25th ANNIVERSARY OF CLONING BY SOMATIC-CELL NUCLEAR TRANSFER Twenty-five years after Dolly: how far have we come?

Bernhard Klinger and Angelika Schnieke

¹Department of Animal Sciences, Chair of Livestock Biotechnology, School of Life Sciences Weihenstephan, Technical University Munich, Freising, Germany

Correspondence should be addressed to A Schnieke; Email: angelika.schnieke@wzw.tum.de

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Abstract

For more than a century, the scientific consensus stated that a nucleus from a terminally differentiated cell would not be able to control the development of offspring. This theory was refuted by the birth of Dolly, the first animal generated by nuclear transfer using an adult somatic cell as a nuclear donor. Following this paradigm shift, a wide variety of animals has been cloned using somatic cell nuclear transfer. Coupled with modern genome engineering technology, somatic cell nuclear transfer has become the method of choice for the generation of genetically modified farm animals. This has opened new opportunities to study the function of genes and has led to the establishment of animal models for a variety of human conditions and diseases or to improve the health of livestock animals.

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Introduction

The concept of somatic cell nuclear transfer (SCNT) was originally introduced in 1938 as a means to study cell differentiation (Spemann 1938). Pioneering work by Briggs and King (1952) conducted in amphibians led to the generation of the first cloned animals from cells at the blastocyst stage (Briggs & King 1952) but failed when using cells from the late gastrulation stage (King & Briggs 1955). That led to the conclusion that irreversible chromosomal changes had occurred and nuclear totipotency had been lost. In 1962 Gurdon conducted the first successful SCNT – epithelial cells from tadpoles – to generate living tadpoles (Gurdon 1962). It took 21 more years until the first nuclear transfer was performed in mice (McGrath & Solter 1983). The donor cell was derived from a single cell embryo, the recipient cell was a zygote. In contrast, blastomeres from four-cell or later stage embryos did not support embryo development. Again, it was stated that 'the inability of cell nuclei from these stages to support development reflects rapid loss of totipotency'. A couple of years later, the birth of the first sheep cloned from an eight-cell stage blastomere was reported using unfertilised oocytes instead of zygotes as recipient cells (Willadsen 1986). Other mammalian species such as rabbits (Stice & Robl 1988), pigs (Prather *et al.* 1989), and cattle (Prather *et al.* 1987) followed shortly thereafter. However, all of these animals were

cloned using blastomeres from early embryos as nuclear donors. In 1996 the birth of sheep derived from cultured inner cell mass cells was reported (Campbell *et al.* 1996). In the same year Dolly, the first cloned offspring generated from an adult-derived somatic cell population, was born (Wilmut *et al.* 1997). The birth of Dolly irrevocably dispelled the longstanding hypothesis that cell differentiation involves irreversible modifications that make cell dedifferentiation and therefore cloning of mammals impossible (Weismann *et al.* 1893).

Despite its success, technical problems have made the practical application of somatic cell nuclear transfer (SCNT) technology challenging. First, Dolly was the only offspring obtained from 277 reconstructed embryos (efficiency of 0.3%) (Wilmut *et al.* 1997). Since then, the efficiency of the cloning procedure has improved for some species (e.g. cattle) but remained low (<5%) for many other species (e.g. sheep, mouse, pig, rabbit) (Meissner & Jaenisch 2006). Second, malformations are frequently observed in cloned embryos and their extraembryonic tissues (Ogura *et al.* 2013). Both are caused by faulty or insufficient epigenetic reprogramming. Mechanisms involved in reprogramming have been reviewed by Matoba *et al.* (Matoba & Zhang 2018) and insights gained have led to approaches on how to overcome these defects/problems (Cibelli & Gurdon 2018).

SCNT is first of all a reproductive technology that enables the preservation and rederivation of elite animals (Galli *et al.* 2003) or endangered species (Lanza *et al.* 2000). However, its greatest potential has been a simple fact that cells could be cultured, expanded, and precisely modified prior to nuclear transfer. It opened novel avenues for livestock breeding, fast-track introduction of known (hornless cattle (Carlson *et al.* 2016)), or novel traits such as disease resistance for example, porcine reproductive and respiratory syndrome virus (PRRSV) (Prather *et al.* 2017). By facilitating the generation of genetically modified animals, SCNT also holds great potential for biomedical research (Rogers 2016). Here the pig is especially well suited due to its similar size, anatomy, and physiology to humans (Perleberg *et al.* 2018).

In this review, we give a brief historical perspective on what has been achieved in the field of SCNT in the last 25 years. We discuss its advantages and disadvantages as a tool for the generation of genetically modified animals, provide examples of its application, and ask if novel technologies have superseded SCNT.

Cloning technology 25 years of incremental advances

SCNT is a multi-step process that comprises the generation of a cytoplast via enucleation of an oocyte, selection of a suitable donor cell, embryo reconstruction, activation, and culture. Mature oocytes in metaphase II of the second meiotic division (MII) are the cytoplast donor of choice as their use results in the highest developmental potential. In the past MII oocytes for the production of cytoplasts had to be sourced from donor animals but for many species, they can now be replaced by *in vitro* matured oocytes. This is especially relevant for domestic animals where large numbers of oocytes can be obtained from slaughterhouse material. The quality of *in vivo* derived cytoplasts is higher but commonly involves superovulation regimes to increase yield, which adversely impacts embryo development and foetal growth (Van der Auwera I & D'Hooghe 2001). In accordance with the 3R principle, the substitution of *in vivo* with *in vitro* matured oocytes significantly reduces the number of experimental animals required (Yuan & Krisher 2012). If the goal is the preservation of endangered species with an inevitable lack of oocytes, those from closely related species can serve as suitable cytoplast donors (Lanza *et al.* 2000). A timeline depicting the development of cloning related technology is shown in Fig. 1.

Enucleation

Cytoplasts are generated in a process termed enucleation, in which the genetic material is removed

from the recipient cell. Enucleation can be performed blindly by removing the first polar body and about one-third of the cytoplasm beneath it with a glass pipette. However, in many cases the metaphase plate is not located close to the polar body (Nour & Takahashi 1999). Furthermore, the removal of a significant proportion of the cytoplasm reduces the oocytes' capacity for epigenetic reprogramming and subsequent embryonic development. Successful enucleation can be confirmed by exposing recipient oocytes to UV light after staining with DNA specific fluorochromes but this approach decreases the viability of the resulting cytoplast (Dominko *et al.* 2000). Enucleation can also be performed chemically, for example with ethanol and demecolcine, but this reduces embryonic development compared to mechanical enucleation methods (Gasparrini *et al.* 2003).

Embryo reconstruction, activation and culture

Embryo reconstruction by fusion of donor and recipient cells using a DC electric pulse is the method of choice in most species. Piezo- or laser-assisted microinjection is the most common technique in rodents because electrofusion was less successful in those species due to the smaller size and fragility of the oocytes (Wakayama *et al.* 1998). The reconstructed embryo then has to be activated to exit meiotic arrest and initiate embryonic development. Chemical activation can be achieved by exposure to strontium in mice and ionomycin in cattle or sheep (Akagi *et al.* 2003). Electrical activation remains the most widely used method in livestock (Polejaeva *et al.* 2000). The exact activation stimuli, timings, and post-activation treatments must be optimised for each individual protocol and species.

Embryo culture conditions and the stage at which reconstructed embryos are transferred to recipients are species-specific. *In vitro* cultivation of embryos increases the incidence of epigenetic defects, which is typically associated with developmental abnormalities such as large offspring syndrome (LOS) (Young *et al.* 1998). LOS is characterised by overlarge foetuses, placental malformations, reduced pregnancy rates, dystocia, and pulmonary dysfunction (Fleming *et al.* 2004). Pathologies based on an altered epigenome are largely contributed to suboptimal culture conditions, such as medium containing serum (Hill 2014). The adverse consequences of *in vitro* culture can be minimised by transferring reconstructed embryos at an early stage of development to the oviduct of the recipient. This approach is practicable in mice and pigs (Wakayama *et al.* 1998, Polejaeva *et al.* 2000). However, for most domestic animals, this is not an option because small litter sizes, high costs, and long gestation intervals make the availability of recipients the limiting factor.

Figure 1 The development of SCNT and related technology.

Therefore, reconstructed embryos must be cultured to the blastocyst stage *in vitro* to ensure that only viable embryos will be transferred.

Handmade cloning

Handmade cloning (HMC) is an alternative cloning technique that forgoes the use of micromanipulators. The characteristic feature of this method is that enucleation of oocytes is conducted with a handheld blade after partial zona pellucida digestion (Vajta *et al.* 2003). The site of the metaphase plate is indicated by the polar

body or can be determined by demecolcine treatment which leads to the formation of a protrusion cone (Vajta *et al.* 2003). Bisection is conducted manually with an embryo-splitting blade to generate a cytoplasm and a karyoplast. Another distinguishing aspect of HMC is that two cytoplasts are fused with the donor cell instead of one. This counteracts the typical loss of cytoplasm during enucleation, which has a positive effect on embryo development (Sayaka *et al.* 2008). Zona-free embryos require special culture conditions in a well of the well system before they can be transferred to recipients at the blastocyst stage. With this approach, mitochondria from

three different animals are introduced into one individual but so far, no deleterious effects of this heteroplasmy have been reported (Steinborn *et al.* 2000). HMC has the advantage that only minimal equipment is required, reducing the cost of cloning while providing similar cloning efficiency (Tecirlioglu *et al.* 2005).

A method for all mammals

Other than the development of zona-free approaches mentioned above, cloning technology has remained largely the same since it was first described in 1986 (Willadsen 1986). The greatest advance has been the adaptation of SCNT technology to other species. For decades embryonic stem cells (ESCs) had been the only cell-based method for the generation of animals. Shortly after the publication of Dolly, SCNT technology expanded first to other domestic species, followed by wild cats, wolves, and other endangered species (Gomez *et al.* 2004, Kim *et al.* 2007). Certain species are notoriously hard to clone. Dogs, for example, have a very limited breeding period, *in vitro* maturation is difficult and their oocytes are coated with fat which impedes enucleation (Lee *et al.* 2005). In pigs at least four foetuses are required to maintain a pregnancy (Prather 2000). Consequently, most technological advances have been species-specific. The main obstacle for some species remains the lack of sufficient numbers of oocytes. To date, a large variety of mammalian species including monkeys (Liu *et al.* 2018) have been cloned by SCNT using a wide range of donor cell types from foetal and adult tissues (see Fig. 2).

Beyond reproductive cloning

In mice pluripotent ESC based chimera technology offers an elegant way for the generation of Genetically modified (GM) animals (Capecchi 1989b). ESCs are genetically modified in cell culture and injected into a recipient blastocyst followed by transfer to a pseudo-pregnant female to gestate chimeric offspring (Capecchi 1989b). Appropriate breeding results in mice carrying the desired genotype (Koller & Smithies 1992). ESCs are a powerful tool for gene targeting because they maintain pluripotency and a normal karyotype during long term culture and support homologous recombination (HR) at a high frequency (Capecchi 1989a). Pluripotent ESCs with proven germline transmission are currently still unavailable in livestock even though there are promising reports for sheep, cattle, and pigs (Bogliotti *et al.* 2018). It has been the lack of ESCs which spurred research into SCNT as an alternative method for the generation of GM offspring in domestic species.

Genetically modified (GM) animals hold great potential for agriculture and biomedical research. Mice are the most widely used laboratory animals

because they are inexpensive to house and techniques for their genetic modification are well established. However, rodents often do not mimic human disease pathology or phenotypes accurately which makes large animal models indispensable (Mak *et al.* 2014). Similarities in body size, organ anatomy, diet, and pathophysiology make the pig the animal of choice to model human diseases. Pigs are highly fertile, housing conditions including specific-pathogen-free (SPF) are well established and public acceptance for the use of livestock in animal experiments is less controversial than for primate species or companion animals. Porcine disease models have been generated for cancer research (Flisikowska *et al.* 2013), diabetes (Wolf *et al.* 2014), cystic fibrosis (Rogers *et al.* 2008), and Duchenne muscular dystrophy (Klymiuk *et al.* 2013). Pigs with multiple genetic modifications may in the near future serve as donors for xeno-organ transplantation into humans (Fischer *et al.* 2016). Furthermore, Cattle, sheep, and goats have been proposed as bioreactors for the production of pharmaceutically important proteins (Schnieke *et al.* 1997). Genome engineering also has the potential to revolutionise animal breeding (Ruan *et al.* 2017), improve productivity, animal welfare, reduce the use of antibiotics, and protect the environment (Laible *et al.* 2015).

Genetic engineering in livestock

Pronuclear DNA microinjection was the first method for the generation of GM large animals but this approach is inefficient, facilitates only random integration of transgenes, and frequently leads to mosaicism (Hammer *et al.* 1985). Viral vectors provided a more efficient alternative, but they only allow for limited transgene size and the preparation and concentration of viral particles is time and labour-intensive (Reichenbach *et al.* 2010). Transposon-mediated transgenesis is efficient and has advantages over viral vectors regarding cargo size, ease of implementation, and biosafety. However, this approach depends on microinjection as a delivery method and again only allows for random integration of transgenes (Zhao *et al.* 2016). Sperm-mediated gene transfer (SMGT) employs the natural ability of sperm to introduce DNA into oocytes to co-transfer exogenous DNA. Theoretically, this makes micromanipulation, culture, and transfer of embryos obsolete but despite its simplicity, successful implementation of SMGT has been limited to few laboratories worldwide, rendering its validity questionable (Brinster *et al.* 1989).

In contrast to the methods mentioned above, SCNT enabled both the generation of transgenic animals (Schnieke *et al.* 1997) and, more importantly, gene-targeting (McCreath *et al.* 2000). The method, first established in sheep, was soon extended to other species (Polejaeva *et al.* 2000) and remained the most

Figure 2 Timeline of cloned mammalian species over the last 25 years.

frequently used tool for the generation of GM domestic animals. Extensive selection and screening of donor cells ensure that 100% of offspring carry solely and precisely the intended modification (Wolf *et al.* 2000). This is a considerable advantage over ESC technology because the injection of ESCs into blastocysts results in chimeric offspring. Consequently, germline transmission has to be assessed by breeding which is not realistic in species with single offspring and long gestation times.

A major disadvantage of SCNT has been the fact that gene targeting in somatic cells has been orders of

magnitude less efficient than in pluripotent stem cells. This changed with the emergence of nuclease-based genome editing technology, which has further expanded the toolbox for the generation of genetically modified animals. Site-specific endonucleases, such as zinc finger nucleases, transcription activator-like effector nucleases, and the CRISPR/Cas9 system facilitate the introduction of double strand breaks (DSBs) into predetermined sites within the host genome to trigger DNA repair mechanisms, thus enabling efficient genome engineering (Gaj *et al.* 2013). The power of this

technology has been demonstrated by the inactivation of multiple endogenous copies of the same retroviral gene (Niu *et al.* 2017) or simultaneous inactivation of several different host genes (Fischer *et al.* 2019) in somatic cells, followed by the generation of cloned animals. Due to their high-efficiency genome editing reagents, for example, CRISPR/Cas9 can also be delivered directly to early embryos by intracytoplasmic microinjection (Carbery *et al.* 2010) or electroporation (Hashimoto & Takemoto 2015). Genome editing in zygotes is a powerful method for the inactivation of genes but had been less efficient at introducing modifications via homology directed repair (HDR). New strategies are

being developed to improve efficiencies (Nambiar *et al.* 2019). One limitation is the high incidence of mosaicism which is especially problematic in livestock due to the long generation interval (Ryu *et al.* 2018). Another downside of Cas9 and other site-specific nucleases are possible off-target effects, that is, the induction of DSBs at unwanted locations (Anderson *et al.* 2018). Genome editing in combination with SCNT allows for comprehensive *in vitro* off-target analysis prior to the generation of animals, avoiding undesirable mutations as well as mosaicism (Zhang *et al.* 2015). The high adaptability, usability, and efficiency of modern genome engineering technology has revolutionised the

Figure 3 The toolkit for the generation of genetically modified livestock. (A) Pronuclear DNA microinjection leads to random transgene integration but does not facilitate gene targeting. Viral vectors and transposons can be used to increase the frequency of transgenesis. (B) Cytoplasmic microinjection of site-specific endonucleases facilitates targeted genome engineering directly in early embryos. (C) Genetically engineered somatic cells selected for the presence of the desired modification. Single cells are inserted into the perivitelline space of enucleated oocytes, followed by cell fusion and activation to generate reconstructed embryos for embryo transfer.

production of GM animals (Sander & Joung 2014). The individual strengths and weaknesses of both approaches complement each other well, and together, they provide an efficient toolkit for the generation of GM livestock (see Fig. 3).

SCNT – has it been superseded?

SCNT is a reproduction method. In the absence of ES cells, it has been the only method to re-derive animals from cultured cells. After more than 30 years of effort, one can be cautiously optimistic that fully functional ES cells might soon be available for all livestock species. However, except for the pig, the generation of chimeric animals is not a realistic option for most large animal species. The alternative could be tetraploid aggregation to obtain non-chimeric offspring, which is the gold standard to assess ES cell functionality (Nagy *et al.* 1990). Time will tell if this can be achieved for livestock ES cells. Until then, livestock ES cells or their differentiated derivatives might serve as donor cells for SCNT. In contrast to somatic cells, ES cells have the advantage that they grow permanently in culture and allow for efficient targeted modification.

When it comes to precise genome engineering, genome editing has become the method of choice. It is developing at a fast-pace and has replaced SCNT for some applications. More often, for complex genome modifications as required for xeno-organ transplantation, it is a combination of tools, including SCNT and CRISPR/Cas9, that is required.

A recent publication suggested donor-derived spermatogenesis following stem cell transplantation in sterile *NANOS2* knockout male pigs, goats, and cattle as a tool for biomedical research, preservation of endangered species, and dissemination of desirable genetics (Ciccarelli *et al.* 2020). While this approach is limited to the preservation of male genetic material, it could provide an alternative to reproductive cloning once efficiencies of donor-derived spermatogenesis have been improved (Ciccarelli *et al.* 2020). In the future, it may also allow for the generation of GE livestock. Culture conditions for spermatogonial stem cells exist for some species and after transduction with viral vectors, and transplantation into the testis murine spermatogonial stem cells supported the development of transgenic offspring (Kubota *et al.* 2004).

In 2008 the Wakayama's group reported the resurrection of a dead mouse, frozen at -20°C for 16 years by establishing an ESC line from cloned embryos using brain nuclei as nuclear donors followed by a second nuclear transfer using the ES cells (Wakayama *et al.* 2008). The result was somewhat surprising as brain cells are generally poor donor cells; on the other hand, the high levels of endogenous brain glucose may have acted as a cryoprotectant. This amazing achievement brought the notion that extinct species might also be brought

back to life by SCNT, possibly from samples conserved in permafrost (e.g. mammoth) or using a combination of genetic engineering and SCNT (Sherkow & Greely 2013). *Resurrection biology* made the headlines but due to technical, environmental, and especially ethical considerations, it may never be realised for prehistoric mammals. However, for some species at the brink of extinction SCNT is a reproductive option (Folch *et al.* 2009).

Soon after the birth of Dolly, SCNT was suggested for the generation of patient-specific ES cells (ntESCs) (Tachibana *et al.* 2013). At the time, it was the only method to obtain autologous pluripotent stem cells for regenerative medicine or the correction of congenital defects. Besides technical and ethical hurdles, the shortage of human oocytes was the main obstacle. In 2006 Yamanaka showed that a transient expression of four transcription factors (Oct4, Sox2, Myc, Klf4) is sufficient to dedifferentiate cells to an induced pluripotent stem (iPS) cell state (Takahashi & Yamanaka 2006). IPS cells are functionally equivalent to ES cells and can be isolated from patients' cells without any of the drawbacks of ntESCs which makes therapeutic cloning obsolete. However, cloning technology is still relevant for the treatment of human inherited mitochondrial diseases (Zhang *et al.* 2017). Mitochondria are the powerhouse of the cell, mitochondrial DNA (mtDNA) is maternally inherited and dysfunction due to mutations (e.g. Leigh's syndrome) can therefore be detrimental to foetal development. It is avoidable if the mother's genome is transferred into an enucleated healthy recipient oocyte (spindle transfer) followed by fertilisation.

Concluding remarks

The birth of Dolly 25 years ago proved to the world that somatic cell differentiation is reversible. It was exciting science and on a personal note taught me the importance of open discussions of science in progress. Hearing from Jim McWhir over a cup of tea about the experiment that later resulted in the birth of the cloned sheep Megan and Morag (Campbell *et al.* 1996) got me involved in the project as a Ph.D. student. Talking to Alex Kind about expressing milk proteins in cell culture led to the use of mammary cells as the nuclear donor that gave rise to Dolly. Those more experienced than myself were sceptical because it was a high-risk project. Luckily it succeeded – with a sample size of one – thanks to the work of Ian Wilmut's and Keith Campbell's group. It couldn't have been realised without financial support which was granted by Alan Colman who was at the time scientific director of PPL-Therapeutics. If I – a youngish unknown scientist – would have had to apply for a public grant to disprove a dogma, would it have been approved? I doubt it. Dolly was the result of teamwork. All involved, including all technical personnel, played

an essential part. Sadly, two people, Keath and Jim, are no longer with us.

A quarter of a century ago, when the Dolly paper was published, my young son was baffled – why all the excitement about one more sheep when there were so many sheep in Scotland? It was a very special sheep, it had a name, and Dolly caught the imagination not only of the scientific world but also the public. It spurred an interest in science. It taught us that all cells can be reprogrammed, which ultimately led to the development of iPS cells. Until recently cloning has been the only option to precisely alter the genome of many species. Has it become an obsolete technology with the rise of CRISPR/Cas9 or livestock ES cells? Possibly at some point in the future but not yet. If you are visiting Scotland, go and see Dolly in the Royal Museum in Edinburgh.

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