2015). A variety of strategies have been trialled in attempts to augment mitochondrial function in SCNT embryos; these include antioxidant treatments to support mitochondrial integrity (An *et al.*, 2019), and supplementation with additional mtDNA to increase mtDNA copy number and enhance energy production (Srirattana & St John, 2018). Further research in this exciting field is bound to generate progress in SCNT efficiency in the coming years.

While the mechanisms of epigenetic reprogramming and mitochondrial function in SCNT are still under investigation, SCNT is establishing itself in the realms of commercial livestock production and animal conservation. As demonstrated by recent progress in equine SCNT, it is likely that future iterations of the technology will increasingly overlap with the developing stem cell and induced pluripotency fields.

(4) Scope and limitations for SCNT application in endangered animal conservation

SCNT has been used in attempts to propagate genetics of endangered species but success has been limited, primarily because this often requires iSCNT, where an oocyte from one species is used as the recipient for a nucleus from another species. Thus far, most efforts involving interspecies transfers have not been successful in generating healthy live offspring. Only a few cases of iSCNT between closely related species have resulted in the actual production of offspring, with varying health outcomes. In many cases these animals have not thrived and died soon after birth, reflecting the immaturity of iSCNT as an applied technique. A gaur (*Bos gaurus*) calf was produced by transplantation of a gaur nucleus into a bovine oocyte but died shortly after birth (Vogel, 2001). More recent attempts have seen gradual improvements in success; a cloned gaur calf reached full-term development but died within 12 h after birth due to respiratory defects (Srirattana *et al.*, 2012). Some success has been achieved in wild felids, using domestic cat oocytes as recipient cytoplasts. Offspring have been produced using iSCNT-derived embryos in two small wild cat species (sand cat *Felis margarita* and African wild cat *Felis silvestris lybica*), with domestic cats used as surrogates (Gómez *et al.*, 2008, 2004); although not listed as endangered, successful cloning of these species is an important step towards the use of SCNT in other felid species. Intergeneric SCNT was attempted with marbled cat *Pardofelis marmorata* and flat-headed cat *Prionailurus planiceps* DNA transferred to domestic cat cytoplasts and embryos transferred into domestic cat surrogates, but embryos only survived to the blastocyst stage (Thongphakdee *et al.*, 2010).

An endangered ungulate species, the mouflon (*Ovis orientalis musimon*), has been cloned successfully by iSCNT using oocytes collected from the closely related domesticated sheep. Blastocyst-stage cloned embryos transferred into sheep surrogates established two pregnancies, one of which produced an apparently normal mouflon (Loi *et al.*, 2001), facilitating genetic rescue from an animal found dead. Most recently, endangered black-footed ferrets (*Mustela nigripes*) have been cloned with the support of the domestic ferret (San Diego Zoo Global *et al.*, 2021) and the Przewalski's horse has been cloned with the support of domestic horses for iSCNT (San Diego Zoo Global *et al.*, 2020). The latter is a colt cloned from a cell line stored since 1980, from a stallion born in 1975 that died in 1998. The cloned colt is expected to provide a valuable infusion of genetic diversity for the Przewalski's horse population. Likewise, the cloning of the black-footed ferret is expected to introduce crucial genetic diversity into a small population of animals. Such milestones exemplify the utility of SCNT for the re-establishment of important genetic lines that would otherwise have been lost from the genetic pool. While cloning by SCNT is often criticised as a labour-intensive technique that only contributes a single individual at great effort, it is important to consider the genetic diversity that can be unlocked by cloning biobanked samples as relative to the genetic diversity available in the live population, rather than in absolute terms. For example, the living population of northern white rhinoceros consists of two individuals, further, the two animals are related, both female, and unable to breed naturally. Meanwhile, fibroblast cell lines have been biobanked from 12 individuals of this species in San Diego Zoo Global's Frozen Zoo®; eight of these animals are unrelated, and five have never reproduced (Korody *et al.*, 2021). The genetic variation captured in the biobanked population is evidently greater than in the remaining live animals and sequencing demonstrates sufficient diversity to facilitate re-establishment of a viable population *via* ARTs such as cloning (Korody *et al.*, 2021).

Among canids, wolves (*Canis lupus*) and coyotes (*Canis latrans*) have been cloned using domestic dog oocytes and surrogates, with healthy live pups born and surviving to adulthood although in both species some pups were aborted, morphologically abnormal, stillborn, or died soon after birth (Hwang *et al.*, 2013; Kim *et al.*, 2007; Oh *et al.*, 2008). The abnormal coyotes predominantly displayed defects in muscle development as well as other organ malformations, while an abnormal wolf pup had brain malformations and died soon after birth. Interestingly in both the wolf and coyote SCNT reports there was an instance of sex reversal, where cloning a male animal resulted in a female offspring phenotype despite an XY karyotype (Oh *et al.*, 2008; Hwang *et al.*, 2013); the mechanism is not clear but presumably involves failure of X-inactivation following mutations in the *SRY* gene (Kang *et al.*, 2009*a*).

Other interspecies donor–cytoplast combinations have resulted in embryos developing to various stages – most commonly the blastocyst stage – but are yet to generate a term pregnancy and viable offspring, for example banteng *Bos javanicus*/domestic cattle (Sansinena *et al.*, 2005), Tibetan antelope *Pantholops hodgsonii*/domestic goat (Zhao *et al.*, 2007), and giant panda *Ailuropoda melanoleuca*/rabbit (Chen *et al.*, 2002). The challenges posed by iSCNT have unearthed new but fundamental questions about evolution and interspecies compatibility. Some proposed reasons for limited iSCNT success include incomplete activation of the embryonic genome and nuclear–mitochondrial incompatibilities (Lagutina *et al.*, 2013). The compatibility between such hybrid embryos and the surrogate species, particularly the question of how great a taxonomic distance can still afford implantation and adequate placental function, is also poorly understood. We can expect to see paradigm shifts in coming years that defy our assumptions about species compatibility as we explore the capabilities of iSCNT further under the pressure of a global extinction crisis.

In SCNT, mitochondria come predominantly from the oocyte donor and therefore are not of the same origin as the animal being cloned. Since the oocyte is poised for totipotency, the mitochondria found in the enucleated oocyte to be used for SCNT are perfectly aligned with this cell state and are well suited to supporting the metabolic demands of embryo development. Variably, some mitochondria from the nuclear donor are also transferred along with the nucleus, leading to mixing of mitochondria from two different donors and therefore with different mtDNA genomes, known as heteroplasmy (Takeda, 2019). This is typically seen as a pitfall of SCNT; a different perspective is that SCNT is an opportunity to correct or alleviate mitochondrial defects, insufficient mtDNA copy number or the potential detrimental effects of aged mitochondria. This is illustrated *via* human IVF where mitochondrial replacement therapy, albeit still experimental, has now entered clinical practice, achieving pregnancies and effectively reducing the mtDNA mutational load in embryos at risk of inheriting lethal or debilitating mtDNA-associated disease (Zhang *et al.*, 2017).

Compatibility between the function and genome of the mitochondria and the nucleus is crucially important. Effective mitochondrial function requires that components of the electron transport chain, some of which are nuclear encoded and others mtDNA encoded, are able to function together within the same organelle. Compatibility between the two genomes is also essential for transcription and replication of the mitochondrial genome. If taxonomic divergence between the nuclear and mitochondrial genomes is too great, as is often likely to be the case in iSCNT, essential nucleo-cytoplasmic interactions cannot take place; for example the nucleus cannot communicate with the mitochondrial genome and fails to regulate mtDNA transcription and replication, particularly important upon the initiation of mtDNA replication in the developing embryo (St. John *et al.*, 2004). Indeed, mitochondrial mismatch and/or heteroplasmy appear to be chiefly responsible for the developmental arrest seen in attempts at interspecies/intergeneric reconstructed SCNT embryos (Thongphakdee *et al.*, 2008), which show impaired mitochondrial respiration (Dey, Barrientos & Moraes, 2000). This mismatch can potentially be addressed by some applications of iPSCs, as discussed in Section IV.4.

It follows that the origin and inheritance of the mitochondrial genome are key considerations when SCNT cloning is used as a tool to conserve species and enhance genetic diversity. Mitochondria are passed down the female germ line while mitochondria from the spermatozoon do not contribute to the resulting embryo. Thus, female clones will pass on to the next generation not only the intentionally cloned nuclear genome but also the mitochondrial genome introduced *via* the nuclear transfer procedure, originating from the oocyte donor (e.g. slaughterhouse oocyte of unknown health history, or an oocyte from a different species in the case of iSCNT). In the cloned Przewalski's horse example given above, the cloned animal was a stallion, and as such, his mitochondrial genome will not be passed down to subsequent generations. Meanwhile, in the case of the black-footed ferret, the female clone's oocytes will propagate the mitochondrial genome she inherited *via* the domestic ferret donor oocyte.

In a conservation context, the IUCN recognises the difference between cloned animals and their donors, and has designated cloned animals as 'proxies' for the species in question. Its policy on the use of such proxies in conservation highlights the potentially unpredictable effects of inheriting heteroplasmic mtDNA in iSCNT along with effects of epigenetics, the influence of the rearing environment, the absence of appropriate conspecific learning opportunities, and other factors. The IUCN Species Survival Commission considers that the creation and release of such a proxy of an extinct species will pose risks, but has the potential to derive a conservation benefit – provided a clear set of guiding principles are adhered to. As such, successful SCNT remains an important goal in assisting conservation efforts in many species. Although outside the scope of this mammal-focused review, the ongoing active efforts to preserve amphibian biodiversity *via* SCNT deserve mention. Up to half of all amphibian species may be at imminent risk of extinction and preservation of genetic material *via* biobanking forms a key mitigation strategy in response to the scale and rapid pace of decline (Silla & Byrne, 2019). Despite the central role of frog species in enabling the early progress of nuclear transfer (McKinnell & Di Berardino, 1999), successful SCNT in endangered and extinct frogs has remained elusive. To date, viable adults have been generated using nuclei from tadpole intestinal cells (Gurdon & Uehlinger, 1966) and tadpole epidermis (Kobel, Brun & Fischberg, 1973), but not from adult somatic tissues. Conversely, offspring that have been generated from adult tissues have not survived to adulthood (see Gurdon & Byrne, 2003). Once again, the challenges seem to emanate chiefly from incomplete reprogramming, as well as aneuploidy likely resulting from the short time that amphibian oocytes allow for chromosome replication (Gurdon, Byrne & Simonsson, 2003). Notable nuclear transfer attempts resulted in the production of embryos of the extinct gastric brooding frog *Rheobatrachus vitellinus*, although these did not survive beyond a few days of development (J. Clulow, personal communication). Several research groups continue to actively pursue nuclear transfer as a means of species rescue in amphibians, recognising that while the SCNT technique itself is yet to become feasible in this taxon, perfecting the biobanking strategies that will allow preserving genetic material to be used for cloning in future years is a major priority in this field (Clulow & Clulow, 2016).

In summary, the role of cloning by SCNT is indeed limited in conservation at present, but as the list of endangered and functionally extinct mammals grows, so does the niche for this increasingly efficient technique. In certain scenarios, cloning is not only very useful but may be the only viable option for restoring genetic diversity or facilitating de-extinction of a species. Its use must be determined on a case-by-case basis, taking into consideration the genetic diversity of both cryobanked and living populations, their ability to breed naturally or *via* the use of other ARTs, and the available fundamental knowledge of reproductive biology of the species in question and its closest relatives.

IV. CELL REPROGRAMMING AND INDUCED PLURIPOTENCY

(1) Historical and biological context

The cloning of Dolly by SCNT was a major milestone in understanding how the genetic information stored inside a fully differentiated cell can be 'reset' to its most primal totipotent state. Even if the technique had its imperfections in terms of practical applications for domestic livestock, it was now clear that the nuclei of somatic cells have the capacity to dictate embryonic development if provided with the right conditions. This had major implications for regenerative medicine and highlighted the remarkable power of the oocyte to reprogram the nucleus, opening up the possibility that the processes involved in reprogramming could be pinned down with greater precision and then used to convert somatic cells to pluripotent ones *en masse*. As a result, a field of research emerged in biology set on deciphering the factors that affect the growth of stem cells and early-embryo cells. Genetic manipulations in mice revealed that the transcription factors *OCT4* (or *POU5F1*) and *SOX2* are both necessary for maintaining pluripotency in the early stages of embryonic growth (Nichols *et al.*, 1998; Avilion *et al.*, 2003). This body of research morphed into the idea that the activation of a very specific subset of genes defined the identity of stem cells and suggested that these factors could also be used to turn differentiated cells into immature, undifferentiated, and therefore pluripotent, cells.

With this goal in sight, Takahashi & Yamanaka (2006) identified a pool of 24 pluripotency-associated candidate genes from the literature and screened these to identify transcriptional regulators that could reprogram adult cells into pluripotent cells, termed 'induced pluripotent stem cells' or iPSCs. Through successive rounds of elimination, they identified a core set of four genes minimally required to induce and maintain stem cell characteristics: *Klf4*, *Sox2*, *c-Myc*, and *Pou5f1* (*Oct4*). iPSCs generated using introduction of these transcription factors generated teratomas when injected subcutaneously into immunocompromised mice, and contributed to different tissues of developing embryos upon blastocyst injection, thus fulfilling some of the criteria defining pluripotency. Validating the hypothesis raised by the cloning of Dolly exactly a decade earlier, this was a major breakthrough, and the set of transcription factors subsequently became known as the 'Yamanaka cocktail'. However, these initial iPSCs did not exhibit the same level of pluripotency as embryonic stem cells (ESCs); they expressed lower levels of several key pluripotency genes, showed incomplete promoter demethylation of ESC regulators such as *Pou5f1* (*Oct4*), and failed to generate postnatal chimeras or contribute to the germline.

To be considered equivalent to ESCs, iPSCs would have to demonstrate the ability to contribute to every cell lineage except for the extraembryonic endoderm and trophoblast (which goes on to form the placenta) and to successfully dictate the coordinated development of all tissues through embryonic development to live birth. Thus, the ultimate test of pluripotency is the injection of the pluripotent cells into a blastocyst that has had its ICM removed, and successful development of this newly formed chimeric blastocyst to term. Because complete removal of the ICM is difficult to guarantee and any remaining ICM cells from the donor blastocyst can contribute to or override the iPSCs, an alternative approach is first to create a tetraploid embryo to use as the blastocyst donor; tetraploid cells, where the nuclei from two diploid cells have been fused, can support development of a normal trophoblast and placenta, but cannot contribute to the ICM and embryo proper. This is termed 'tetraploid complementation' and the technique represents the most stringent method for validating pluripotency (Nagy *et al.*, 1990). When iPSCs or ESCs are injected into a tetraploid blastocyst, the resulting conceptus is a chimera of the blastocyst's and the injected cells' genomes while it develops *in utero*, but once the conceptus reaches term and sheds the placenta at birth, the offspring remains a genetic copy of the iPSC or ESC donor. Using this technique, true pluripotency of iPSC lines has been confirmed following injection of the cells into tetraploid blastocysts, resulting in the generation of mice derived entirely from iPSCs (Boland *et al.*, 2009; Kang *et al.*, 2009*b*; Stadtfeld *et al.*, 2010; Zhao *et al.*, 2009), thus indicating that these iPSC lines have a developmental potency equivalent to ESCs.

The primary reason for prolific enthusiasm surrounding iPSCs is their potential to contribute to regenerative medicine, providing an autologous source of biotherapeutics, cells and potentially even organs. Recently iPSCs have been successfully differentiated *in vitro* into germ cells, opening new horizons for fertility restoration; such an application also has clear implications for restoring populations of rare, endangered and even extinct mammals. While tetraploid complementation with iPSCs has its origins as a method to confirm pluripotency and is not typically considered as an end-goal in itself, it is clearly a new means to generate genetically identical animals and has the capacity to be used for this application. At present, induction of pluripotency remains a relatively young technology and still carries several concerns and limitations, but also several potential advantages over 'traditional' cloning by SCNT.

(2) Inducing pluripotency and generating iPSC-derived offspring: the process

The processes of iPSC derivation, embryo complementation and *in vitro* gametogenesis are outlined in Fig. 4. For generation of iPSCs, cells are initially collected from a donor and cultured *in vitro*. A variety of cell types have been successfully

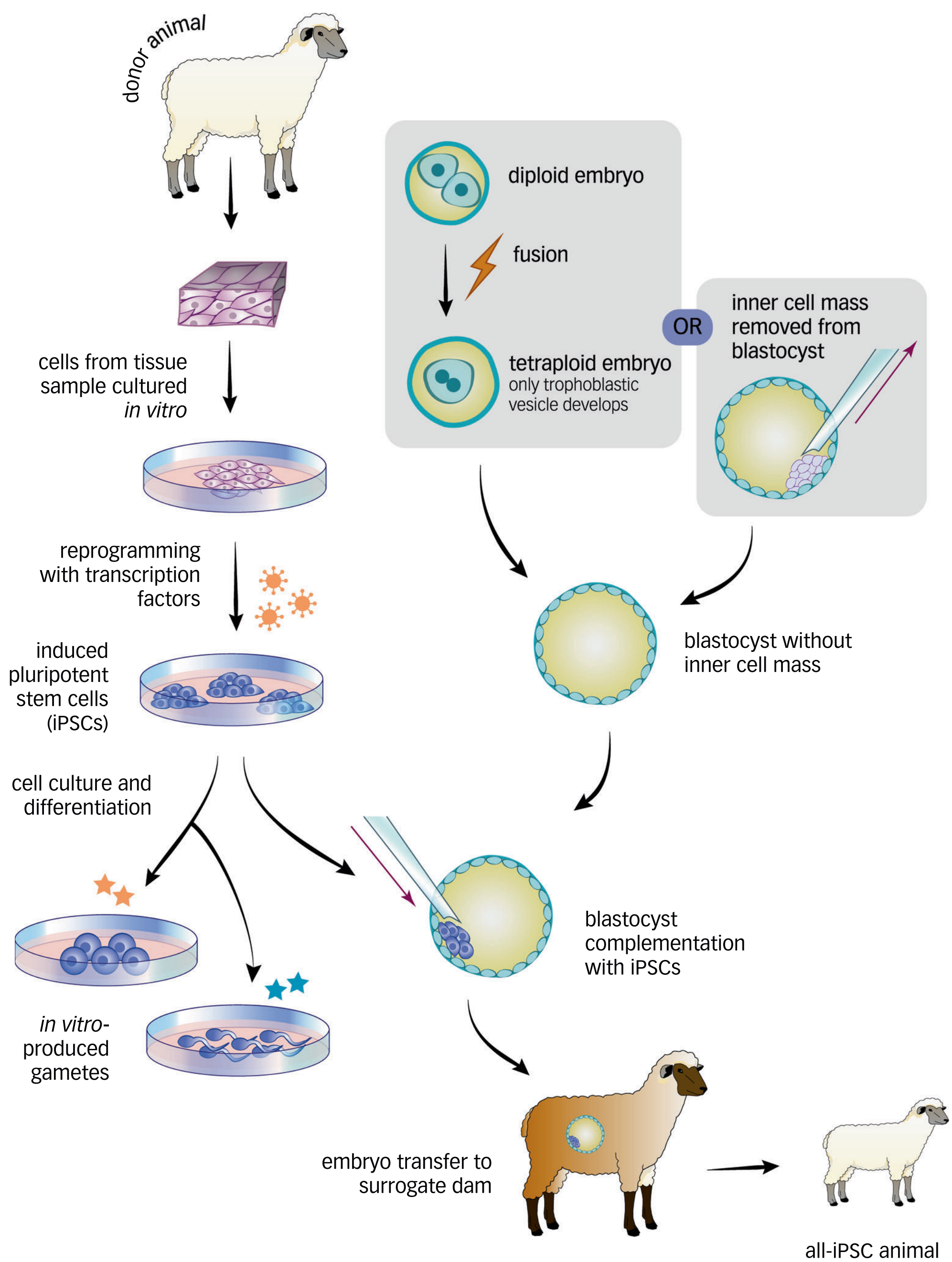

Fig. 4. Schematic illustration of generation of induced pluripotent stem cell (iPSC)-derived animals and *in vitro* gametogenesis.

used to derive iPSCs. The cocktail of transcription factors (usually *POU5F1/OCT4*, *SOX2*, *KLF4*, and *MYC* or their species-appropriate homologues) is then introduced using a viral vector. Ideally only transient overexpression is required before self-sustaining pluripotency can be achieved, in which case a non-integrating vector (e.g. adenovirus) is used. Integrating vectors – lentiviral and retroviral – can also be used, and are very efficient but may result in long-term expression of the transgenes, which is not always desirable but may be necessary in some cases. Following introduction of the reprogramming factors and provided an appropriate cell culture environment is established, a subpopulation of cells should convert to a pluripotent phenotype. Not all cells within a population will successfully express the introduced factors, and of those that do, not all will be successfully reprogrammed; as such, reprogramming efficiency remains very low even in the best-studied species and cell types, typically between 0.1 and 10% of cells successfully form iPSC colonies for most somatic cell types (Stadtfeld & Hochedlinger, 2010), although some protocols have attained efficiencies as high as 80% (e.g. Vidal *et al.*, 2014). Ideally stringent clonal selection should take place at multiple stages in the process, identifying the cells that take on the transcription factors and the subpopulation expressing other markers of pluripotency, and a selection step to propagate this population, generally based on cell colony morphology.

For tetraploid complementation, the blastomeres of a two-cell cleavage-stage embryo are fused to induce tetraploidy; this can be done chemically, using Sendai virus or electrofusion. Alternatively, cleavage can be inhibited in a two-cell embryo using cytokinesis inhibitors. Tetraploids are then cultured further to develop to blastocyst stage. In mice, tetraploidy restricts development of the embryo proper to the first few days of development but beyond this, can only facilitate development of the extra-embryonic tissues. iPSCs are then injected into the tetraploid blastocyst and, if truly pluripotent, will take over the ICM and develop into the embryo proper contained within the tetraploid trophoblast. Such chimeric or 'tetraploid-complemented' blastocysts can then be transferred to a surrogate for development to term, as per normal embryo transfer procedure. Ultimately, if successful, the procedure results in a fetus derived entirely from the iPSCs with the placenta derived from the tetraploid blastocyst.

(3) State of the art of iPSC technology in domestic animals

At present, the use of iPSCs has been studied most in the mouse, with human iPSCs being actively investigated as potential sources of biotherapeutics. In both of these species, protocols have now been established that permit stable maintenance of self-renewing populations of iPSCs that can be clonally propagated and even directed to differentiate into specific, functional cell lineages. Importantly, this can be achieved following transient expression of the reprogramming factors, after which a select population of cells engage in further signalling that sustains pluripotency without depending on continued ectopic expression of the reprogramming genes. Whilst routine in mice, studies targeting induction of iPSCs in other species (domestic livestock and wildlife) are much fewer. Nevertheless, iPSCs have been generated for numerous species [for recent detailed reviews see Scarfone *et al.* (2020) and Su *et al.* (2020)], although the validation of pluripotency is generally less extensive than in the mouse. In short, all-iPSC animals derived *via* tetraploid complementation have only been successfully created in the mouse. The most stringent assessments of pluripotency are chimera formation/blastocyst complementation and germline transmission of the pluripotent cell donor's genetics to the next generation. There has been little evidence of germline transmission in the study of domestic animal iPSCs (excluding mice), and generation of chimeras has been difficult to achieve. Injected cells tend to only be incorporated into an embryo to a limited extent and subsequently the resulting offspring often are either not chimeric or contain a very low proportion of stem cell donor cells (Fujishiro *et al.*, 2013; Sartori *et al.*, 2012; Zhang *et al.*, 2015). The significant variation seen in iPSC integration among different species indicates that much work remains to be done both to improve the stable maintenance of pluripotency and to develop the techniques used to validate it (Nagasaka *et al.*, 2017; Bai *et al.*, 2016). Reports of successful germline transmission of the iPSC donor genome, such as that achieved for porcine iPSCs (West *et al.*, 2011), hold promise for the imminent practical application of this technology.

Evidently, inducing definitive pluripotency has proved more challenging in domestic animals than in rodent models. While the necessary reprogramming factors appear to be highly conserved, their transient expression is often insufficient to generate a self-sustaining pluripotent population, and ongoing expression is required. This has been addressed by using integrating vectors to deliver the transgenes, but this in turn brings its own drawbacks and limits the applied use of iPSCs in these species. Continued expression of the reprogramming genes in the iPSCs and their downstream applications (e.g. differentiated cell lines, chimeric animals) is not a desired outcome, potentially interfering with cell differentiation and function, and causing tumour growth (Ezashi, Yuan & Roberts, 2016). Excitingly, several new approaches are presently being explored as delivery vectors for the transgenes. Use of a Sendai virus system (Fusaki *et al.*, 2009; Lieu *et al.*, 2013) has demonstrated production of transgene-free iPSCs with high reprogramming efficiency, while non-viral strategies using piggyBac transposons permit excision of the transgenes following iPSC generation (Woodard & Wilson, 2015) and have been applied in a range of species such as cattle, goats, and baboons (Kawaguchi & Cho, 2016; Hanna *et al.*, 2020; Rodriguez-Polo *et al.*, 2019). Other studies herald the prospect that genetic reprogramming could eventually be replaced entirely with a series of small molecules capable of activating the signalling pathways required to induce a pluripotent state (Borgohain *et al.*, 2019; Desponts & Ding, 2010). Other DNA-free reprogramming techniques that have the potential to derive iPSCs without genomic manipulation include recombinant proteins, miRNAs (Ma *et al*., 2014; Qiao *et al*., 2019), and synthetic messenger RNA; these have achieved partial reprogramming thus far. Further research on these fronts is set to enhance and optimise the practical application of iPSCs technology in applied clinical, production animal and other settings beyond the investigation of fundamental pluripotency mechanisms.

Identifying a growth medium that adequately supports the pluripotent state is also a species-specific endeavour, as different species seem to have different requirements for growth factors (reviewed in detail by Ezashi *et al*., 2016); further elucidation of the signalling pathways involved in maintaining pluripotency and self-renewal is underway and will help to develop tailored protocols for iPSCs in each species.

While the struggle to generate and validate true and self-sustaining pluripotency in cells from large animal species remains the primary overarching obstacle in this field, in addition to these practical limitations there are also safety concerns that preclude the release of this technology beyond the research sphere and into applied use. These include an observed tendency of iPSC-derived cells to form tumours, as has been observed for iPSC-derived neural cells transplanted into the brains of immunocompromised mice (Miura *et al*., 2009), and accelerated ageing, for example in human iPSC-derived early blood progenitor cells that undergo premature senescence (Feng *et al*., 2010). As with SCNT, there is also the open question of whether complete erasure of the epigenetic memory of the original cell type used to generate the iPSCs can be achieved: differences in DNA methylation (Deng *et al*., 2009; Doi *et al*., 2009; Pick *et al*., 2009) and in the expression of mRNAs and miRNAs (Chin *et al*., 2009; Wilson *et al*., 2009) have been reported between ESCs and iPSCs in the human and mouse. The implications of these findings for the generation and health of cloned progeny remain unclear at this time.

A key development in the stem cell field that must be addressed here is the emerging capability to induce *in vitro* differentiation of pluripotent cells into germ cells and mature gametes, namely mature oocytes and spermatozoa. Recently, mouse oocyte-like cells, capable of being fertilised and cleaving, have been generated *in vitro* from ESCs and iPSCs (Hamazaki *et al*., 2020; Hikabe *et al*., 2016), and even been fertilised to produce live offspring. This milestone achievement heralds major transformations for reproductive medicine, practical aspects of SCNT and tetraploid complementation with iPSCs, and an imminent expansion of 'cloning' possibilities. Assuming such *in vitro*-produced oocytes are fertilised with spermatozoa and not used for SCNT, this technique will not be generating adult genetic copies of the donor individual. It would, however, enable the potential creation of numerous germ cells from an individual enabling the propagation, and thus the genetics, of individuals otherwise not able to reproduce by natural means or standard artificial breeding techniques. Without creating an entire genetically identical individual, it still fulfils one of the key, and most elusive, criteria of true pluripotency: contribution to the germ line. It is also a step towards induction of totipotency – the ability of a cell to develop autonomously into a complete animal (i.e. without the assistance of blastocyst complementation, nuclear transfer, etc.), a feature entirely unique to the oocyte among all cell types. Ultimately, the ability to generate germ cells from iPSCs or ESCs means we can extract reproductive potential from biobanked non-reproductive tissue or cell lines, much the same way that biobanked fibroblasts could be used to produce cloned offspring of the donor by SCNT (Hildebrandt *et al*., 2021). As such, *in vitro* gametogenesis must be considered alongside SCNT and blastocyst complementation by iPSCs in all future discussions centred on 'cloning' and the biological and ethical consequences thereof.

(4) Scope and limitations for application for iPSC-based cloning in endangered animal conservation

The capacity to preserve and exploit non-reproductive tissues in order to generate offspring from animals that are deceased or unable to reproduce naturally holds great potential for conservation of endangered species and even de-extinction/restoration of species already lost. Notwithstanding the overall immaturity of stem cell technologies in their capacity to produce healthy offspring efficiently and safely, a series of milestones need to be attained for each species before any attempt at generating individuals *via* cell reprogramming can even begin. Evidently the primary milestone is the successful generation of true iPSCs for a given species. Then, if the goal is to produce offspring through all-iPSC blastocyst complementation, recovery and provision of compatible donor blastocysts, and successful induction of tetraploidy must all be established; meanwhile in seeking to attain *in vitro* gametogenesis/IVF, protocols need to be developed for the successful differentiation of iPSCs into both oocytes and spermatozoa, along with those for oocyte maturation, sperm capacitation and finally IVF or ICSI. In addition, underpinning these technical steps will be a thorough knowledge of the species' reproductive physiology and oestrus cycles, customised *in vitro* culture media and conditions, and the availability of surrogate animals of a compatible species to complete gestation to term.

Amid the excitement generated by iPSC technologies and the possibilities they raise for use of biobanked tissue of endangered and extinct species, steps have been taken toward iPSC generation in a number of non-domestic species. Remarkably, the reprogramming factors needed to generate iPSCs appear highly conserved in diverse species, and iPSCs/iPSC-like cells for the mouse, human, primate (macaque), rat, pig, dog, horse, sheep and cow have all been generated using almost identical methods, although in most cases, beyond the mouse and human models, iPSCs can only be sustained by continuous expression of the exogenous factors.

Despite the range of challenges still being tackled, as described here and in Section IV.3, iPSC-like cells have successfully been generated across various taxa including felids, canids, ungulates, and primates (Table 1). Felids have been particularly difficult to breed in captivity, partly due to very limited genetic diversity, which could be rescued with the aid of pluripotent stem cells in combination with biobanking. The first promising attempt at deriving iPSCs in a felid was the induction of pluripotency in snow leopard *Panthera uncia* fibroblasts (Verma *et al*., 2012), validated by teratoma formation representing the three germ layers. iPSC-like cells have also been generated for an endangered primate, the silver-maned drill *Mandrillus leucophaeus*, and the functionally extinct northern white rhinoceros

Table 1. Reports of generation of iPSC-like cells in non-domestic species

Reference	Species	IUCN species status	Reprogramming method	Transcription factors used for reprogramming	Validation of pluripotency
Liu *et al.* (2008)	Rhesus monkey (*Macaca mulatta*)	Least concern	pMX retroviral vector	Monkey OCT4, SOX2, KLF4, cMYC	Expression of stem cell marker genes; teratoma formation.
Ben-Nun *et al.* (2011)	Northern white rhinoceros (*Ceratotherium simum cottoni*)	Critically endangered	retro-VSV.G viruses	Human POU5F1 (OCT4), SOX2, KLF4 and cMYC	Expression of stem cell marker genes; formation of embryoid bodies expressing markers of the three germ layers.
Ben-Nun *et al.* (2011)	Silver-maned drill (*Mandrillus leucophaeus*)	Endangered	Retroviral vector	Human OCT4, SOX2, KLF4, cMYC	Expression of stem cell marker genes; formation of embryoid bodies expressing markers of the three germ layers; teratoma formation.
Korody *et al.* (2021)	Northern white rhinoceros (*Ceratotherium simum cottoni*)	Critically endangered	Non-integrating Sendai virus	Human OCT4, SOX2, KLF4, cMYC	Embryoid body formation; expression of pluripotency markers; differentiation into cardiomyocytes.
Verma *et al.* (2013)	Bengal tiger (*Panthera tigris*), serval (*Leptailurus serval*), and jaguar (*Panthera onca*)	Endangered; least concern; near threatened	Moloney-based retroviral vectors (pMXs)	Human OCT4, SOX2, KLF4, cMYC + NANOG	Formation of embryoid bodies expressing markers of the three germ layers; teratoma formation.
Mo *et al.* (2014)	Little brown bat (*Myotis lucifugus*)	Endangered	PiggyBac system	Human OCT4, SOX2, KLF4, cMYC, NANOG, LIN28, NR5A2 and bat MIR302/367	Formation of embryoid bodies expressing markers of the three germ layers; teratoma formation.
Ramaswamy *et al.* (2015)	Orangutan (*Pongo abelii*)	Critically endangered	Retroviral vectors	Human OCT4, SOX2, KLF4, and cMYC	Formation of embryoid bodies expressing markers of the three germ layers; teratoma formation.
Menzorov *et al.* (2015)	Mink (*Neovison vison*)	Least concern	Lentiviral vectors	Human OCT4, SOX2, KLF4, and cMYC	Formation of *in vivo* teratomas with cell types representing all three germ layers.
Katayama *et al.* (2016)	Prairie vole (*Microtus ochrogaster*)	Least concern	PiggyBac system	Mouse Oct4, Sox2, Klf4, cMyc, Lin28 and Nanog	Expression of stem cell marker genes; teratoma formation.
Weeratunga *et al.* (2018)	Tasmanian devil (*Sarcophilus harrisii*)	Endangered	Lentiviral vector	Human OCT4, SOX2, KLF4, cMYC, NANOG, and LIN28A	Formation of embryoid bodies expressing markers of the three germ layers; teratoma formation.
Whitworth *et al.* (2019)	Platypus (*Ornithorhynchus anatinus*)	Near threatened	Lentiviral vectors	Human OCT4, SOX2, KLF4, cMYC, NANOG, and LIN28	Embryoid bodies; teratomas *in vitro*, expressing ectoderm and mesoderm but not endoderm. (Mice could not be used for *in vivo* teratoma assay as platypus body temperature is 32 °C).
Verma *et al.* (2012)	Snow leopard (*Panthera uncia*)	Vulnerable	Moloney-based retroviral vectors (pMXs)	Human OCT4, SOX2, KLF4, cMYC + NANOG	Formation of teratomas containing tissues representative of the three germ layers.

(Ben-Nun *et al.*, 2011). The authors reprogrammed cryopreserved fibroblasts from both species by retroviral expression of the human genes *POU5F1/OCT4*, *SOX2*, *KLF4* and *MYC* to generate iPSC lines, and demonstrated pluripotency using molecular markers and tri-lineage differentiation. iPSC-like cells have also been generated for mink *Neovison vison*, prairie voles *Microtus ochrogaster*, little brown bats *Myotis lucifugus*, orangutans *Pongo abelis*, Tasmanian devils *Sarcophilus harrisii* and platypus *Ornithorhynchus anatinus* (see Table 1), similarly validated by expression of stemness markers and teratoma formation. As for domestic non-rodent species, these cell lines have yet to demonstrate pluripotency conclusively according to the most stringent criteria of blastocyst complementation and contribution to the germ line. This is understandable as these validation techniques are often completely untested in such non-conventional species, and resources to optimise and perform them are rarely available. Progress in domestic large animals will be crucial to supporting the development of these techniques in rarer species.

The need for a source of blastocysts to facilitate tetraploid complementation to generate cloned offspring is clearly an overwhelming limiting factor. In an endangered species context, if blastocysts are available they are unlikely to then be sacrificed to provide only the placental tissues for an iPSC 'clone'; typically each viable blastocyst is in itself very valuable and has a greater chance of survival if allowed to develop as it is rather than to support tetraploid complementation of iPSCs, a technique that in the foreseeable future will continue to exhibit very low efficiency. There are two possible ways this issue can be overcome: (1) use of embryos from a closely related species where abundant blastocysts can be produced (e.g. by IVF); and (2) future developments that will give rise to alternative sources of extraembryonic tissues. At present, mixed evidence exists about whether ESCs can produce trophectoderm; there are some indications that human ESCs are able to produce trophoblast (Amita *et al.*, 2013; Das *et al.*, 2007), and that some subpopulations of mouse ESCs can differentiate as trophectoderm derivatives (Canham *et al.*, 2010; Macfarlan *et al.*, 2012; Morgani *et al.*, 2013). Other studies indicate that mouse ESCs can be induced to differentiate into trophectoderm derivatives by manipulation of specific genes (Lu, Yang & Jin, 2011) or by exposing them to specific culture conditions (Hayashi *et al.*, 2010; Mfopou *et al.*, 2014). Thus, if iPSCs are truly equivalent in potency to ESCs, there is the theoretical possibility that future approaches will allow some iPSC populations to support development of extraembryonic tissues and perhaps negate the need for blastocyst complementation.

Until such innovations abolish the need for an abundant source of blastocysts for complementation to create all-iPSC animals, applications in conservation would likely require the formation of interspecies chimeras, where the trophoblast/placenta are provided by a closely related species, to be shed at parturition with the offspring derived fully from the donor iPSCs. This raises the issue of interspecies compatibility between the embryo and the extraembryonic tissues, and whether the maternal environment and placenta from one species can support development of offspring from another. Blastocyst complementation has been used to create chimeric (intraspecies) animals in pigs, goats, sheep and cattle following injection of (diploid) blastocysts with iPSCs or ESCs (reviewed in Mascetti & Pedersen, 2016). Efficiency is generally low and success inconsistent; the stem cells only contribute partially to the ICM, and complementation achieving all-iPSC or all-ESC animals is yet to be demonstrated. This is partly due to the methods of iPSC reprogramming being inadequate but also because of limitations on the side of the blastocyst – the generation of tetraploid blastocysts, and their developmental potential are poorly studied. Interspecies chimeras in which the placental tissues derive from one species and the entire embryo proper from another have been generated using tetraploid complementation of mouse pluripotent cells (ESCs and iPSCs) injected into rat tetraploid blastocysts and *vice versa* (Yamaguchi *et al.*, 2018). Such embryos developed normally but only until the initiation of placental development, with xenogenic incompatibilities such as inappropriate timing and site for cellular interactions, mismatched ligand–receptor interactions, affinity differences in adhesion molecules, and other cell factors proposed as the possible mechanisms limiting further development. As such, interspecies complementation is only likely to be successful in very closely related species.

While euploid blastocysts can be used for complementation, inducing tetraploidy is preferable as this should ensure that the entire embryo proper is derived from the (injected) stem cell population since the tetraploid cells can only contribute to the extraembryonic tissues. Therefore, techniques used to induce and confirm tetraploidy need to be established for each species. Inducing tetraploidy is a relatively consistent process in the mouse but appears to be more challenging in large animal species. Most protocols trialled in cattle and pigs still result in a significant proportion of fused embryos remaining diploid, as well as hexaploid and mosaic (2n and 4n) karyotypes (Iwasaki *et al.*, 1989; Curnow, Gunn & Trounson, 2000; Procházka *et al.*, 2004; He *et al.*, 2013). Reassuringly, the distribution of ploidy in the pig tetraploids appears similar to that in the mouse, whereby the tetraploid cells contribute mostly to the trophectoderm and the diploid cells to ICM (He *et al.*, 2013). Precisely how this non-random distribution occurs – although widely exploited due to lethality of tetraploid embryonic cells – is not yet understood, nor is it known how conserved this phenomenon is across different species. Optimisation of tetraploid generation, improved development rates and characterisation of ploidy distribution will be needed before this technique can be deployed to support iPSC-based cloning.

Notwithstanding the limited capability to induce tetraploidy, there have been a small number of attempts to complement bovine stem cells with tetraploid embryos. Bovine embryonic stem-like (ES-like) cells were aggregated with tetraploid compacting morulae (two per aggregate) (Iwasaki *et al.*, 2000). Here, 28 (36.4%) of 77 aggregates developed to blastocysts and ultimately six term pregnancies were achieved following transfer to surrogate cows. Interestingly, chromosome analysis showed some of the calves to have a mixture of diploid and tetraploid cells, and all calves had phenotypic features indicative of contribution from the putative tetraploid embryos. Contribution of ES-like cells was not observed in the testes from the three male calves. In short, this particular line of ES-like cells contributed to chimera formation at a low level but could not, at this stage, generate all-ESC embryos using the tetraploid aggregation technique. In fact, in four of the six embryos, ES-like cells did not contribute to the calves' genotype, moreover, in some calves they contributed only to the placenta and not the embryo. As complete tetraploidy could not be confirmed before proceeding with the aggregation, and given the lower success of inducing tetraploidy in bovine as compared to mouse embryos, the inconsistency of this step together with the uncertainty around the pluripotency status of the ES-like cells both seem to underlie the low and variable contribution of the ES-like donor cells to the offspring genotype and germ line. Aggregation of tetraploid *Bos indicus* embryos with diploid *Bos taurus* embryos has also been attempted and resulted in four putative chimeric blastocysts (4n + 2n) obtained from 31 attempts; however, tetraploid status was not confirmed following fusion and tissue distribution was not examined since blastocysts were not transferred to surrogates to develop to term (Razza *et al.*, 2016). Nonetheless this is an important development, possibly representing interspecies complementation within closely related domestic animal species. Altogether the scarcity of reports of tetraploid embryo complementation in large animal species and the variable success therein demonstrate that this technique is not at the same stage of optimisation as it is in the laboratory rodent species. Substantial progress in this aspect as well as in the induction and maintenance of a stable pluripotent state in large animal iPSCs will be required before all-iPSC animals can realistically be produced in domestic or endangered mammals.

As in SCNT cloning, consideration must be given to the increasingly recognised role of mitochondria. In generating iPSCs, mitochondria from the original cell donor are retained. A transition away from mitochondrial oxidative phosphorylation (OXPHOS) towards glycolytic metabolism is required as part of the induction of pluripotency (Folmes *et al.*, 2012), and correspondingly iPSCs exhibit immature mitochondrial morphology and a reduced reliance on OXPHOS to meet energetic demands (Prigione *et al.*, 2010); meanwhile mature oocytes are heavily OXPHOS-reliant and thus have a very different metabolic profile to iPSCs. Metabolism and mitochondrial function evidently vary with stem cell dynamics but it is still not clear to what extent mitochondria determine stem cell properties and fate decisions, or *vice versa*. Mitochondrial activity also varies considerably between cells within iPSC colonies (Bukowiecki, Adjaye & Prigione, 2014) and we do not yet know whether selection of pluripotent cells based on mitochondrial characteristics may assist with improving efficiency of iPSC-based applications, or which cells are more appropriate for generation of all-iPSC animals or differentiation for *in vitro* gametogenesis. Another key question that is yet to be examined is whether the appropriate mitochondrial transformations occur during generation of gametes from iPSCs, and how closely these mimic *in vivo* oogenesis and spermatogenesis; this may prove to be an important aspect of this emerging technology in the near future.

In creating all-iPSC animals, mitochondria of course would come from the same cell donor as the nucleus, so within the embryo proper the issue of mtDNA mismatch is avoided. A different issue arises, however, since blastocyst complementation is required for an embryo to develop to term, and the source of this blastocyst may pose a new set of compatibility obstacles pertaining to functional interaction between the cells of the embryo proper and the extraembryonic tissues. Intraspecies complementation should pose minimal problems, although the rates of success are quite low even within the mouse model, and whilst this is not a thoroughly studied phenomenon it would not be surprising to find that compatibility issues emerge at both the nuclear and mtDNA levels for many species. Experiments in interspecies complementation have been reviewed in some detail above; again, intuitively one might expect that placental tissue from one species combined with embryo proper from another would face unsurmountable challenges in development and relegate interspecies all-iPSC complementation to the distant future, if indeed it is possible. In this light, using iPSCs to generate gametes *in vitro* presents a tantalising alternative approach for propagating a given genome into the next generation, without several of the complications encountered in all-iPSC animal production. Nonetheless this technique is in its infancy, with proof-of-concept attained only in the mouse thus far.

For both iPSC and SCNT applications, where an aged animal is the cell donor, the detrimental effects of aged mitochondria could be significant. Mitochondria have long been suspected to be involved in the ageing process, with the details of this interaction recently beginning to be unravelled. The frequency of mtDNA defects is higher in iPSCs generated from aged compared with young people (Kang *et al.*, 2016), and mice with an accelerated rate of accumulation of mtDNA mutations show profound deficits in tissue regeneration and self-renewal. Stem cells recovered from such animals, even at the fetal stage, have reduced self-renewal ability *in vitro* (Ahlqvist *et al.*, 2012) and fibroblasts are more refractory to reprogramming into iPSCs (Hämäläinen *et al.*, 2015). Both these deficits appear to be mediated through an altered redox balance, as they could be rescued by treatment with a reactive oxygen species (ROS) scavenger. Notably, mitochondrial products of metabolism have been shown to be involved directly in somatic stem cell signalling rather than affecting tissue regeneration *via* ROS-induced damage (reviewed in Ahlqvist, Suomalainen & Hämäläinen, 2015). These observations, together with the well-established role of mitochondria in oocyte/embryo viability, suggest that the origin and quality of mitochondria can affect the reprogramming efficiency of iPSCs, the efficiency of all cloning techniques and the developmental competence of embryos generated downstream.

Evidently, beyond mice, cloning by generating all-iPSC animals remains a distant possibility and the urgency surrounding many endangered species is unlikely to afford the experimental numbers to develop this technology in the near future. *In vitro* gametogenesis from iPSCs is likely to be a more pragmatic alternative, and although not yet achieved in species beyond the mouse either, presents fewer foreseeable technical obstacles and has the potential to increase reproductive potential with fewer complications arising from genetic manipulations or interspecies complementation challenges. However, species-specific differentiation and culture requirements, prolonged culture period required for gametogenesis, and determination of safety profiles for resulting offspring are some of the major issues that remain to be addressed.

Cloning by tetraploid complementation and *in vitro* gametogenesis at present are the most obvious uses for iPSC technology in a conservation context, but are yet to achieve concrete success in any species beyond the mouse. This does not exclude the advent of new avenues in the near future or combination with other ARTs as described in Section V. Generation of pluripotent cells represents a fundamental strategy with immense flexibility and an initial step with a wide range of downstream applications, many of which we are yet to see develop in the future.

V. OTHER NOVEL TECHNIQUES AND COMBINED APPROACHES TO PROPAGATE GENOMES *IN VITRO*

Nuclear transfer approaches beyond SCNT may facilitate transfer of genetic material from two male or two female parents. 'Double sperm cloning' involves the transfer of two sperm nuclei into an enucleated oocyte, resulting in diploid androgenetic embryos. Such embryos can develop to the blastocyst stage at around the same rate as fertilised (IVF control) embryos, and a bovine pregnancy has survived as far as day 28 (Lagutina *et al.*, 2004). Term offspring have yet to be generated using this technique and it appears that the main challenges here pertain to an inability to replicate the pattern of maternal and paternal gene imprinting in the absence of crucial genetic and epigenetic contributions from the oocyte (Tucci *et al.*, 2019). Nonetheless double sperm cloning has been flagged as a promising approach to generate stem cells and may facilitate novel strategies for genetic rescue and conservation in the future (Zhang *et al.*, 2020). Live mice have been obtained following injection of hypomethylated haploid ESCs into MII oocytes (bimaternal offspring) or tetraploid complementation of ESCs from diploid androgenetic embryos obtained by double sperm cloning (bipaternal offspring) (Li *et al.*, 2018). The latter study represents significant progress towards generation of bimaternal and bipaternal offspring and has further demonstrated that imprinted genome regions play a major role in uniparental reproduction barriers. Hypomethylation and targeted reprogramming strategies are likely to emerge as key tools for overcoming these barriers in the future.

In addition, various permutations of two or more of the techniques described throughout this review may, in the future, help to overcome some of the key challenges currently being faced. For example, as abnormal placentation appears a common pitfall limiting the successful generation of healthy offspring by SCNT, a proposed solution is to derive the ICM using SCNT but to provide an alternative source of trophectoderm, namely by blastocyst complementation with *in vivo*-derived or IVF-produced embryos. This technique has been attempted in cattle but is yet to generate live offspring, so the potential for it to yield improvement in survival or offspring health is not yet clear (Murakami *et al.*, 2006). ICM-replacement offspring have been generated using two different strains of mice (Zheng *et al.*, 2005), and embryos produced by SCNT have been aggregated with tetraploid blastocysts to reduce epigenetic errors in the placenta and improve pregnancy and implantation rates (Sim & Min, 2014). The ICM-transfer approach has also been suggested to assist with interspecies embryo transfer, as in the case of wild-to-domestic interspecies surrogacy (Saragusty *et al.*, 2020).

Another combined approach is to use iPSCs as nuclear donors for nuclear transfer. This would presumably reduce the epigenetic distance or the amount of reprogramming that needs to be undertaken by the oocyte. Since incomplete reprogramming is one of the major hurdles in attaining higher rates of embryo development following SCNT, such an approach is expected to improve efficiency and the likelihood of generating healthy live offspring. As mentioned in Section III.3, studies examining this hypothesis have yielded mixed conclusions. Nonetheless, using pluripotent blastomeres (*versus* adult somatic cells) as donor cells results in higher cloning efficiency in mice and appears to decrease the incidence of developmental abnormalities, presumably because only minimal nuclear reprogramming is required (Hochedlinger & Jaenisch, 2006). Mouse iPSCs have been successfully used as donor cells to generate nuclear transfer clones and demonstrated an improved cloning efficiency when compared with the original embryonic fibroblast cells used to derive the iPSC population (Zhou *et al.*, 2010). Using this approach the authors were also able to obtain live animals from iPSC lines that are not tetraploid-complementation compatible. Thus, the combination of induced pluripotency and nuclear transfer may be useful for generating cloned animals using cell types that prove recalcitrant to SCNT alone, and scenarios where tetraploid complementation of iPSCs is not feasible.

VI. DISCUSSION

In this review we have summarised the established and emerging biotechnological techniques that result in the production of genetically identical individuals, that is clones.

It becomes evident that SCNT is not the only technique deserving of this label, and that mammalian cloning comprises a rapidly transforming, dynamic field of biological research. While some of these techniques have attained remarkable progress at the cutting edge, progress with respect to endangered species has been slow and thus the diversity of species in which these techniques can realistically be deployed remains heavily constrained by pragmatic or logistical issues, or by lack of fundamental biological knowledge. For example, while oestrus synchronisation and embryo transfer are routine procedures in domestic livestock, these procedures remain experimental in most endangered mammals. The survival of transferred embryos after nuclear transfer is quite poor in many species, with many poor pregnancy outcomes and a high rate of perinatal mortality. Establishing reliable embryo transfer protocols for endangered mammals will require a dedicated phase of experimentation, and can pose an ethical problem where healthy animals are subjected to handling, anaesthesia, and surgical procedures without guaranteed beneficial outcomes for the individual or the species.

Beyond the immediate technical challenges, many features of reproductive physiology are notoriously divergent between species, because the evolution of reproductive mechanisms is subject to strong intra-species selective pressures (Anholt *et al.*, 2020). Induced ovulation, embryonic diapause, and maternal recognition of pregnancy are just a few examples of reproductive strategies that are still poorly understood despite many decades of investigation in fairly well-studied species (Ratto *et al.*, 2019; Renfree & Fenelon, 2017; Swegen, 2021), highlighting that much remains to be done in mapping even the basic reproductive functions across such a diverse group as mammals. The obstacles posed by reproductive diversity indirectly culminate in an ethical challenge, whereby resources for biotech solutions such as ARTs and cloning may flow to species for which those technologies are most immediately feasible (by virtue of conserved reproductive strategies). By the same token these are unlikely to be the most phylogenetically unique species, and thus perhaps not the best species to prioritise if conservation of maximal biodiversity is the end goal (Arponen, 2012). Hence, systematic efforts to understand the reproductive physiology of so-called non-traditional species (beyond laboratory rodents, humans and domestic livestock), establishment of efficient routine procedures (including oocyte collection, oestrous cycle manipulation, embryo transfer and pregnancy detection), and optimisation of species-specific laboratory protocols must all be prioritised before any of the more novel techniques can feasibly be attempted. The urgency of these seemingly mundane research goals is easy to underestimate; it is crucial that such research occurs well before a given species becomes too endangered to enable research on meaningful numbers of animals, and thus some foresight is required in planning research and biobanking samples. Finally, an understanding of the fundamental mechanisms limiting interspecies compatibility, at the nucleo-mitochondrial, embryo–placental unit and surrogate pregnancy levels, must also be attained in order to facilitate conservation efforts seeking to rescue endangered species with the help of more common ones.

We reiterate the importance of habitat preservation, addressing human–wildlife conflict, and attention to socio-economic factors (e.g. stewardship, alleviation of poverty, alternative sources of income to poaching) in wild animal conservation, and highlight that biotech strategies do not replace any of these important measures; rather, they represent additional tools available to contribute to a multifaceted effort. Furthermore, a holistic approach is essential when decisions around cloning and iPSC use are made for functionally extinct and endangered animals, particularly with regard to whether a suitable habitat still exists and can be maintained, whether animals will be physiologically and behaviourally fit for release into the wild, and whether a healthy, genetically diverse population is likely to be sustained into the future. Luckily, biobanks, such as the San Diego Frozen Zoo®, the IZW Berlin Biobank and most recently Nature's SAFE in the UK, exist as a backstop to ensure cellular samples can be stored until the other numerous limitations can be resolved.

VII. CONCLUSIONS

(1) Re-examining the history of 'cloning' reveals that several techniques capable of generating genetically identical individuals are available, including embryo splitting or blastomere separation to generate monozygotic twins, transferring DNA into an enucleated oocyte (SCNT), and tetraploid complementation of iPSCs. In addition, novel approaches and those that combine two or more of these techniques, are becoming possible. All technologies that facilitate the propagation of a nuclear genome, without recombination, to a population of germ cells or to the next generation, must be considered alongside cloning and associated techniques.

(2) The use of these techniques in endangered mammals may help to overcome specific challenges, such as the scarcity of fertile gametes or recalcitrance to superovulation, or augment production of embryos *in vitro*. The use case for cloning in conservation is more sophisticated and varied than that of simply increasing numbers of individuals within a species. In some scenarios, cloning emerges as the only viable option for restoring genetic diversity or facilitating de-extinction of a species. Relevant considerations include the genetic diversity of both cryobanked and living populations, their ability to breed naturally or *via* the use of other ARTs, and the available fundamental knowledge of reproductive biology of the species in question and its closest relatives.

(3) Most conservation-focused cloning efforts will require interspecies biotechnologies. The fundamental mechanisms underpinning interspecies compatibility, at the nucleo-mitochondrial, embryo–placental unit and surrogate pregnancy levels, are surprisingly poorly understood and require investigation. Mitochondrial compatibility, as well as the origin

and inheritance of the mitochondrial genome, are key considerations in immediate progress for cloning by SCNT, iPSCs and derived techniques.

(4) iPSC-based cloning and *in vitro* gametogenesis theoretically offer the capacity to exploit non-reproductive tissues to generate offspring from deceased or infertile animals, promising potential for conservation of endangered species and even de-extinction/restoration of species already lost. Despite remarkable conservation of the transcription factors needed to induce stemness across species, the technology is immature in its capacity to produce healthy offspring efficiently and safely – especially beyond the commonly studied laboratory and domestic animals. A series of milestones need to be attained for each species before any attempt at generating individuals *via* cell reprogramming can even begin. Progress in domestic large animals will be crucial to supporting the development of these techniques in rarer species.

(5) The cloning techniques described here have attained proof-of-concept and some have been applied successfully in livestock, with others limited to rodent models. Progress with respect to endangered species has been slow and the range of species in which these techniques can realistically be deployed remains heavily constrained by pragmatic or logistical issues, or by lack of fundamental biological knowledge. Ongoing systematic efforts to understand the diverse reproductive physiology and anatomy of so-called non-traditional species, establishment of efficient routine reproductive procedures, and optimisation of species-specific laboratory protocols must all be prioritised before any of the more novel techniques can be considered feasible.

VIII. REFERENCES

Ahlqvist, K. J., Hämäläinen, R. H., Yatsuga, S., Uutela, M., Terzioglu, M., Götz, A., Forsström, S., Salven, P., Angers-Loustau, A., Kopra, O. H., Tyynismaa, H., Larsson, N. G., Wartiovaara, K., Prolla, T., Trifunovic, A., *et al.* (2012). Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice. *Cell Metabolism* **15**(1), 100–109.

Ahlqvist, K. J., Suomalainen, A. & Hämäläinen, R. H. (2015). Stem cells, mitochondria and aging. *Biochimica et Biophysica Acta* **1847**(11), 1380–1386.

Akagi, S., Matsukawa, K. & Takahashi, S. (2014). Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *Journal of Reproduction and Development* **60**(5), 329–335.

Alvarenga, M. A., Carmo, M. T. & Landim-Alvarenga, F. d. C. (2008). Superovulation in mares: limitations and perspectives. *Pferdeheilkunde* **24**(1), 88–91.

Amita, M., Adachi, K., Alexenko, A. P., Sinha, S., Schust, D. J., Schulz, L. C., Roberts, R. M. & Ezashi, T. (2013). Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proceedings of the National Academy of Sciences of the United States of America* **110**(13), E1212–E1221.

An, Q., Peng, W., Cheng, Y., Lu, Z., Zhou, C., Zhang, Y. & Su, J. (2019). Melatonin supplementation during in vitro maturation of oocyte enhances subsequent development of bovine cloned embryos. *Journal of Cellular Physiology* **234**(10), 17370–17381.

Anholt, R. R. H., O'Grady, P., Wolfner, M. F. & Harbison, S. T. (2020). Evolution of reproductive behavior. *Genetics* **214**(1), 49–73.

Arponen, A. (2012). Prioritizing species for conservation planning. *Biodiversity and Conservation* **21**(4), 875–893.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N. & Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Development* **17**(1), 126–140.

Bae, H. K., Hwang, I. S., Kim, J. Y., Lee, S. Y., Park, C. K., Yang, B. K. & Cheong, H. T. (2015). Antioxidant treatment during manipulation procedures prevents mitochondrial and DNA damage and enhances nuclear reprogramming of bovine somatic cell nuclear transfer embryos. *Reproduction, Fertility and Development* **27**(7), 1088–1096.

Bai, C., Li, X., Gao, Y., Yuan, Z., Hu, P., Wang, H., Liu, C., Guan, W. & Ma, Y. (2016). Melatonin improves reprogramming efficiency and proliferation of bovine-induced pluripotent stem cells. *Journal of Pineal Research* **61**(2), 154–167.

Behboodi, E., Anderson, G. B., BonDurant, R. H., Cargill, S. L., Kreuscher, B. R., Medrano, J. F. & Murray, J. D. (1995). Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology* **44**(2), 227–232.

Ben-Nun, I. F., Montague, S. C., Houck, M. L., Tran, H. T., Garitaonandia, I., Leonardo, T. R., Wang, Y. C., Charter, S. J., Laurent, L. C., Ryder, O. A. & Loring, J. F. (2011). Induced pluripotent stem cells from highly endangered species. *Nature Methods* **8**(10), 829–831.

Boland, M. J., Hazen, J. L., Nazor, K. L., Rodriguez, A. R., Gifford, W., Martin, G., Kupriyanov, S. & Baldwin, K. K. (2009). Adult mice generated from induced pluripotent stem cells. *Nature* **461**(7260), 91–94.

Borgohain, M. P., Haridhasapavalan, K. K., Dey, C., Adhikari, P. & Thummer, R. P. (2019). An insight into DNA-free reprogramming approaches to generate integration-free induced pluripotent stem cells for prospective biomedical applications. *Stem Cell Reviews and Reports* **15**(2), 286–313.

Bortolus, R., Zanardo, V. & Trevisanuto, D. (2001). Epidemiology of identical twin pregnancy. *La Pediatria Medica e Chirurgica* **23**(3–4), 153–158.

Brackett, B. G., Bousquet, D., Boice, M. L., Donawick, W. J., Evans, J. F. & Dressel, M. A. (1982). Normal development following in vitro fertilization in the cow. *Biology of Reproduction* **27**(1), 147–158.

Briggs, R. & King, T. J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proceedings of the National Academy of Sciences* **38**(5), 455–463.

Bukowiecki, R., Adjaye, J. & Prigione, A. (2014). Mitochondrial function in pluripotent stem cells and cellular reprogramming. *Gerontology* **60**(2), 174–182.

Campbell, K. H., McWhir, J., Ritchie, W. A. & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**(6569), 64–66.

Canham, M. A., Sharov, A. A., Ko, M. S. & Brickman, J. M. (2010). Functional heterogeneity of embryonic stem cells revealed through translational amplification of an early endodermal transcript. *PLoS Biology* **8**(5), e1000379.

Chen, D. Y., Wen, D. C., Zhang, Y. P., Sun, Q. Y., Han, Z. M., Liu, Z. H., Shi, P., Li, J. S., Xiangyu, J. G., Lian, L., Kou, Z. H., Wu, Y. Q., Chen, Y. C., Wang, P. Y. & Zhang, H. M. (2002). Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. *Biology of Reproduction* **67**(2), 637–642.

Chin, M. H., Mason, M. J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiuwu, O., Richter, L., Zhang, J., Khvorostov, I., Ott, V., Grunstein, M., Lavon, N., Benvenisty, N., *et al.* (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell* **5**(1), 111–123.

Clulow, J. & Clulow, S. (2016). Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians and reptiles: bringing the ARTs up to speed. *Reproduction, Fertility and Development* **28**(8), 1116–1132.

Comizzoli, P. & Holt, W. V. (2019). Breakthroughs and new horizons in reproductive biology of rare and endangered animal species. *Biology of Reproduction* **101**(3), 514–525.

Cosby, N. C. & Dukelow, W. R. (1990). Microencapsulation of single, multiple, and zona pellucida-free mouse preimplantation embryos in sodium alginate and their development in vitro. *Journal of Reproduction and Fertility* **90**(1), 19–24.

Craig, J. M., Calais-Ferreira, L., Umstad, M. P. & Buchwald, D. (2020). The value of twins for health and medical research: a third of a century of progress. *Twin Research and Human Genetics* **23**(1), 8–15.

Curnow, E. C., Gunn, I. M. & Trounson, A. O. (2000). Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts in vitro. *Molecular Reproduction and Development* **56**(3), 372–377.

Czernik, M., Anzalone, D. A., Palazzese, L., Oikawa, M. & Loi, P. (2019). Somatic cell nuclear transfer: failures, successes and the challenges ahead. *International Journal of Developmental Biology* **63**(3–4–5), 123–130.

Das, P., Ezashi, T., Schulz, L. C., Westfall, S. D., Livingston, K. A. & Roberts, R. M. (2007). Effects of fgf2 and oxygen in the bmp4-driven differentiation of trophoblast from human embryonic stem cells. *Stem Cell Research* **1**(1), 61–74.

Deng, J., Shoemaker, R., Xie, B., Gore, A., LeProust, E. M., Antosiewicz-Bourget, J., Egli, D., Maherali, N., Park, I. H., Yu, J., Daley, G. Q., Eggan, K., Hochedlinger, K., Thomson, J., Wang, W., *et al.* (2009). Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nature Biotechnology* **27**(4), 353–360.

Desponts, C. & Ding, S. (2010). Using small molecules to improve generation of induced pluripotent stem cells from somatic cells. *Methods in Molecular Biology* **636**, 207–218.

Dey, R., Barrientos, A. & Moraes, C. T. (2000). Functional constraints of nuclear mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *Journal of Biological Chemistry* **275**(40), 31520–31527.

Doi, A., Park, I. H., Wen, B., Murakami, P., Aryee, M. J., Irizarry, R., Herb, B., Ladd-Acosta, C., Rho, J., Loewer, S., Miller, J., Schlaeger, T., Daley, G. Q. & Feinberg, A. P. (2009). Differential methylation of tissue- and cancer-specific CpG Island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nature Genetics* **41**(12), 1350–1353.

Dominko, T., Mitalipova, M., Haley, B., Beyhan, Z., Memili, E., McKusick, B. & First, N. L. (1999). Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. *Biology of Reproduction* **60**(6), 1496–1502.

Elsheikh, A. S., Takahashi, Y., Hishinuma, M., Nour, M. S. & Kanagawa, H. (1997). Effect of encapsulation on development of mouse pronuclear stage embryos in vitro. *Animal Reproduction Science* **48**(2–4), 317–324.

Ezashi, T., Yuan, Y. & Roberts, R. M. (2016). Pluripotent stem cells from domesticated mammals. *Annual Reviews in Animal Biosciences* **4**, 223–253.

Feng, Q., Lu, S. J., Klimanskaya, I., Gomes, I., Kim, D., Chung, Y., Honig, G. R., Kim, K. S. & Lanza, R. (2010). Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence. *Stem Cells* **28**(4), 704–712.

Folmes, C. D., Nelson, T. J., Dzeja, P. P. & Terzic, A. (2012). Energy metabolism plasticity enables stemness programs. *Annals of the New York Academy of Sciences* **1254**, 82–89.

Forsberg, E. J., Strelchenko, N. S., Augenstein, M. L., Betthauser, J. M., Childs, L. A., Eilertsen, K. J., Enos, J. M., Forsythe, T. M., Golueke, P. J., Koppang, R. W., Lange, G., Lesmeister, T. L., Mallon, K. S., Mell, G. D., Misica, P. M., *et al.* (2002). Production of cloned cattle from in vitro systems. *Biology of Reproduction* **67**(1), 327–333.

Fujishiro, S. H., Nakano, K., Mizukami, Y., Azami, T., Arai, Y., Matsunari, H., Ishino, R., Nishimura, T., Watanabe, M., Abe, T., Furukawa, Y., Umeyama, K., Yamanaka, S., Ema, M., Nagashima, H., *et al.* (2013). Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. *Stem Cells and Development* **22**(3), 473–482.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. & Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proceedings of the Japan Academy Series B: Physical and Biological Sciences* **85**(8), 348–362.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. & Lazzari, G. (2003). Pregnancy: a cloned horse born to its dam twin. *Nature* **424**(6949), 635.

Galli, C. & Lazzari, G. (2021). Current applications of SCNT in advanced breeding and genome editing in livestock. *Reproduction* **162**(1), F23–F32.

Gómez, M. C., Pope, C. E., Giraldo, A., Lyons, L. A., Harris, R. F., King, A. L., Cole, A., Godke, R. A. & Dresser, B. L. (2004). Birth of African Wildcat cloned kittens born from domestic cats. *Cloning and Stem Cells* **6**(3), 247–258.

Gómez, M. C., Pope, C. E., Kutner, R. H., Ricks, D. M., Lyons, L. A., Ruhe, M., Dumas, C., Lyons, J., López, M., Dresser, B. L. & Reiser, J. (2008). Nuclear transfer of sand cat cells into enucleated domestic cat oocytes is affected by cryopreservation of donor cells. *Cloning and Stem Cells* **10**(4), 469–483.

Gurdon, J. B. & Byrne, J. A. (2003). The first half-century of nuclear transplantation. *Proceedings of the National Academy of Sciences* **100**(14), 8048–8052.

Gurdon, J. B., Byrne, J. A. & Simonsson, S. (2003). Nuclear reprogramming and stem cell creation. *Proceedings of the National Academy of Sciences* **100**(suppl_1), 11819–11822.

Gurdon, J. B. & Uehlinger, V. (1966). "Fertile" intestine nuclei. *Nature* **210**(5042), 1240–1241.

Hämäläinen, R. H., Ahlqvist, K. J., Ellonen, P., Lepistö, M., Logan, A., Otonkoski, T., Murphy, M. P. & Suomalainen, A. (2015). MtDNA mutagenesis disrupts pluripotent stem cell function by altering redox signaling. *Cell Reports* **11**(10), 1614–1624.

Hamazaki, N., Kyogoku, H., Araki, H., Miura, F., Horikawa, C., Hamada, N., Shimamoto, S., Hikabe, O., Nakashima, K., Kitajima, T. S., Ito, T., Leitch, H. G. & Hayashi, K. (2020). Reconstitution of the oocyte transcriptional network with transcription factors. *Nature* **589**(7841), 264–269.

Hanna, M., Sahito, R. G. A., Rateb, M., Kachiwal, A. B., Seddiek, H. A., Bhutto, B. & Hescheler, J. (2020). Generation of transgene-free induced pluripotent stem cells from cardiac fibroblasts of goat embryos. *Journal of Stem Cells and Regenerative Medicine* **16**(2), 34–43.

Hashiyada, Y. (2017). The contribution of efficient production of monozygotic twins to beef cattle breeding. *Journal of Reproduction and Development* **63**(6), 527–538.

Hayashi, Y., Furue, M. K., Tanaka, S., Hirose, M., Wakisaka, N., Danno, H., Ohnuma, K., Oeda, S., Aihara, Y., Shiota, K., Ogura, A., Ishiura, S. & Asashima, M. (2010). BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin. *In Vitro Cellular & Developmental Biology - Animal* **46**(5), 416–430.

He, W., Kong, Q., Shi, Y., Xie, B., Jiao, M., Huang, T., Guo, S., Hu, K. & Liu, Z. (2013). Generation and developmental characteristics of porcine tetraploid embryos and tetraploid/diploid chimeric embryos. *Genomics, Proteomics & Bioinformatics* **11**(5), 327–333.

Herrick, J. R. (2019). Assisted reproductive technologies for endangered species conservation: developing sophisticated protocols with limited access to animals with unique reproductive mechanisms. *Biology of Reproduction* **100**(5), 1158–1170.

Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M. & Hayashi, K. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature* **539**(7628), 299–303.

Hildebrandt, T. B., Hermes, R., Goeritz, F., Appeltant, R., Colleoni, S., de Mori, B., Diecke, S., Drukker, M., Galli, C., Hayashi, K., Lazzari, G., Loi, P., Payne, J., Renfree, M., Seet, S., *et al.* (2021). The ART of bringing extinction to a freeze – history and future of species conservation, exemplified by rhinos. *Theriogenology* **169**, 76–88.

Hochedlinger, K. & Jaenisch, R. (2006). Nuclear reprogramming and pluripotency. *Nature* **441**(7097), 1061–1067.

Holoch, D. & Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. *Nature Reviews. Genetics* **16**(2), 71–84.

Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E. S., Golub, T. R. & Jaenisch, R. (2002). Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proceedings of the National Academy of Sciences* **99**(20), 12889–12894.

Hwang, I., Jeong, Y. W., Kim, J. J., Lee, H. J., Kang, M., Park, K. B., Park, J. H., Kim, Y. W., Kim, W. T., Shin, T., Hyun, S. H., Jeung, E. B. & Hwang, W. S. (2013). Successful cloning of coyotes through interspecies somatic cell nuclear transfer using domestic dog oocytes. *Reproduction, Fertility and Development* **25**(8), 1142–1148.

Illmensee, K., Kaskar, K. & Zavos, P. M. (2006). In vitro blastocyst development from serially split mouse embryos and future implications for human assisted reproductive technologies. *Fertility and Sterility* **86**(4 Suppl), 1112–1120.

Inoue, K., Noda, S., Ogonuki, N., Miki, H., Inoue, S., Katayama, K., Mekada, K., Miyoshi, H. & Ogura, A. (2007). Differential developmental ability of embryos cloned from tissue-specific stem cells. *Stem Cells* **25**(5), 1279–1285.

Iwasaki, S., Campbell, K. H., Galli, C. & Akiyama, K. (2000). Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. *Biology of Reproduction* **62**(2), 470–475.

Iwasaki, S., Kono, T., Fukatsu, H. & Nakahara, T. (1989). Production of bovine tetraploid embryos by electrofusion and their developmental capability in vitro. *Gamete Research* **24**(3), 261–267.

Jonsson, H., Magnusdottir, E., Eggertsson, H. P., Stefansson, O. A., Arnadottir, G. A., Eiriksson, O., Zink, F., Helgason, E. A., Jonsdottir, I., Gylfason, A., Jonasdottir, A., Jonasdottir, A., Beyter, D., Steingrimsdottir, T., Norddahl, G. L., *et al.* (2021). Differences between germline genomes of monozygotic twins. *Nature Genetics* **53**(1), 27–34.

Juanpanich, T., Suttirojpattana, T., Takayama, M., Liang, Y., Dochi, O., Parnpai, R. & Imai, K. (2018). Survival and developmental competence of bovine embryos at different developmental stages and separated blastomeres after vitrification in different solutions. *Animal Science Journal* **89**(1), 42–51.

Kamath, M. S., Antonisamy, B. & Sunkara, S. K. (2020). Zygotic splitting following embryo biopsy: a cohort study of 207 697 single-embryo transfers following IVF treatment. *British Journal of Obstetrics and Gynaecology* **127**(5), 562–569.

Kang, E., Wang, X., Tippner-Hedges, R., Ma, H., Folmes, C. D., Gutierrez, N. M., Lee, Y., Van Dyken, C., Ahmed, R., Li, Y., Koski, A., Hayama, T., Luo, S., Harding, C. O., Amato, P., *et al.* (2016). Age-related accumulation of somatic mitochondrial DNA mutations in adult-derived human iPSCs. *Cell Stem Cell* **18**(5), 625–636.

Kang, J. T., Oh, H. J., Hong, S. G., Park, J. E., Kim, D. Y., Jang, G., Kim, M. K. & Lee, B. C. (2009*a*). 89 male-to-female sex reversal in wolf derived from somatic cell nuclear transfer with 78, XY karyotype. *Reproduction Fertility and Development* **21**(1), 145.

Kang, L., Wang, J., Zhang, Y., Kou, Z. & Gao, S. (2009*b*). iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell* **5**(2), 135–138.

Katayama, M., Hirayama, T., Horie, K., Kiyono, T., Donai, K., Takeda, S., Nishimori, K. & Fukuda, T. (2016). Induced pluripotent stem cells with six reprogramming factors from prairie vole, which is an animal model for social behaviors. *Cell Transplantation* **25**(5), 783–796.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. & Tsunoda, Y. (1998). Eight calves cloned from somatic cells of a single adult. *Science* **282**(5396), 2095–2098.

Kato, Y., Tani, T. & Tsunoda, Y. (2000). Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *Journal of Reproduction and Fertility* **120**(2), 231–237.

Kawaguchi, T. & Cho, D. (2016). Derivation of induced trophoblast cell lines in cattle by doxycycline-inducible piggyBac vectors. *PLoS One* **11**(12), e0167550.

Keefer, C. L. (2015). Artificial cloning of domestic animals. *Proceedings of the National Academy of Sciences* **112**(29), 8874–8878.

Kim, M. K., Jang, G., Oh, H. J., Yuda, F., Kim, H. J., Hwang, W. S., Hossein, M. S., Kim, J. J., Shin, N. S., Kang, S. K. & Lee, B. C. (2007). Endangered wolves cloned from adult somatic cells. *Cloning and Stem Cells* **9**(1), 130–137.

Kishigami, S., Wakayama, S., Thuan, N. V., Ohta, H., Mizutani, E., Hikichi, T., Bui, H. T., Balbach, S., Ogura, A., Boiani, M. & Wakayama, T. (2006). Production of cloned mice by somatic cell nuclear transfer. *Nature Protocols* **1**(1), 125–138.

Kobel, H. R., Brun, R. B. & Fischberg, M. (1973). Nuclear transplantation with melanophores, ciliated epidermal cells, and the established cell-line A-8 in *Xenopus laevis*. *Journal of Embryology and Experimental Morphology* **29**(3), 539–547.

Korody, M. L., Ford, S. M., Nguyen, T. D., Pivaroff, C. G., Valiente-Alandi, I., Peterson, S. E., Ryder, O. A. & Loring, J. F. (2021). Rewinding extinction in the northern white rhinoceros: genetically diverse induced pluripotent stem cell bank for genetic rescue. *Stem Cells and Development* **30**(4), 177–189.

Krentz, K. J., Nebel, R. L., Canseco, R. S. & McGilliard, M. L. (1993). In vitro and in vivo development of mouse morulae encapsulated in 2% sodium alginate or 0.1% poly-l-lysine. *Theriogenology* **39**(3), 655–667.

Lacy, R. C. (2013). Achieving true sustainability of zoo populations. *Zoo Biology* **32**(1), 19–26.

Lagutina, I., Fulka, H., Lazzari, G. & Galli, C. (2013). Interspecies somatic cell nuclear transfer: advancements and problems. *Cellular Reprogramming* **15**(5), 374–384.

Lagutina, I., Lazzari, G., Duchi, R. & Galli, C. (2004). Developmental potential of bovine androgenetic and parthenogenetic embryos: a comparative study. *Biology of Reproduction* **70**(2), 400–405.

Leemans, B., Gadella, B. M., Stout, T. A., De Schauwer, C., Nelis, H., Hoogewijs, M. & Van Soom, A. (2016). Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/fertilization. *Reproduction* **152**(6), R233–R245.

Lees, C. M. & Wilcken, J. (2009). Sustaining the Ark: the challenges faced by zoos in maintaining viable populations. *International Zoo Yearbook* **43**(1), 6–18.

Li, J., Greco, V., Guasch, G., Fuchs, E. & Mombaerts, P. (2007). Mice cloned from skin cells. *Proceedings of the National Academy of Sciences* **104**(8), 2738–2743.

Li, Z.-K., Wang, L.-Y., Wang, L.-B., Feng, G.-H., Yuan, X.-W., Liu, C., Xu, K., Li, Y.-H., Wan, H.-F., Zhang, Y., Li, Y.-F., Li, X., Li, W., Zhou, Q. & Hu, B.-Y. (2018). Generation of Bimaternal and Bipaternal Mice from Hypomethylated Haploid ESCs with Imprinting Region Deletions. *Cell Stem Cell* **23**(5), 665–676.e4.

Lieu, P. T., Fontes, A., Vemuri, M. C. & Macarthur, C. C. (2013). Generation of induced pluripotent stem cells with CytoTune, a non-integrating Sendai virus. *Methods in Molecular Biology* **997**, 45–56.

Liu, H., Liu, J., Chen, S., Kang, X., Du, H. & Li, L. (2018*a*). Elevated incidence of monozygotic twinning is associated with extended embryo culture, but not with zona pellucida manipulation or freeze-thaw procedure. *Fertility and Sterility* **109**(6), 1044–1050.

Liu, H., Zhu, F., Yong, J., Zhang, P., Hou, P., Li, H., Jiang, W., Cai, J., Liu, M., Cui, K., Qu, X., Xiang, T., Lu, D., Chi, X., Gao, G., *et al.* (2008). Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell* **3**(6), 587–590.

Liu, S., Hong, Y., Cui, K., Guan, J., Han, L., Chen, W., Xu, Z., Gong, K., Ou, Y., Zeng, C., Li, S., Zhang, D. & Hu, D. (2018*b*). Four-generation pedigree of monozygotic female twins reveals genetic factors in twinning process by whole-genome sequencing. *Twin Research and Human Genetics* **21**(5), 361–368.

Liu, X. & Shi, J. (2021). Maternal age is associated with embryo splitting after single embryo transfer: a retrospective cohort study. *Journal of Assisted Reproduction and Genetics* **38**(1), 79–83.

Loi, P., Ptak, G., Barboni, B., Fulka, J. Jr., Cappai, P. & Clinton, M. (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nature Biotechnology* **19**(10), 962–964.

Lu, R., Yang, A. & Jin, Y. (2011). Dual functions of T-box 3 (Tbx3) in the control of self-renewal and extraembryonic endoderm differentiation in mouse embryonic stem cells. *Journal of Biological Chemistry* **286**(10), 8425–8436.

Ma, K., Song, G., An, X., Fan, A., Tan, W., Tang, B., Zhang, X. & Li, Z. (2014). miRNAs promote generation of porcine-induced pluripotent stem cells. *Molecular and Cellular Biochemistry* **389**(1–2), 209–218.

Macfarlan, T. S., Gifford, W. D., Driscoll, S., Lettieri, K., Rowe, H. M., Bonanomi, D., Firth, A., Singer, O., Trono, D. & Pfaff, S. L. (2012). Embryonic stem cell potency fluctuates with endogenous retrovirus activity. *Nature* **487**(7405), 57–63.

MacKenna, A., Schwarze, J. E., Crosby, J. & Zegers-Hochschild, F. (2020). Factors associated with embryo splitting and clinical outcome of monozygotic twins in pregnancies after IVF and ICSI. *Human Reproduction Open* **2020**(1), hoaa024.

Mardini, A. C., Pereira, F. S., Schuler-Faccini, L. & Matte, U. (2017). Analysis of polymorphism rs1042522 in TP53 gene in the mothers of twins and of singletons: a population-based study in Rio Grande do Sul, Brazil. *Twin Research and Human Genetics* **20**(2), 132–136.

Mascetti, V. L. & Pedersen, R. A. (2016). Contributions of mammalian chimeras to pluripotent stem cell research. *Cell Stem Cell* **19**(2), 163–175.

Matte, U., Le Roux, M. G., Bénichou, B., Moisan, J. P. & Giugliani, R. (1996). Study on possible increase in twinning rate at a small village in south Brazil. *Acta Geneticae Medicae et Gemellologiae (Roma)* **45**(4), 431–437.

McKinnell, R. G. & Di Berardino, M. A. (1999). The biology of cloning: history and rationale. *Bioscience* **49**(11), 875–885.

Meng, L., Ely, J. J., Stouffer, R. L. & Wolf, D. P. (1997). Rhesus monkeys produced by nuclear transfer. *Biology of Reproduction* **57**(2), 454–459.

Menzorov, A. G., Matveeva, N. M., Markakis, M. N., Fishman, V. S., Christensen, K., Khabarova, A. A., Pristyazhnyuk, I. E., Kizilova, E. A., Cirera, S., Anistoroaei, R. & Serov, O. L. (2015). Comparison of American mink embryonic stem and induced pluripotent stem cell transcriptomes. *BMC Genomics* **16**(13), S6.

Mfopou, J. K., Geeraerts, M., Dejene, R., Van Langenhoven, S., Aberkane, A., Van Grunsven, L. A. & Bouwens, L. (2014). Efficient definitive endoderm induction from mouse embryonic stem cell adherent cultures: a rapid screening model for differentiation studies. *Stem Cell Research* **12**(1), 166–177.

Miura, K., Okada, Y., Aoi, T., Okada, A., Takahashi, K., Okita, K., Nakagawa, M., Koyanagi, M., Tanabe, K., Ohnuki, M., Ogawa, D., Ikeda, E., Okano, H. & Yamanaka, S. (2009). Variation in the safety of induced pluripotent stem cell lines. *Nature Biotechnology* **27**(8), 743–745.

Mizutani, E., Ohta, H., Kishigami, S., Van Thuan, N., Hikichi, T., Wakayama, S., Kosaka, M., Sato, E. & Wakayama, T. (2006). Developmental ability of cloned embryos from neural stem cells. *Reproduction* **132**(6), 849–857.

Mo, X., Li, N. & Wu, S. (2014). Generation and characterization of bat-induced pluripotent stem cells. *Theriogenology* **82**(2), 283–293.

Morgani, S. M., Canham, M. A., Nichols, J., Sharov, A. A., Migueles, R. P., Ko, M. S. & Brickman, J. M. (2013). Totipotent embryonic stem cells arise in ground-state culture conditions. *Cell Reports* **3**(6), 1945–1957.

Mrowiec, P., Bugno-Poniewierska, M. & Młodawska, W. (2021). The perspective of the incompatible of nucleus and mitochondria in interspecies somatic cell nuclear transfer for endangered species. *Reproduction in Domestic Animals* **56**(2), 199–207.

Mullen, R., Whitten, W. & Carter, S. (1970). Studies on chimeric mice and half-embryos. *Annual Report of the Jackson the Laboratory* 67–68.

Murakami, M., Ferguson, C. E., Perez, O., Boediono, A., Paccamonti, D., Bondioli, K. R. & Godke, R. A. (2006). Transfer of inner cell mass cells derived from bovine nuclear transfer embryos into the trophoblast of bovine in vitro-produced embryos. *Cloning and Stem Cells* **8**(1), 51–60.

Nagasaka, R., Matsumoto, M., Okada, M., Sasaki, H., Kanie, K., Kii, H., Uozumi, T., Kiyota, Y., Honda, H. & Kato, R. (2017). Visualization of morphological categories of colonies for monitoring of effect on induced pluripotent stem cell culture status. *Regenerative Therapy* **6**, 41–51.

Nagatomo, H., Yao, T., Araki, Y., Mizutani, E. & Wakayama, T. (2017). Agarose capsules as new tools for protecting denuded mouse oocytes/embryos during handling and freezing-thawing and supporting embryonic development in vivo. *Scientific Reports* **7**(1), 17960.

Nagy, A., Gócza, E., Diaz, E. M., Prideaux, V. R., Iványi, E., Markkula, M. & Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**(3), 815–821.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H. & Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**(3), 379–391.

Ogonuki, N., Inoue, K., Yamamoto, Y., Noguchi, Y., Tanemura, K., Suzuki, O., Nakayama, H., Doi, K., Ohtomo, Y., Satoh, M., Nishida, A. & Ogura, A. (2002). Early death of mice cloned from somatic cells. *Nature Genetics* **30**(3), 253–254.

Oh, H. J., Kim, M. K., Jang, G., Kim, H. J., Hong, S. G., Park, J. E., Park, K., Park, C., Sohn, S. H., Kim, D. Y., Shin, N. S. & Lee, B. C. (2008). Cloning endangered gray wolves (*Canis lupus*) from somatic cells collected postmortem. *Theriogenology* **70**(4), 638–647.

Olivera, R., Moro, L. N., Jordan, R., Luzzani, C., Miriuka, S., Radrizzani, M., Donadeu, F. X. & Vichera, G. (2016). In vitro and in vivo development of horse cloned embryos generated with iPSCs, mesenchymal stromal cells and fetal or adult fibroblasts as nuclear donors. *PLoS One* **11**(10), e0164049.

Otsuki, J., Iwasaki, T., Katada, Y., Sato, H., Furuhashi, K., Tsuji, Y., Matsumoto, Y. & Shiotani, M. (2016). Grade and looseness of the inner cell mass may lead to the development of monochorionic diamniotic twins. *Fertility and Sterility* **106**(3), 640–644.

Palomino, J. M., Cervantes, M. P., McCorkell, R. B., Mapletoft, R. J. & Adams, G. P. (2016). Superovulation in wood bison (*Bison bison athabascae*): effects of progesterone, treatment protocol and gonadotropin preparations for the induction of ovulation. *Animal Reproduction Science* **167**, 31–39.

Patel, H., Chougule, S., Chohan, P., Shah, N. & Bhartiya, D. (2014). An ideal oocyte activation protocol and embryo culture conditions for somatic cell nuclear transfer using sheep oocytes. *Indian Journal of Experimental Biology* **52**(10), 925–934.

Pick, M., Stelzer, Y., Bar-Nur, O., Mayshar, Y., Eden, A. & Benvenisty, N. (2009). Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells. *Stem Cells* **27**(11), 2686–2690.

Polejaeva, I. A., Chen, S. H., Vaught, T. D., Page, R. L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D. L., Colman, A. & Campbell, K. H. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407**(6800), 86–90.

Polge, C., Rowson, L. E. & Chang, M. C. (1966). The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. *Journal of Reproduction and Fertility* **12**(2), 395–397.

Prigione, A., Fauler, B., Lurz, R., Lehrach, H. & Adjaye, J. (2010). The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem Cells* **28**(4), 721–733.

Procházka, R., Vodicka, P., Zudová, D., Rybár, R. & Motlík, J. (2004). Development of in vivo derived diploid and tetraploid pig embryos in a modified medium NCSU 37. *Theriogenology* **62**(1–2), 155–164.

Prodöhl, P. A., Loughry, W. J., McDonough, C. M., Nelson, W. S. & Avise, J. C. (1996). Molecular documentation of polyembryony and the micro-spatial dispersion of clonal sibships in the nine-banded armadillo, *Dasypus novemcinctus*. *Proceedings: Biological Sciences* **263**(1377), 1643–1649.

Qiao, S., Deng, Y., Li, S., Yang, X., Shi, D. & Li, X. (2019). Partially reprogrammed induced pluripotent stem cells using microRNA cluster mir-302s in Guangxi Bama minipig fibroblasts. *Cellular Reprogramming* **21**(5), 229–237.

Ramaswamy, K., Yik, W. Y., Wang, X. M., Oliphant, E. N., Lu, W., Shibata, D., Ryder, O. A. & Hacia, J. G. (2015). Derivation of induced pluripotent stem cells from orangutan skin fibroblasts. *BMC Research Notes* **8**, 577.

Ratto, M. H., Berland, M., Silva, M. E. & Adams, G. P. (2019). New insights of the role of β-NGF in the ovulation mechanism of induced ovulating species. *Reproduction* **157**(5), R199–R207.

Razza, E. M., Satrapa, R. A., Emanuelli, I. P., Barros, C. M. & Nogueira, M. F. (2016). Screening of biotechnical parameters for production of bovine inter-subspecies embryonic chimeras by the aggregation of tetraploid *Bos indicus* and diploid crossbred *Bos taurus* embryos. *Reproductive Biology* **16**(1), 34–40.

Renfree, M. B. & Fenelon, J. C. (2017). The enigma of embryonic diapause. *Development* **144**(18), 3199–3210.

Rodriguez-Polo, I., Stauske, M., Becker, A., Bartels, I., Dressel, R. & Behr, R. (2019). Baboon induced pluripotent stem cell generation by piggyBac transposition of reprogramming factors. *Primate Biology* **6**(2), 75–86.

San Diego Zoo Global, Revive & Restore & Viagen Equine (2020). Birth of cloned Przewalski's foal offers genetic diversity for this endangered species. (media release: https://science.sandiegozoo.org/news/birth-cloned-przewalski%E2%80%99s-foal-offers-genetic-diversity-endangered-species)

San Diego Zoo Global, Us Fish And Wildlife Service, Revive & Restore, Viagen Pets & Equine (2021). Innovative genetic research boosts black-footed ferret conservation efforts by USFWS and partners. (media release: https://www.fws.gov/press-release/2021-02/genetic-research-boosts-black-footed-ferret-conservation-efforts)

Sansinena, M. J., Hylan, D., Hebert, K., Denniston, R. S. & Godke, R. A. (2005). Banteng (*Bos javanicus*) embryos and pregnancies produced by interspecies nuclear transfer. *Theriogenology* **63**(4), 1081–1091.

Saragusty, J., Ajmone-Marsan, P., Sampino, S. & Modlinski, J. A. (2020). Reproductive biotechnology and critically endangered species: merging in vitro gametogenesis with inner cell mass transfer. *Theriogenology* **155**, 176–184.

Sartori, C., DiDomenico, A. I., Thomson, A. J., Milne, E., Lillico, S. G., Burdon, T. G. & Whitelaw, C. B. (2012). Ovine-induced pluripotent stem cells can contribute to chimeric lambs. *Cellular Reprogramming* **14**(1), 8–19.

Scarfone, R. A., Pena, S. M., Russell, K. A., Betts, D. H. & Koch, T. G. (2020). The use of induced pluripotent stem cells in domestic animals: a narrative review. *BMC Veterinary Research* **16**(1), 477.

Sellers, R., Castillo, J. C., Ten, J., Rodríguez, A., Ortiz, J. A., Sellers, F., Llácer, J. & Bernabeu, R. (2020). Monozygotic twinning following embryo biopsy at the blastocyst stage. *JBRA Assisted Reproduction* **25**(1), 122–127.

Shull, G. H. (1912). Genotypes," "biotypes," "pure lines" and "clones. *Science* **35**(888), 27–29.

Silla, A. J. & Byrne, P. G. (2019). The role of reproductive technologies in amphibian conservation breeding programs. *Annual Reviews in Animal Biosciences* **7**(1), 499–519.

Sim, B.-W. & Min, K.-S. (2014). Production of cloned mice by aggregation of tetraploid embryo. *Animal Cells and Systems* **18**(5), 324–332.

Srirattana, K., Imsoonthornruksa, S., Laowtammathron, C., Sangmalee, A., Tunwattana, W., Thongprapai, T., Chaimongkol, C., Ketudat-Cairns, M. & Parnpai, R. (2012). Full-term development of gaur-bovine interspecies somatic cell nuclear transfer embryos: effect of trichostatin A treatment. *Cellular Reprogramming* **14**(3), 248–257.

Srirattana, K. & St John, J. C. (2018). Additional mitochondrial DNA influences the interactions between the nuclear and mitochondrial genomes in a bovine embryo model of nuclear transfer. *Scientific Reports* **8**(1), 7246.

Srirattana, K. & St John, J. C. (2019). Transmission of dysfunctional mitochondrial DNA and its implications for mammalian reproduction. *Advances in Anatomy, Embryology and Cell Biology* **231**, 75–103.

St. John, J. C., Lloyd, R. E. I., Bowles, E. J., Thomas, E. C. & El Shourbagy, S. (2004). The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. *Reproduction* **127**(6), 631–641.

Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T. & Hochedlinger, K. (2010). Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature* **465**(7295), 175–181.

Stadtfeld, M. & Hochedlinger, K. (2010). Induced pluripotency: history, mechanisms, and applications. *Genes & Development* **24**(20), 2239–2263.

Steensma, D. P. (2017). The origin and evolution of the term "clone". *Leukemia Research* **57**, 97–101.

Su, Y., Zhu, J., Salman, S. & Tang, Y. (2020). Induced pluripotent stem cells from farm animals. *Journal of Animal Science* **98**(11).

Sung, L. Y., Gao, S., Shen, H., Yu, H., Song, Y., Smith, S. L., Chang, C. C., Inoue, K., Kuo, L., Lian, J., Li, A., Tian, X. C., Tuck, D. P., Weissman, S. M., Yang, X., *et al.* (2006). Differentiated cells are more efficient than adult stem cells for cloning by somatic cell nuclear transfer. *Nature Genetics* **38**(11), 1323–1328.

Swegen, A. (2021). Maternal recognition of pregnancy in the mare: does it exist and why do we care? *Reproduction* **161**(6), R139–R155.

Tachibana, M., Amato, P., Sparman, M., Gutierrez, N. M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H. S., Sritanaudomchai, H., Masterson, K., Larson, J., Eaton, D., Sadler-Fredd, K., Battaglia, D., *et al.* (2013). Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* **153**(6), 1228–1238.

Tagawa, M., Matoba, S., Narita, M., Saito, N., Nagai, T. & Imai, K. (2008). Production of monozygotic twin calves using the blastomere separation technique and Well of the Well culture system. *Theriogenology* **69**(5), 574–582.

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**(4), 663–676.

Takeda, K. (2019). Functional consequences of mitochondrial mismatch in reconstituted embryos and offspring. *Journal of Reproduction and Development* **65**(6), 485–489.

Thongphakdee, A., Kobayashi, S., Imai, K., Inaba, Y., Tasai, M., Tagami, T., Nirasawa, K., Nagai, T., Saito, N., Techakumphu, M. & Takeda, K. (2008). Interspecies nuclear transfer embryos reconstructed from cat somatic cells and bovine ooplasm. *Journal of Reproduction and Development* **54**(2), 142–147.

Thongphakdee, A., Siriaroonrat, B., Manee-in, S., Klincumhom, N., Kamolnorranath, S., Chatdarong, K. & Techakumphu, M. (2010). Intergeneric somatic cell nucleus transfer in marbled cat and flat-headed cat. *Theriogenology* **73**(1), 120–128.

Tian, X. C., Kubota, C., Enright, B. & Yang, X. (2003). Cloning animals by somatic cell nuclear transfer--biological factors. *Reproductive Biology and Endocrinology* **1**, 98.

Tucci, V., Isles, A. R., Kelsey, G. & Ferguson-Smith, A. C. (2019). Genomic imprinting and physiological processes in mammals. *Cell* **176**(5), 952–965.

Verma, R., Holland, M. K., Temple-Smith, P. & Verma, P. J. (2012). Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid. *Theriogenology* **77**(1), 220–228.

Verma, R., Liu, J., Holland, M. K., Temple-Smith, P., Williamson, M. & Verma, P. J. (2013). Nanog is an essential factor for induction of pluripotency in somatic cells from endangered felids. *BioResearch Open Access* **2**(1), 72–76.

Vidal, S. E., Amlani, B., Chen, T., Tsirigos, A. & Stadtfeld, M. (2014). Combinatorial modulation of signaling pathways reveals cell-type-specific requirements for highly efficient and synchronous iPSC reprogramming. *Stem Cell Reports* **3**(4), 574–584.

Vogel, G. (2001). Endangered species. Cloned gaur a short-lived success. *Science* **291**(5503), 409.

Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**(6691), 369–374.

Wang, X., Qu, J., Li, J., He, H., Liu, Z. & Huan, Y. (2020). Epigenetic reprogramming during somatic cell nuclear transfer: recent progress and future directions. *Frontiers in Genetics* **11**, 205–205.

Weeratunga, P., Shahsavari, A., Ovchinnikov, D. A., Wolvetang, E. J. & Whitworth, D. J. (2018). Induced pluripotent stem cells from a marsupial, the tasmanian devil (*Sarcophilus harrisii*): insight into the evolution of mammalian pluripotency. *Stem Cells and Development* **27**(2), 112–122.

West, F. D., Uhl, E. W., Liu, Y., Stowe, H., Lu, Y., Yu, P., Gallegos-Cardenas, A., Pratt, S. L. & Stice, S. L. (2011). Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. *Stem Cells* **29**(10), 1640–1643.

Whitworth, D. J., Limnios, I. J., Gauthier, M. E., Weeratunga, P., Ovchinnikov, D. A., Baillie, G., Grimmond, S. M., Graves, J. A. M. & Wolvetang, E. J. (2019). Platypus induced pluripotent stem cells: the unique pluripotency signature of a monotreme. *Stem Cells and Development* **28**(3), 151–164.

Willadsen, S. M. (1979). A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* **277**(5694), 298–300.

Willadsen, S. M. (1986). Nuclear transplantation in sheep embryos. *Nature* **320**(6057), 63–65.

Willadsen, S. M. & Polge, C. (1981). Attempts to produce monozygotic quadruplets in cattle by blastomere separation. *Veterinary Record* **108**(10), 211–213.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**(6619), 810–813.

Wilson, K. D., Venkatasubrahmanyam, S., Jia, F., Sun, N., Butte, A. J. & Wu, J. C. (2009). MicroRNA profiling of human-induced pluripotent stem cells. *Stem Cells and Development* **18**(5), 749–758.

Wolstenholme, G. E. W. (2009). *Man and His Future*. Wiley, New Jersey, USA.

Wood, C. (2001). Embryo splitting: a role in infertility? *Reproduction, Fertility and Development* **13**(1), 91–93.

Woodard, L. E. & Wilson, M. H. (2015). piggyBac-ing models and new therapeutic strategies. *Trends in Biotechnology* **33**(9), 525–533.

Wu, F., Liu, Y., Wu, Q., Li, D., Zhang, L., Wu, X., Wang, R., Zhang, D., Gao, S. & Li, W. (2018). Long non-coding RNAs potentially function synergistically in the cellular reprogramming of SCNT embryos. *BMC Genomics* **19**(1), 631.

Yamaguchi, T., Sato, H., Kobayashi, T., Kato-itoh, M., Goto, T., Hara, H., Mizuno, N., Yanagida, A., Umino, A., Hamanaka, S., Suchy, F., Masaki, H., Ota, Y., Hirabayashi, M. & Nakauchi, H. (2018). An interspecies barrier to tetraploid complementation and chimera formation. *Scientific Reports* **8**(1), 15289.

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I. & Sinclair, K. D. (2001). Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genetics* **27**(2), 153–154.

Zhang, J., Liu, H., Luo, S., Lu, Z., Chávez-Badiola, A., Liu, Z., Yang, M., Merhi, Z., Silber, S. J., Munné, S., Konstantinidis, M., Wells, D., Tang, J. J. & Huang, T. (2017). Live birth derived from oocyte spindle transfer to prevent mitochondrial disease. *Reproductive Biomedicine Online* **34**(4), 361–368.

Zhang, W., Pei, Y., Zhong, L., Wen, B., Cao, S. & Han, J. (2015). Pluripotent and metabolic features of two types of porcine iPSCs derived from defined mouse and human es cell culture conditions. *PLoS One* **10**(4), e0124562.

Zhang, Z.-P., Zhang, J.-T., Huang, S.-C., He, X.-Y. & Deng, L.-X. (2020). Double sperm cloning (DSC) is a promising strategy in mammalian genetic engineering and stem cell research. *Stem Cell Research & Therapy* **11**(1), 388.

Zhao, X. Y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C. L., Ma, Q. W., Wang, L., Zeng, F. & Zhou, Q. (2009). iPS cells produce viable mice through tetraploid complementation. *Nature* **461**(7260), 86–90.

Zhao, Z. J., Li, R. C., Cao, H. H., Zhang, Q. J., Jiang, M. X., Ouyang, Y. C., Nan, C. L., Lei, Z. L., Song, X. F., Sun, Q. Y. & Chen, D. Y. (2007). Interspecies nuclear transfer of Tibetan antelope using caprine oocyte as recipient. *Molecular Reproduction and Development* **74**(4), 412–419.

Zheng, Y. L., Jiang, M. X., OuYang, Y. C., Sun, Q. Y. & Chen, D. Y. (2005). Production of mouse by inter-strain inner cell mass replacement. *Zygote* **13**(1), 73–77.

Zhong, X., Cui, P., Cai, Y., Wang, L., He, X., Long, P., Lu, K., Yan, R., Zhang, Y., Pan, X., Zhao, X., Li, W., Zhang, H., Zhou, Q. & Gao, P. (2019). Mitochondrial dynamics is critical for the full pluripotency and embryonic developmental potential of pluripotent stem cells. *Cell Metabolism* **29**(4), 979–992.e4.

Zhou, S., Ding, C., Zhao, X., Wang, E., Dai, X., Liu, L., Li, W., Liu, Z., Wan, H., Feng, C., Hai, T., Wang, L. & Zhou, Q. (2010). Successful generation of cloned mice using nuclear transfer from induced pluripotent stem cells. *Cell Research* **20**(7), 850–853.

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