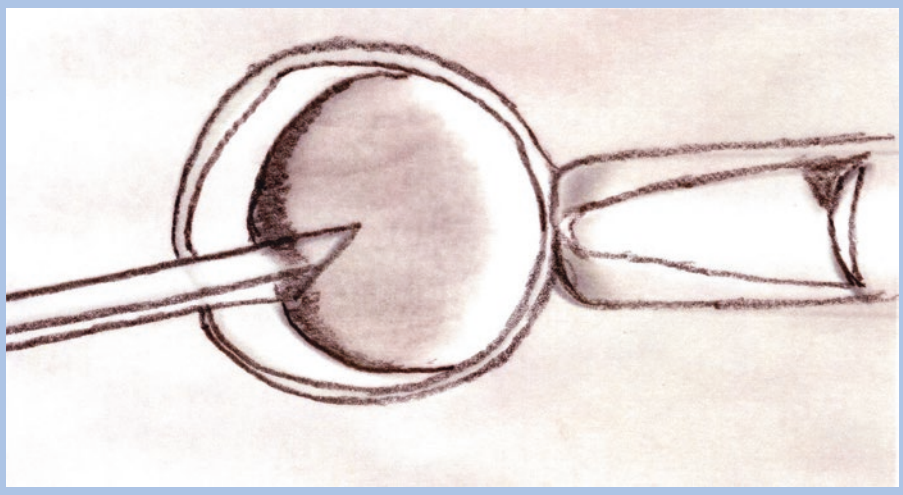
Cloning

Bernard A. J. Roelen



Contents

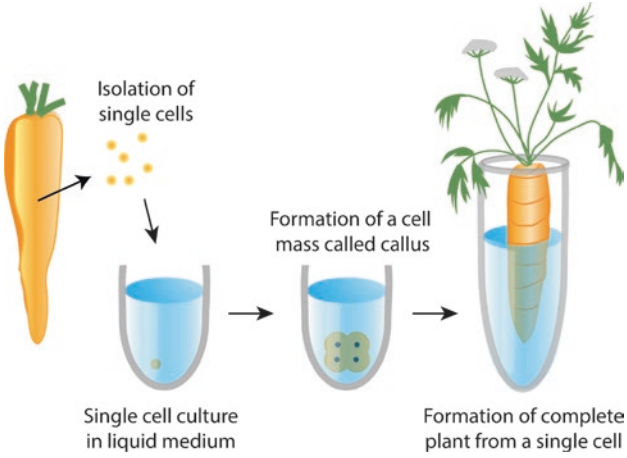
G. Rodrigues, B. A. J. Roelen (eds.), *Concepts and Applications of Stem Cell Biology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-030-43939-2_6

What Will You Will Learn in This Chapter?

Cloning is the generation of a genetically identical organism from an existing organism. The best known example of cloning is taking plant cuttings, but also in various animals; genetically identical clones can be formed from cells or tissues, for example in some types of flatworms. Differentiation of cells in more complex animals leads to loss of developmental capacity and is caused by epigenetic events: methylation of cytosines in CpG dinucleotides and histone tail modifications. Differentiated cells can be reprogrammed to a totipotent state when introduced into an enucleated oocyte, a process known as somatic cell nuclear transfer. Although inefficient, an embryo developed with this method can give rise to offspring. The sheep Dolly was the first clone born after somatic cell nuclear transfer using an adult cell as donor. Since the birth of Dolly, several animal species have been cloned, including pets and commercially valuable animals. In therapeutic cloning, cells of a patient are injected into a human oocyte to form a blastocyst of which embryonic stem cells are generated rather than transplantation of the embryo to the uterus. These patient-specific pluripotent cells may be used for drug screening, study of the disease, or regenerative medicine.

6.1 What Is Cloning?

Before every cell division, the genomic DNA is precisely copied so that the progeny cells contain the same biological information. This implies that in a living organism almost all cells have the capacity to form all cells and tissues of that organism. Indeed it has been demonstrated that in plants individual root cells have the capacity to grow out to a complete plant with roots, leaves, and flowers [1]. The newly formed plant would be genetically identical to the plant from which the individual cell was taken; they would be clones (◘ Fig. 6.1).Taking cuttings to make more of your favorite plants, albeit not from individual cells, is a way of cloning: the asexual generation of genetically identical organisms that descend from a single cell or organism.



◘ **Fig. 6.1** Plant cloning. Schematic representation of the cloning of a carrot plant from a single cell. Parts of a carrot root are isolated, fractionated to single cells, and culture in a suitable medium. The cells dedifferentiate to form a clump of totipotent cells called callus. The callus can develop to a complete plant with differentiated structures such as roots, stem, leaves, and flowers

6.2 Regeneration

Not all asexual reproduction is cloning. Particularly in more complex bisexual organisms, including snakes, lizards, sharks, and domestic fowl, asexual reproduction occurs by oocytes that develop without being fertilized. In 2006, parthenogenetic offspring was reported from two independent female Komodo dragons (*Varanus komodeonsis*) that were kept individually in separate zoos (Fig. 6.2). These animals each produced clutches of more than 20 eggs from which about one third hatched. The offspring were all male, and although the offspring from one mother were homozygous, they were not clones. The exclusive male offspring can be explained by the sex chromosomes in these lizards, where females are heterozygous for the sex chromosomes Z and W, while males are homozygous ZZ. Since the parthenogenetic mechanism can only produce homozygotes and WW is not viable, the only viable parthenotes will be ZZ males [2]. Similar observations have been done in other species and even in wild non-captive snakes. This could be a functional strategy to maintain a population when females become isolated from males. The exact mechanism of the parthenogenesis is probably different in various vertebrates and can include fusion of cells before or after meiosis [3].

Less complex organisms demonstrate a regenerative ability similar to that of plants. A classic example is the freshwater polyp *Hydra*, belonging to the Phylum Cnidaria, that can regenerate a complete organism from small fragments and dissociated cells [4, 5]. Another well-known example for studying regeneration is the group of freshwater planarians, types of flatworms in the Phylum Platyhelminthes and the Class Turbellaria. These are relatively simple animals but more complex than *Hydra* as they are composed of three germ layers: ectodermal nerve cells, endodermal intestinal cells, and mesodermal muscle. Planarians have formidable regenerative capacities; when the animal is cut in half between the head and tail, the tail-part will form a new head and the head part will form a new tail. Similarly, when a planarian is cut lengthwise, each half will form a new half. These newly formed animals are all clones of the original dissected animal. Although many planarians are hermaphrodites and can reproduce sexually, there are also triploid populations that reproduce asexually

Fig. 6.2 Komodo dragon. Photograph of a preserved Komodo dragon (*Varanus komodoensis*) at a museum display. This large lizard's natural habitat is in the Indonesian archipelago. Examples exist of female Komodo dragons living in captivity that laid unfertilized eggs of which only male animals hatched. Whether this parthenogenesis occurs in the wild or only during captivity is not known

by cloning. They do so by simply tearing themselves into a head and a tailpiece that each produces a new worm. Intriguingly the process of fission is socially controlled by a mechanism in the brain and fission is promoted after decapitation of the worm (Fig. 6.3).

It has been identified that the tissue regeneration, either when externally disrupted or after fission, is mediated via pluripotent neoblasts that accumulate at the wound edge and can give rise to fully patterned body parts [6]. Not all planarians are equal; however, there are various planaria species that can form a new tail from a head but lack the capacity to form a new head from a tail. Intriguingly, in these regeneration-non-competent species, downregulating Wnt/β-catenin signaling could lead to head regeneration from regeneration-deficient tails [7–9]. The question of how the neoblasts are instructed to form the correct tissue types of appropriate sizes is yet to be resolved.

Various vertebrate species such as the South African clawed frog *Xenopus laevis* and the axolotl *Ambystoma mexicanum* have similar, although more limited, regeneration potential (Fig. 6.4). *Xenopus* tadpoles can regenerate their tails including nerves and muscles, *Xenopus* froglets can partly regenerate their limb to form a carti-

Fig. 6.3 Planaria. Freshwater planarians of the class of Turbellaria have a remarkable regenerative capacity. In this particular species, rudimentary eyes are visible and the ear-like auricles that function as sensory organs

Fig. 6.4 Axolotl. The axolotl (*Ambystoma mexicanum*) can regenerate an entire limb including digits

laginous protrusion, while the axolotl is well-known for its capacity to grow a new completely functional limb after injury [10]. For the axolotl, it has been established that amputation induces the formation of a blastema with a heterogeneous set of progenitor cells that are not pluripotent but instead have a restricted developmental potential [11]. Programmed cell death or apoptosis, plays a key role in tissue regeneration of both planarians and amphibians as it controls the exact numbers of cells needed to form the various tissues [12].

6.3 Somatic Cell Nuclear Transfer

The regeneration of appendages in amphibians indicate that, at least some, vertebrate cells maintain or regain the capacity to differentiate into derivatives of the three germ layers.

6.3.1 Frog Cloning

In the 1950s, Briggs and King tested whether nuclei of differentiated cells were intrinsically changed by transplanting nuclei of various stages of embryos into enucleated eggs. For their experiments, they made use of eggs and embryos of the American leopard frog *Rana pipiens*. These nuclear transfer experiments were partially successful and Briggs and King obtained feeding stage larva from blastula nuclei, but not from nuclei of later stages. It was concluded that although nuclei of early blastula stage embryos are pluripotent, nuclei from neurula stage embryos onwards are restricted in developmental capacity [13, 14]. Similar experiments were made by Gurdon, only he used *Xenopus* eggs and nuclei for the transplantation experiments. By that time *Xenopus* was widely distributed in European and American labs for its use as a pregnancy test since female adult *Xenopus* frogs injected with urine of a pregnant woman started to lay eggs the following day, the so-called Hogben test [15, 16]. *Xenopus* had several advantages above *Rana* and was becoming the preferred model organism: *Xenopus* is a wholly aquatic animal making it easier to maintain in a laboratory, it can produce eggs year-round and has a relatively short life cycle. Most importantly for nuclear transfer experiments, different *Xenopus* strains could be identified by the number of nucleoli, enabling to distinguish between donor and recipient cells. Nuclei from the intestinal epithelium of *Xenopus* feeding tadpoles were transplanted to eggs from which the chromosomes had been destroyed after ultraviolet light exposure. These transplant embryos could develop to swimming tadpoles and even become adult fertile frogs, although in a low percentage [17, 18]. Similar experiments were performed using cells from adult frogs as nuclear donor, but although the formed embryos did develop to healthy appearing tadpoles, these never transformed into adult frogs [19]. From these data, it was concluded that, at least in amphibians, embryonic nuclei are unrestricted in their developmental potential but that the hereditary material in the nucleus does not remain intact during differentiation.

6.3.2 Dolly

Experiments similar to those performed in frogs were performed with mouse embryonic cells as donor and fertilized and enucleated one-cell zygotes as recipients. This led to controversial results; while one research group claimed the birth of healthy mice from transplanted nuclei of embryonic cells [20], another group claimed that it is biologically impossible to clone mammals by somatic cell nuclear transfer [21].

The birth of the Scottish cloned sheep Dolly in 1996 [22], therefore, came for many as a complete surprise, but there had been several tell-tale signs (◘ Fig. 6.5).

Already in 1986, it was published that sheep can be produced by transplantation of nuclei of the blastomeres from eight cell embryos to enucleated unfertilized eggs [23]. With the development of pluripotent embryonic stem cell lines, combined with homologous recombination and chimaera formation it had become possible to generate mice with targeted mutations [24–26]. That, together with the availability of inbred strains limited the interest in the cloning technology for mouse embryologists. For other animal species including farm animals, embryonic stem cell lines were not available. Therefore the theoretical possibility of replicating those animals with favorable genetic characteristics, or rapidly enhancing those characteristics using cloning, spurred the research on cloning particularly in farm animals. Before the birth of Dolly, live calves and sheep had already been born by transfer of nuclei from cultured embryonic cells [27, 28]. It was Keith Campbell, then at the Roslin Institute in Scotland, who realized that the cell cycle stage of the donor cell and the recipient oocyte should match [29]. In subsequent somatic cell nuclear transfer experiments the donor cells were therefore induced to a state of quiescence by serum starvation (◘ Fig. 6.6). Eventually, this led to the birth of the sheep Dolly using cultured udder cells from a 6-year-old ewe as a donor [22].

◘ **Fig. 6.5** Dolly. The most famous sheep in the world Dolly has been stuffed after her death in 2003 and is now at display at the National Museum of Scotland in Edinburgh

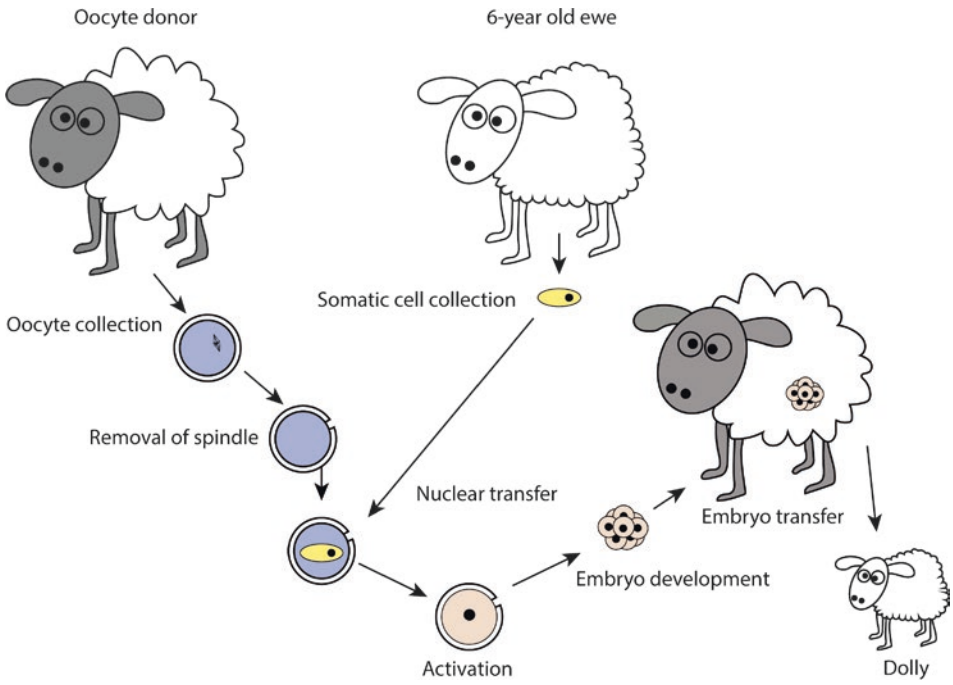


Fig. 6.6 How to clone a sheep? Schematic representation of the somatic cell nuclear transfer experiments that led to the birth of Dolly. Oocytes were collected from Scottish blackface sheep. The genomic DNA was removed by aspiration of the meiotic spindle. Somatic fibroblasts were isolated from the mammary gland of a Finn Dorset ewe and cultured in vitro for a maximum of six passages before being microinjected into the enucleated oocyte. Electrical pulses induced fusion of the donor cell and the enucleated oocyte. Embryos developed to morula and blastocyst stages were transferred to Scottish Blackface recipient ewes. Of the total 434 fused oocytes, 247 embryos were formed which led to 29 blastocysts that were transferred to 13 recipient ewes. One animal was pregnant after embryo transfer and gave birth to Dolly

6.4 Epigenetics

The successful nuclear reprogramming of an adult cell and the birth of an intact organism from that nucleus unequivocally demonstrated that even in cells of complex organisms the DNA sequence remains intact during differentiation. In the 1940s, Conrad Waddington coined the term 'epigenetics' for complex developmental processes that take place in a one-way direction during differentiation that gradually become more severe. Waddington envisioned cellular differentiation as a marble that rolls down a hill that is pulled by gravity and steered by ridges and canals. On top of the hill, the marble can roll to many directions, but as the ball rolls further down the hill the alternative routes become more and more limited. This route is clearly unidirectional as gravity and ridges prevent the marble from changing the path [30].

The contemporary view of epigenetics is that of hereditable changes in gene function or activity that are not due to changes in the DNA sequence. The most well-known epigenetic modifications are methylation of DNA at position 5 of cysteine in CG dinucleotides (Fig. 6.7), and histone tail modifications. DNA within the

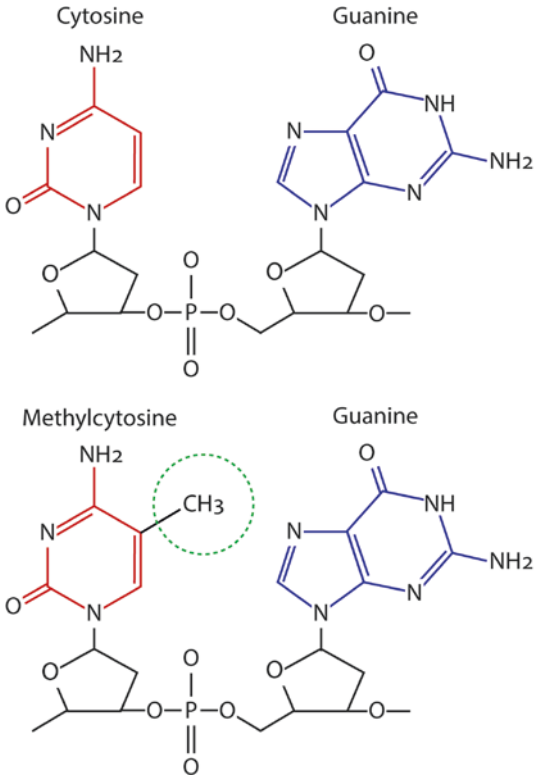


Fig. 6.7 DNA methylation. Skeletal formula of a cytosine (red) nucleotide followed by a guanine (blue) nucleotide separated by a phosphate group of the DNA backbone, a so-called CpG dinucleotide (top). The cytosine in a CpG dinucleotide can be methylated at the 5-position (green dotted circle) to form methylcytosine (bottom). Methylation of CpG sites at promotor regions leads to gene silencing

nucleus is not bare but wrapped around protein complexes to form repeating units known as nucleosomes.

The core proteins that constitute the nucleosome occur as octamers containing 2 copies each of histones H2A, H2B, H3, and H4.Transcriptional activity from the chromatin can be regulated by covalent reversible modifications of the amino acid tails protruding from the central histone proteins. Biochemical modification of the histone N-terminal amino acid sequences can change the accessibility of the DNA for transcription or by promoting the association of chromatin-binding proteins. The best known modifications in this respect are methylation of lysine and arginine residues, ubiquitination of lysine residues, and acetylation of lysine residues. To add more complexity to this system, lysine residues can be mono-, di-, or trimethylated that can have different roles in gene regulation [31]. DNA methylation represses transcription, while histone tail modifications such as acetylation and methylation can be repressive or activating, depending on the type of modification (Fig. 6.8).

Nowadays, there is some debate on how strict heritability fits within the definition of epigenetics since, for instance, histone modifications are reversible and not all stable during cell divisions [32]. Also, the birth of Dolly the sheep unequivocally demonstrated that epigenetic changes are reversible, albeit rather inefficiently. In this respect, the epigenetic landscape resembles a pinball machine: without action the pinball rolls down the table but player-controlled flippers can get the ball back to the top. No matter how good you are at pinball, it seems virtually impossible to control the ball, quite similar to cloning by somatic cell nuclear transfer.

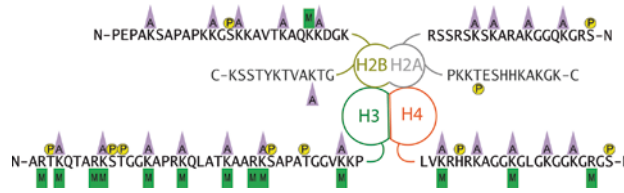


Fig. 6.8 Histone tail modifications. Schematic representation of various reversible modifications that can occur on the amino acids that compose the N- and C-tails of the core histone proteins and that are involved in chromatin reorganization. Methylation is indicated with a green rectangle, acetylation with a purple triangle, and phosphorylation with a yellow circle. Note that residues can carry multiple modifications, for instance, H3K27 can carry three methylation marks. Other modifications that can occur, such as ubiquitination, sumoylation and propionylation, are not indicated in this scheme

6

6.5 Ways in Which Cloning Might Be Useful

The publication of 'Dolly' evoked many emotions and sparked many questions from scientists, students, and the press. What is the real 'age' of Dolly? Why sheep? Will this lead to human cloning? Should these types of experiments be allowed?

6.5.1 Cloning and Age

Some of these questions have been answered in the years since the publication of the 'Dolly' paper, others are still left unanswered. The birth of a healthy living animal after somatic cell nuclear transfer demonstrated that nuclei from adult differentiated cells can be fully reprogrammed to a totipotent state. The procedure was, and still is, highly inefficient, however. In the case of Dolly, 277 oocytes were injected with a somatic cell leading to the birth of one healthy lamb [22]. In the years, since Dolly, quite a few animal species have been cloned (Table 6.1), but the efficiency has remained low.

One way of determining the 'age' of a cell is by examining the repetitive DNA sequences that decorate ends of chromosomes, the so-called telomeres. It is generally believed that in somatic cells telomeres shorten with each cell division in a way that telomeres are shorter in aged individuals [33] (for further information see ▶ Chap. 5). When Dolly was one-year old, her telomeres indeed were significantly smaller than those from age-matched control sheep, which could mean that the telomeres could prematurely reach a critical size [34]. Telomere sizes of cloned cattle and pigs, however, were normal or even slightly increased, suggesting that telomere lengths are restored after nuclear transfer [35, 36]. When mice were repeatedly recloned, telomere size did not differ compared with controls, not even after 23 generations of cloning [37], strengthening the hypothesis that genomic reprogramming leads to a resetting of telomere size and argues against premature aging of cloned animals. Indeed the life span of cloned and repeatedly recloned mice is similar to that of naturally conceived mice [37].

The world famous sheep, Dolly was rather corpulent during her life which may have been caused by the excess food she received to perform well for the press. She was a celebrity after all. Perhaps because of the weight problem she developed arthritis, but arthritis is also a sign of aging. On Valentine's day 2003, when Dolly was

Table 6.1 List of mammalian species cloned by somatic cell nuclear transfer that led to live births

Common name	Scientific name	Reference
African wild cat	*Felis silvestris lybica*	[60]
Bactrian camel	*Camelus bactrianus*	[61]
Banteng	*Bos javanicus*	[78]
Brown rat	*Rattus norvegicus*	[62]
Cattle	*Bos taurus*	[63]
Coyote	*Canis latrans*	[64]
Cynomolgus monkey[a]	*Macaca fascicularis*	[57]
(Crab eating macaque)		
Domestic cat	*Felis silvestris catus*	[40]
Dog	*Canis lupus familiaris*	[41]
Dromedary	*Camelus dromedaries*	[65]
European mouflon	*Ovis orientalis musimon*	[66]
Ferret	*Mustela putorius furo*	[67]
Gaur[b]	*Bos gaurus*	[68]
Goat	*Capra aegagrushircus*	[69]
Horse	*Equus caballus*	[47]
House mouse	*Mus musculus*	[70]
Mule	*Equus asinus* × *Equus caballus*	[46]
Pig	*Sus scrofa*	[71, 72]
Pyrenean ibex[c]	*Capra pyrenaica pyrenaica*	[43]
Rabbit	*Oryctolagus cuniculus*	[73]
Red Deer	*Cervus elaphus*	[74]
Sand cat	*Felis margarita*	[75]
Sheep	*Ovis aries*	[22]
Swamp buffalo	*Bubalus bubalis*	[76]
Wolf	*Canis lupus*	[77]

[a]From embryonic cells only
[b]Died 2 days after birth
[c]Died minutes after birth

6.5 years old (she was born 5 July 1996), she was euthanized after veterinarians confirmed that she suffered from a contagious lung cancer caused by a virus. There is no indication that this disease was related to cloning, and in fact several other normal sheep at the Roslin institute had gone down with it. Importantly, Dolly herself had given birth to 6 healthy lambs through natural matings as a demonstration of her vitality. Even after her death, Dolly remains in the limelight and is exhibited in the National Museum of Scotland in Edinburgh.

Cloning by somatic cell nuclear transfer can have several applications. It can teach us about cell function and differentiation. It can be used to generate commercially or emotionally valuable animals; it can be used to reproduce animals that are almost extinct or even already extinct; and it can possibly be used to clone humans either for reproduction or for therapeutics.

6.5.2 Cloning to Understand Cell Biology

A great deal of research has been devoted to making the somatic cell nuclear transfer procedure more efficient and to decipher how many healthy cloned animals are similar to naturally born animals. By the use of various chemical histone modifiers, cloning has been made more efficiently, albeit only marginally [38]. In addition, procedures have been described that facilitate nuclear transfer without the use of expensive micromanipulators, the so-called handmade cloning. In this process, rather than injecting somatic cells into oocytes, the zona pellucida is first removed from the oocyte after which the cell is denuded by hand with a disposable blade. A somatic cell is subsequently combined with the cytoplast by electrofusion [39]. Another way of making cloning more efficient by somatic cell nuclear transfer could be by identification of the most suitable oocytes. Using in vitro fertilization, the efficiency of oocytes that can develop to blastocyst is 30–50% in cattle and globally the success rate of human IVF is between 20% and 40%, in terms of babies born. This indicates that there is a large variability in the developmental capacity in oocytes. Understanding what causes this variability could increase the efficiency of IVF and also cloning by somatic cell nuclear transfer.

6.5.3 Pet Cloning

Shortly after the cloning of a sheep, it was suggested that cloning could be used to commercially ‘restore’ pets that are critically ill or had already deceased. Similarly, cloning was suggested as a way to reproduce dogs or cats that, for instance, have great intelligence and a gentle temperament but that are unable to reproduce naturally because they have been neutered. Indeed both cats [40] and dogs [41] have been cloned from adult cells. Cloning of dogs poses an additional complication since dog oocytes are ovulated at the first meiotic prophase and mature in the oviduct for another 48–72 hours before reaching the metaphase II stage. Therefore, oocytes have to be retrieved by flushing oviducts by laparotomy. Interestingly, for the first successful cloning of a cat, a cumulus cell from a calico cat was used. In cats, coat color is partly caused by genes on the X-chromosomes, and since in queens one of the X-chromosomes in every cell is randomly inactivated, coat patches originated from

different cells with either the paternal or maternal X inactivated can have different colors in calico cats. Copy Cat, as is the name of the first cloned cat, was phenotypically not an exact copy of her twin mother because of the random events associated with the coat color. She was predominantly white with tabby grey patches while her donor/twin sister Rainbow had orange, white, and black fur patches. Scientifically very interesting, but for those interested in creating an exact copy of their beloved pet animal, it is less appealing. Similarly, in cloned dogs, the spot pattern may vary. The possible differences in appearance, together with the high costs of cloning, are among the factors by which commercial pet cloning never became a success, although in South Korea you can still have your favorite dog cloned commercially for around 100,000 euros. Applications of dog cloning as a help to study disease are likely to be more supported by the society, for instance cloning transgenic dogs that show hallmarks of Alzheimer's disease.

6.5.4 Cloning of Commercially Valuable Animals

Cloning has also been associated with the food industry, particularly for meat and milk production. Elite animals can be reproduced by cloning as sires for pigs, beef and dairy cattle. This would seem to be economically more valuable than using the meat or milk from cloned animals directly. Either way, eventually the meat or milk from cloned animals, directly or after several generations, subsequently enters the food chain. In the USA, already in 2008, the US Food and Drug administration approved the use of milk and meat from the offspring of pig, cattle, and goat clones. Interestingly, the use of milk and meat from other animal species, including sheep was not recommended due to lack of sufficient information regarding safety. Since cloned food cannot be recognized as such, no special labeling is required. In 2015, the European parliament has banned cloned meat and milk of all farm animals from the market. The European Commission decided that labeling of meat from offspring of clones is unrealistic and it is therefore inevitable that semen or embryos from cloned animals or their descendants will enter the agricultural market in Europe as well.

6.5.5 Cloning Endangered and Extinct Animal Species

In January 2001, a Gaur (*Bos gaurus*), a wild ox from Southeast Asia that faces extinction, was born from a regular cow (*Bos taurus*). The Gaur was cloned using Gaur skin cells and enucleated oocytes from a cow. Not only was this the first cloned Gaur, but it was also the first cloned animal born from interspecies somatic cell nuclear transfer where the species of donor cell and recipient egg and carrying mother are different [42]. Although the Guar calf, Noah, was initially healthy she died within two days after birth from scours, a disease characterized by diarrhea. More embryos were produced and transplanted to foster cow mothers but the majority never implanted into the cow's womb and those that did experience spontaneous abortions, except for Noah.

Not only endangered animals but also animal species or at least a subspecies that are extinct have been cloned. The Pyrenaan ibex *Capra pyrenaica* is a subspecies of the Spanish ibex of which in the late 1990s reportedly only one animal, a female, was

Fig. 6.9 Thylacine. Taxidermy specimen of a thylacine (Tasmanian tiger) at a museum. It seems unlikely that DNA from taxidermy specimens is sufficiently intact to allow cloning, but fetal animals preserved in ethanol may be useful

left. Since no males were known to exist, the subspecies was doomed to extinction. Fortunately, the last remaining animal could be captured and a skin sample secured. The Pyrenean ibex became extinct when in 2000 the last remaining specimen died in 2000 by a falling tree that crashed her skull. Cells from the skin sample were used in an attempt to clone the animal. Since obviously no oocytes were available, oocytes from domestic goats were used for nuclear transfer and Spanish ibex and hybrids of Spanish ibex with domestic goats were used as a surrogate mother. One hybrid goat pregnancy continued and a morphologically normal animal was born by caesarian section. Unfortunately, the animal died within minutes due to respiratory stress resulting in a second extinction of the subspecies [43].

Bringing back extinct animals by ways of cloning has been repeatedly discussed in popular press and the topic is one of the favorites of the entertainment industry. One necessity for cloning extinct animals would be the availability of cells, and therefore two animal species top the list of animals to be cloned: the Woolly mammoth (*Mammuthus primigenius*) and the Thylacine or Tasmanian tiger (*Thylacinus cynocephalus*) (Fig. 6.9).

Cells of the Woolly mammoth can be obtained from specimens that have been fairly conserved in the American and Russian permafrost. An important question is whether the DNA of tissues that have been frozen without cryoprotectants would be intact enough for the generation of viable offspring after nuclear transfer. Viable mice have been born from an animal that had been frozen for 16 years without any cryoprotection, thereby demonstrating the feasibility of cloning from frozen bodies [44]. An additional problem in cloning extinct animals is the oocyte for reprogramming and embryo formation and a surrogate that could accommodate the cloned embryo. In the case of the Wooly mammoth, the Asian elephant (*Elephas maximus*) would be a logical choice based on evolutionary relationship and size. With the inefficiency of cloning it can be argued, however, whether sufficient numbers of oocytes and surrogate elephant cows are available or even present. The question remains as to why we would want to de-extinct animals. It has been proposed that it is our obligation, as many animals became extinct because of humans. Others argue that it would be better to invest time and money in trying to prevent extinction of plants and animal species that are now critically endangered.

Recently a large part of the Thylacine genome has been sequenced from a pouch young specimen [45]. Whether the DNA is of sufficient quality for nuclear reprogramming is not known. Another difficulty with this animal is the oocyte and surrogate mother. The closest living relative of the Thylacine seems to be the Numbat (*Myrmecobius fasciatus*) but this animal by itself is endangered.

6.5.6 Cloning of Equids

It may be worthwhile to clone valuable animals that cannot reproduce in the normal fashion. The first cloned equid was a mule, a hybrid from the breeding of a male donkey (*Equus asinus*) with a horse (*Equus caballus*) mare [46]. Mules are by definition sterile but can give offspring via cloning using horse oocytes and a mare as recipient. Mules can be commercially valuable when used for sports. Indeed, the first mule clone, Idaho Gem, was cloned from a fetus that could have been a race winner and has already won races himself.

For valuable mares that have been successful in sports but are too old for breeding, or champion geldings, cloning might be an interesting option. Shortly after the birth of the first cloned mule, the first cloned horse was born. This animal was not only the first of its species, but was also the first animal that was carried by her 'twin' sister: adult cell donor and surrogate mother were the same animal [47]. This demonstrated that recognition of the embryo by the mother and maintenance of gestation is not dependent on immunological recognition. Whether horse cloning will have a future is partly dependent on the breeding associations, as many of these associations do not allow admittance of cloned horses and participation to some equestrian sports requires listing in a breed registry. Horse cloning could be valuable on the other hand for the preservation of genetic lines [48]. Irrespectively, horse cloning can teach us many aspects of equine peri-implantation development and maternal-fetal interactions.

6.5.7 Generation of Transgenic Animals

For the generation of transgenic or knockin/knockout animals, the use of embryonic stem cell lines targeted using homologous recombination in combination with chimera formation has been very successful indeed [49]. For mammalian species other than rodents or primates, however, it has been demonstrated to be extremely difficult to derive and maintain pluripotent stem cell lines [50]. Transgenic farm animals can be made by microinjection of DNA into zygotes optionally in combination with, for instance, CRISPR/Cas9 technology. Selection of animals with the correct transgenes and subsequent production of F1 animals from founders is, however, a time-consuming process in animals with long generation intervals. When the aim is, for instance, to produce a pharmaceutically active human protein in the milk of a cow, the process can take years. Once such an animal has been generated, cloning by somatic cell nuclear transfer would be a relatively efficient strategy to enhance the numbers of animals [51].

6.6 Human Cloning

The creation of Dolly ignited many scientific and ethical discussions on the possibilities of human cloning and their consequences. Animal cloning is already inefficient, unreliable, and risky, so what about human cloning? First of all, why cloning humans? To clone humans for reproductive purposes, for instance, as an alternative for subfertile couples, seems unrealistic. The predictable inefficiency resulting in the large numbers of human oocytes and surrogate mothers needed and the expected occurrences of spontaneous abortions exclude human cloning as a way of reproduction. Besides reproductive cloning, in combination with embryonic stem cell culture, human cloning could be useful in (regenerative) medicine. Instead of transferring a cloned human embryo to a womb, embryonic stem cells can be generated from a cloned embryo. When adult cells of a patient are used for cloning, the clone-derived pluripotent stem cells would carry the patient's genotype and could be used for autologous transplantation without being rejected. Maybe even more important, the patient-specific pluripotent cells could be used to study disease progression and to test drug efficacy for personalized medicine.

Due to the inefficiency of cloning by somatic cell nuclear transfer, large numbers of oocytes are needed for cloning. Human oocytes are scarce, however, and retrieval is not without risks for women as they can develop ovarian hyperstimulation syndrome [52]. Most women who qualify for hyperstimulation are those who do so for immediate in vitro fertilization procedures or for egg freezing and in vitro fertilization at a later stage. Surplus eggs of such procedures could be used after informed consent of the women. Alternatively, eggs may be donated altruistically or after commercial payment, depending on the country's legislation.

Although human oocytes can reprogram somatic cells to a pluripotent state, removal of the oocyte's genetic material (metaphase II spindle) led to developmental arrest at the early morula stage following nuclear transfer [53]. Apparently, in human oocytes, critical factors for development are physically associated with the meiotic spindle apparatus. Removal of these factors leads to spontaneous exit from meiosis, thereby disturbing reprogramming and development. Enucleation and somatic cell fusion in the presence of caffeine, functioning as a phosphatase inhibitor, protects the oocyte from premature meiosis exit. This technique has enabled the generation of patient-specific human embryonic stem cell lines [54] (◘ Fig. 6.10). Patient-specific pluripotent stem cell lines can, however, also be produced using induced pluripotent stem (iPS) cell technology and with fewer ethical and legal barriers [55] (for further information, read ► Chap. 4). It has been suggested, however, that human ES cells derived after somatic cell nuclear transfer are more faithfully reprogrammed and contain less genomic errors than iPS cells [56].

A slightly different approach was adapted for the first successful reproductive cloning of the crabeating macaque *Macaca fascicularis*, a primate. Oocytes from these animals were retrieved by laparoscopy after ovarian superovulation. The meiotic spindle of the oocytes was visualized and removed using a spindle imaging microscopic system. Critical for the procedure was the treatment of the enucleated cells with the histone deacetylase inhibitor trichostatin A. In addition, after the nuclear transfer procedure, the embryos were injected with human *KDM4D* mRNA, coding for a histone demethylase with the incentive of opening up the chromatin to

facilitate nuclear reprogramming. Similar to the cloning of other mammals, the procedure was highly inefficient; injection of 127 oocytes with somatic cells from a 61-day-old aborted fetal monkey led to the birth of two healthy individuals from 79 transferred embryos. Interestingly, no live births were obtained when cells from adult animals were used [57].

Human-cloned embryos have been generated to study whether a disease caused by a genetic mutation can be corrected with CRISPR/ Cas9 technology. For this, skin cells from a patient suffering from the genetic disease β-Thalassemia were used to produce cloned zygotes. The patient was homozygous for the disease and thus carried two abnormal copies of the *HBB* gene. Since the zygotes were genetically identical to the patient, they carried the same homozygous mutation, and base-editing technology with a modified CRISPR/ Cas9 protocol was used to correct the disease. Sequencing of the blastomeres after embryo culture revealed that indeed the gene was corrected in 8 out of 20 embryos in at least one copy, albeit with a high degree of mosaicism, meaning that in most blastomeres the gene was still defective [58]. Cloning was used in this study as a tool to generate sufficient embryos with a specific mutation to test a gene-editing system. Both cloning and gene editing of human embryos are controversial and not allowed in many countries.

When it comes to reproductive cloning, monozygotic twins can help us understand what it means to have a genetic 'copy' or how it may limit, or enhance, selfness. Monozygotic twins are by definition of the same age and share the same mitochondrial DNA, while clones from somatic cell nuclear transfer would be of dissimilar age and have different mitochondrial DNA. Not surprisingly, monozygotic twins are less likely to object to human cloning [59].

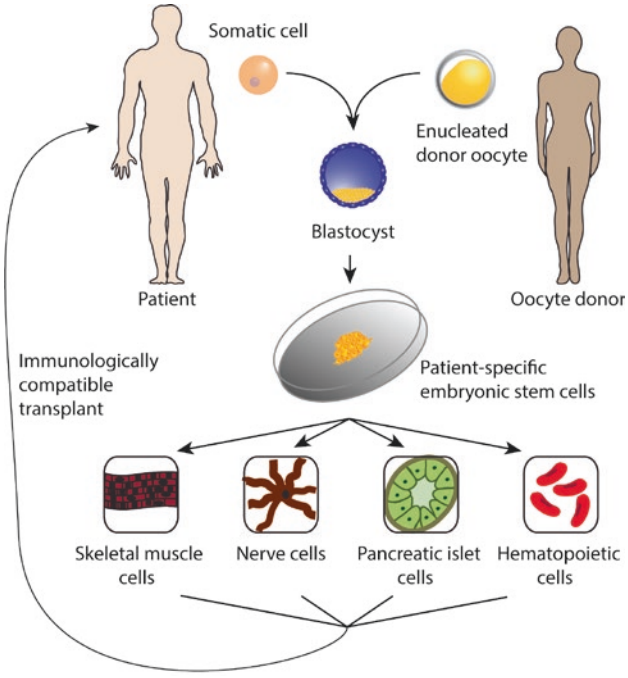


Fig. 6.10 Therapeutic cloning. Schematic representation of therapeutic human cloning. Theoretically, a somatic cell from a patient can be injected into an enucleated oocyte which can result in a blastocyst stage embryo that is genetically identical to the patient (a clone). Instead of transfer of the embryo to a uterus, embryonic stem cells can be derived from the inner cell mass of the blastocyst. Theoretically pluripotent stem cells can be used to generate tissues that can be transplanted to the patient. The tissues would not be immune rejected by the patient

Take-Home Message

This chapter covers the following topics:

- A clone is a genetically identical organism that descends from a single cell or organism.
- Cloning is the generation of clones.
- Various examples are provided of asexual reproduction in vertebrates.
- Experiments with frogs demonstrated that the nucleus of a differentiated embryonic cell can be reprogrammed by the cytoplasm of an oocyte. The method is known as somatic cell nuclear transfer.
- Dolly, the sheep, was the first animal cloned from a single cell of an adult animal by somatic cell nuclear transfer.
- Cloning is an inefficient procedure.
- Various mammalian species have been cloned.
- During differentiation, the genetic code stays intact but access to the DNA changes: epigenetics.
- The most important epigenetic changes are methylation of cytosines in CG dinucleotides and histone tail modifications.
- Cloning by somatic cell nuclear transfer has been used to clone pets and economically valuable animals and is considered as a possibility to repopulate endangered species.
- Human cloning is a method of generating patient-specific pluripotent stem cells.
- Cloning is not allowed in most countries.

References

1. Steward FC. Totipotency, variation and clonal development of cultured cells. Endeavour. 1970;29(108):117–24.
2. Watts PC, Buley KR, Sanderson S, Boardman W, Ciofi C, Gibson R. Parthenogenesis in Komodo dragons. Nature. 2006;444(7122):1021–2.
3. Booth W, Smith CF, Eskridge PH, Hoss SK, Mendelson JR 3rd, Schuett GW. Facultative parthenogenesis discovered in wild vertebrates. Biol Lett. 2012;8(6):983–5.
4. Gierer A, Berking S, Bode H, David CN, Flick K, Hansmann G, et al. Regeneration of hydra from reaggregated cells. Nat New Biol. 1972;239(91):98–101.
5. Gierer A. The Hydra model - a model for what? Int J Dev Biol. 2012;56(6–8):437–45.
6. Wagner DE, Wang IE, Reddien PW. Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science. 2011;332(6031):811–6.
7. Liu SY, Selck C, Friedrich B, Lutz R, Vila-Farre M, Dahl A, et al. Reactivating head regrowth in a regeneration-deficient planarian species. Nature. 2013;500(7460):81–4.
8. Sikes JM, Newmark PA. Restoration of anterior regeneration in a planarian with limited regenerative ability. Nature. 2013;500(7460):77–80.
9. Umesono Y, Tasaki J, Nishimura Y, Hrouda M, Kawaguchi E, Yazawa S, et al. The molecular logic for planarian regeneration along the anterior-posterior axis. Nature. 2013;500(7460):73–6.
10. Suzuki M, Yakushiji N, Nakada Y, Satoh A, Ide H, Tamura K. Limb regeneration in Xenopus laevis froglet. Sci World J. 2006;6(Suppl 1):26–37.
11. Kragl M, Knapp D, Nacu E, Khattak S, Maden M, Epperlein HH, et al. Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature. 2009;460(7251):60–5.
12. Tseng AS, Adams DS, Qiu D, Koustubhan P, Levin M. Apoptosis is required during early stages of tail regeneration in Xenopus laevis. Dev Biol. 2007;301(1):62–9.

13. Briggs R, King TJ. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc Natl Acad Sci U S A. 1952;38(5):455–63.
14. King TJ, Briggs R. Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. Proc Natl Acad Sci U S A. 1955;41(5):321–5.
15. Hogben L. History of the Hogben test. Br Med J. 1946;2:554.
16. Gurdon JB, Hopwood N. The introduction of Xenopus laevis into developmental biology: of empire, pregnancy testing and ribosomal genes. Int J Dev Biol. 2000;44(1):43–50.
17. Gurdon JB. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol. 1962;10:622–40.
18. Gurdon JB, Uehlinger V. "Fertile" intestine nuclei. Nature. 1966;210(5042):1240–1.
19. Laskey RA, Gurdon JB. Genetic content of adult somatic cells tested by nuclear transplantation from cultured cells. Nature. 1970;228(5278):1332–4.
20. Illmensee K, Hoppe PC. Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. Cell. 1981;23(1):9–18.
21. McGrath J, Solter D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science. 1984;226(4680):1317–9.
22. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997;385(6619):810–3.
23. Willadsen SM. Nuclear transplantation in sheep embryos. Nature. 1986;320(6057):63–5.
24. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292(5819):154–6.
25. Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, et al. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proc Natl Acad Sci U S A. 1989;86(22):8927–31.
26. Capecchi MR. Altering the genome by homologous recombination. Science. 1989;244(4910):1288–92.
27. Sims M, First NL. Production of calves by transfer of nuclei from cultured inner cell mass cells. Proc Natl Acad Sci U S A. 1994;91(13):6143–7.
28. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature. 1996;380(6569):64–6.
29. Campbell KH, Ritchie WA, Wilmut I. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod. 1993;49(5):933–42.
30. Waddinngton CH. The strategy of genes: a discussion of some aspects of theoretical biology. London: George Allen and Unwin; 1957.
31. Berger SL. The complex language of chromatin regulation during transcription. Nature. 2007;447(7143):407–12.
32. Bird A. Perceptions of epigenetics. Nature. 2007;447(7143):396–8.
33. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature. 2010;464(7288):520–8.
34. Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I, Colman A, et al. Analysis of telomere lengths in cloned sheep. Nature. 1999;399(6734):316–7.
35. Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM, et al. Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. Science. 2000;288(5466):665–9.
36. Jiang L, Carter DB, Xu J, Yang X, Prather RS, Tian XC. Telomere lengths in cloned transgenic pigs. Biol Reprod. 2004;70(6):1589–93.
37. Wakayama S, Kohda T, Obokata H, Tokoro M, Li C, Terashita Y, et al. Successful serial recloning in the mouse over multiple generations. Cell Stem Cell. 2013;12(3):293–7.
38. Vajta G. Cloning: a sleeping beauty awaiting the kiss? Cell Reprogram. 2018;20(3):145–56.
39. Vajta G. Handmade cloning: the future way of nuclear transfer? Trends Biotechnol. 2007;25(6):250–3.
40. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, et al. A cat cloned by nuclear transplantation. Nature. 2002;415(6874):859.
41. Lee BC, Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, et al. Dogs cloned from adult somatic cells. Nature. 2005;436(7051):641.
42. Vogel G. Endangered species. Cloned gaur a short-lived success. Science. 2001;291(5503):409.

43. Folch J, Cocero MJ, Chesne P, Alabart JL, Dominguez V, Cognie Y, et al. First birth of an animal from an extinct subspecies (Capra pyrenaica pyrenaica) by cloning. Theriogenology. 2009;71(6):1026–34.
44. Wakayama S, Ohta H, Hikichi T, Mizutani E, Iwaki T, Kanagawa O, et al. Production of healthy cloned mice from bodies frozen at −20 degrees C for 16 years. Proc Natl Acad Sci U S A. 2008;105(45):17318–22.
45. Feigin CY, Newton AH, Doronina L, Schmitz J, Hipsley CA, Mitchell KJ, et al. Genome of the Tasmanian tiger provides insights into the evolution and demography of an extinct marsupial carnivore. Nat Ecol Evol. 2018;2(1):182–92.
46. Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, et al. A mule cloned from fetal cells by nuclear transfer. Science. 2003;301(5636):1063.
47. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, et al. Pregnancy: a cloned horse born to its dam twin. Nature. 2003;424(6949):635.
48. Church SL. Nuclear transfer saddles up. Nat Biotechnol. 2006;24(6):605–7.
49. Mak TW. Gene targeting in embryonic stem cells scores a knockout in Stockholm. Cell. 2007;131(6):1027–31.
50. Ezashi T, Yuan Y, Roberts RM. Pluripotent stem cells from domesticated mammals. Ann Rev Anim Biosci. 2016;4:223–53.
51. Niemann H, Lucas-Hahn A. Somatic cell nuclear transfer cloning: practical applications and current legislation. Reprod Domest Anim. 2012;47(Suppl 5):2–10.
52. Beeson D, Lippman A. Egg harvesting for stem cell research: medical risks and ethical problems. Reprod Biomed Online. 2006;13(4):573–9.
53. Noggle S, Fung HL, Gore A, Martinez H, Satriani KC, Prosser R, et al. Human oocytes reprogram somatic cells to a pluripotent state. Nature. 2011;478(7367):70–5.
54. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, et al. Human embryonic stem cells derived by somatic cell nuclear transfer. Cell. 2013;153(6):1228–38.
55. Mummery CL, Roelen BA. Stem cells: cloning human embryos. Nature. 2013;498(7453):174–5.
56. Ma H, Morey R, O'Neil RC, He Y, Daughtry B, Schultz MD, et al. Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature. 2014;511(7508):177–83.
57. Liu Z, Cai Y, Wang Y, Nie Y, Zhang C, Xu Y, et al. Cloning of macaque monkeys by somatic cell nuclear transfer. Cell. 2018;172(4):881–7 e7.
58. Liang P, Ding C, Sun H, Xie X, Xu Y, Zhang X, et al. Correction of beta-thalassemia mutant by base editor in human embryos. Protein Cell. 2017;8(11):811–22.
59. Prainsack B, Cherkas LF, Spector TD. Attitudes towards human reproductive cloning, assisted reproduction and gene selection: a survey of 4600 British twins. Hum Reprod. 2007;22(8):2302–8.
60. Gomez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King AL, et al. Birth of African wildcat cloned kittens born from domestic cats. Cloning Stem Cells. 2004;6(3):247–58.
61. Wani NA, Vettical BS, Hong SB. First cloned Bactrian camel (Camelus bactrianus) calf produced by interspecies somatic cell nuclear transfer: a step towards preserving the critically endangered wild Bactrian camels. PLoS One. 2017;12(5):e0177800.
62. Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, et al. Generation of fertile cloned rats by regulating oocyte activation. Science. 2003;302(5648):1179.
63. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, et al. Eight calves cloned from somatic cells of a single adult. Science. 1998;282(5396):2095–8.
64. Hwang I, Jeong YW, Kim JJ, Lee HJ, Kang M, Park KB, et al. Successful cloning of coyotes through interspecies somatic cell nuclear transfer using domestic dog oocytes. Reprod Fertil Dev. 2013;25(8):1142–8.
65. Wani NA, Wernery U, Hassan FA, Wernery R, Skidmore JA. Production of the first cloned camel by somatic cell nuclear transfer. Biol Reprod. 2010;82(2):373–9.
66. Loi P, Ptak G, Barboni B, Fulka J Jr, Cappai P, Clinton M. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol. 2001;19(10):962–4.
67. Li Z, Sun X, Chen J, Liu X, Wisely SM, Zhou Q, et al. Cloned ferrets produced by somatic cell nuclear transfer. Dev Biol. 2006;293(2):439–48.
68. Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, et al. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. Cloning. 2000;2(2):79–90.

69. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, et al. Production of goats by somatic cell nuclear transfer. Nat Biotechnol. 1999;17(5):456–61.
70. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature. 1998;394(6691):369–74.
71. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, et al. Pig cloning by microinjection of fetal fibroblast nuclei. Science. 2000;289(5482):1188–90.
72. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, et al. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. 2000;407(6800):86–90.
73. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol. 2002;20(4):366–9.
74. Berg DK, Li C, Asher G, Wells DN, Oback B. Red deer cloned from antler stem cells and their differentiated progeny. Biol Reprod. 2007;77(3):384–94.
75. Gomez MC, Pope CE, Kutner RH, Ricks DM, Lyons LA, Ruhe M, et al. Nuclear transfer of sand cat cells into enucleated domestic cat oocytes is affected by cryopreservation of donor cells. Cloning Stem Cells. 2008;10(4):469–83.
76. Shi D, Lu F, Wei Y, Cui K, Yang S, Wei J, et al. Buffalos (Bubalus bubalis) cloned by nuclear transfer of somatic cells. Biol Reprod. 2007;77(2):285–91.
77. Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hwang WS, et al. Endangered wolves cloned from adult somatic cells. Cloning Stem Cells. 2007;9(1):130–7.
78. Jansen DL, Edwards ML, Koster JA, Lanza RP, Ryder OA. Postnatal management of cryptorchid Banteng calves cloned by nuclear transfer utilizing frozen fibroblast cultures and enucleated cow ova. Reprod Fertil Dev. 2004;16:206.