25th ANNIVERSARY OF CLONING BY SOMATIC-CELL NUCLEAR TRANSFER

Nuclear transfer and the development of genetically modified/ gene edited livestock

Ramiro Alberio¹ and Eckhard Wolf²

¹School of Biosciences, University of Nottingham, Nottingham, UK and ²Gene Center and Department of Veterinary Sciences, LMU Munich, Munich, Germany

Correspondence should be addressed to R Alberio or E Wolf; Email: Ramiro.Alberio@nottingham.ac.uk or ewolf@genzentrum.lmu.de

This paper forms part of an anniversary issue on the 25th Anniversary of cloning by somatic-cell nuclear transfer. The Guest Editor for this section was Professor Kevin Sinclair, University of Nottingham, UK

Abstract

The birth and adult development of 'Dolly' the sheep, the first mammal produced by the transfer of a terminally differentiated cell nucleus into an egg, provided unequivocal evidence of nuclear equivalence among somatic cells. This ground-breaking experiment challenged a long-standing dogma of irreversible cellular differentiation that prevailed for over a century and enabled the development of methodologies for reversal of differentiation of somatic cells, also known as nuclear reprogramming. Thanks to this new paradigm, novel alternatives for regenerative medicine in humans, improved animal breeding in domestic animals and approaches to species conservation through reproductive methodologies have emerged. Combined with the incorporation of new tools for genetic modification, these novel techniques promise to (i) transform and accelerate our understanding of genetic diseases and the development of targeted therapies through creation of tailored animal models, (ii) provide safe animal cells, tissues and organs for xenotransplantation, (iii) contribute to the preservation of endangered species, and (iv) improve global food security whilst reducing the environmental impact of animal production. This review discusses recent advances that build on the conceptual legacy of nuclear transfer and – when combined with gene editing – will have transformative potential for medicine, biodiversity and sustainable agriculture. We conclude that the potential of these technologies depends on further fundamental and translational research directed at improving the efficiency and safety of these methods.

Reproduction (2021) **162** F59–F68

What did Dolly teach us?

The germ-plasm theory by August Weismann proposed that cells of a developing organism lose developmental plasticity during differentiation (Weismann *et al.* 1889). Observations in the roundworm *Parascaris equorum* made by Theodor Boveri, showing chromosome diminution in the somatic compartment whilst a full chromosome set was retained in the germline, contributed to Weismann's concept. Hans Spemann proposed that transferring the nucleus of a cell into an egg would be a 'fantastical experiment' that would put this idea to the test (Spemann 1938). Early experimental attempts in amphibians supported this idea, as embryonic development failed after the nuclear transfer of gastrula-derived somatic cells into oocytes (King & Briggs 1955). Furthermore, when primordial germ cells (PGCs) from the same stages were used as nuclear donors, normal tadpoles developed, implying that developmental plasticity was restricted

in cells adopting a somatic identity (Smith 1965). However, subsequent work by using more advanced developmental stages, such as embryonic gut epithelial cells, resulted in the development of normal adult frogs after the nuclear transfer (NT). Although the efficiency was very low (~1%), this experiment offered the first evidence that the genetic content of differentiated cells was equivalent to that of an undifferentiated blastomere (Gurdon & Uehlinger 1966). However, adult frogs were never obtained after NT with adult cells, thus a demonstration of complete reprogramming of an adult somatic cell remained unanswered for another three decades. A report of four cloned cattle made from NT embryos reconstructed with cultured inner cell mass (ICM) cells suggested that partially differentiated donor cells supported full-term development in mammals (Sims & First 1994). The year 1996 saw the culmination of extensive efforts by many groups over previous decades in overcoming the technical challenges of performing NT in mammals. For the first time, cultured

cells from established cell lines from the embryonic disc of a sheep embryo were successfully used as nuclear donors. These experiments resulted in the birth of two lambs, Megan and Morag, who grew to fertile adults (Campbell *et al.* 1996b). Following this experiment, the team used cells isolated from the mammary gland of a 6-year-old *Finn Dorset* sheep and performed 277 NT experiments, from which one lamb, *Dolly*, was born (Wilmut *et al.* 1997). A key insight for the success of these experiments was the understanding of the critical need for cell-cycle coordination between the donor cell and the recipient oocyte (Campbell & Alberio 2003). Previous work by Campbell and colleagues had established the importance of using cells in the non-replicative phase of the cell cycle for NT into metaphase II oocytes (Campbell *et al.* 1996a). This was achieved very ingeniously via the 'starvation' of cells for several days, in order to slow down cell-cycle progression and enrich for cells in G0. As a result, reconstructed embryos had normal ploidy. This was in contrast to embryonic blastomeres, which have a long S-phase and undergo DNA damage after transfer into an MII oocyte (Campbell *et al.* 1993). DNA damage contributes to widespread chromosomal abnormalities, consistent with those reported in early studies in amphibians.

The biological significance of *Dolly* the sheep, the first cloned mammal by using an adult somatic cell, was far-reaching. First, it answered the long-standing question of genetic/cellular equivalence among cells in the adult organism, which had occupied the minds of scientists for over a century. Secondly, it represented a new dawn for biotechnological applications in medicine and agriculture. Importantly, NT carried out under optimized conditions can erase epigenetic memory of somatic cells enabling multiple rounds of re-cloning without loss of developmental potential (Wakayama *et al.* 2013), emphasizing the powerful reprogramming capacity of oocytes (Alberio *et al.* 2006, Halley-Stott *et al.* 2013). Indeed, the physiological parameters of 6-year-old *Dolly* clones were equivalent to age-matched control animals, which indicates that NT does not have long-term detrimental effects on aging (Sinclair *et al.* 2016). Thus, although the overall efficiency of NT remains low, the animals that develop full-term can be clinically healthy and fertile.

Genetic modification in livestock

Genetic modification of animals has primarily relied on the genetic modification of mouse embryonic stem cells (ESC) and the generation of chimeric founders that are bred to homozygosity (Doetschman *et al.* 1987, Thomas & Capecchi 1987). Mice have a short intergenerational interval and stem cell technologies have been available since 1981 (Evans & Kaufman 1981, Martin 1981). Hitherto, these methods have

not been adopted in livestock because germline competent ESC were not available. However, two recent reports suggest that this may no longer be a limitation. They demonstrated two novel sources of embryonic stem cells derived from pig and horse pre-implantation embryos capable to robustly generate PGCs *in vitro* and can contribute to chimeric fetuses (Gao *et al.* 2019, Yu *et al.* 2020). The authors indicate that this technology can also work in other species, which would offer exciting opportunities for genetic engineering (GE) in livestock species. However, demonstration of efficient germline contribution in chimeric offspring is still needed before the broad use of livestock stem cells for GE can be realized. Thus far, however, NT has been a valuable alternative strategy for the generation of GE livestock. It has been used for multiple purposes, such as the generation of animal models of disease, the development of genetically multi-modified organ donor pigs for xenotransplantation, the production of nutraceuticals, preservation of endangered species, and as a platform technology for enhancing livestock genetic selection. Examples of these applications and some of the challenges and future directions are presented in the following sections.

Tailored large animal models for human diseases

Livestock species share many anatomical and physiological characteristics with humans, such as large body size, similar metabolism and long lifespan, which are desirable when modelling human development and studying disease. Furthermore, livestock species are mostly outbred, making phenotypic observations more relevant to humans (Fig. 1). Among livestock, the pig stands out as the species of choice for human disease modelling due to similarities in organ anatomy, size and physiology. Genetically engineered pig models of cardiovascular disease (Schneider *et al.* 2020), diabetes (Renner *et al.* 2010, 2020), cystic fibrosis (Rogers *et al.* 2008, Bartlett *et al.* 2016, Caballero *et al.* 2021), several types of cancer (Perleberg *et al.* 2018), Duchenne muscular dystrophy (DMD) (Klymiuk *et al.* 2013, Moretti *et al.* 2020) and neurodegenerative disorders (such as Huntington's disease and spinal muscular atrophy) have shown to closely recapitulate the physiopathology of these human diseases (Yang *et al.* 2010, Baxa *et al.* 2013, Holm *et al.* 2016). Importantly, these models are currently being used as platforms for developing new treatments and diagnostic tools (Renner *et al.* 2016, Regensburger *et al.* 2019, Moretti *et al.* 2020).

Notably, all these models have been generated via NT by using genetically modified somatic cells. The creation of gene-targeted animals by using somatic cells is very laborious, requires intensive cell screening, multiple rounds of NT, and results in only low numbers

Figure 1 Nuclear transfer using genetically modified cells as technological pipeline for the generation of large animal models for translational medicine, organ donor pigs for xenotransplantation, and new developments for sustainable animal agriculture. PUFA, polyunsaturated fatty acid; *hfat1*, humanized version of the *C. elegans fat1* desaturase gene; *app*, expression cassette for microbial phytase; *BgEgXyAp*, polycistronic expression cassette for beta-glucanase, xylanase, and phytase.

of viable healthy offspring, which explains the relatively small numbers of animals generated so far. However, the development of gene-editing techniques by using the CRISPR/Cas9 system promises to drastically increase the efficacy of gene modification in somatic cells as well as directly in zygotes, which would remove the need for NT. For example, a new DMD pig model that displayed robust disease phenotype has been created by zygotic injection of Cas9 mRNA and guide RNA (Yu *et al.* 2016). However, DNA repair following double-strand breaks (DSB) caused by CRISPR endonucleases in zygotes is primarily driven by non-homologous end-joining (NHEJ), which results in a high proportion of mosaic embryos. One way to reduce mosaicism has been to optimize the injection of the Cas9/gRNA complex during the first few hours (~5 h in pig and ~10 h in bovine), before the onset of the S-phase in the zygote, greatly reducing mosaicism (Park *et al.* 2017, Lamas-Toranzo *et al.* 2019). Another key aim of gene editing is the generation of a targeted knock-in via homologous recombination (HR). Since homology-directed repair (HDR) is not the preferred mechanism for DSB repair, methods that promote this process are needed. Complementation of Cas9/gRNA complex with RAD18, a component of the post-

replicative repair pathway, increases HDR in cell lines, however, no data exist for embryos (Nambiar *et al.* 2019). The use of chemical compounds promoting HDR in bovine embryos has shown promising results yielding >50% gene targeting (Lamas-Toranzo *et al.* 2020). The simplicity of zygotic injection represents a major technological advantage over NT for the generation of gene-targeted animals and may replace the need of the latter in the future. It is also possible that microinjection may become less critical as methods for delivering one or multiple ribonucleoprotein complexes by electroporation are becoming more efficient in livestock (Tanihara *et al.* 2016, Hirata *et al.* 2020). A note of caution, however, needs to be made with regards to the high frequency of whole- or segmental-chromosome loss determined after DSB caused by the on- and off-target Cas9 cleavage during the zygote gene editing (Zuccaro *et al.* 2020). These observations call for the use of alternative approaches that do not require DSB to convert a targeted DNA into a new desired sequence, such as base or prime editors (Anzalone *et al.* 2020). Recently, transgenic chickens and pigs expressing Cas9 were reported as new resources for genome editing in livestock (Rieblinger *et al.* 2021).

Table 1 Genetic modifications of donor pigs of cells, tissues and organs for xenotransplantation.

Aim	Genetic modification
Deletion of sugar moieties of pig cells with pre-formed recipients' antibodies	Knockout of the <i>GGTA1</i> , <i>CMAH</i> , <i>B4GALNT2</i> genes
Inhibition of complement activation	Transgenic expression of human complement regulatory proteins (hCD46, hCD55, hCD59)
Prevention of coagulation dysregulation	Transgenic expression of human THBD, EPCR, TFPI, ENTPD1, CD73 (NT5E)
Prevention of T-cell mediated rejection	Transgenic expression of human CTLA4-Ig, LEA29Y, PD-L1; knockout or knockdown of swine leukocyte antigens
Inhibition of natural killer cells	Transgenic expression of HLA-E/human β 2-microglobulin
Inhibition of macrophages	Transgenic expression of human CD47
Prevention of inflammation	Transgenic expression of human TNFAIP3 (A20), HO-1, soluble TNFRI-Fc
Reduction of the risk of transmission of porcine endogenous retroviruses (PERV)	Knockdown of PERV expression; genome-wide knockout of the PERV <i>pol</i> gene

Genetically multi-modified donor pigs for xenotransplantation

The 'opt-out' system introduced as part of the changes to organ donation law in several countries was supposed to alleviate the waiting lists for organ transplantation. However, data from the UK shows that demand for organ transplants is growing at 1% per year, and the number of suitable donors is decreasing at a rate of 1–4% per annum. Reasons for the decline in available organs include older age of donors and obesity, both factors that contribute to adverse effects on transplant outcomes. The highest organ demand is for kidneys, pancreata, hearts and livers. The use of animal organs from livestock, such as pigs, offers a possible solution to this growing problem (Fig. 1). The pig has many advantages, including human-like size and physiology, broad availability and breeding characteristics (large litters and short reproductive cycles). Furthermore, pigs are amenable to advanced reproductive and genetic engineering platforms (reviewed in Kemter *et al.* 2020). Public acceptance for using pigs as organ sources is growing, although strict regulatory measures that ensure safe and ethically sustainable sourcing of organs are imperative (Kogel & Marckmann 2020). The technology has now reached a stage that offers hopes for clinical applications in the not-so-distant future.

Pig heart transplantation to non-human primates has been widely used over decades as a model system (Cooper *et al.* 2014). NT technology was used to produce multi-modified pigs devoid of α -1,3-galactosyltransferase (*GGTA1*), and expressing human complement regulatory protein CD46 and human thrombomodulin. Hearts from these triple-modified pigs survived for over 900 days after heterotopic abdominal transplantation in baboons (Mohiuddin *et al.* 2016). Remarkably, pig hearts with these genetic modifications showed consistent life-supporting function after orthotopic transplantation in baboons with survival times of up to 195 days (Langin *et al.* 2018, Reichart *et al.* 2020). Survival for a longer period was mainly limited by the continued growth of the pig hearts in the small chests of the recipient baboons. Inactivation of the growth hormone receptor gene (*GHR*) in the donor pigs is a potential strategy to overcome this problem (Hinrichs *et al.* 2021).

Besides the heart, multi-modified pigs (*GGTA1*-deficient/human *CD55* transgenic) made by NT have been used for kidney transplantation in a baboon that survived for 136 days (Iwase *et al.* 2017a, Iwase *et al.* 2017b). Moreover, transplantation of similar kidneys into macaques resulted in more than 1-year survival (Kim *et al.* 2009). For liver and lung xenotransplantation, the survival is more limited, however, the best results were obtained by using multi-modified pigs. Multiple other genetic modifications have been proposed to overcome humoral and cellular rejection of xenotransplants, to prevent coagulation disorders and other physiological incompatibilities, and to reduce/eliminate the risk of transmission of porcine endogenous retroviruses (Table 1; reviewed in Kemter *et al.* 2020).

Recently, the combined use of CRISPR/Cas9 technology plus transposon-mediated transgenesis in somatic cells has been used to create NT-engineered pigs with multiple gene knockouts and human transgenes (Yue *et al.* 2020). The efficacy of these modifications was demonstrated in *in vitro* assays, but transplantation experiments in non-human primates still need to be performed. Newly created multi-modified pigs carrying eight human transgenes encoding for coagulation regulators, negative regulators of the immune response and complement system, plus the inactivation of three pig xeno-antigens were successfully used as donors of skin xenografts in *Cynomolgus* monkeys without the need of immunosuppressants for 25 days (Zou *et al.* 2020).

Thus, future milestones directed to reducing the size of the organs produced, compatible with humans, and increased tolerance through targeted gene modifications will pave the way to clinical assessment of these organs (Sykes & Sachs 2019, Reichart *et al.* 2020). The recent progress demonstrates the critical need for NT technologies for the generation of these donor animals for xenotransplantation that can now be enhanced by the incorporation of efficient gene-editing and -targeting technologies.

Another avenue that is being explored for autologous transplantation considers the creation of human organs in organogenesis-disabled pigs through interspecies chimerism. The proof of concept for this idea comes

from a study showing the creation of a mouse with a rat pancreas following blastocyst complementation and interspecies chimerism (Kobayashi *et al.* 2010). Notably, in a reciprocal experiment, a mouse pancreas was created in a rat by blastocyst complementation. Isolated islets of Langerhans transplanted into a diabetic mouse restored normal glycaemia, demonstrating functional complementation (Yamaguchi *et al.* 2017). This experiment demonstrated the potential for creating functional organs using interspecies chimeras. As an alternative for human organogenesis, pigs and sheep are the desired host species. An initial study showed very limited contribution of human cells into pig chimeric foetuses following blastocyst complementation (Wu *et al.* 2017). The causes of the very limited chimerism are unclear, but they could be due to differences in the developmental stages represented by the stem cells and the host embryo (Mascetti & Pedersen 2016). The type of stem cell and the culture conditions can also determine the viability of the cells in interspecies chimeras (Fu *et al.* 2020, Aksoy *et al.* 2021). It is also noteworthy that the greater evolutionary divergence between pigs and humans (>90 million years) compared to that between mice and rats (<20 million years) renders this approach incompatible under these experimental conditions. However, a better understanding of the signalling pathways and pluripotency features operating in the early embryo could lead to developing better stage-matched complementation strategies. Comparative embryology using scRNASeq has revealed that conventional and naïve human cells are more closely matched to late pig blastocysts, suggesting that more advanced stages of pig development could be better hosts for human cells (Ramos-Ibeas *et al.* 2019).

The combination of strategies aiming at the 'humanization' of pig organs via (i) elimination of pig antigens, (ii) introduction of human immunomodulatory genes, and (iii) genetic ablation of pig organs followed by embryo complementation constitute avenues that are technically possible. Indeed, a recent study shows the generation of human–pig chimeric embryos using a combination of techniques. First, ablation of the *ETV2* gene, a master regulator of haemato-endothelium, was performed in porcine somatic cells. *ETV2* mutant embryos created by NT were then complemented with human-induced pluripotent stem cells (iPSC). Remarkably, the embryos generated contained blood vessels made exclusively of human endothelial cells (Das *et al.* 2020). The colonization of pig embryos was facilitated by the overexpression of *BCL2*, an anti-apoptotic gene. Previous work showed that inhibition of *BCL2* can overcome the staged-related barriers to colonization in chimeric embryos (Masaki *et al.* 2016). More recently, a new approach for increasing interspecies chimerism was reported, based on the creation of *Igf1r* mutant mouse embryos complemented with WT rat ESC (Nishimura *et al.* 2021). This resulted in the generation

of neonatal mice having the extensive contribution of rat cells in diverse organs, predominantly those in which IGF signalling is very important, including the kidney, heart, lung and thymus. Although future studies should focus on the phenotypic analysis of such animals, this work shows the prospect of using pigs for creating organs containing autologous human vasculature, thus greatly reducing the chances of immune rejection.

The impact of gene editing in animal production and welfare

The challenges of improving sustainable animal production whilst meeting the increased demand for healthier and nutritious animal products by the growing world population demand the use of new approaches to enhance the quality and productivity of livestock (Fig. 1). One approach suggested to improve the health benefits of meat consumption is to modify the proportion of unsaturated fats. Since dietary interventions to feed animals with sources of such fats are not environmentally friendly, transgenic approaches could be used. Examples of cattle, sheep and pigs expressing the *C. elegans fat1* desaturase gene in fibroblasts prior to NT have been reported (Lai *et al.* 2006, Wu *et al.* 2012, Zhang *et al.* 2013). These animals had a richer content in omega-3 fatty acids, making them a prime example of a nutraceutical produced by NT. Other examples include the generation of hypoallergenic milk through the abolition of β -lactoglobulin production in cows (Jabed *et al.* 2012), and lactoferrin and lysozyme-containing cow milk (Kaiser *et al.* 2017). However, current regulations for the consumption of products from genetically modified animals are very restrictive.

Another strategy for improving specific traits not reliant on transgenesis is to incorporate specific mutations that can alter animal phenotypes. For example, the naturally occurring mutation at the *MSTN* gene is the cause of the 'double muscle' phenotype in some cattle (Grobet *et al.* 1997, McPherron & Lee 1997) and sheep (Clop *et al.* 2006) breeds, resulting in 20% more muscle mass. These mutations were introduced not only into other sheep and cattle breeds (Proudfoot *et al.* 2015) but also into pigs (Wang *et al.* 2015a) and goats (Wang *et al.* 2015b) by gene editing via NT or zygotic injections. Gene editing offers an alternative means for the accelerated introduction of naturally occurring alleles, albeit in different species. From a regulatory and consumer perspective, the acceptability of such products may be less controversial.

Importantly, herd productivity is also dependant on the robust health status of the animals reared. Gene editing can be used to generate disease-resistant animals. Porcine reproductive and respiratory syndrome virus (PRRSV)-resistant pigs were created following the mutation of *CD163*, which prevents viral infection (Whitworth *et al.* 2016, Burkard *et al.* 2017). Similarly,

transmissible gastroenteritis virus (TGEV)-resistant pigs were created by editing the gene for the putative viral receptor ANPEP (Whitworth *et al.* 2019). Other strategies include the use of gene introgressions to create disease-resistant pigs. The *RELA* gene from the warthog, naturally resistant to African swine fever, was introgressed into domestic pigs (Lillico *et al.* 2016). Although a delayed onset of infection was determined, this introgression did not confer complete protection against the clinical symptoms, suggesting that additional modifications may be needed (McCleary *et al.* 2020). Another recent study reports the use of a CRISPR/Cas9 nickase strategy combined with NT to generate cattle with a targeted insertion of a natural resistance-associated macrophage protein-1 (NRAMP1) expression cassette making them resistant to tuberculosis (Gao *et al.* 2017).

Part of improving production systems will require changes in the ways in which animals are reared. A good example of a step towards improving welfare is the propagation of the *POLLED* genotype across cattle breeds. By using gene editing and NT, *POLLED* alleles were introgressed and resulted in the birth of homozygous polled bulls (Carlson *et al.* 2016), which after crossing with horned cows delivered hornless offspring (Young *et al.* 2020).

Probably the most important goal of future animal production is environmental sustainability. Genetic engineering was thus used to overcome inefficient feed digestion in pigs, which results in excessive release of phosphorus and nitrogen to the environment. Transgenic pigs expressing microbial phytase in the salivary glands had an increased ability to digest phosphorus from dietary phytate and showed a markedly reduced faecal phosphorus concentration (Golovan *et al.* 2001). Recently, this approach was extended to transgenic pigs expressing beta-glucanase, xylanase, and phytase in the salivary glands. As a consequence, digestion of non-starch polysaccharides and phytate was enhanced, faecal nitrogen and phosphorus outputs were reduced (23–45%), and growth rate (~25%) and feed conversion rate (12–15%) were significantly improved (Zhang *et al.* 2018).

These examples demonstrate that favourable traits (e.g. disease resistance and feed conversion efficiency) can be rapidly introduced to improve sustainable production, health and welfare of animal production systems.

Advanced animal breeding and genetic selection

The long generation intervals in domestic animals hinder the progress of genomic selection requiring novel approaches to accelerate the pace at which new animal phenotypes can be created. As discussed above, the combination of robust and safe gene/base-editing methods and reproductive techniques, such as *in vitro* fertilization and NT can drastically accelerate

the rate of genetic gain. However, the bottleneck of meiosis remains. This critical step in the reproductive cycle of animals ensures genetic diversity, however, in breeding programmes, it represents a major hurdle due to the long time needed to generate mature gametes in livestock. Breeders and geneticists have been working on technological solutions to shorten this period for decades. A vision for utilizing *in vitro* systems for growing gametes as a means to reduce generation intervals, also known as 'velogenetics', was proposed in the early '90s when genotype databases and assisted reproduction were beginning to be used (Georges & Massey 1991). More recently, these ideas have resurfaced as a result of developments in genetic selection, stem cell technologies and the possibilities of *in vitro* gamete production in domestic animals (Rexroad *et al.* 2019). *In vitro* breeding (IVB) was proposed as a platform combining the use of quantitative trait loci (QTL) datasets and reproductive techniques as a method of enhanced genetic selection (Goszczynski *et al.* 2019). This approach would yield a ten-fold increase in genetic selection without genetic manipulation. The use of gene/base-editing methods could further enhance the rate of genetic gain of this platform by multiplexing the incorporation of new alleles (Jenko *et al.* 2015).

However, this technology is contingent on a complete *in vitro* system for making gametes. Recent advances in our understanding of gamete development in large mammals are paving the way for the generation of mature gametes (Kobayashi *et al.* 2017). Remarkably, there are close similarities in the transcriptional programme between human, pig and cattle germline development (Soto & Ross 2021, Zhu *et al.* 2021), which offers advantages when it comes to translating findings from one species to another. This is particularly important because progress in human germ cell differentiation shows that mature oogonia can be generated, albeit at very low efficiency (Yamashiro *et al.* 2018). Detailed molecular understanding of the biology of germ cells has also enabled the creation of oocyte-like cells directly from mouse embryonic stem cells by the expression of eight specific transcription factors (Hamazaki *et al.* 2021). These oocyte-like cells were capable of chromosome segregation but failed to undergo normal meiosis. Nevertheless, these advances offer exciting opportunities for adapting some of these conditions for the generation of animal gametes from novel stem cells.

Considering the challenges of accomplishing meiosis *in vitro*, the technology of surrogate sires could serve as an alternative. The idea is to generate males that lack their own spermatogonia, but their testis can act as a developmental niche for spermatogonia from other males. Testis of *NANOS2* KO males support allogeneic spermatogonial transplantation and full spermatogenesis (Ciccarelli *et al.* 2020). The use of this technology could not only significantly increase the genetic merit of sires

used in breeding programs (Gottardo *et al.* 2019) but can also serve as a tool for the genetic preservation of endangered species.

Preservation of endangered species

Nuclear transfer offers an avenue for the rescue of endangered species, provided a compatible cytoplasm can be obtained from closely related species. Some successes were reported with wild African cats and grey wolves, but limited success was reported with an extinct subspecies of goat (Gomez *et al.* 2004, Kim *et al.* 2007, Folch *et al.* 2009). A critical aspect of this approach is the reliance on compatible cytoplasts. Importantly, a major breakthrough in the generation of synthetic cytoplasts from embryonic stem cells was recently reported (Hamazaki *et al.* 2021). Future experiments will determine whether this technology can be used as an alternative source of compatible cytoplasts suitable for nuclear transfer. This platform would offer a reproductive alternative for rescuing species such as the Northern White rhinoceros (Hildebrandt *et al.* 2018). In this case, *in vitro* fertilized embryos and embryonic stem cells have been produced from the last two female specimens of the species. The generation of gametes from these stem cells as well as the generation of cytoplasts would enable the propagation of these last remaining animals by both natural reproduction and nuclear transfer. Although a limited gene pool could represent an obstacle for expanding endangered species, the establishment of induced pluripotent stem cells from frozen tissues/blood could offer an alternative route for the generation of gametes that could be used for *in vitro* breeding.

Concluding remarks

In this review, we summarized the critical impact that nuclear transfer had in facilitating the generation of genetically modified animals over the past 25 years. The impact of this technology has undoubtedly been more significant in livestock species due to the lack of embryonic stem cells, the standard route of gene targeting in mice. Thus, gene modification of donor cells prior to NT has been the preferred avenue for creating genetically modified livestock. Although the technique remains quite labour intensive, significant progress has been made in the procedures resulting in better efficiency. New sources of livestock embryonic stem cells have been reported, suggesting that gene manipulation and chimera generation may be simplified in the future. However, the long generation interval of livestock species makes the process of breeding to homozygosity of chimeric animals a lengthy and expensive process compared to mice. In contrast, NT allows the instant generation of transgenic animals, suggesting that it will remain a valuable technology for years to come.

We have come a long way since this landmark experiment, with improved knowledge of the molecular mechanisms of cellular reprogramming and more efficient techniques of NT. We can look forward to the next 25 years in which NT, in combination with other modern gene manipulation technologies, can offer solutions to urgent biomedical needs, improve sustainable animal production and facilitate species conservation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (grant number BB/S000178/1) to R A and by the German Research Council (DFG; TRR127) and the German Center for Diabetes Research (DZD; 82DZD00802) to E W.

Author contribution statement

RA and EW performed literature research and wrote the manuscript. EW prepared the figure and table. Both authors reviewed and approved the final version of the article.

Acknowledgements

The authors apologize to authors whose work was not cited due to space limitations. RA states that writing this review brought back many memories of the excitement and the mystique that surrounded the *Dolly* the sheep experiment. Doubts were soon dispelled and a race to develop a better understanding of NT started in many labs around the world. The author having just started his PhD studies (RA), struggled to fully envision the impact of the discovery, but realized the magnitude of the findings for the understanding of cell plasticity. Only 2 years after starting as Chair for Molecular Animal Breeding and Biotechnology at LMU Munich (EW), he was incredibly lucky to have Valeri Zakhartchenko in the team who was among the first worldwide to establish NT in bovine. EW is also very grateful to Hiroshi Nagashima for sharing his porcine NT technology that was the basis of their pipeline of genetically modified pig models.

References

- Aksoy I, Rognard C, Moulin A, Marcy G, Masfaraud E, Wianny F, Cortay V, Bellemin-Menard A, Doerflinger N, Dirheimer M *et al.* 2021 Apoptosis, G1 phase stall, and premature differentiation account for low chimeric competence of human and rhesus monkey naive pluripotent stem cells. *Stem Cell Reports* **16** 56–74. (<https://doi.org/10.1016/j.stemcr.2020.12.004>)
- Alberio R, Campbell KH & Johnson AD 2006 Reprogramming somatic cells into stem cells. *Reproduction* **132** 709–720. (<https://doi.org/10.1530/rep.1.01077>)

- Anzalone AV, Koblan LW & Liu DR 2020 Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nature Biotechnology* **38** 824–844. (<https://doi.org/10.1038/s41587-020-0561-9>)
- Bartlett JA, Ramachandran S, Wohlford-Lenane CL, Barker CK, Pezzulo AA, Zabner J, Welsh MJ, Meyerholz DK, Stoltz DA & McCray Jr PB 2016 Newborn cystic fibrosis pigs have a blunted early response to an inflammatory stimulus. *American Journal of Respiratory and Critical Care Medicine* **194** 845–854. (<https://doi.org/10.1164/rccm.201510-2112OC>)
- Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, Miyanochara A, Nejime T, Klima J, Macakova M *et al.* 2013 A transgenic minipig model of Huntington's disease. *Journal of Huntington's Disease* **2** 47–68. (<https://doi.org/10.1233/JHD-130001>)
- Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, Whitelaw CB & Archibald AL 2017 Precision engineering for PRRSV resistance in pigs: macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. *PLoS Pathogens* **13** e1006206. (<https://doi.org/10.1371/journal.ppat.1006206>)
- Caballero I, Ringot-Destrez B, Si-Tahar M, Barbry P, Guillon A, Lantier I, Berri M, Chevalerey C, Fleuret I, Barc C *et al.* 2021 Evidence of early increased sialylation of airway mucins and defective mucociliary clearance in CFTR-deficient piglets. *Journal of Cystic Fibrosis* **20** 173–182. (<https://doi.org/10.1016/j.jcf.2020.09.009>)
- Campbell KH & Alberio R 2003 Reprogramming the genome: role of the cell cycle. *Reproduction* **61** 477–494. (<https://doi.org/10.1530/bioscioprocs.5.035>)
- Campbell KH, Ritchie WA & Wilmut I 1993 Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. *Biology of Reproduction* **49** 933–942. (<https://doi.org/10.1095/biolreprod49.5.933>)
- Campbell KH, Loi P, Otaegui PJ & Wilmut I 1996a Cell cycle co-ordination in embryo cloning by nuclear transfer. *Reviews of Reproduction* **1** 40–46. (<https://doi.org/10.1530/ror.0.0010040>)
- Campbell KH, McWhir J, Ritchie WA & Wilmut I 1996b Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380** 64–66. (<https://doi.org/10.1038/380064a0>)
- Carlson DF, Lancto CA, Zang B, Kim ES, Walton M, Oldeschulte D, Seabury C, Sonstegard TS & Fahrenkrug SC 2016 Production of hornless dairy cattle from genome-edited cell lines. *Nature Biotechnology* **34** 479–481. (<https://doi.org/10.1038/nbt.3560>)
- Ciccarelli M, Giassetti MI, Miao D, Oatley MJ, Robbins C, Lopez-Biladeau B, Waqas MS, Tibary A, Whitelaw B, Lillico S *et al.* 2020 Donor-derived spermatogenesis following stem cell transplantation in sterile NANOS2 knockout males. *PNAS* **117** 24195–24204. (<https://doi.org/10.1073/pnas.2010102117>)
- Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, Bouix J, Caiment F, Elsen JM, Eychenne F *et al.* 2006 A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics* **38** 813–818. (<https://doi.org/10.1038/ng1810>)
- Cooper DK, Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab MB & Schuurman HJ 2014 Progress in pig-to-non-human primate transplantation models (1998–2013): a comprehensive review of the literature. *Xenotransplantation* **21** 397–419. (<https://doi.org/10.1111/xen.12127>)
- Das S, Koyano-Nakagawa N, Gafni O, Maeng G, Singh BN, Rasmussen T, Pan X, Choi KD, Mickelson D, Gong W *et al.* 2020 Generation of human endothelium in pig embryos deficient in ETV2. *Nature Biotechnology* **38** 297–302. (<https://doi.org/10.1038/s41587-019-0373-y>)
- Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S & Smithies O 1987 Targeted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330** 576–578. (<https://doi.org/10.1038/330576a0>)
- Evans MJ & Kaufman MH 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292** 154–156. (<https://doi.org/10.1038/292154a0>)
- Folch J, Cocero MJ, Chesne P, Alabart JL, Dominguez V, Cognie Y, Roche A, Fernandez-Arias A, Marti JJ, Sanchez P *et al.* 2009 First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning. *Theriogenology* **71** 1026–1034. (<https://doi.org/10.1016/j.theriogenology.2008.11.005>)
- Fu R, Yu D, Ren J, Li C, Wang J, Feng G, Wang X, Wan H, Li T, Wang L *et al.* 2020 Domesticated cynomolgus monkey embryonic stem cells allow the generation of neonatal interspecies chimeric pigs. *Protein and Cell* **11** 97–107. (<https://doi.org/10.1007/s13238-019-00676-8>)
- Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q, Cui C, Liu X, Zhang J & Zhang Y 2017 Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. *Genome Biology* **18** 13. (<https://doi.org/10.1186/s13059-016-1144-4>)
- Gao X, Nowak-Imialek M, Chen X, Chen D, Herrmann D, Ruan D, Chen ACH, Eckersley-Maslin MA, Ahmad S, Lee YL *et al.* 2019 Establishment of porcine and human expanded potential stem cells. *Nature Cell Biology* **21** 687–699. (<https://doi.org/10.1038/s41556-019-0333-2>)
- Georges M & Massey JM 1991 Velogenetics, or the synergistic use of marker assisted selection and germ-line manipulation. *Theriogenology* **35** 151–159. ([https://doi.org/10.1016/0093-691X\(91\)90154-6](https://doi.org/10.1016/0093-691X(91)90154-6))
- Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard JW, Fan MZ, Hayes MA *et al.* 2001 Pigs expressing salivary phytase produce low-phosphorus manure. *Nature Biotechnology* **19** 741–745. (<https://doi.org/10.1038/90788>)
- Gomez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King AL, Cole A, Godke RA & Dresser BL 2004 Birth of African Wildcat cloned kittens born from domestic cats. *Cloning and Stem Cells* **6** 247–258. (<https://doi.org/10.1089/clo.2004.6.247>)
- Goszczynski DE, Cheng H, Demyda-Peyras S, Medrano JF, Wu J & Ross PJ 2019 In vitro breeding: application of embryonic stem cells to animal production. *Biological Reproduction* **100** 885–895. (<https://doi.org/10.1093/biolre/boy256>)
- Gottardo P, Gorjanc G, Battagin M, Gaynor RC, Jenko J, Ros-Freixedes R, Bruce A, Whitelaw C, Mileham AJ, Herring WO & Hickey JM 2019 A strategy to exploit surrogate sire technology in livestock breeding programs. *G3* **9** 203–215. (<https://doi.org/10.1534/g3.118.200890>)
- Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J *et al.* 1997 A deletion in the bovine myostatin gene causes the double-muscling phenotype in cattle. *Nature Genetics* **17** 71–74. (<https://doi.org/10.1038/ng0997-71>)
- Gurdon JB & Uehlinger V 1966 'Fertile' intestine nuclei. *Nature* **210** 1240–1241. (<https://doi.org/10.1038/2101240a0>)
- Halley-Stott RP, Pasque V & Gurdon JB 2013 Nuclear reprogramming. *Development* **140** 2468–2471. (<https://doi.org/10.1242/dev.092049>)
- Hamazaki N, Kyogoku H, Araki H, Miura F, Horikawa C, Hamada N, Shimamoto S, Hikabe O, Nakashima K, Kitajima TS *et al.* 2021 Reconstitution of the oocyte transcriptional network with transcription factors. *Nature* **589** 264–269. (<https://doi.org/10.1038/s41586-020-3027-9>)
- Hildebrandt TB, Hermes R, Colleoni S, Diecke S, Holtze S, Renfree MB, Stejskal J, Hayashi K, Drukker M, Loi P *et al.* 2018 Embryos and embryonic stem cells from the white rhinoceros. *Nature Communications* **9** 2589. (<https://doi.org/10.1038/s41467-018-04959-2>)
- Hinrichs A, Riedel EO, Klymiuk N, Blutke A, Kemter E, Langin M, Dahlhoff M, Kessler B, Kurome M, Zakharchenko V *et al.* 2021 Growth hormone receptor knockout to reduce the size of donor pigs for preclinical xenotransplantation studies. *Xenotransplantation* **28** e12664. (<https://doi.org/10.1111/xen.12664>)
- Hirata M, Wittayarat M, Namula Z, Le QA, Lin Q, Nguyen NT, Takebayashi K, Sato Y, Tanihara F & Otoi T 2020 Evaluation of multiple gene targeting in porcine embryos by the CRISPR/Cas9 system using electroporation. *Molecular Biology Reports* **47** 5073–5079. (<https://doi.org/10.1007/s11033-020-05576-3>)
- Holm IE, Alstrup AK & Luo Y 2016 Genetically modified pig models for neurodegenerative disorders. *Journal of Pathology* **238** 267–287. (<https://doi.org/10.1002/path.4654>)
- Iwase H, Hara H, Ezzelarab M, Li T, Zhang Z, Gao B, Liu H, Long C, Wang Y, Cassano A *et al.* 2017a Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts. *Xenotransplantation* **24** e12293 (<https://doi.org/10.1111/xen.12293>)
- Iwase H, Liu H, Schmelzer E, Ezzelarab M, Wijkstrom M, Hara H, Lee W, Singh J, Long C, Lagasse E *et al.* 2017b Transplantation of hepatocytes

- from genetically engineered pigs into baboons. *Xenotransplantation* **24** e12289. (<https://doi.org/10.1111/xen.12289>)
- Jabed A, Wagner S, McCracken J, Wells DN & Laible G 2012 Targeted microRNA expression in dairy cattle directs production of beta-lactoglobulin-free, high-casein milk. *PNAS* **109** 16811–16816. (<https://doi.org/10.1073/pnas.1210057109>)
- Jenko J, Gorjanc G, Cleveland MA, Varshney RK, Whitelaw CB, Woolliams JA & Hickey JM 2015 Potential of promotion of alleles by genome editing to improve quantitative traits in livestock breeding programs. *Genetics, Selection, Evolution* **47** 55. (<https://doi.org/10.1186/s12711-015-0135-3>)
- Kaiser GG, Mucci NC, Gonzalez V, Sanchez L, Parron JA, Perez MD, Calvo M, Aller JF, Hozbor FA & Mutto AA 2017 Detection of recombinant human lactoferrin and lysozyme produced in a bitransgenic cow. *Journal of Dairy Science* **100** 1605–1617. (<https://doi.org/10.3168/jds.2016-11173>)
- Kemter E, Schnieke A, Fischer K, Cowan PJ & Wolf E 2020 Xeno-organ donor pigs with multiple genetic modifications – the more the better? *Current Opinion in Genetics and Development* **64** 60–65. (<https://doi.org/10.1016/j.gde.2020.05.034>)
- Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hwang WS, Hossein MS, Kim JJ, Shin NS, Kang SK *et al.* 2007 Endangered wolves cloned from adult somatic cells. *Cloning and Stem Cells* **9** 130–137. (<https://doi.org/10.1089/clo.2006.0034>)
- Kim SC, Mathews DV, Breeden CP, Higginbotham LB, Ladowski J, Martens G, Stephenson A, Farris AB, Strobert EA, Jenkins J *et al.* 2019 Long-term survival of pig-to-rhesus macaque renal xenografts is dependent on CD4 T cell depletion. *American Journal of Transplantation* **19** 2174–2185. (<https://doi.org/10.1111/ajt.15329>)
- King TJ & Briggs R 1955 Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. *PNAS* **41** 321–325. (<https://doi.org/10.1073/pnas.41.5.321>)
- Klymiuk N, Blutke A, Graf A, Krause S, Burkhardt K, Wuensch A, Krebs S, Kessler B, Zakhartchenko V, Kurome M *et al.* 2013 Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Human Molecular Genetics* **22** 4368–4382. (<https://doi.org/10.1093/hmg/ddt287>)
- Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, Sato H, Lee YS, Usui J, Knisely AS *et al.* 2010 Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* **142** 787–799. (<https://doi.org/10.1016/j.cell.2010.07.039>)
- Kobayashi T, Zhang H, Tang WWC, Irie N, Withey S, Klisch D, Sybirna A, Dietmann S, Contreras DA, Webb R *et al.* 2017 Principles of early human development and germ cell program from conserved model systems. *Nature* **546** 416–420. (<https://doi.org/10.1038/nature22812>)
- Kogel J & Marckmann G 2020 'Xenotransplantation challenges us as a society': what well-informed citizens think about xenotransplantation. *EMBO Reports* **21** e50274. (<https://doi.org/10.15252/embr.202050274>)
- Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A *et al.* 2006 Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nature Biotechnology* **24** 435–436. (<https://doi.org/10.1038/nbt1198>)
- Lamas-Toranzo I, Galiano-Cogolludo B, Cornudella-Ardiaca F, Cobos-Figueroa J, Ousinde O & Bermejo-Alvarez P 2019 Strategies to reduce genetic mosaicism following CRISPR-mediated genome editing in bovine embryos. *Scientific Reports* **9** 14900. (<https://doi.org/10.1038/s41598-019-51366-8>)
- Lamas-Toranzo I, Martínez-Moro A, O Callaghan E, Millán-Blanca G, Sánchez JM, Lonergan P & Bermejo-Álvarez P 2020 RS-1 enhances CRISPR-mediated targeted knock-in in bovine embryos. *Molecular Reproduction and Development* **87** 542–549. (<https://doi.org/10.1002/mrd.23341>)
- Langin M, Mayr T, Reichart B, Michel S, Buchholz S, Guethoff S, Dashkevich A, Baehr A, Egerer S, Bauer A *et al.* 2018 Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature* **564** 430–433. (<https://doi.org/10.1038/s41586-018-0765-z>)
- Lillico SG, Proudfoot C, King TJ, Tan W, Zhang L, Mardjuki R, Paschon DE, Rebar EJ, Urnov FD, Mileham AJ *et al.* 2016 Mammalian interspecies substitution of immune modulatory alleles by genome editing. *Scientific Reports* **6** 21645. (<https://doi.org/10.1038/srep21645>)
- Martin GR 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS* **78** 7634–7638. (<https://doi.org/10.1073/pnas.78.12.7634>)
- Masaki H, Kato-Itoh M, Takahashi Y, Umino A, Sato H, Ito K, Yanagida A, Nishimura T, Yamaguchi T, Hirabayashi M *et al.* 2016 Inhibition of apoptosis overcomes stage-related compatibility barriers to chimera formation in mouse embryos. *Cell Stem Cell* **19** 587–592. (<https://doi.org/10.1016/j.stem.2016.10.013>)
- Mascetti VL & Pedersen RA 2016 Contributions of mammalian chimeras to pluripotent stem cell research. *Cell Stem Cell* **19** 163–175. (<https://doi.org/10.1016/j.stem.2016.07.018>)
- McCleary S, Strong R, McCarthy RR, Edwards JC, Howes EL, Stevens LM, Sanchez-Cordon PJ, Nunez A, Watson S, Mileham AJ *et al.* 2020 Substitution of warhog NF-kappaB motifs into RELA of domestic pigs is not sufficient to confer resilience to African swine fever virus. *Scientific Reports* **10** 8951. (<https://doi.org/10.1038/s41598-020-65808-1>)
- McPherron AC & Lee SJ 1997 Double muscling in cattle due to mutations in the myostatin gene. *PNAS* **94** 12457–12461. (<https://doi.org/10.1073/pnas.94.23.12457>)
- Mohiuddin MM, Singh AK, Corcoran PC, Thomas Iii ML, Clark T, Lewis BG, Hoyt RF, Eckhaus M, Pierson Iii RN, Belli AJ *et al.* 2016 Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nature Communications* **7** 11138. (<https://doi.org/10.1038/ncomms11138>)
- Moretti A, Fonteyne L, Giesert F, Hoppmann P, Meier AB, Bozoglu T, Baehr A, Schneider CM, Sinnecker D, Klett K *et al.* 2020 Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy. *Nature Medicine* **26** 207–214. (<https://doi.org/10.1038/s41591-019-0738-2>)
- Nambiar TS, Billon P, Diedenhofen G, Hayward SB, Taglialatela A, Cai K, Huang JW, Leuzzi G, Cuella-Martin R, Palacios A *et al.* 2019 Stimulation of CRISPR-mediated homology-directed repair by an engineered RAD18 variant. *Nature Communications* **10** 3395. (<https://doi.org/10.1038/s41467-019-11105-z>)
- Nishimura T, Suchy FP, Bhadury J, Igarashi KJ, Charlesworth CT & Nakauchi H 2021 Generation of functional organs using a cell-competitive niche in intra- and inter-species rodent chimeras. *Cell Stem Cell* **28** 141–149.e3. (<https://doi.org/10.1016/j.stem.2020.11.019>)
- Park KE, Powell A, Sandmaier SE, Kim CM, Mileham A, Donovan DM & Telugu BP 2017 Targeted gene knock-in by CRISPR/Cas ribonucleoproteins in porcine zygotes. *Scientific Reports* **7** 42458. (<https://doi.org/10.1038/srep42458>)
- Perleberg C, Kind A & Schnieke A 2018 Genetically engineered pigs as models for human disease. *Disease Models and Mechanisms* **11** dmm030783. (<https://doi.org/10.1242/dmm.030783>)
- Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, Lillico SG, Mileham AJ, McLaren DG, Whitelaw CB *et al.* 2015 Genome edited sheep and cattle. *Transgenic Research* **24** 147–153. (<https://doi.org/10.1007/s11248-014-9832-x>)
- Ramos-Ibeas P, Sang F, Zhu Q, Tang WWC, Withey S, Klisch D, Wood L, Loose M, Surani MA & Alberio R 2019 Pluripotency and X chromosome dynamics revealed in pig pre-gastrulating embryos by single cell analysis. *Nature Communications* **10** 500. (<https://doi.org/10.1038/s41467-019-08387-8>)
- Regensburger AP, Fonteyne LM, Jungert J, Wagner AL, Gerhalter T, Nagel AM, Heiss R, Flenkenthaler F, Qurashi M, Neurath MF *et al.* 2019 Detection of collagens by multispectral optoacoustic tomography as an imaging biomarker for Duchenne muscular dystrophy. *Nature Medicine* **25** 1905–1915. (<https://doi.org/10.1038/s41591-019-0669-y>)
- Reichart B, Langin M, Denner J, Schwinzer R, Cowan PJ & Wolf E 2020 Pathways to clinical cardiac xenotransplantation. *Transplantation In press*. (<https://doi.org/10.1097/TP.0000000000003588>)
- Renner S, Fehlings C, Herbach N, Hofmann A, von Waldthausen DC, Kessler B, Ulrichs K, Chodnevskaja I, Moskalenko V, Amselgruber W *et al.* 2010 Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes* **59** 1228–1238. (<https://doi.org/10.2337/db09-0519>)
- Renner S, Blutke A, Streckel E, Wanke R & Wolf E 2016 Incretin actions and consequences of incretin-based therapies: lessons from complementary animal models. *Journal of Pathology* **238** 345–358. (<https://doi.org/10.1002/path.4655>)
- Renner S, Blutke A, Clauss S, Deeg CA, Kemter E, Merkus D, Wanke R & Wolf E 2020 Porcine models for studying complications and organ

- crosstalk in diabetes mellitus. *Cell and Tissue Research* **380** 341–378. (<https://doi.org/10.1007/s00441-019-03158-9>)
- Rexroad C, Vallet J, Matukumalli LK, Reecy J, Bickhart D, Blackburn H, Boggess M, Cheng H, Clutter A, Cockett N *et al.* 2019 Genome to phenome: improving animal health, production, and well-being – a new USDA Blueprint for Animal Genome Research 2018–2027. *Front Genet* **10** 327.
- Rieblinger B, Sid H, Duda D, Bozoglu T, Klinger R, Schlickerrieder A, Lengyel K, Flisikowski K, Flisikowska T, Simm N *et al.* 2021 Cas9-expressing chickens and pigs as resources for genome editing in livestock. *PNAS* **118** e2022562118. (<https://doi.org/10.1073/pnas.2022562118>)
- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA *et al.* 2008 Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* **321** 1837–1841. (<https://doi.org/10.1126/science.1163600>)
- Schneider JW, Oommen S, Qureshi MY, Goetsch SC, Pease DR, Sundsbak RS, Guo W, Sun M, Sun H, Kuroyanagi H *et al.* 2020 Dysregulated ribonucleoprotein granules promote cardiomyopathy in RBM20 gene-edited pigs. *Nature Medicine* **26** 1788–1800. (<https://doi.org/10.1038/s41591-020-1087-x>)
- Sims M & First NL 1994 Production of calves by transfer of nuclei from cultured inner cell mass cells. *PNAS* **91** 6143–6147. (<https://doi.org/10.1073/pnas.91.13.6143>)
- Sinclair KD, Corr SA, Gutierrez CG, Fisher PA, Lee JH, Rathbone AJ, Choi I, Campbell KH & Gardner DS 2016 Healthy ageing of cloned sheep. *Nature Communications* **7** 12359. (<https://doi.org/10.1038/ncomms12359>)
- Smith LD 1965 Transplantation of the nuclei of primordial germ cells into enucleated eggs of *Rana pipiens*. *PNAS* **54** 101–107. (<https://doi.org/10.1073/pnas.54.1.101>)
- Soto DA & Ross PJ 2021 Similarities between bovine and human germline development revealed by single-cell RNAseq. *Reproduction* **161** 239–253. (<https://doi.org/10.1530/REP-20-0313>)
- Spemann H 1938 Embryonic development and induction. In *American Journal of the Medical Sciences*, p. 401. Ed H Milford. New Haven: Oxford University Press. (<https://doi.org/10.1097/0000441-193811000-00047>)
- Sykes M & Sachs DH 2019 Transplanting organs from pigs to humans. *Science Immunology* **4** eaau6298. (<https://doi.org/10.1126/sciimmunol.aau6298>)
- Tanihara F, Takemoto T, Kitagawa E, Rao S, Do LT, Onishi A, Yamashita Y, Kosugi C, Suzuki H, Sembon S *et al.* 2016 Somatic cell reprogramming-free generation of genetically modified pigs. *Science Advances* **2** e1600803. (<https://doi.org/10.1126/sciadv.1600803>)
- Thomas KR & Capecchi MR 1987 Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51** 503–512. ([https://doi.org/10.1016/0092-8674\(87\)90646-5](https://doi.org/10.1016/0092-8674(87)90646-5))
- Wakayama S, Kohda T, Obokata H, Tokoro M, Li C, Terashita Y, Mizutani E, Nguyen VT, Kishigami S, Ishino F *et al.* 2013 Successful serial recloning in the mouse over multiple generations. *Cell Stem Cell* **12** 293–297. (<https://doi.org/10.1016/j.stem.2013.01.005>)
- Wang K, Ouyang H, Xie Z, Yao C, Guo N, Li M, Jiao H & Pang D 2015a Efficient generation of myostatin mutations in pigs using the CRISPR/Cas9 system. *Scientific Reports* **5** 16623. (<https://doi.org/10.1038/srep16623>)
- Wang X, Yu H, Lei A, Zhou J, Zeng W, Zhu H, Dong Z, Niu Y, Shi B, Cai B *et al.* 2015b Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. *Scientific Reports* **5** 13878. (<https://doi.org/10.1038/srep13878>)
- Weismann A, Poulton EBS & Shipley AES 1889 Essays upon heredity and kindred biological problems. Authorised translation, edited by E. B. Poulton, S. Schönland, and A. E. Shipley. Oxford, Clarendon Press, 1891–1892.
- Whitworth KM, Rowland RR, Ewen CL, Tribble BR, Kerrigan MA, Cino-Ozuna AG, Samuel MS, Lightner JE, McLaren DG, Mileham AJ *et al.* 2016 Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nature Biotechnology* **34** 20–22. (<https://doi.org/10.1038/nbt.3434>)
- Whitworth KM, Rowland RRR, Petrovan V, Sheahan M, Cino-Ozuna AG, Fang Y, Hesse R, Mileham A, Samuel MS, Wells KD *et al.* 2019 Resistance to coronavirus infection in amino peptidase N-deficient pigs. *Transgenic Research* **28** 21–32. (<https://doi.org/10.1007/s11248-018-0100-3>)
- Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KH 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385** 810–813. (<https://doi.org/10.1038/385810a0>)
- Wu X, Ouyang H, Duan B, Pang D, Zhang L, Yuan T, Xue L, Ni D, Cheng L, Dong S *et al.* 2012 Production of cloned transgenic cow expressing omega-3 fatty acids. *Transgenic Research* **21** 537–543. (<https://doi.org/10.1007/s11248-011-9554-2>)
- Wu J, Platero-Luengo A, Sakurai M, Sugawara A, Gil MA, Yamauchi T, Suzuki K, Bogliotti YS, Cuella C, Morales Valencia M *et al.* 2017 Interspecies chimerism with mammalian pluripotent stem cells. *Cell* **168** 473.e15–486.e15. (<https://doi.org/10.1016/j.cell.2016.12.036>)
- Yamaguchi T, Sato H, Kato-Itoh M, Goto T, Hara H, Sanbo M, Mizuno N, Kobayashi T, Yanagida A, Umino A *et al.* 2017 Interspecies organogenesis generates autologous functional islets. *Nature* **542** 191–196. (<https://doi.org/10.1038/nature21070>)
- Yamashiro C, Sasaki K, Yabuta Y, Kojima Y, Nakamura T, Okamoto I, Yokobayashi S, Murase Y, Ishikura Y, Shirane K *et al.* 2018 Generation of human oogonia from induced pluripotent stem cells in vitro. *Science* **362** 356–360. (<https://doi.org/10.1126/science.aat1674>)
- Yang D, Wang CE, Zhao B, Li W, Ouyang Z, Liu Z, Yang H, Fan P, O'Neill A, Gu W *et al.* 2010 Expression of Huntington's disease protein results in apoptotic neurons in the brains of cloned transgenic pigs. *Human Molecular Genetics* **19** 3983–3994. (<https://doi.org/10.1093/hmg/ddq313>)
- Young AE, Mansour TA, McNabb BR, Owen JR, Trott JF, Brown CT & Van Eenennaam AL 2020 Genomic and phenotypic analyses of six offspring of a genome-edited hornless bull. *Nature Biotechnology* **38** 225–232. (<https://doi.org/10.1038/s41587-019-0266-0>)
- Yu HH, Zhao H, Qing YB, Pan WR, Jia BY, Zhao HY, Huang XX & Wei HJ 2016 Porcine zygote injection with Cas9/sgRNA results in DMD-modified pig with muscle dystrophy. *International Journal of Molecular Sciences* **17** 1668. (<https://doi.org/10.3390/ijms17101668>)
- Yu L, Wei Y, Sun HX, Mahdi AK, Pinzon Arteaga CA, Sakurai M, Schmitz DA, Zheng C, Ballard ED, Li J *et al.* 2020 Derivation of intermediate pluripotent stem cells amenable to primordial germ cell specification. *Cell Stem Cell* **28** 550.e12–567.e12. (<https://doi.org/10.1016/j.stem.2020.11.003>)
- Yue Y, Xu W, Kan Y, Zhao HY, Zhou Y, Song X, Wu J, Xiong J, Goswami D, Yang M *et al.* 2020 Extensive germline genome engineering in pigs. *Nature Biomedical Engineering* **5** 134–143. (<https://doi.org/10.1038/s41551-020-00613-9>)
- Zhang P, Liu P, Dou H, Chen L, Chen L, Lin L, Tan P, Vajta G, Gao J, Du Y *et al.* 2013 Handmade cloned transgenic sheep rich in omega-3 fatty acids. *PLoS ONE* **8** e55941. (<https://doi.org/10.1371/journal.pone.0055941>)
- Zhang X, Li Z, Yang H, Liu D, Cai G, Li G, Mo J, Wang D, Zhong C, Wang H *et al.* 2018 Novel transgenic pigs with enhanced growth and reduced environmental impact. *eLife* **7** e34286. (<https://doi.org/10.7554/eLife.34286>)
- Zhu Q, Sang F, Withey S, Tang W, Dietmann S, Klisch D, Ramos-Ibeas P, Zhang H, Requena CE, Hajkova P *et al.* 2021 Specification and epigenomic resetting of the pig germline exhibit conservation with the human lineage. *Cell Reports* **34** 108735. (<https://doi.org/10.1016/j.celrep.2021.108735>)
- Zou L, Zhang Y, He Y, Yu H, Chen J, Liu D, Lin S, Gao M, Zhong G, Lei W *et al.* 2020 Selective germline genome edited pigs and their long immune tolerance in non human primates. *bioRxiv* 2020.2001.2020.912105.
- Zuccaro MV, Xu J, Mitchell C, Marin D, Zimmerman R, Rana B, Weinstein E, King RT, Palmerola KL, Smith ME *et al.* 2020 Allele-specific chromosome removal after Cas9 cleavage in human embryos. *Cell* **183** 1650.e15–1664.e15. (<https://doi.org/10.1016/j.cell.2020.10.025>)

Received 22 February 2021

First decision 15 March 2021

Revised manuscript received 15 April 2021

Accepted 4 May 2021