Chapter 3
Generation of Gene Edited Pigs

S. Navarro-Serna, C. Piñeiro-Silva, R. Romar, J. Parrington, and J. Gadea

Abstract

Background:

The porcine species (*Sus scrofa domesticus*) has had a great impact as a source of food worldwide, but also it is a very useful animal for biomedical applications. Genetic engineering involves modifying the sequence of DNA. Nowadays, genetic engineering is a common procedure in many laboratories, in part due to the development of simple, accessible, cheap, and effective programmable endonucleases such as those in the CRISPR/Cas9 approach. A key advantage of gene editing is the possibility of producing pigs with desired characteristics

Mayor Advances:

Here we review the latest advances in the production of genetically modified pigs with a particular focus on the use of CRISPR/Cas9 gene editing in the porcine species, as a way to produce genetically modified pigs, and with a consideration of advantages and limitations, as well as new approaches, with regard to this technology.

Keywords Endonucleases · Embryo microinjection · Oocyte electroporation · Somatic cell nuclear transfer · Disease resistance · Animal production · Human disease bio-models · Bioproducts

---

Authors Sergio Navarro-Serna and Celia Piñeiro-Silva have contributed equally to this work.

S. Navarro-Serna · R. Romar · J. Gadea (✉)
Physiology of Reproduction Group, Department of Physiology, Universidad de Murcia, Murcia, Spain

Instituto Murciano de Investigación Biosanitaria, IMIB-Arrixaca-UMU, Murcia, Spain
e-mail: jgadea@um.es

C. Piñeiro-Silva
Physiology of Reproduction Group, Department of Physiology, Universidad de Murcia, Murcia, Spain

J. Parrington
Department of Pharmacology, University of Oxford, Oxford, UK

© The Author(s), under exclusive license to Springer Nature Switzerland AG 2022
V. K. Yata et al. (eds.), *Sustainable Agriculture Reviews 57*, Sustainable Agriculture Reviews 57, https://doi.org/10.1007/978-3-031-07496-7_3

3.1 Introduction

The porcine species (*Sus scrofa domesticus*) has had a great impact in the meat industry worldwide, being the second most consumed meat source in the world in 2020 after poultry according to the Food and Agriculture Organization of the United Nations (FAO 2020). Since its domestication 9000 years ago from the wild boar (*Sus scrofa*) (Giuffra et al. 2000), humans have been selecting for breeding those porcine variants with desired characteristics for food production and animal husbandry. This genetic selection has most recently allowed a significant increase of pig production in comparison with that obtained a few decades ago (Rocadembosch et al. 2016; Koketsu et al. 2017). However, classical breeding and genetic selection techniques are slow processes that carry an economic and time cost which can be avoided by the use of genetic engineering (Yang and Wu 2018). In recent decades, techniques have been developed to accelerate genetic selection by gene editing. Genetic engineering involves modifying the sequence of DNA, which makes it possible to introduce new genes or change the pattern of gene expression in edited cells, including modifications in sequences that encode proteins, regulatory sequences or sequences that produce non-coding RNAs (Yang and Wu 2018).

Nowadays, genetic engineering is a common procedure in many laboratories, in part due to the development of simple, accessible, cheap, and effective programmable endonucleases such as those in the CRISPR/Cas9 approach. The contribution of Jennifer Doudna and Emmanuelle Charpentier to the development of this approach, discovered in microorganisms but now used to edit the genome of practically any organism (Jinek et al. 2012) was acknowledged by the award of the Nobel Prize in Chemistry to them in 2020 and has opened the door to easier genetic modification of mammals, and specifically in our case, of pigs.

Since the first generation of gene edited pigs using CRISPR/Cas9 in 2014 (Hai et al. 2014), new findings have been published relating to strategies for the efficient production of gene edited pigs. A key advantage of gene editing is the possibility of producing pigs with desired characteristics, for example, pigs with better meat production (Wang et al. 2015a), less fat deposition (Zheng et al. 2017) or resistance to viral diseases (Whitworth et al. 2016), far more rapidly than would be possible using traditional breeding methods. Furthermore, genetically modified pigs can be produced for use in biomedical research due to the similarity of pigs and humans in relation to physiology, anatomy, size, and metabolic profile (Zettler et al. 2020). In addition, pigs have advantages over other animal models like non-human primates because they are cheaper to produce, are associated with less ethical issues, and because of better development of porcine embryo manipulation techniques (Yang and Wu 2018). Reflecting this, porcine models of human diseases like diabetes mellitus (Tanihara et al. 2020b; Zettler et al. 2020), neuromuscular diseases (Crociara et al. 2019) and ones for xenotransplantation (Hein et al., 2020), have recently been produced.

In recent reported studies, differences in procedure have been found with respect to the type of methodology and the strategies used to obtain modified embryos, the molecular nature of Cas9, the method and conditions of CRISPR/Cas9 delivery into embryos, and the embryo transfer protocol. Up till now, there has not been a standard procedure for generation of gene edited pigs, so it is very important to study all the factors that have had an influence on the efficiency of this technique, to determine what are the best options in the design of protocols to optimize the CRISPR/Cas9 approach. For that reason, the objective of this chapter is to summarize the knowledge about the advances of production of genetically modified pigs with the use of CRISPR/Cas9 system in the porcine species, focusing on the production of genetically modified pigs, its advantages and limitations, as well as the new approaches for this system.

3.2 History of Gene Editing in Pigs

3.2.1 Gene Editing Before Programmable Endonucleases

The first strategy to produce genetically modified mammals was pronuclear injection which involves the direct introduction of a DNA construct into the pronuclei of the fertilized egg that can integrate randomly in the genome of the zygote. Using this approach, transgenic mice were produced in the early 1980s (Gordon et al. 1980; Gordon and Ruddle 1981; Palmiter et al. 1982) followed by other mammals such as pigs, sheep or rabbits in 1985 (Brem et al. 1985; Hammer et al. 1985) (Fig. 3.1). Other approaches used to introduce foreign DNA into pig embryos were developed, such as the use of retroviruses (Petters et al. 1987), lentiviruses (Hofmann et al. 2003) or sperm-mediated DNA transfer (Sperandio et al. 1996; Lavitrano et al. 1997; Garcia-Vazquez et al. 2010). These approaches were limited by the fact that the foreign DNA randomly inserted into the host genome and did not allow the specific editing of endogenous genes, so other approaches were needed. In mice, homologous recombination was used to genetically modify embryonic stem cells (ESCs) in culture and these were then introduced into early embryos to produce chimeras. Despite the low efficiency of this approach, the generation of genetically modified animals by homologous recombination was a significant improvement in terms of the on-target nature of the introduced mutation and the increase in mutation rates (Robertson et al. 1986; Hooper et al. 1987).

The lack of success in obtaining ESCs from other mammalian species, apart from rats and humans, made it impossible to apply this technique to mammals in general (Yang and Wu 2018). However, the development of cloning by somatic cell nuclear transfer, which involves the introduction of somatic cells into enucleated oocytes, solved the problem and made it possible to generate fully genetically modified animals in one generation by first performing homologous recombination in these somatic cells (McCreath et al. 2000). Although the first attempt to generate

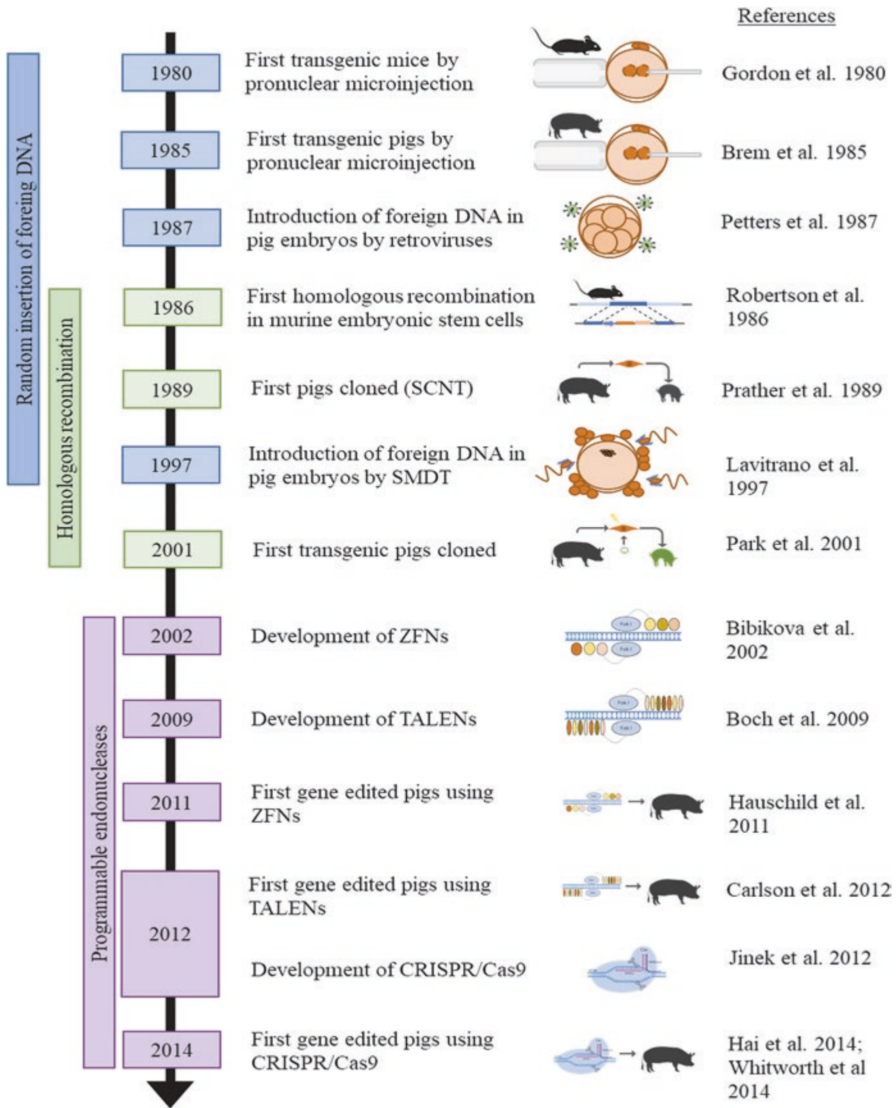

Fig. 3.1 **History of pig gene editing:** Timeline of milestones in the history of gene editing in swine species

genetically modified animals using homologous recombination was made in the 1990s, it was not until 2001 that the first application of this technique in pigs was reported (Park et al. 2001; Dai et al. 2002; Lai et al. 2002). While being a great advance in terms of gene editing in livestock, a limitation was that the efficiency was still low.

3.2.2 Programable Endonucleases

The production of genetically modified mammals has been greatly helped by the development of programmable endonucleases. Three such types of endonucleases have been developed so far: ZFNs (zinc finger nucleases), TALENs (transcription activator- like effector nucleases) and the CRISPR/Cas system (CRISPR-associated protein). All of them are composed principally of two domains: a DNA binding domain that recognises the target DNA sequence and a cleavage domain that produces a double-strand break (DSB) in the target DNA sequence (Petersen 2017).

Genetic modifications mediated by programmable endonucleases are due to mistakes produced by the cell machinery in the process of repairing the DSB. After the DSB occurs, the cells can repair these breakages by two cellular mechanisms: non-homologous end joining (NHEJ), or homology-directed repair (HDR) (Fig. 3.2). In most cases (over 90%), this repair happens via the NHEJ mechanism. During this process in which the two ends of the break are brought together and ligated, small insertions or deletions (indels) often occur, which can modify the reading frame of the gene (if the indel is not a multiple of three) causing a premature appearance of a stop codon and producing a knock-out. However, if a donor DNA is introduced the repair can occur via the HDR pathway. In this case, part of an endogenous gene can be subtly changed or alternatively one or more complete genes can be introduced, producing a knock-in (Whitelaw et al. 2016; Petersen 2017).

The first programmable endonuclease used to produce genetically modified animals was ZFNs, as first reported in a Drosophila model in 2002 (Bibikova et al. 2002).However, it was not until 2011 that this type of endonuclease was used to

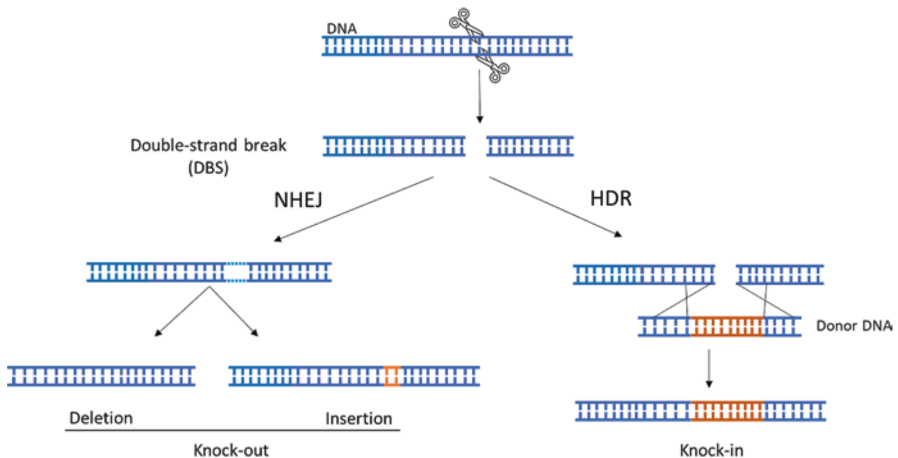

Fig. 3.2 DNA repair mechanism: Schematic showing the DNA repair process after cutting by an endonuclease. Two types of repair can occur after the double-strand break: non-homologous end joining (NHEJ) in the absence of a donor DNA sequence or homology-directed repair (HDR) in the presence of a donor DNA sequence. NHEJ produces insertions or deletions that can cause knock-out and HDR can generate knock-ins through the integration of exogenous DNA

generate knock-out pigs (Hauschild et al. 2011). ZFNs consist of the nuclease domain of the restriction enzyme FokI and multiple zinc finger protein sequences combined to recognize a specific DNA sequence. Each zinc finger protein is formed by 30 amino acids which recognizes a sequence of 3 bp (Wolfe et al. 2000; Bibikova et al. 2001). FokI must dimerize to produce a DSB, so ZFNs needs to be designed and used as pairs (Vanamee et al. 2001; Miller et al. 2007).

The second type of programmable endonuclease developed (Boch et al. 2009; Christian et al. 2010) and used to produced genetically modified pigs was TALENs (Carlson et al. 2012). This type of nuclease consist of the FokI restriction enzyme domain as is the case with ZFNs; in contrast however, the DNA binding domain is formed by peptides that can recognize a single base pair, and which are called transcription activator-like effectors (Boch et al. 2009; Christian et al. 2010). The fact that TALEN domains can recognize a single base allows greater design possibilities with TALEN nucleases, these being more specific, easy to use, and efficient than ZFNs, which recognize groups of three bases (Hockemeyer et al. 2011; Moore et al. 2012). In addition, the use of TALENs seems to be less cytotoxic than ZFNs (Yao et al. 2016).

After ZFNs and TALENs, the development of a third type of programmable endonuclease called CRISPR/Cas9 was reported (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013). The CRISPR/Cas9 system was identified in archaea and eubacteria as an adaptative immune mechanism to protect the cell from viral infections (Mojica et al. 2000; Mojica et al. 2005). CRISPR/Cas systems can be classified into two classes, and six types, but the one most used for gene editing involves the Cas9 from *Streptococcus pyogenes*, and is classified as class 2, type II.

In contrast to the ZFNs and TALENs, which are composed of proteins, the CRISPR/Cas9 system consists of Cas9 protein and a single guide RNA (sgRNA). Cas9 protein has two nuclease domains, RuvC and HNH, which cut the target and complementary sequences respectively to generate DSBs. The sgRNA allows the endonuclease to cut at the target sequence and contains a sequence of 20 nucleotides complementary to the target sequence, cis-repressed RNA (crRNA), and a Cas9 binding sequence (trans-activating crRNA). To allow cleavage of the DNA sequence, the sgRNA should contain a DNA sequence that is complementary to the sgRNA and an adjacent protospacer-adjacent motif (PAM) (Yuk et al. 2014). The PAM sequence differs depending on the species, and the system from *S. pyogenes* needs a 5′-NGG-3′ PAM sequence (Petersen 2017; Yang and Wu 2018).

Compared with ZFNs and TALENs, the CRISPR/Cas9 system is highly efficient and adaptable as a way to produce mutations in developing embryos in part due to the difficult of designing and generating novel ZFN and TALEN endonucleases (Hai et al. 2014; Zhou et al. 2016). The first time that the use of the CRISPR/Cas9 system was described in pigs was in 2014 (Hai et al. 2014), when it was used to produce knock-out pigs for the v-WF gene, whose deficiency in humans causes severe von Willebrand disease, by microinjection of Cas9 mRNA and sgRNA into the cytoplasm of one-cell zygote obtained by *in vitro* fertilization. Since that time, a large number of gene edited pigs have been reported with a number of different applications, as will be explained further in Sect. 3.4.

3.3 How to Generate a Gene Edited Pig?

3.3.1 Methodology

3.3.1.1 Editing of Somatic Cells and Somatic Cell Nuclear Transfer (SCNT)

Briefly, the SCNT technique consists of the introduction of a genetically modified somatic cell into an enucleated oocyte to produce an embryo with the desired genetic characteristics. The first cloned piglets (non-genetically modified) were produced in 1989 by Prather *et al*. using as donor cells porcine embryo blastomeres (Prather et al. 1989).. Subsequently, the first genetically modified piglets were produced in 2001, by introducing the GFP gene contained in a retroviral vector into foetal fibroblasts (Park et al. 2001), but it was not until 2014 that the CRISPR/Cas9 system was first used to produced genetically modified pigs by SCNT (Sato et al. 2014; Whitworth et al. 2014). This technique is widely used, but it has low efficiency (10-30% of blastocyst rate and 0.5-5% piglets/transferred embryos) due to the difficulty of the procedure and epigenetic dysregulations in cloned embryos, which can cause development defects in the offspring such as weakness or low birth weight (Beaujean et al. 2015).

On the other hand, the mayor advantage of this procedure over the direct modification of embryos is that the genotype of the offspring is known as the donor cells with the desired genotype are chosen. This is a great advantage when multiple genes are targeted or when the purpose is a knock-in or a conditional transgenesis (Li et al. 2015; Whitelaw et al. 2016). In general, the SCNT procedure can be divided into three steps: (a) selection, culture and genetic modification of donor somatic cells, (b) oocyte enucleation and nuclear transfer and (c) embryo culture (Fig. 3.3).

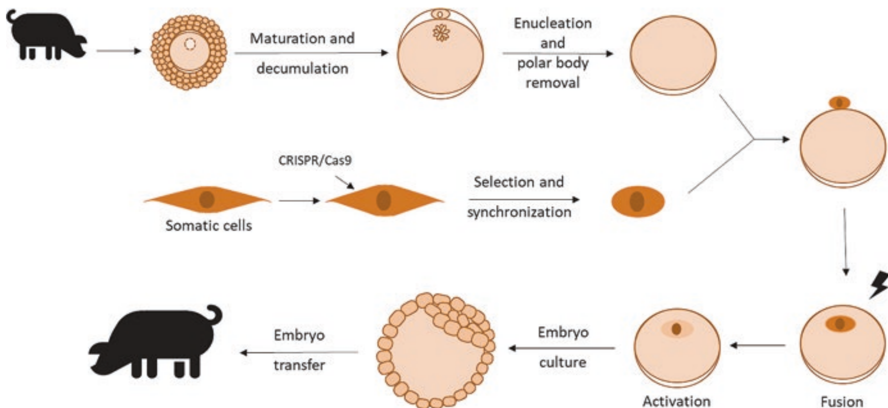

Fig. 3.3 Schematic of the process of somatic cell nuclear transfer (SCNT). Somatic cells are edited by the CRISPR/Cas9 system. After being selected and synchronized, cells are fused with *in vitro* matured enucleated oocytes. Subsequently, cloned zygotes are cultured *in vitro* until the time of embryo transfer

Selection, Culture, and Genetic Modification of Donor Cells

This step involves the selection and culture of the donor somatic cells. The first thing to consider is the donor cell type. In general, these cells need to have a correct karyotype and long lifespan, exhibit good proliferation, and they should be transfectable (Beaujean et al. 2015).More than 200 different cell types have been used as donor cells to produce cloned pigs, each one presenting advantages and disadvantages (Liu et al. 2015). Theoretically, the less differentiated cells are the ones easiest to reprogram. This reprogramming involves essential epigenetic changes that occur during embryo development (Lee and Prather 2014; Liu et al. 2015).

Although there are few studies that demonstrate that one cellular type is better than another, foetal fibroblasts are the most common cell type used for SCNT to produce genetically modified pigs (Whitworth et al. 2014; Chen et al. 2020; Li et al. 2020a); they are highly dedifferentiated cells (Liu et al. 2015). In addition, adult fibroblast cells can be obtained from valuable animals like genetically modified pigs, so they can be used to bypass one step of the process (Sheets et al. 2018; Xu et al. 2020).

Liu et al. achieved a greater overall efficiency in terms of live birth rate using foetal fibroblasts as donor cells in comparison with adult fibroblasts, observing less abnormalities in the piglets produced (deformities, mummified and stillbirth piglets) (Liu et al. 2015). In addition, Zhai et al. observed a higher blastocyst rate using bone marrow- derived mesenchymal stem cells in comparison with foetal fibroblasts. Furthermore, cloned embryos using as donor cells bone marrow-derived mesenchymal stem cells were more similar to those produced by conventional *in vitro* fertilization (IVF) in terms of expression of pluripotency genes, degree of apoptosis, and number of cells per blastocyst (Zhai et al. 2018). One thing to consider is that both studies were performed without transfected somatic cells.

Once a suitable cell type is chosen, the cells are cultured to purify them (when obtained from an animal) and to achieve the necessary cell concentration. Next, these cells need to be transfected (Kurome et al. 2015). There are many transfection methods available: viral vectors (adenovirus, retrovirus…), chemical transfection methods (nanoparticles, lipofection…) and physical transfection methods (electroporation, magnetotransfection…) (Fajrial et al. 2020). Regarding the production of genetically modified pigs, the authors usually prefer the electroporation method, being the one most used to generate knock-out and knock-in models (Lai et al. 2016; Cho et al. 2018; Han et al. 2020; Xu et al. 2020; Zhang et al. 2020) and because it is simpler, more efficient and reproducible (Fajrial et al., 2020). This method involves subjecting the cells to an electric field that induces the formation of pores in the cell membrane through which the components of the CRISPR/Cas9 system can enter the cell (Fajrial et al. 2020). However, some authors use other methods like lipofection (Luo et al. 2019; Fischer et al. 2020; Zhu et al. 2020a). This method is based on the use of lipid molecules that encapsulate the CRISPR/Cas9 system components forming vesicles. These vesicles are introduced into the cell by endocytosis, releasing the components. One thing to consider when using this method is that the components in the vesicle are only going to reach the cytoplasm

so it is not the best option when using plasmid DNA because that needs to reach the nucleus to function (Fajrial et al. 2020).

Something that needs to be taken into account is the format in which the CRISPR/Cas9 system is delivered. This can be plasmid DNA (plasmid encoding Cas9 and sgRNA), mRNA (mRNA encoding for Cas9 and separate sgRNA) and ribonucleoprotein (Cas9 protein and sgRNA) (Fajrial et al. 2020). When using DNA vectors, these require previous transcription and translation to produce functional ribonucleoprotein in the cell. This increases the lag time between the transfection and the expression of the system, which increases the risk of off-target events in addition to the high persistence of the system. Furthermore, plasmid DNA can be inserted into the genome of the cell, so this possibility should be assessed when selecting the most suitable donor cell to perform SCNT (Wang et al. 2017a; Fajrial et al. 2020).

In contrast, mRNA does not have risk of insertional mutagenesis and, as it does not require the entry into the nucleus to function, its expression is faster. Furthermore, the use of mRNA results in transient expression, reducing the persistence time of the system in the cell, which reduces the risk of off-target events (Wang et al. 2017a; Fajrial et al. 2020). In general, when talking about SCNT the most common delivery format is DNA, with only one group performing the transfection using mRNA to produce piglets by SCNT (Guo et al. 2019a). In addition, some authors have used ribonucleoprotein to transfect different porcine somatic cells, so this delivery format could be used as well to produce cloned pigs (Park et al. 2016; Elkhadragy et al. 2021).

Once the cells are edited, the next step is select a colony with the desired mutation. The cells are cultured to produce a single-cell colony (monoclonal colony) and once they reach the appropriate cell density, part of the colony is used to detect (usually by PCR and sequencing) the presence of mutations while the other part continues in culture. The colonies with the desired mutation are used as donor cells (Beaujean et al. 2015; Xie et al. 2020b; Zhang et al. 2020). Sometimes a selection procedure can be performed when the plasmid used has a selection marker. Some authors use antibiotics like geneticin (Han et al. 2020; Huang et al. 2020a) and puromycin (Fischer et al. 2020; Zhu et al. 2020b), others a fluorescent marker like the enhanced green fluorescent protein (EGFP), to select the transfected cells (Li et al. 2020b; Shi et al. 2020).

Oocyte Enucleation and Nuclear Transfer

In this phase mature oocytes are enucleated. The oocytes are usually collected from ovaries from prepuberal gilts obtained from local slaughterhouses, and then *in vitro* matured (Beaujean et al. 2015). Some authors have shown that a selection of the best oocytes can be helpful to achieve a better cloning efficiency. For example, treatment with a hyperosmotic medium with sucrose assists the selection of high-quality oocytes and improves enucleation efficiency due to the formation of a swelling around the metaphase plate (Dang-Nguyen et al. 2018).

Once mature, the meiotic spindle and the polar body of the oocytes are removed**.** There are two enucleation techniques. The first one is called blind enucleation and is performed with micromanipulation equipment (Polejaeva et al. 2000; Liu et al. 2017) (Fig. 3.4). The second one is called handmade cloning and consists of the bisection of 1/3 of the oocyte (where the polar body is located) with a splitting blade (Du et al. 2007; Liu et al. 2017; Li et al. 2019b). Although Liu et al. achieved a greater blastocyst rate when performing handmade cloning and it is cheaper and easier (Liu et al. 2017), blind enucleation is the most common enucleation method used to produce genetically modified pigs.

With both methods, the manipulation medium needs to be supplemented with cytochalasin B in order to avoid the extrusion of the pseudo-second polar body and to maintain the diploidy of the resulting embryos (Beaujean et al. 2015). In addition, in order to increase the enucleation rate, a treatment with demecolcine can be used. With this treatment a swelling around the meiotic spindle occurs and it becomes easier to remove (Gao et al. 2019). The next phase is the nuclear transfer itself. First, the somatic cells need to be synchronised. Usually, cells in the G0/G1 phase of the cell cycle are used to perform SCTN because that way the diploidy of the resulting embryo is maintained (if another phase is used, the re-replication of the DNA can occur before the first cell cycle of the embryo). This synchronisation is usually performed by serum starvation (Hatada 2017). Next, one donor cell is placed in the perivitelline space of the enucleated oocyte with micromanipulator equipment (if blind enucleation is performed) (Kurome et al. 2015; Cho et al. 2018). If handmade cloning is performed, two cytoplasts are attached to a single donor cell (Du et al. 2007; Liu et al. 2017).

These cytoplast-cell complexes are then fused and activated. The fusion allows the nucleus of the donor cell to enter the oocyte cytoplasm and the activation releases the oocyte from its state of meiotic arrest state (Hatada 2017). The fusion is performed by electric pulses (Kurome et al. 2015). The activation can be electrical or chemical, but the first method is more common (Whitworth et al. 2009). Furthermore, it can be performed at the same time as the fusion (simultaneous activation) or a few

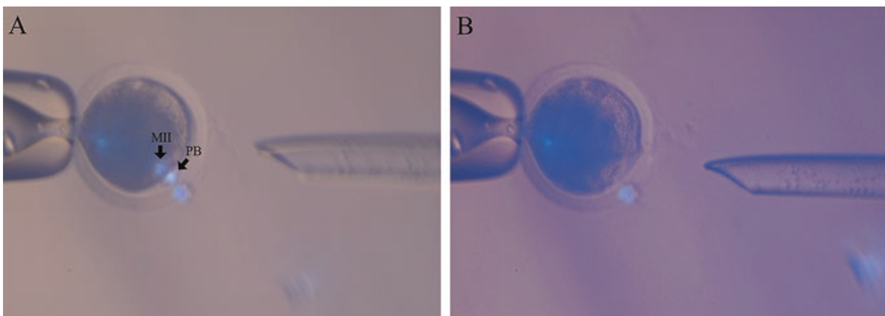

Fig. 3.4 Mature oocyte enucleation. Enucleation process for somatic cell nuclear transfer in porcine oocytes. (**a**) Mature oocyte stained with Hoechst 33342. (**b**) Mature oocyte enucleated without a metaphase plate and polar body. MII: Metaphase II; PB: polar body

hours later (delayed activation) (Hatada 2017; Guo et al. 2019b). Guo et al. in their meta-analysis observed that the delayed activation increases blastocyst rate (without improving cell number per blastocyst) to a greater degree than the simultaneous activation, probably because the cytoplasm of the oocyte needs time to mix with the cytoplasm and the nucleus of the donor cell (Guo et al. 2019b), but simultaneous activation is more commonly used when producing genetically modified pigs.

Embryo Culture

After fusion and activation, the reconstructed embryos are cultured until embryo transfer. For the first 3 hours the medium needs to contain cytochalasin B to avoid the extrusion of the pseudo-second polar body (Hatada 2017). Next, embryos are cultured in *in vitro* culture mediums such as porcine zygote medium (PZM) or North Carolina State University 23 medium (NCSU-23) until ready for embryo transfer (Im et al. 2004; Lee and Prather 2014).

During embryo culture, the remodelling of the chromatin of the somatic cell occurs. This process is required to achieve the totipotent state that the embryo needs to reach to develop correctly, but it is dysregulated in cloned embryos which leads to the low efficiency of the process (Vajta et al. 2007; Lee and Prather 2014). DNA methylation and histone acetylation/deacetylation play important roles in embryo development and cellular function and errors in these processes contribute to the low efficiency of the SCNT procedure (Diao et al. 2013; Kumar et al. 2013). For that reason, donor cells and embryos have been treated with different compounds like DNA methyltransferases inhibitors and histone deacetylase inhibitors (Zhao et al. 2009; Diao et al. 2013; Kumar et al. 2013).

The additive most commonly used in the production of genetically modified pigs is Scriptaid, a histone deacetylase inhibitor (Whitworth et al. 2014; Cho et al. 2018; Boettcher et al. 2020; Zhu et al. 2020b). This compound induces histone acetylation, which changes the structure of the chromatin (more relaxed form), allowing transcription. Furthermore, it promotes DNA demethylation, a key process in nuclear reprogramming and remodelling (Zhao et al. 2009; Park et al. 2012).

The use of other treatments has led to an improvement of the efficiency of the SCNT procedure, but they have not yet been used to produce genetically modified pigs. These compounds include other histone deacetylase inhibitors such as oxamflatin, which led to an improvement in blastocyst rate, embryo quality (total cell number per blastocyst and gene expression), and overall efficiency (offspring) in comparison with control cloned embryos and cloned embryos treated with Scriptaid (Park et al. 2012); sodium butyrate, with which a greater blastocyst rate and a positive effect on embryo gene expression were achieved (Liu et al. 2012); valproic acid, which led to an increase in blastocyst rate and embryo quality (total cell number per blastocyst and gene expression) (Miyoshi et al. 2016; Lv et al. 2020) and suberoylanilide hydroxamic acid, which led to an improvement in blastocyst rate and embryo quality (gene expression) (Sun et al. 2017).

Regarding DNA methyltransferase inhibitors, the treatment with 5-aza-2′-deoxycytidine and zebularine led to improved blastocyst rate and embryo quality (gene expression) in comparison with a control group (Huan et al. 2013; Taweechaipaisankul et al. 2019). Furthermore, donor cells can also be treated with these compounds to improve embryo development. For example, sodium butyrate, 5-aza-2′-deoxycytidine, zebularine and RG108 (DNA methyltransferase inhibitor) were used (Diao et al. 2013), leading to improved blastocyst rate and less DNA fragmentation in comparison with the control cloned embryos.

3.3.1.2 Editing of Gametes and Embryos

The delivery of CRISPR/Cas9 components into *in vivo* or *in vitro* derived zygotes either as mRNAs or ribonucleoprotein makes it possible to achieve gene editing in embryos without the need to perform a cloning step, thereby making this an easier process compared to SCNT due to no need for enucleation and cell fusion. Therefore, this methodology makes it possible to obtain a higher yield in terms of quality and quantity of embryos compared to SCNT (Tanihara et al. 2016).

Gene editing in gametes and embryos has disadvantages such as not knowing the resulting sequence of the target gene after CRISPR/Cas9 cutting and subsequent random DNA strand repair. As a consequence, after the process of embryo production, gene editing and embryo transfer, animals may have no mutation, or be heterozygous and a high percentage of animals might be mosaics, having cells and tissues with different combinations of wild type and knock-out alleles. Also, if the objective of gene editing is to generate a knock- in pig, there is the problem that homologous recombination is less frequent than non- homologous end joining, and the percentage of embryo knock-ins will therefore be low compared to embryos obtained by SCNT. Since the first generation of genetically modified pigs with CRISPR/Cas9 in 2014 (Hai et al. 2014; Whitworth et al. 2014), intracytoplasmic injection has been the most used method for releasing CRISPR/Cas9 into the embryo. Recently, other methods of CRISPR/Cas9 delivery into zygotes such as electroporation have been developed and are in the process of being optimized (Tanihara et al. 2016; Nishio et al. 2018).

CRISPR/Cas9 Delivery by Intracytoplasmic Microinjection

Intracytoplasmic microinjection is a process similar to intracytoplasmic sperm injection (ICSI) (Fig. 3.5). While a holding pipette stabilizes the zygote, a thin micropipette is used to pierce the zona pellucida and the cytoplasmic membrane, and Cas9 and sgRNA is delivered into the cytoplasm. Although this method is less aggressive towards the integrity of the zygote than SCNT, intracytoplasmic microinjection can cause stress which affects embryo quality and therefore the percentage of embryos able to give rise to a piglet (Hai et al. 2014; Wang et al. 2015c).

Fig. 3.5 Mature oocyte microinjection: *In vitro* matured oocyte being subjected to intracytoplasmic microinjection with CRISPR/Cas9 gene-editing components

The importance of producing embryos with high quality to be transferred is one of the critical steps. In the literature, we find that both *in vivo* (Hai et al. 2014; Peng et al. 2015; Wang et al. 2015b; Petersen et al. 2016; Wang et al. 2016; Burkard et al. 2017; Chuang et al. 2017; Park et al. 2017) and *in vitro* zygotes (Whitworth et al. 2014; Park et al. 2017; Whitworth and Prather 2017; Hinrichs et al. 2018) have been used for intracytoplasmic microinjection.

In vivo embryo production consists of collecting zygotes by natural mating or artificial insemination after oestrus detection. Around 14-24 hours after insemination, the zygotes have been flushed from oviducts and collected. These embryos can be obtained from sows or gilts sacrificed at the slaughterhouse (Petersen et al. 2016; Chuang et al. 2017) or also by surgical collection (Peng et al. 2015; Wang et al. 2015c; Burkard et al. 2017; Park et al. 2017). Both *in vivo* zygote production using slaughtered sows or via surgery present some difficulties in terms of accurately and reliably obtaining newly formed zygotes because the ovulation takes place over a 4 hour windows of time in synchronized sows (Park et al. 2017). Timing is an important problem in *in vivo* production because is very probable that DNA replication takes place before injection or when zygote cleavage happens, in which case there are only two options: discard the embryo or inject both cells knowing that you would thereby produce a mosaic (Peng et al. 2015).

In vivo production of embryos is an intensive labour and numerous sows are required to obtain a sufficient number of *in vivo* derived zygotes (Wang et al. 2015b). Consequently, other authors have used *in vitro* produced embryos (Whitworth et al. 2014; Park et al. 2017; Whitworth and Prather 2017; Hinrichs et al. 2018). This

method involves obtaining oocytes from ovaries obtained from the slaughterhouse and performing *in vitro* maturation (IVM). After cumulus-oocyte complexes are matured, *in vitro* fertilization (IVF) is performed and next, putative zygotes are *in vitro* cultured until embryo transfer. The use of *in vitro* produced embryos offers the opportunity to improve the timing control of different parts of the early development of the embryo. *In vitro* embryo production makes it possible to know approximately the time of fertilization better than with *in vivo* embryo collection (Whitworth and Prather 2017). This can help to regulate the timing of DNA editing with respect to *in vitro* fertilization and DNA replication. One strategy to reduce mosaicism is to microinject as soon as possible, even before fertilization, with the aim of being that the ribonucleoprotein is present in the cytoplasm to cut the target DNA before the first DNA replication in the zygote (Lamas-Toranzo et al. 2019b). It has already been shown that the microinjection of CRISPR/Cas9 into oocytes before fertilization (Navarro-Serna et al. 2021) or parthenote embryos just at the moment of activation (Sato et al. 2018), does not affect the mutation rate, nevertheless CRISPR/Cas9 delivery into the cytoplasm before *in vitro* fertilization reduces the mosaicism rate (Navarro-Serna et al. 2021).

Despite improvements in porcine *in vitro* embryo production such as the addition of reproductive fluids (Canovas et al. 2017; Paris-Oller et al. 2021), cytokines (Yuan et al. 2017), or culture media (Redel et al. 2016), embryo quality has not yet been assessed similar to embryos produced *in vivo.* One problem with *in vitro* produced embryos is the high rate of polyspermy that takes place in *in vitro* fertilization process in swine species (Romar et al. 2019). Due to this problem, some authors have used other IVF options, for instance the use of *in vivo* matured oocytes, but this approach is very laborious and difficult and requires a large number of animals similar to that for *in vivo* zygote production (Park et al. 2017).

Regarding the molecular nature of CRISPR/Cas9, this has been microinjected in porcine embryos as DNA, mRNA, or ribonucleoprotein. There is no evidence that there are differences in mutation rate using such different strategies (Navarro-Serna et al. 2021) but due to the possibility of genome integrity, microinjected plasmids have been the least used. Despite this risk, DNA integrations were not found in studies in which CRISPR/Cas9 plasmids were used in pigs (Petersen et al. 2016; Chuang et al. 2017). The most used forms for microinjection have been Cas9 mRNA and sgRNA (Hai et al. 2014; Kwon et al. 2015; Peng et al. 2015; Burkard et al. 2017; Hinrichs et al. 2018; Chen et al. 2019a; Ostedgaard et al. 2020; Xie et al. 2020a). The combination of Cas9 protein and sgRNA as an injected ribonucleoprotein is also used (Sheets et al. 2016; Park et al. 2017; Sheets et al. 2018) and has the advantage that the activity is instantaneous after ribonucleoprotein formation without the need for Cas9 protein to be synthesized in the zygote.

In addition to differences in the molecular nature of the CRISPR/Cas9, variations have also been found in the concentration and/or proportion of Cas9 and sgRNA microinjected. There is not a standard concentration because the amount of CRISPR/Cas9 microinjected varies depending on the efficiency of the designed sgRNA and the molecular environment in which the target sequence is found. In the literature, the potential toxic effect of CRISPR/Cas9 microinjection in embryo development

has been tested by performing injection with water or Cas9 mRNA and sgRNA in parthenogenetic activated oocytes. The blastocyst rate was similar in both groups, so CRISPR/Cas9 does not seem to have toxic effects (Hai et al. 2014; Whitworth et al. 2014; Wang et al. 2015b; Yu et al. 2016). However intracytoplasmic microinjection can produce some defects in embryo development (Yu et al. 2016). Thus, CRISPR/Cas9 has been microinjected at a concentration of between 25-1000ng/µl in porcine embryos.

Another difference besides the method of embryo injection is the site in which zygotes were injected. In almost all studies, CRISPR/Cas9 components were injected into the cytoplasm (Hai et al. 2014; Whitworth et al. 2014; Zhou et al. 2016; Burkard et al. 2017; Whitworth and Prather 2017) but in Chuang *et al.* 2017, the zygotes were injected in the pronucleus (Chuang et al. 2017). Pronuclear microinjection is difficult in embryos of domestic animals due to the cytoplasm being opaque due to lipid droplets present in the cytoplasm and the pronucleus microinjection requires centrifugation of the zygote to expose the pronucleus (Wall et al. 1985).

In addition, other strategies have been described such as combining SCNT and intracytoplasmic microinjection (Sheets et al. 2016; Sheets et al. 2018). In Sheets *et al.* 2016, SCNT was performed with foetal fibroblasts and matured oocytes with the aim being to microinject CRISPR/Cas9 components as with *in vivo* or *in vitro* derived embryos (Sheets et al. 2016) and in Sheets *et al.* 2018, genetically modified zygotes were produced by *in vivo* zygote microinjection and these were used to obtain genetically modified foetal fibroblast cells which were used to obtain piglets by SCNT (Sheets et al. 2018). Despite the limitation of *in vitro* embryo production, the quality and quantity of *in vitro* produced embryos is sufficient for authors to report the generation of healthy piglets.

CRISPR/Cas9 Delivery by Electroporation

In addition to microinjection, the increasing use of electroporation as a method of introducing CRISPR/Cas9 into the cytoplasm of porcine zygotes has been of significant importance in the last few years (Tanihara et al. 2016; Nishio et al. 2018). This technique involving placing the zygotes in media with Cas9 (protein or mRNA) and sgRNA and subjecting the zygotes to an electric current that allows the formation of membrane pores through which CRISPR/Cas9 components can enter (Fig. 3.6).

Transfection by electroporation has been in development for many types of mammalian cells, for example foetal fibroblasts for SCNT but to penetrate the zygote membrane is more difficult because the zona pellucida must be penetrated before this membrane. To reduce this barrier, the zona pellucida can be partially digested by acid Tyrode's solution in mouse zygotes, improving CRISPR/Cas9 entry (Chen et al. 2016) but in porcine studies, CRISPR/Cas9 editing by electroporation has been reported without zona pellucida digestion. Another option to improve the penetration of the zona pellucida is to use molecules with a smaller size

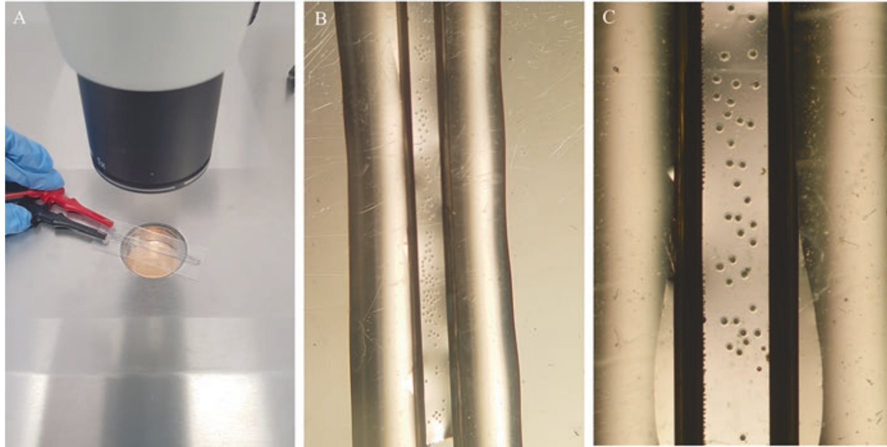

Fig. 3.6 Mature oocyte electroporation: *In vitro* matured oocyte during electroporation process. (**a**) plate electroporation device. (**b**) oocytes in electroporation device at low magnification. (**c**) oocytes in electroporation device at higher magnification

than Cas9 protein (160 kDa) with respect to Cas9 mRNA (~1500 kDa), this can improve mutation rate (Tanihara et al. 2016). Another limitation is that the need to put the zygotes into a medium with a mixture of Cas9 and sgRNA requires using more sgRNA and Cas9 than for CRISPR/Cas9 microinjection.

Since 2016, the successful generation of genetically modified pigs using this technique has been reported (Tanihara et al. 2016; Nishio et al. 2018; Tanihara et al. 2018; Hirata et al. 2019; Tanihara et al. 2019a; Hirata et al. 2020; Le et al. 2020; Tanihara et al. 2020a; Yamashita et al. 2020). Some protocols using different electroporation devices, and variations in the number and polarity of pulses, pulse length, Cas9 and single guide RNA proportion and concentration, electroporation medium and voltage strength have been described.

Two main devices for electroporation have been used: electroporation plates and electroporation cuvettes. The use of the last one has not been reported for porcine zygotes, so there seems to be a preference for the use of electroporation plates. Some electroporation devices offer the opportunity to perform bipolar pulses (Nishio et al. 2018; Yamashita et al. 2020). However, it has been reported that the use of bipolar pulses does not increase the mutation rate but produces a decrease of blastocyst rate compared to that with unipolar pulses (Nishio et al. 2018). In addition to polarity, machines such as the NEPA21 Super Electroporator (NEPAGENE, Ichikawa, Japan) offer the option of making two types of pulses: first an electroporation pulse, which involves performing a high voltage pulse to destabilize the membrane and later transfer pulses to create an input current of CRISPR/Cas9 present in the electroporation media (Yamashita et al. 2020).

Regarding pulses, a balance must be found between the damage caused to embryo quality and quantity and the proportion of mutant embryos obtained. A greater number of pulses (Tanihara et al. 2016), voltage strength (Nishio et al. 2018) and pulse length (Tanihara et al. 2016) can negatively affect embryo development.

Currently, the electroporation parameters used are between 3-5 pulses, 20-30 V and 1ms of pulse length.

Similar to microinjection, electroporation also makes it possible to deliver CRISPR/Cas9 into *in vitro* matured oocytes rather than zygotes (Hirata et al. 2019). As with microinjection, in addition to reducing the amount of mosaicism, this strategy offers other benefits. The zona pellucida of the mature oocytes has a higher permeability than the zona pellucida of zygotes, allowing molecules of up to 170 kDa to cross it, compared to those of 110 kDa that can cross the zona pellucida of zygotes (Legge 1995). This situation makes the membrane of mature oocytes more permeable and, as a consequence, a high concentration of ribonucleoprotein can enter the cytoplasm and therefore a higher mutation rate can be obtained (Hirata et al. 2019).

Compared with electroporation, CRISPR/Cas9 microinjection and SCNT requires greater and more laborious work because micromanipulation needs more qualified personnel to microinject cell after cell in the shortest possible time, and this also requires a lot of time (Navarro-Serna et al. 2019). In contrast, groups of between 50-100 zygotes can be electroporated at the same time, allowing more rapid work and a shorter time of exposure of the embryos to a hostile environment outside the incubator.

3.3.1.3 Embryo Transfer

Once produced, gene-edited embryos can be transferred to recipient animals. Commercial application of embryo transfer (ET) in swine is not as well developed as other assisted reproductive techniques such as artificial insemination or oestrous synchronization. ET was considered unfeasible for decades mainly because of the requirements of surgical techniques for embryo collection and embryo deposition into recipients, alongside challenges in the preservation of embryos (Martinez et al. 2019). This situation has drastically changed in recent decades with the current technology allowing non-surgical ET (nsET) in a non-sedated recipient (Li et al. 1996). Despite piglets having been born from *in vitro*-produced embryos following nsET (Yoshioka et al. 2003; Yoshioka et al. 2012), when working with gene-edited *in vitro*-derived embryos, the conventional surgical ET (sET) by mid laparotomy is the most common methodology followed by researchers due to the compromised quality and limited number of embryos obtained. As such, the low quality of *in vitro*-produced porcine embryos is compounded by the difficulties arising from a surgical intervention. Therefore, in order to maximize sET efficiency with gene edited embryos it is crucial to investigate the most relevant ET- related factors impacting the final pregnancy rate and fertility such as the selection of recipients, embryo handling, age and number of embryo transferred, ET position, and the use of aiding parallel strategies such as the use of co-transferred helper embryos or the infusion of seminal plasma.

Whenever possible, females with an excellent reproductive history should be used as recipients since reproductive performance is one of the well-known factors influencing ET success in the pig. In fact, when ET is performed in first-oestrus gilts, the survival of embryos is approximately 20% lower compared with that from older gilts (reviewed by (Youngs 2001)). The precise control and detection of oestrous in the recipient animal and the timing of *in vitro* fertilization (IVF) to produce embryos is another key factor for a successful ET. It has been demonstrated that after sET the efficiency of piglet production (percentage number of piglet(s) born based on the number of embryos transferred) is greater in recipients whose oestrous cycle is asynchronous to that of donors with a 1-day delay (8.3%) compared to those recipients with a 2-day (1.5%) or 3-day (0.9%) delay (Yoshioka et al. 2012). The same has been described when transferring *in vivo*-derived embryo by nsET (Angel et al. 2014). From the practical point of view, it means that oocytes must be inseminated in the lab 24-48 hours before oestrous beginning in the recipient animal. This leads to an environment where uterine histotrophic secretions from a “younger” uterus are compatible with metabolic needs of the “older” embryos. Following this schedule, our group has achieved around 35% of transferred recipients reaching pregnancy to term after sET of wild type *in vitro*-derived embryos (Paris-Oller et al. 2021) and CRISPR/Cas9 gene-edited embryos (Navarro-Serna et al. 2021). The final yield of ET also depends on the protocols followed to generate the embryo. Thus, the current efficiency of sET with SCNT-embryo is very low with results indicating that transfer of cloned embryos cultured for a longer time after reconstruction (22-24 hours vs. 4-6 hours) decreases the recipient's pregnancy rate and farrowing rate suggesting that long *in vitro* culture time negatively affects the development of transferred cloned porcine embryos (Shi et al. 2015).

Another factor to consider for ET is the embryo’s age, which is linked to the ET site, and the number of embryos to transfer. In an ideal situation, once gene-edited zygotes are produced, it is advisable to reduce the culture time to minimize the stress derived from *in vitro* culture conditions (Garcia-Vazquez et al. 2010). That would mean transferring 2-4 cell stage embryos into the recipient’s oviduct. Despite there not being extended studies in pigs relating to unilateral or bilateral transfer, it has been shown after sET of cloned embryos into double oviducts that there is an increase in pregnancy rate, farrowing rate of recipients, and the developmental rate of transferred embryos, compared to the situation with unilateral oviduct transfer (Shi et al. 2015). Based on these findings, splitting the total number of embryos to transfer, and performing a bilateral deposition should result in a higher efficiency with sET compared to a unilateral approach. As for morulae or blastocysts, the site for sET is the uterus, preferably within 30 cm from the tip of the uterine horn. Although there are not extensive studies relating to the most suitable sET site for embryos, it seems clear that the uterine body should be avoided since under *in vivo* conditions morula/blastocysts remain near the tip of the uterine horn until day 6 to 7 of the oestral cycle, progressing subsequently toward the uterine body (Hunter et al. 1967; Dziuk 1985). Information in the literature about the optimum total number of transferred embryo varies with numbers ranging from 14 to 50 wild type *in vitro*-derived blastocysts and litter sizes of 8-10 piglets (Marchal et al. 2001;

Kikuchi et al. 2002; Yoshioka et al. 2002; Yoshioka et al. 2003; Somfai et al. 2014; Paris-Oller et al. 2021) although some groups report 100% pregnancy rates after sET with only 20-25 blastocysts per recipient (Yoshioka et al. 2002). However, when transferring gene-edited *in vitro*- derived embryos, it is necessary to increase these numbers up 80-200 per recipient (Onishi et al. 2000; Shi et al. 2015; Navarro-Serna et al. 2021).

Although porcine embryos can develop well *in vitro* to the blastocyst stage, their subsequent development *in utero* after ET in the uterine horns of surrogates can be improved. One strategy to achieve this is to use helper embryos since typically pigs require at least four foetuses for a successful pregnancy and the presence of helper embryos might assist the full development of gene-edited embryos (Onishi et al. 2000). This strategy consists in transferring gene-edited embryo concomitantly with other embryos with the objective being to help establish and/or maintain pregnancy in recipients. Recently, the use of co-transferred helper embryos has been used in an attempt of increase efficiency of sET using cloned embryos (Shi et al. 2015). These authors employed helper embryos (50 per recipient) with different origins such as parthenogenetic, *in vitro*- and *in vivo*-derived (by inseminating the recipient before sET) embryos but either type of helper embryos could aid establishment and/or maintenance of pregnancy with SCNT embryos. On the other hand, another strategy would be the use of seminal plasma. It has been reported that infusion of heterologous seminal plasma prior to AI of recipients upregulates the expression of embryo development related genes in day 6 wild type pig embryos (Martinez et al. 2020; Tajima et al. 2020). However, the likely beneficial effect of this strategy to increase sET efficiency with gene-edited embryos remains to be determined.

3.3.2 How to Detect Mutations?

In the literature, we can find different methods to detect DNA modifications, which change depending on the information that we want to obtain: detection of mutations, identification of the mutant sequence, or evaluation of mosaicism.

3.3.2.1 Electrophoresis-Based Techniques

The simplest method is to perform PCR and then agarose gel electrophoresis. This method is used to evaluate large insertions or deletions for example when the strategy is to generate large deletions and two sgRNAs are designed to remove part of an exon or even complete exons (Whitworth and Prather 2017; Wu et al. 2017; Hirata et al. 2020; Koppes et al. 2020). PCR product digestion with mismatch-sense endonucleases, such as T7 endonuclease I are also used to detect mutations produced by CRISPR/Cas9 and other programmable endonucleases (Wang et al. 2015a; Kang et al. 2016; Bloom et al. 2017; Li et al. 2017a; Xie et al. 2017). This

method involves performing a PCR of the target region of the embryo/animal sample. Next, the PCR product is mixed with a wild type amplicon, denatured, and reannealed. As a result, a heteroduplex will be formed with one wild type chain and one sample chain. When a sample of DNA has mutations, a heteroduplex with mismatches are formed and these mismatches are detected and cut by T7 endonuclease I after incubation with the endonuclease. Finally, agarose gel electrophoresis is performed with the digestion product. When the band has the size of the PCR product, this signifies that the sample is wild type and when a smaller band appears, T7 endonuclease I has cut it, so the sample is mutant (Bloom et al. 2017). This method makes it possible to obtain results quickly and easily, however the T7 endonuclease I assay only gives information about the presence of mutations in the sample.

Other methods make it possible not only to detect mutations in the sample but also to detect the number of bases that are inserted or deleted in the alleles and the number of alleles in a sample. The fluorescent PCR-capillary gel electrophoresis technique is accurate enough to differentiate one base-pair differences between alleles, so this technique can indicate the presence or absence of a frameshift in the coding sequence of the gene (Ramlee et al. 2017). This technique involves performing a conventional PCR with one primer labelled with a fluorochrome and later the sample is run in a capillary gel electrophoresis device linked to a genetic analyser (Ramlee et al. 2015). In order to discriminate the wild type allele in comparison to the other alleles in a sample, a PCR product of the sample is mixed with another wild type amplicon labelled with a different fluorochrome (Ramlee et al. 2017).

The fluorescent PCR-capillary gel electrophoresis technique allows detection of not only the presence of mutations, but also the number of alleles (Ramlee et al. 2017). The detection of alleles, based on their size, makes it possible to differentiate between wild type samples, heterozygous samples with one allele being wild type and another mutant, heterozygous samples with two different alleles mutated (Ramlee et al. 2017) or also mosaicism with the presence of more than two alleles of different sizes (Navarro-Serna et al. 2021). In addition, the knowledge of the size of alleles makes it possible to know if the difference in the number of nucleotides causes a frameshift and gives rise to a knock-out phenotype. In an interesting example, wild type (WT) sample and edited samples (Fig. 3.7a) as well as wild type (WT) sample and homozygotic edited samples (Fig. 3.7b) were shown difference in the migration time and sequence.

Fig. 3.7 (continued) bottom of each figure. (**a**) sequence and capillary electrophoresis graph of WT sample and edited sample with mosaicism. In the capillary electrophoresis graph three alleles can be seen with the edited sample in blue and WT control sample in green. (**b**) sequence of wild type sample and homozygotic edited sample. In capillary electrophoresis graph one allele can be seen with the edited sample in blue and the WT control sample in green

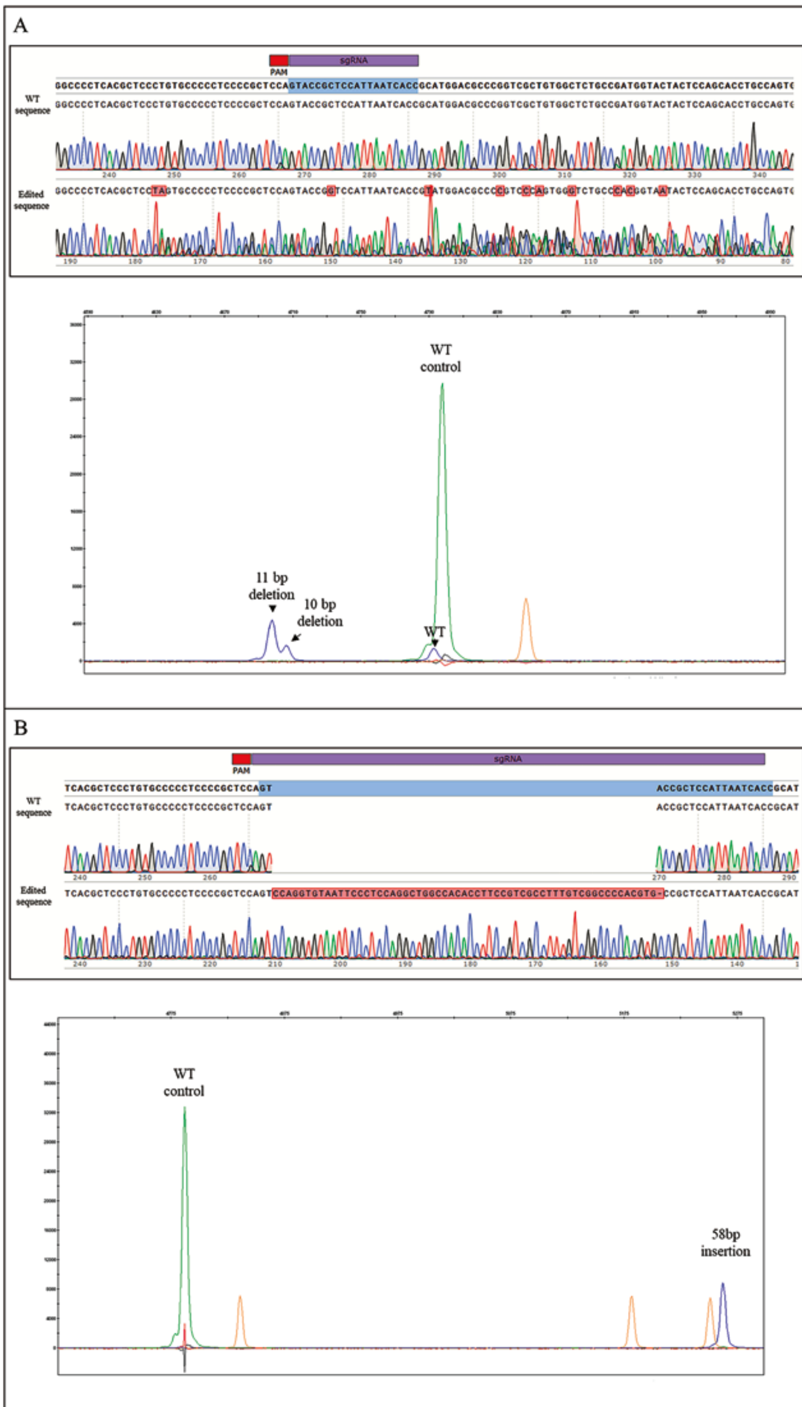

Fig. 3.7 Evaluation of mutation by sanger sequencing and capillary electrophoresis: Comparison of WT and edited sequences at the top and capillary electrophoresis graph at the

3.3.2.2 Sequencing Techniques

Although electrophoresis-based techniques offer a lot of useful information, they do not make it possible to know the sequences of alleles detected. For this, it is necessary to perform sequencing. Sanger sequencing is the method most reported in the literature for detection of mutations (Burkard et al. 2017; Whyte et al. 2018; Chen et al. 2019a; Whitworth et al. 2019; Hirata et al. 2020). In this method, the target sequence is amplified by PCR and then sequenced directly (Sakurai et al. 2014). Sequencing the PCR product directly produces a convoluted spectrum with overlapping peaks in the Cas9 cut region, so, this makes it possible to detect mutations in the sample, but the sequence cannot be known if the sample is not from a homozygous organism (Dehairs et al. 2016).

To solve this, the PCR product can be purified and amplicons can be cloned into vectors for bacterial propagation and single colony cloning sequencing (Lamas-Toranzo et al. 2019a). Clonal sequencing makes it possible to detect the different alleles present in a sample, and discern between wild type samples, heterozygous samples with one allele wild type and another mutant, heterozygous samples with two different alleles mutated, and mosaic samples. The difference with respect to the fluorescent PCR-capillary gel electrophoresis technique is that clonal sequencing makes it possible to know the sequence, also giving information about base substitutions and differences between alleles with the same number of inserted or deleted base pairs but with different sequences.

This method requires more laborious work; however the development of software to analyse sequences with overlapping peaks produced by DNA repair after Cas9 cleavage has allowed the analysis *in silico* of mutation without the need for clonal sequencing. Software such as Tracking of Indels by Decomposition (TIDE) () makes it possible to determinate the spectrum and frequency of targeted mutations designed to analyse a pool of cells (Brinkman et al. 2018). The ability of the software to decompose sequences into as many alleles as detected makes it possible not only to evaluate mutations (Le et al. 2020; Tanihara et al. 2020b) but also to detect mosaicism (Yamashita et al. 2020).

Finally, the most complete and accurate method is next-generation sequencing. This method makes it possible to obtain information about all the alleles present in a sample (Tanihara et al. 2019a); nevertheless, it is more expensive than other methods mentioned above unless a large number of samples are analysed. This technique consists of performing PCR on samples adding a barcode to identify each sample (Hirata et al. 2019; Tanihara et al. 2019a; Le et al. 2020). Next, all samples to be analysed are mixed together in a pool and sequenced. Finally, the data are analysed and interpreted. This method is a good option when a large number of samples have to be analysed, such as with candidate regions for off-target mutations (Le et al. 2020).

In conclusion, we currently have a large number of options for evaluating mutations after gene editing using CRISPR/Cas9, which can be chosen based on the information required for the research.

3.3.3 Negative Aspects of Gene Editing

3.3.3.1 Mosaicism

Mosaicism is the most important problem for gene editing in embryos (Hai et al. 2014). This involves the presence of two or more cell lines with different alleles of the target gene in one organism due to the activity of Cas9 in the zygote after the first DNA replication or even after the subsequent cell divisions. A problem with the generation of mosaic organisms is that they cannot be used to provide samples to study because the distribution and proportion of the different alleles with different functionalities around the organism is unknown (Navarro-Serna et al. 2020). Furthermore, there is a possibility that the edited alleles are not found in the germ line and therefore the animal cannot be used as a founder of a colony. On the other hand, there may be mosaic organism that have all the alleles edited and therefore these organisms could present the desired phenotype (Yamashita et al. 2020).

In species that reach reproductive age quickly, such as mice, mosaicism is not an important problem because a second generation can be obtained after crossing the animals obtained in the first generation. Therefore, a second generation can be obtained with the guarantee that these are not mosaics and that the allele detected in one tissue is the same as that found in all tissues of the organism. However, in a livestock species such as the pig, the time to reach reproductive age and gestation time is longer, so mosaicism leads to a greater time being required to obtain piglets with the required mutation (Mehravar et al. 2019).

One way to solve this problem is to perform embryo production by SCNT because these embryos have the required mutation, which is previously analysed in cell culture. However, due to the difficulties associated with cloning, gene editing directly in zygotes is of great interest despite disadvantages such as mosaicism. In the literature, several strategies have been described to try to reduce the mosaicism such as, performing the delivery of CRISPR/Cas9 as early as possible with respect to the first DNA replication in the zygote (Lamas-Toranzo et al. 2019b; Navarro-Serna et al. 2021), the enhancement of repair mechanisms after the Cas9 cut to decrease mosaicism (Yamashita et al. 2020), modifications of Cas9 protein (Tu et al. 2015), or editing strategies using multiple sgRNAs for the same target gene.

The timing of CRISPR/Cas9 delivery into the zygote cytoplasm with respect to the first DNA replication in an important factor that affects the degree of mosaicism (Navarro- Serna et al. 2021). In *in vivo* collected embryos, the control of timing is so low that even unfertilized oocytes or two-cell embryos may be obtained (Peng et al. 2015). Due to this lack of control, it is unknown whether at the time of microinjection the first DNA replication of the zygote has already taken place or not. Due to this lack of control, the *in vivo* collected zygotes are injected just after being collected (Hai et al. 2014; Petersen et al. 2016; Burkard et al. 2017; Gadea et al. 2018) and therefore, this is the reason that high rates of mosaicism in live-born piglets are reported by many authors (Peng et al. 2015; Wang et al. 2015c; Yu et al. 2016; Zhou et al. 2016). On the other hand, DNA replication in porcine zygotes produced

in vitro has been described to begin between 8-9 hours post- insemination (Jeong et al. 2007; Navarro-Serna et al. 2021). Despite this, in most studies, *in vitro* produced zygotes have been microinjected between 5 (Park et al. 2017) and 14 hours post-insemination (Whitworth et al. 2014; Whitworth and Prather 2017).

The effect of CRISPR/Cas9 microinjection time has been studied in both parthenogenetic pig embryos (Tao et al. 2016) and *in vitro* produced embryos (Navarro-Serna et al. 2021). When an oocyte is activated by parthenogenetic activation, DNA synthesis occurs between 5-6 hours post-activation (Liu et al. 1996). In a study which used parthenogenetic pig embryos, activated oocytes were injected at 3, 8 or 18 hours after activation. In this study, the time of microinjection did not affect cleavage and mutation rate but the percentage of embryo development was less in the 3 hours group (Tao et al. 2016). Despite the mutation rate not being affected by the timing, the biallelic mutation rate was influenced by the microinjection time after parthenogenetic activation in pigs and the rate was higher when microinjection was performed earlier, that is at 3 and 8 hours post- activation (Tao et al. 2016). The mosaicism rate was also affected, and 1/3 of mosaic embryos were obtained when the microinjection occurred before DNA replication and 100% after DNA replication (Tao et al. 2016). The same results were obtained when *in vitro* produced embryos were microinjected at three different times: in oocytes before *in vitro* insemination, 5-6 hours after insemination and 10-11 hours after insemination (Navarro-Serna et al. 2021). *In vitro* embryo production and mutation rate was not affected by the time of microinjection, but mosaicism was lower in embryos microinjected as soon as possible after the first zygotic DNA replication (Navarro-Serna et al. 2021). These results suggest that performing the microinjection time earlier may be a good strategy to reduce mosaicism, such that even the microinjection of matured oocytes before insemination is a good option.

When zygotes are obtained by SCNT and then microinjected, timing is also an important factor as following somatic cell injection an oocyte at metaphase-like stage after 2 hours and pronuclear structure is formed within 6 hours (Bui et al. 2006). In Sheets *et al*. 2016 the time of microinjection into cloned zygotes was 4 hours after fusion (Sheets et al. 2016). In addition to delivering CRISPR/Cas9 components as soon as possible, other strategies have been described to reduce mosaicism, such as reducing the half-life of Cas9 protein by tagging Cas9 with ubiquitin-proteasomal degradation signals (Tu et al. 2017). The reduction of the half-life of Cas9 leads to the concentration of the protein deceasing earlier than wild-type Cas9, so that the protein concentration is reduced when the successive DNA replications take place. As a consequence, this study reports a decrease in mosaicism after using the modified Cas9 in primate embryos (Tu et al. 2017).

Another approach described to reduce mosaicism is to shorten the repair time of the cuts produced by CRISPR/Cas9 (Yamashita et al. 2020). The authors that used this strategy concluded that not only should Cas9 cut the target DNA before the first DNA replication, but the repair and consequent creation of the mutation should also take place before the DNA replication. If the repair occurs after DNA replication, each replicated chain will have a different degree of repair, resulting in more than two alleles and in the production of a mosaic organism. To solve this problem, the

authors decided to introduce murine three-prime repair exonuclease 2 (mTrex2) into porcine zygotes at the same time as CRISPR/Cas9 by electroporation. The results showed that co-delivery of CRISPR/Cas9 and the repair protein mTrex2 can reduce mosaicism (Yamashita et al. 2020).

Despite the improvements described, currently there is not any methodology to completely remove the mosaicism in gene editing directly in embryos so, another option described is to use multiple guides for the same target gene in order to generate knock- out piglets in which at least all cells of the organism have a loss of objective protein function and a knock-out phenotype, even it is due to different alleles with different mutations (Whitworth and Prather 2017; Wu et al. 2017; Zuo et al. 2017; Hirata et al. 2020).

In conclusion, currently, the only method that allows the generation of one hundred percent homozygous knock-out pigs edited by CRISPR/Cas9 is through SCNT. However, if the goal is to generate a KO colony, one-step gene editing in embryos is enough to achieve this.

3.3.3.2 Off-Target Mutations

The main advantage of programmable endonucleases is their high specificity for the target sequence to be modified. However, there is the possibility that DNA sequences similar to the sgRNA have sufficient consensus. This could mean that the Cas9 protein cuts in other regions of the genome different than the target sequence. These modifications outside the target region are called off-target mutations. The editing of other sequences in the genome of the organism could affect the expression or integrity of other genes that could lead to phenotypic variations (Wang et al. 2015b). This could complicate the phenotypic study of the animal models generated because the phenotype that is observed might not be due to the modification of the target gene but instead be due to alterations in other similar sequences in other genes.

Strategies to increase the specificity of editing have been designed, such as the development of Cas9 nickases. Unlike the wild type Cas9, which generates a double- strand break in the target site that is then repaired by NHEJ or HDR, Cas9 nickase produces a single-strand nick which is repaired without mistakes. This system needs sgRNA pairs to produce two nicks in close proximity and generate a double-strand break. The requirement to design two sgRNAs that recognize the target gene reduces the possibility of off-target editing occurring but the likelihood of HDR taking place is less than for double-strand breaks generated with wild-type Cas9 editing (Shen et al. 2014). Previous studies showed that the CRISPR/Cas9 system might cut sequences with divergences from the PAM sequence (Wang et al. 2016) and these mistakes could cause off-target editing, nevertheless the presence of off-target mutations in genetically modified piglets produced by CRISPR/Cas9 has not been reported (Navarro-Serna et al. 2020). Studies of off-target mutations in pigs detected *de novo* mutations in genetically modified pigs with a frequency close to that previously estimated in humans, which indicates that in this study, CRISPR/Cas9 does not significantly increase the rate of off- target mutations (Wang et al.

2016). Furthermore, another study in porcine embryos found that the frequency of off-target mutations was very low, even when using a high concentration of CRISPR/Cas9 complex (Le et al. 2020).

In conclusion, the low incidence of off-target mutations reported is due to the use of bioinformatic guide design tools and the knowledge available about whole genome sequences of *Sus scrofa* in data bases. This has made it possible to design sgRNAs associated with a very low possibility of producing off-target mutations(Le et al. 2020).

3.3.3.3 Chromosome Aberrations

The generation of genetically modified pigs using CRISPR/Cas9 involves a complicated gamete manipulation process. In addition to gene editing, other processes are involved such as oocyte *in vitro* maturation, collection and *in vitro* culture of embryos produced *in vivo*, cloning, microinjection, electroporation, *in vitro* fertilization and culture, and embryo transfer.

In the literature, it has been reported that assisted reproductive techniques in pigs can produce chromosome aberrations such as aneuploidies (Hornak et al. 2009) and chromosome translocations (Garcia-Vazquez et al. 2011). Studies in porcine blastocysts showed that around forty percent of *in vitro* produced embryos had chromosomal aberrations (Ulloa Ullo et al. 2008; Hornak et al. 2009). Although it is possible that many of these abnormalities are not compatible with generating a liveborn piglet, some of them are compatible. Indeed, in our studies we have detected the presence of reciprocal chromosomal translocation in piglets produced by ICSI (Garcia-Vazquez et al. 2011) and also when we generated gene edited pigs, a double chromosomal translocation was detected in one homozygous knock-out pig produced *in vitro* (Navarro-Serna et al. 2021). Despite the risk of chromosomal aberrations, the karyotype has not been analyzed in other studies in which CRISPR/Cas9 gene editing pigs were produced. This means that there may be chromosomal alterations that cause health problems in animal models which are falsely attributed to the desired gene modification.

3.4 Applications of Gene Edited Pigs

The ability to edit genes allows insertion of exogenous sequences (knock-in) or elimination of gene function (knock-out), which could have unlimited applications for use in basic science, agriculture, and biomedicine. In these areas, the production of genetically modified pigs makes it possible to investigate areas of interest such as gene expression, protein structure, intracellular mechanisms and gene functions (Whitelaw et al. 2016; Yao et al. 2016; Burkard et al. 2017; Wells and Prather 2017). The use of gene edited pigs is higher in biomedicine than in agriculture. This is due

to the severe restriction of consumption of food products derived from genetic modified organisms (GMO's). This topic will be revised in Sect. 3.6 of this chapter.

3.4.1 Basic Science

Knock-out and knock-in pigs are used to study the function of different genes or proteins as complementary studies with murine models in different areas of knowledge (Table 3.1). For example in the developmental biology area, a knock-out model for OCT4 was developed to study the function of this transcription factor in the early development of the pig embryo (Kwon et al. 2015) and Lai *et al.* produced a knock-in model introducing the coding gene of a fluorescent protein under the

Table 3.1 Gene edited pigs with applications in basic science

Research field	Gene	Study	Methodology	Model	References
Development biology	OCT4	Preimplantation embryo development	Injection	KO	Kwon et al. (2015)
Development biology	OCT4	Pluripotency cells reporter	SCNT	KI	Lai et al. (2016)
Immunology	PBD2	Protection against infection	SCNT	KI	Huang et al. (2020a)
Immunology	IgM	B-cell deficiency	SCNT	KO	Chen et al. (2015)
Metabolism	GRB10	Insulin resistance and obesity	Injection	KO	Sheets et al. (2016)
Metabolism	MC3R	Fat metabolism, energy homeostasis	SCNT	KO	Yin et al. (2019)
Metabolism	IRX3	Body mass, fat metabolism and obesity	SCNT	KO	Zhu et al. (2020a)
Organogenesis	NGN3	Pancreas development	Injection and SCNT	KO	Sheets et al. 2018)
Organogenesis	SIX1 and SIX4	Kidney development	SCNT	KO	Wang et al. (2019b)
Organogenesis	EDA	Submucosal glands development	Injection	KO	Ostedgaard et al. (2020)
Organogenesis	ETV2	Hematoendotelial linages	SCNT	KO	Das et al. (2020)
Reproduction	PTGS2	Embryonic development and luteal function	SCNT	KO	Pfeiffer et al. (2020)
Reproduction	BMP15	Female fertility and follicular development	SCNT	KO	Shi et al. (2020))
Reproduction	SRY	Sex determination	Injection	KO	Kurtz et al. (2021)
Cellular biology	TPC2	Calcium signalling	Injection	KO	Navarro-Serna et al. (2021)

SCNT Somatic cell nuclear transfer. Injection: Intracytoplasmic microinjection of oocytes/embryos

control of the promoter of OCT4 in order to localize and monitor pluripotency (Lai et al. 2016).

Different models have been developed to study the role of different genes in organogenesis. For example, pigs with a knock-out in NGN3 (Sheets et al. 2018), SIX1 and SIX4 (Wang et al. 2019b), ETV2 (Das et al. 2020) or EDA (Ostedgaard et al. 2020) were used to study pancreas, kidney, hematoendotelial and submucosal gland development, respectively. Other genetically modified pigs were produced to investigate the functions of different genes in fat metabolism, insulin resistance and obesity, such as GRB10 (Sheets et al. 2016), MC3R (Yin et al. 2019) and IRX03 (Zhu et al. 2020a), respectively.

Other examples of models produced in the area of basic science are pigs with knock-out in PTGS2 to study its function in embryonic development and luteal function (Pfeiffer et al. 2020), in BMP15 to investigate its function on follicular development (Shi et al. 2020) and in PBD2, a gene with antimicrobial activity (Huang et al. 2020a). We have developed a TPC2 KO pig model to explore the functional role of TPC2 in calcium signalling pathways (Navarro-Serna et al. 2021). The information generated with the use of gene edited pig models will be complementary to knowledge derived from cell and rodent models (Hryhorowicz et al. 2020). Most of the pig models generated are KO (Table 3.1), probably because the first steps in the study of the function of a gene is the knock out and on the other hand the efficiency for KO models is higher than for KI models.

3.4.2 *Agricultural Production*

In terms of agricultural applications, the CRISPR/Cas9 system can be used with the objective of creating animals with an improved carcass composition, decreasing input requirements, animals with an improved milk production, or animals that are resistant to diseases (Gadea and Garcia Vazquez 2010; Murray and Maga 2016; Wells and Prather 2017; Yang and Wu 2018).

3.4.2.1 Animal Health

Porcine infectious diseases are a great problem for pig production, with huge economic costs and a blockade of the international market and interchange. One of the main applications of genome modification in animals for agriculture is the generation of animals resistant to diseases (Whitelaw and Sang 2005; Lassnig and Müller 2015). (Table 3.2). In order to improve the general immune defence of the animals, Han *et al.* generated knock-in porcine models for lactoferrin, an immune-active protein with antimicrobial and antiviral activity, to improve milk quality and subsequently piglet development (Han et al. 2020).

In relation to viral diseases, two main strategies could be applied using CRISPR/Cas9 technology to inhibit or block viral infection, one of them involves targeting

Table 3.2 Gene edited pigs with applications in animal health

Gene	Study	Methodology	Model	References
CD163 and CD1D	Resistance to PRRS	SCNT and Injection	KO	Whitworth et al. (2014)
CD163	Resistance to PRRS	Injection	KO	Burkard et al. (2017)
TMPRRSS2	Resistance to influenza virus	Injection	KO	Whitworth et al. (2017)
"shRNA"	Resistance to classical swine fever virus	SCNT	KI	Xie et al. (2018)
CD163	Resistance to PRRS	SCNT	KO	Yang et al. (2018)
CD163	Resistance to PRRS	SCNT	KO	Wang et al. (2019a)
CD163	Resistance to PRRS	SCNT	KO	Guo et al. (2019a)
CD163	Resistance to PRRS	Electroporation	KO	Tanihara et al. (2019b)
CD163/ hCD163	Resistance to PRRS	SCNT	KO/ KI	Chen et al. (2019b)
pAPN	Resistance to coronavirus	Injection	KO	Whitworth et al. (2019)
pAPN	Resistance to transmissible gastroenteritis virus (TGEV)	SCNT	KO	Luo et al. (2019)
CD163 & pAPN	Resistance to PRRS and TGE	SCNT	KO*2	Xu et al. (2020)
pRSAD2	Resistance to classical swine fever and pseudorabies	SCNT	KI	Xie et al. (2020b)
Lactoferrin	antibacterial activities in milk	SCNT	KI	Han et al. (2020)

SCNT: Somatic cell nuclear transfer. Injection: Intracytoplasmic microinjection of oocytes/embryos

host genes that are essential for viral infection (cell receptors, etc) and the other directly targeting viral DNA as a potential antiviral strategy (Soppe and Lebbink 2017). Porcine reproductive and respiratory syndrome (PRRS) is one of the most important panzootic infectious viral diseases in pigs and due to the generation of late-term abortions and stillbirths, this disease causes important economic losses to the farming industry, (Wells and Prather 2017). For that reason, the most common model for disease resistance produced using CRISPR/Cas9 is the one resistant to PRRS.

The first attempt to modify the receptors of the virus in porcine alveolar macrophages involved generating a KO pig for CD169 (Prather et al. 2013). However, the KO pigs suffered the same course of the PRRS disease. The same group later generated other KO models for CD163 and CD1D as a possible way to block the interaction between the virus and the macrophages (Whitworth et al. 2014). CD163 has been identified as a putative fusion receptor for the PRRS virus, so different authors from different labs produced knock-out pigs for CD163 that are resistant to PRRS

(Burkard et al. 2017; Yang et al. 2018; Guo et al. 2019a; Tanihara et al. 2019b; Wang et al. 2019a). Furthermore, Whitworth *et al.* and Xu *et al.* disrupted CD1D (a major histocompatibility complex protein) and pAPN (receptor of transmissible gastroenteritis (TGE) virus), respectively, producing double knock-out animals resistant to PRRS and the ones in the second study resistant to TGE, as well (Xu et al. 2020). Another strategy to achieve this resistance is replacing the SRCR5 domain of the CD163 gene with the corresponding domain of human CD163 (Chen et al. 2019b).

In addition to PRRS, other diseases were targeted such as those linked to infection by different viruses including coronaviruses. With this aim, Whitworth *et al.* and Luo *et al.* produced porcine models lacking aminopeptidase-N, a protein present on the surface of epithelial cells that has been suggested as a receptor for different coronaviruses (Luo et al. 2019; Whitworth et al. 2019). Going further, Xie *et al.* produced a knock-in model, piglets that produced pRSAD2, an enzyme with antiviral activities against a wide range of viruses such as classic swine fever virus or pseudorabies virus (Xie et al. 2020b).

Another indirect application of the CRISPR/Cas9 system in the control of viral infections that is not directly via generation of resistant pigs, involves developing mutations in the virus that could facilitate the design of valuables vaccines or by the use of CRISPR/Cas9 for the diagnosis of the disease. A clear example of these alternatives is in African swine fever (ASF), by use of this methodology for vaccines (Borca et al. 2018; Hubner et al. 2018) and diagnosis (Bai et al. 2019; He et al. 2020).

One strategy to control viral diseases is the use of specific small hairpin RNAs (shRNA) to reduce the susceptibility to infection by very contagious viral diseases that leads to important economic losses in the pig industry, such as foot and mouth disease virus (Hu et al. 2015). With the application of CRISPR/Cas9 technology, shRNA and SCNT the knock-in (KI) animals are protected against classical swine fever virus (Xie et al. 2018).

3.4.2.2 Animal Production Improvement

In addition, animal production can be improved by targeting different genes that do not involve disease resistance. The most common one is the gene encoding for myostatin, a negative regulator of muscle growth. Different authors have developed knock-out models for myostatin which exhibit greater muscle mass, enhancing in this way the quality of the product (Wang et al. 2015a; Tanihara et al. 2016; Li et al. 2020b; Zhu et al. 2020b). Another approach for improving muscle development is the one proposed by Liu *et al.,* who disrupted the insulin growth factor 2 (IGF2) gene (Liu et al. 2019) (Table 3.3).

Other authors have developed models involving the introduction of one or more foreign genes. Knock-in pigs for Fat-1 have been produced to improve meat quality. This gene encodes a fatty acid desaturase which converts n-6 polyunsaturated fatty acids to n-3 poly- unsaturated fatty acids, that provide more health benefits (Li et al. 2018). Another example is the knock-in for uncoupling protein 1 (UCP1). This protein is located in the inner mitochondrial membrane and regulates heat production,

Table 3.3 Gene edited pigs with applications in animal production improvement

Research field	Gene	Study	Methodology	Model	References
Growth	MSTN	Muscle development	SCNT and Talen	KO	Kang et al. (2017)
Growth	MSTN	Muscle development	SCNT	KO	Wang et al. (2015a)
Growth	MSTN	Muscle development	SCNT	KO	Wang et al. 2017b)
Growth	MSTN	Muscle development	SCNT	KO	Li et al. (2020b)
Growth	MSTN	Muscle development	SCNT	KO	Zhu et al. (2020b)
Growth	MSTN	Muscle development	Electroporation	KO	Tanihara et al. (2016)
Growth	IGF2	Muscle development	SCNT	KO	(Liu et al. 2019)
Metabolism	UCP1	Thermoregulation	SCNT	KI	Zheng et al. (2017)
Meat quality	Fat-1	Fatty acids n-3PUFAs	SCNT	KI	Li et al. (2018)
Pollution reduction	β-glucanase, xylanase, and phytase	Production of digestive enzymes	SCNT	KI*3	Li et al. (2020a)

SCNT: Somatic cell nuclear transfer

but it is absent in pigs. These knock-in animals can better maintain their body temperature and showed decreased fat deposition, improving in this way production efficiency (Zheng et al. 2017).

3.4.2.3 Pollution Reduction

Pigs lack several enzymes in their digestive tract that hydrolyse plant cell walls to release the nutrients that could be absorbed during digestion. An interesting approach is the integration of bacterial enzymes in the salivary glands of the pig to hydrolyse the complex carbohydrates such as the phytate that contains phosphorus in the diet. The objective of this methodology is to increase the intestinal absorption of these nutrients and reduce the presence of this compound in the manure, this being an ecological contamination problem in some areas.

These models have been previously produced by other technologies different than endonucleases like pronuclear injection (Golovan et al. 2001). Using the PSP/APPA transgene (parotid secretary protein promoter linked to the *Escherichia coli* appA phytase gene) one study produced pigs that expressed the functional enzyme in the saliva with a resulting increase in phosphorus digestibility and reduction of

phosphorus in the manure (Golovan et al. 2001; Forsberg et al. 2014a). This model was known as the Enviropig and supported by the Guelph University in Canada. The Enviropig's characteristics have been described (Murray et al. 2007; Golovan et al. 2008; Forsberg et al. 2013; Forsberg et al. 2014a; Forsberg et al. 2014b), as has the manure that they produce (Mao et al. 2008). The animals were terminated in 2012 (Clark 2015).

Later using electroporation and SCNT another study generated pigs with three microbial enzymes, β-glucanase, xylanase, and phytase in the salivary glands (Zhang et al. 2018b). The expression of these enzymes led to a reduction in nitrogen and phosphorous in manure and an increase in growth rates (Zhang et al. 2018b). In another study PiggyBac Transposons and SNCT were used to generate pigs that expressed in their saliva four enzymes; pectinase, xylanase, phytase, and TeEGI (cellulase and β-glucanase) using somatic cell cloning (Wang et al. 2020).

Recently, the application of CRISPR/Cas9 and SCNT led to the generation of a triple knock-in (Li et al. 2020a). The authors integrated into the porcine genome three genes encoding three microbial enzymes (β-glucanase, xylanase, and phytase), which are produced in the salivary glands of the knock-in pigs. This model improves feed efficiency and reduces environmental impact because these enzymes degrade non-starch polysaccharides and phytate in plants, which can significantly promote the digestion of nitrogen and phosphorus in formula feed (Li et al. 2020a).

3.4.3 Biomedicine

The third group of applications is related to biomedicine. This group includes models produced to improve xenotransplantation, to produce different bioproducts and to mimic and study several human diseases.

3.4.3.1 Xenotransplantation

Organ transplantation is the only option for patients with severe organ failure, but there are not sufficient donors to cover the large number of patients that need one (Niemann and Petersen 2016). Xenotransplantation is a potential approach to solve this problem. The pig is considered the most suitable species for this purpose due to its ease of breeding and the similarities with humans regarding physiology and organ size and function. The major problem for clinical application of xenotransplants is the adverse immune reaction of the host (Niemann and Petersen 2016; Naeimi Kararoudi et al. 2018; Niu et al. 2021). The first immune reaction of the host body is the hyperacute rejection (HAR), which is induced by pre-existing antibodies that principally target α-Gal antigens that exist on the surface of porcine cells (Fu et al. 2020). Different knock-out pigs were produced to eliminate these antigens in order to decrease the HAR. With this purpose, the GGTA1 gene, encoding for a galactosyltransferase that catalyses the formation of the α-Gal antigen, was

disrupted producing GGTA1 knock-out pigs (Petersen et al. 2016; Chuang et al. 2017; Tanihara et al. 2020a). In addition to this modification, other genes were targeted at the same time to produce triple and quadruple knock-out pigs (Zhang et al. 2018a; Fischer et al. 2020; Hein et al. 2020; Tanihara et al. 2021).; in addition, there have been other targets (Li et al. 2015; Sake et al. 2019; Fu et al. 2020) (Table 3.4).

Other examples of genetically modified pigs with xenotransplantation applications are the ones produced by SCNT by Hinrichs *et al.* who disrupted the growth hormone receptor (GHR) in GGTA1-deficient cells expressing the human cluster of differentiation (hCD46) and human thrombomodulin (hTHBD) to reduce the size of organ donor pigs for preclinical studies (Hinrichs et al. 2020) or the pigs with severe combined immunodeficiency produced by Boettcher et al., who depleted the IL2RG gene in pigs within a naturally occurring disruption of DCLRE1C (ARTEMIS) background (Boettcher et al. 2020).

In addition to problems related to immune system responses against xenotransplants, the transplantation of organs from one species to another may be associated with other difficulties such as the transmission of endogenous retroviruses. Porcine endogenous retroviruses (PERV) are gamma retroviruses which can infect human cells and integrate into the human genome in cell culture (Yang et al. 2015). Even though no study has observed PERV transmission to humans, they could potentially integrate into the host genome and lead to immunodeficiency and tumorigenesis, so these retroviruses need to be annulled in order to generate a pig that can be used as an organ donor. For this reason, some studies have focused on generating pigs free of PERV (Niu et al. 2017; Li et al. 2019a; Niu et al. 2021).

In the case of liver transplantation, coagulation and blood factors are crucial for the success of the transplant so Li *et al*. proposed a knock-in model to solve this problem. They produced by SCNT, pigs expressing the humanized liver proteins

Table 3.4 Gene edited pigs with applications in xenotransplantation

Gene	Methodology	Model	Year	References
GGTA1, CMAH, iGb3S	SCNT	KO*3	2015	Li et al. (2015)
GGTA1	Injection	KO	2016	Petersen et al. (2016)
Porcine endogenous retroviruses	SCNT	KO	2017	Niu et al. (2017)
GGTA1	Injection	KO	2017	Chuang et al. (2017)
GGTA1, β4GalNT2 and CMAH	SCNT	KO*3	2018	Zhang et al. (2018a)
hF7 and hAlbumin	SCNT	KI*2	2019	Li et al. (2019a)
B-2-microglobulin	SCNT	KO	2019	Sake et al. (2019)
GHR	SCNT	KO	2020	Hinrichs et al. (2020)
GGTA1, CMAH, β4GalNT2 and β2M	SCNT	KO * 4	2020	Fischer et al. (2020)
GGTA, CIITA and β2M	SCNT	KO*3	2020	Fu et al. (2020)
GGTA1, CMAH and β2M	SCNT	KO*3	2020	Hein et al. (2020)
IL2RG	SCNT	KO	2020	Boettcher et al. (2020)
GGTA1	Electroporation	KO	2020	Tanihara et al. (2020a)

SCNT: Somatic cell nuclear transfer. Injection: Intracytoplasmic microinjection of oocytes/embryos

blood-coagulation factor VII (hF7) and albumin (hALB), replacing the previous background of pig F7, with a negative PERV background (Li et al. 2019a).

3.4.3.2 Bioproducts

Pigs can be used as bioreactors to produce different bioproducts (reviewed by (Gadea and Garcia Vazquez 2010; Bertolini et al. 2016)), but this application is the least developed so far. For now, only two genetically modified pigs were developed using CRISPR/Cas9 to synthetise bioproducts. The first one is the one proposed by Peng *et al.*, a knock-in model for human serum albumin, the most abundant plasma protein which is needed for essential processes such as maintenance of plasma oncotic pressure, or transportation of small molecules. This protein is needed in cases of severe diseases such as liver failure or traumatic shock (Peng et al. 2015).

The second example is a knock-out model for IgM. These animals do not produce B-cells, which is the first step for developing pigs that produce humanized polyclonal antibodies that can be used in clinical medicine (Chen et al. 2015).

3.4.3.3 Models of Human Diseases

Pigs are an excellent animal model for understanding the pathological processes of human diseases and developing therapeutic strategies because of their similarity to humans in terms of anatomy, physiology, and genetics (Perleberg et al. 2018). A lot of different models of human diseases have been developed using CRISPR/Cas9 since 2014, covering diverse areas from oncology to hearing loss (Table 3.5).

The area in which most disease models have been developed is for neuroscience. Parkinson's disease is the most common neurodegenerative movement disorder in the elderly, so Parkinson models have been produced using different strategies. Zhou *et al.* disrupted two genes, PARK2 and PINK1 producing a double knock-out model (Zhou et al. 2015). The PARK2 gene encodes a protein called parkin, a component of multiprotein E3 ubiquitin ligase complex and PINK1 gene encodes PTEN-induced putative kinase 1, a mitochondrial serine/threonine-protein kinase. The depletion of either of these two genes produces autosomal recessive early-onset Parkinson's disease in humans (Zhou et al. 2015). In addition to this combination, Wang *et al.* produced a triple knock-out targeting PARK2, PINK1 and DJ1, to model early-onset Parkinson's disease (Wang et al. 2016).

Duchenne's muscular dystrophy is an X-linked hereditary muscular dystrophy and people who have this disease suffer a severe and progressive clinical course of muscle weakness, loss of ability to move, and finally death, but no treatment has yet been developed. The disease is caused by a mutation in the dystrophin gene. For that reason, knock-out pigs for dystrophin were produced (Yu et al. 2016; Wu et al. 2018). Limb-girdle muscular dystrophy also has no treatment and causes muscle wasting. Expression levels of the FBXO40 gene decrease in limb-girdle muscular dystrophy patients, so a knock-out model of FBXO40 was developed to study this

Table 3.5 Gene edited pigs as model of human diseases

Gene	Human disease	Area	Methodology	Model	Year	References
LMNA	Hutchinson-Gilford progeria syndrome	Ageing	SCNT	KI	2019	Dorado et al. (2019)
ApoE and LDLR	Atherosclerosis	Cardiovascular	SCNT	KO *2	2017	Huang et al. (2017)
OSBPL2	Deafness	Deafness	SCNT	KO	2019	Yao et al. (2019)
Mutant GJB2 CDS	Hearing loss	Dermatology	Injection	KI	2020	Xie et al. (2020a)
HR	Atrichia	Dermatology	SCNT	KO	2019	Gao et al. (2019)
vWF	Von Willebrand disease (vWD)	Haematology	Injection	KO	2014	Hai et al. (2014)
F9/hF9	Haemophilia B	Haematology	SCNT	KO/KI	2020	Chen et al. (2020)
TYR, IL2RG, and RAG1	Albinism and immunodeficiency	Immunology	Injection	KO*3	2019	Chen et al. (2019a)
NLRP3	Cryopyrin-associated periodic syndrome	Immunology	SCNT	KO	2020	Li et al. (2020c)
TYR	Albinism	Melanin biosynthesis	SCNT	KO	2015	Zhou et al. (2015)
MITF	Hypopigmentation, deafness, Waardenburg and Tietz syndromes	Melanin biosynthesis	Injection	KO	2015	Wang et al. (2015b)
INS	Diabetes mellitus	Metabolism	SCNT	KO	2018	Cho et al. (2018)
GHR	Laron Syndrome	Metabolism	Injection	KO	2018	Hinrichs et al. (2018)
hIAPP	Diabetes mellitus	Metabolism	SCNT	KI	2019	Zou et al. (2019)
PDX1	Diabetes mellitus	Metabolism	Electroporation	KO	2020	Tanihara et al. (2020b)
PARK2 and PINK1	Parkinson	Neurology	SCNT	KO*2	2015	Zhou et al. (2015)
parkin, DJ-1, PINK1	Parkinson	Neurology	Injection	KO *3	2016	Wang et al. (2016)
DMD	Muscle dystrophy	Neurology	Injection	KO	2016	Yu et al. (2016)
TPH2	Neuropsychiatric disorders	Neurology	SCNT	KO	2017	Li et al. (2017b)

(continued)

Table 3.5 (continued)

Gene	Human disease	Area	Methodology	Model	Year	References
SCNA	Parkinson	Neurology	SCNT	KO	2018	Zhu et al. (2018)
DMD	Muscle dystrophy	Neurology	SCNT	KO	2018	Wu et al. (2018)
FBXO40	Muscle dystrophy	Neurology	SCNT	KO	2018	Zou et al. (2018)
Phenylalanine hydroxylase (PHA)	Phenylketonuria (PKU)	Neurotoxicity	Injection	KO	2020	Koppes et al. (2020)
RUNX3	Cancer	Oncology	SCNT	KO	2016	Kang et al. (2016)
MITF	Hypopigmentation, deafness, Waardenburg and Tietz syndromes	Oncology	Injection	KO	2017	Hai et al. (2017)
TP53	Cancer	Oncology	Electroporation	KO	2018	Tanihara et al. (2018)
COL2A1	Type II collagenopathy	Skeletal development	SCNT	KO	2020	Zhang et al. (2020)

SCNT: Somatic cell nuclear transfer. Injection: Intracytoplasmic microinjection of oocytes/embryos

disease and the function of FBXO40 in skeletal muscle development (Zou et al. 2018).

Another group of models are the one related to metabolic diseases such as diabetes mellitus. Diabetes mellitus is a chronic disease, characterized by high blood glucose levels, polyuria, polydipsia, and weight loss. It is one of the most common public health problems worldwide and large animal models for the evaluation of different treatments are required. The major cause of diabetes is the deficiency in functional insulin because of abnormal insulin secretion and/or decreased physiological responses to insulin. For that reason, different models of genetically modified pigs were produced (Zettler et al. 2020). Cho *et al.* produced insulin deficient pigs by disrupting the INS gene, so the piglets were not able to produce insulin (Cho et al. 2018). Tanihara *et al.* targeted the pancreatic duodenal homeobox 1 (PDX1) gene, producing a monoallelic disruption. The biallelic

mutation of this gene causes abnormal development of the pancreas and death during infanthood, but a monoallelic mutation of the PDX1 gene impairs insulin secretion from pancreatic β-cells, causing diabetes (Tanihara et al. 2020b). Islet amyloid polypeptide (IAPP) is a polypeptide hormone that has a toxic effect on β-cells when it aggregates which leads to the progressive failure of insulin secretion. Human IAPP is one of the most highly aggregated polypeptides and some studies considered the amyloidosis of human IAPP as a potentially important cause of

diabetes mellitus type II. Therefore, Zou *et al.* developed a knock-in model that express human IAPP to study the pathogenesis of diabetes mellitus type II (Zou et al. 2019).

Cancer is one of the most common cause of death worldwide so suitable animal models are needed to study this group of diseases and its treatment. Runt-related transcription factor 3 (RUNX3) is known as a tumour suppressor gene which, when absent, contributes to gastrointestinal cancer development, so a knock-out model for RUNX3 was developed to study this type of cancer (Kang et al. 2016). In addition, the TP53 gene encodes a transcription factor that acts as a tumour suppressor by promoting senescence or apoptosis following DNA damage induced by cell stress. Mutations in this gene are associated with cancer in humans, in particular with Li Fraumeni multiple cancer syndrome. To study this disease, Tanihara *et al.* produced knock-out piglets by disrupting TP53 (Tanihara et al. 2018).

Haemophilia B is an inherited X-linked bleeding disorder caused by a dysfunction in the F9 gene which encodes the coagulation factor IX, a vitamin K-dependent plasma protein that participates in the intrinsic blood coagulation pathway. In patients with haemophilia B, recurrent spontaneous bleeding mainly occurs in the synovial joints causing chronic pain, immobility, and an important reduction in quality of life. Chen *et al.* developed a pig model to study haemophilia B by depleting the F9 gene. Furthermore, they used the CRISPR/Cas9 system to introduce the human F9 gene into knock-out fibroblasts for F9, to determine if this gene therapy procedure could ameliorate the bleeding phenotype (Chen et al. 2020).

Other examples of production of models for human diseases are summarised in Table 3.5 and include models for deafness (Yao et al. 2019; Xie et al. 2020a), atherosclerosis (Huang et al. 2017), albinism (Zhou et al. 2015; Chen et al. 2019a) and Hutchinson- Gilford progeria syndrome (Dorado et al. 2019), among others.

3.5 Future Directions

3.5.1 Base Editors

In addition to the conventional use of Cas9 to generate INDELs, modifications of this Cas protein have been developed. Among the modified forms of Cas9, base editors stand out. These proteins are synthetic enzymes derived from modifications of *Streptococcus pyogenes* Cas9 which induces single-nucleotide changes in the DNA sequence without cutting the DNA double strand of DNA (Komor et al. 2016; Kim et al. 2017).

Base editors are characterized by the presence of Cas9 with a defective catalytical domain, called dead Cas9 or with an impaired catalytical domine, called Cas9 nickase and fused with a deaminase (Kim et al. 2017). The main advantage of base editor is the ability to generate desired mutations without double-stranded DNA breaks and DNA donor template to produce a knock-in, allowing therefore the

possibility of producing the desired stop codons or precise modifications to make personalized disease models caused by pair base substitution (Kim et al. 2017; Eid et al. 2018).

Depending on the presence of a cytidine deaminase or an adenine deaminase, the base editors system can be classified in two groups: cytosine base editors that convert C:G pairs to T:A pairs and adenine base editors that convert A:T pairs to G:C pairs.

The enzymatic activity of a cytosine base editor involves the conversion of cytosine into uracil by deamination. Thus, a cytosine that pairs with guanine becomes an uracil that has base-paring proprieties of thymine and it pairs with adenine (Kim et al. 2017). A cytosine base editor requires the presence of the target cytosine within a 5-nucleotide window near the PAM sequence, within the position 4 to 8 (Komor et al. 2016). Despite the precision, this 5-nucleotide window can be a problem because the enzyme can modify all cytosine in that range, inducing undesired changes to the target locus (Komor et al. 2016).

In 2016, the first cytosine base editor (BE1) was developed, using rat APOBEC1 as deaminase and a dead Cas9 plus XTEN domain (Komor et al. 2016). Subsequentely, a second generation of base editor (BE2) was designed by the addition of uracil DNA glycosylase inhibitor (UGI) to the complex APOBEC-XTEN-dCas9-UGI (Komor et al. 2016). UGI inhibits uracil DNA glycosylase, which removes uracil from DNA and initiates base-excision repair with the reversion of the U:G pair to C:G pair, decreasing the efficiency of base editors (Komor et al. 2016). Up to this moment, the maximum efficiency that could be achieved was 50% because only one chain of the double stranded DNA was modified.

With the objective of going beyond the limit of inducing changes in the non-edited chain, a third generation was developed (BE3). In this generation, the HNH domain of Cas9 was restored (APOBEC-XTEN-dCas9(A840H)-UGI) (Komor et al. 2016). Therefore, dCas9 was substituted by nickase Cas9 to cut the DNA strand containing the unedited guanine to stimulate the repair of this chain. Therefore, cutting in the non-edited change increases the possibilities of solving the U:G mismatch in U:A respect C:G (Komor et al. 2016).

Subsequently, new versions have been developed. A fourth generation (BE4) of cytidine base editor was designed with the addition of a second copy of UGI to the C terminus of the construct (Komor et al. 2017). Also, to increase the stability of the double strand, the bacteriophage Mu-originated Gam protein was added (BE4-Gam) (Yuan et al. 2020). Gram protein of bacteriophage Mu binds to the end of DSBs and protects them from degradation, as this would reduce the indel formation during the process of base editing (Komor et al. 2017).

The addition of nuclear localization signals (NLSs) to the cytosine base editor was also described (Koblan et al. 2018). This modification increased the mutation rate but simultaneously produced other unwanted mutations (Yuan et al. 2020).

In porcine embryos, the use of cytosine base editors has been described (Xie et al. 2019; Su et al. 2020; Yuan et al. 2020), but not yet adenine base editors. In these studies, cytosine base editors of different generations were used. In all studies, the strategy used was to produce premature stop codons. In this way, knock-out

embryos were generated to model diseases such as Duchenne muscular dystrophy (Xie et al. 2019; Su et al. 2020), albinism (Xie et al. 2019), Hutchinson-Gilford Progeria syndrome (Xie et al. 2019), and the absence of cell of immune system (Xie et al. 2019). In the field of xenotransplantation; cytosine base editors have also been used for also for knock-out porcine endogenous retroviruses (Xie et al. 2019) and simultaneously knock-out three genes (GGTA1, B4GalNT2 and CMAH) to remove the expression of alpha-1,2-galactose in pigs, the major hyperacute rejection xenoantigen (Yuan et al. 2020).

3.5.2 Conditional Models

The Cre–loxP system is a powerful tool for conditional models, that is successfully used for murine models. This system makes it possible to investigate genes of interest in a specific organ/tissue in a specific moment or time (Smedley et al. 2011). The Cre-loxP system needs two elements. First, Cre-driver animals are generated in which Cre recombinase is expressed by a promoter that specifically targets the cell or tissue of interest. Second, specific genes are engineered to be flanked by loxP in specific animals (floxed animals). Conditional knockout pig are generated by breeding the Cre-driver animals with floxed ones. The specificity and timing of recombination are controlled by use of a promoter and/or enhancer.

Although many Cre–loxP mouse models have been established, there are few pig models available. Some Cre pigs have been generated for specific tissues as germ cells by using the VASA promoter (Song et al. 2016), for astrocytes using the promoter of the pig glial fibrillary acidic protein (*pGFAP*) gene (Hwang et al. 2018), alveolar epithelial cells (Luo et al. 2014b) or kidney collecting duct cells (Luo et al. 2014a). Also, different authors have developed models with reporters for monitoring Cre activity in vivo (Li et al. 2009; Li et al. 2014). Additionally, different strategies have been developed for the efficient deletion of the IoxP flanked selectable marker like use of neomycin to avoid possible side effects (Whitworth et al. 2018; Huang et al. 2020b). In pigs, this conditional gene expression strategy has been used to promote oncogenic expression. The Oncopig is a transgenic pig with Cre-inducible TP53R167H and KRASG12D mutations (Schook et al. 2015; Schook et al. 2016). This commercially available model have been used to study liver and pancreatic cancer (Schachtschneider et al. 2017; Boas et al. 2020).

3.6 Legal and Ethical Regulations

The use of gene editing in organisms with the aim of achieving genetic advantages in a short time has been highly controversial. After the application of gene editing technologies, and the development of programmable endonucleases that are as easy

and cheap to use as CRISPR/Cas9, it was necessary to create legislation to regulate the use of this technology.

Currently, genetically modified organisms are covered by the same regulations as transgenic organisms (Lamas-Toranzo et al. 2017; Wasmer 2019). This legislation also includes organisms with simple genetic modifications generated by genetic engineering, such as mutations that affect a single base. However, these mutations cannot be distinguished from organisms bred by conventional techniques, such as those that arise from random mutagenesis (Wasmer 2019). The use of genetically modified organisms is strictly regulated around the world. Next, we will present the legislative situation of genetically modified organisms in Europe, United States of America (USA) and China.

European Union

In the European Union (EU) the regulations covering genetically modified animals for human and animal consumption are somewhat restrictive such that nowadays only genetically modified plants are authorised for that purpose (European Commission Register for Genetically Modified Organisms, https://webgate.ec.europa.eu/dyna/gm_register) . So that a genetically modified organism can be commercialized or released to the environment it needs the approval of the European Food Safety Agency (EFSA). The EFSA assesses the risks that the genetically modified organism may present to the environment, and human health and animal safety, in the EU, and decides if this organism can be approved or not according European regulations on genetically modified food and feed (No 1829/2003 and No 503/2013).

If the genetically modified organism or its bioproducts have medical purposes, it needs to be authorised by the European Medicines Agency (EMA). When the medicinal product contains genetically modified organisms an environmental impact study must be performed and sent to the EMA in addition to the typical reports (administrative, quality, non-clinical and clinical data). However, regarding genetically modified animals, they usually produce a bioproduct that will be part of a medicine. This way, the medical product does not contain a genetically modified organism and does not need an environmental impact study (EMA 2006).

In the case of genetically modified animals that produce bioproducts the EMA provides a guideline on the approaches that should be employed in order to achieve satisfactory quality for biological active substances (EMA 2013). The medicinal product containing components derived from transgenic animals must follow the Regulation (EC) No 726/2004. The effect of the transgene on the health and longevity of the animals must be supervised. Furthermore, a monitoring protocol should be followed in order to assess the health and wellbeing of the animals and check specific infections. It is important to confirm that the bioproduct is free from microorganisms such bacteria, fungi, mycoplasma or virus (EMA 2013). To date, three bioproducts produced by genetically modified animals have been approved in the EU: Antithrombin (ATryn®) from goat milk approved in 2006 but withdrawn in 2018 (Adiguzel et al. 2009; EMA 2019). Human C1- inhibitor (Ruconest) from rabbit milk approved in 2010 (EMA 2020). Sebelipase α (Kanuma) from egg hen approved in 2015 (EMA 2015).

Tissues and organs from genetically modified animals for the objective of xenotransplantation must follow two guidelines: (a) the guideline on xenogeneic cell-based medicinal products and (b) the guideline on the quality, preclinical and clinical aspects of gene transfer medicinal products (EMA 2001, 2009). The therapy must follow the common testing and development procedures, but genetically modified animals should be fully characterised and confirmation of the nature of the inserted, deleted or modified gene must be provided (EMA 2009)

USA

In the USA the regulations covering genetically modified organisms are less strict. To approve the production of an animal with medical and consumption purposes, a new "animal drug application" must be proposed to the Food and Drug Administration describing the characteristics of the animals and its environmental impact and food safety. Furthermore, a compositional and nutritional analysis must be performed if the aim of the product is animal or human consumption in other to compare the composition of the genetically modified animal and the wild-type animal and study any possible toxicological or nutritional hazard to consumers (FDA 2017).

There are two genetically modified animals that have been approved for human consumption in the USA by the Food and Drug Administration the AquAdvantage Salmon (Clifford 2014), which was approved in 2015 and the GalSafe pig, approved in 2020. The last one was also approved for human therapeutics (FDA 2020).

The GalSafe pigs have a disruption of the GGTA1 gene, so they do not produce α-1,3- galactosyltransferase and there are no α-Gal antigens in the surface of their cells. This is an advantage for xenotransplantation, as mentioned above, but this model also has other commercial potential because people that suffer from α-gal syndrome, an allergy to red meat, are able to consume meat from these animals (FDA 2020).

Regarding drugs and biological products derived from genetically modified animals, they should follow the same approval procedure as the ones derived from other sources, providing data of pre-clinical and clinical studies, manufacture and safety (Federal Regulations CFR § 601.2. Applications for biologics licenses; procedures for filing. And CFR §314.5. Applications for FDA approval to market a new drug). There are three products produced by genetically modified animals approved for commercialization and these are the same as those approved in the EU.

China

China has a strict law concerning genetically modified animals. To date, no genetically modified animal has been approved in China for consumption or for medical purposes. The Ministry of Agriculture and Rural Affairs of the State Council of China is the organization that provides the license to a genetically modified organism to be produced and commercialised. To achieve the approval, documents describing the genetically modified animal and its safety must be provided in addition to the usual information (2019).

The economic impact of the application of these new technologies in the pig industry has been evaluated and quantified (Novoselova et al. 2013; Van Eenennaam et al. 2020). According to some authors the delay in the regulation of these animals

will have an economic impact and also a reduction in global food security (Van Eenennaam et al. 2020; Feng and Yang 2019).

3.7 Conclusions

The generation of gene edited pigs with new endonucleases has important applications in the field of agriculture and livestock production and in the biomedicine sector. The develop of more efficient protocols will facilitate the extension and applications to different approaches. These improvements will have worldwide impact in the economy and in the health of the population in terms of food security and control and treatment of human diseases.

Acknowledgements This work was supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and 21105/PDC/19; and FPU fellowship to SNS (FPU16/04480) from Spanish Ministry of Education, Culture and Sport.

References

Adiguzel C, Iqbal O, Demir M, Fareed J (2009) European community and US-FDA approval of recombinant human antithrombin produced in genetically altered goats. Clin Appl Thromb Hemost 15(6):645–651. https://doi.org/10.1177/1076029609339748

Angel MA, Gil MA, Cuello C, Sanchez-Osorio J, Gomis J, Parrilla I, Vila J, Colina I, Diaz M, Reixach J, Vazquez JL, Vazquez JM, Roca J, Martinez EA (2014) An earlier uterine environment favors the in vivo development of fresh pig morulae and blastocysts transferred by a nonsurgical deep-uterine method. J Reprod Dev 60(5):371–376

Bai J, Lin H, Li H, Zhou Y, Liu J, Zhong G, Wu L, Jiang W, Du H, Yang J, Xie Q, Huang L (2019) Cas12a-Based On-Site and Rapid Nucleic Acid Detection of African Swine Fever. Front Microbiol 10:2830. https://doi.org/10.3389/fmicb.2019.02830

Beaujean N, Jammes H, Jouneau A (2015) Nuclear reprogramming: methods and protocols. methods in molecular biology, Methods and protocols,1222, 2nd edn. Springer New York, New York

Bertolini LR, Meade H, Lazzarotto CR, Martins LT, Tavares KC, Bertolini M, Murray JD (2016) The transgenic animal platform for biopharmaceutical production. Transgenic Res 25(3):329–343. https://doi.org/10.1007/s11248-016-9933-9

Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim YG, Chandrasegaran S (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol Cell Biol 21(1):289–297. https://doi.org/10.1128/MCB.21.1.289-297.2001

Bibikova M, Golic M, Golic KG, Carroll D (2002) Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. Genetics 161(3):1169–1175

Bloom K, Ely A, Arbuthnot P (2017) A T7 endonuclease I assay to detect talen-mediated targeted mutation of HBV cccDNA. In: Methods in molecular biology, vol 1540. Humana Press Inc., pp 85–95. https://doi.org/10.1007/978-1-4939-6700-1_8

Boas FE, Nurili F, Bendet A, Cheleuitte-Nieves C, Basturk O, Askan G, Michel AO, Monette S, Ziv E, Sofocleous CT, Maxwell AWP, Schook LB, Solomon SB, Kelsen DP, Scherz A, Yarmohammadi H (2020) Induction and characterization of pancreatic cancer in a transgenic pig model. PLoS One 15(9):e0239391. https://doi.org/10.1371/journal.pone.0239391

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326(5959):1509–1512. https://doi.org/10.1126/science.1178811

Boettcher AN, Li Y, Ahrens AP, Kiupel M, Byrne KA, Loving CL, Cino-Ozuna AG, Wiarda JE, Adur M, Schultz B, Swanson JJ, Snella EM, Ho CS, Charley SE, Kiefer ZE, Cunnick JE, Putz EJ, Dell'Anna G, Jens J, Sathe S, Goldman F, Westin ER, Dekkers JCM, Ross JW, Tuggle CK (2020) Novel Engraftment and T Cell Differentiation of Human Hematopoietic Cells in ART (-/-) IL2RG (-/Y) SCID Pigs. Front Immunol 11:100. https://doi.org/10.3389/fimmu.2020.00100

Borca MV, Holinka LG, Berggren KA, Gladue DP (2018) CRISPR-Cas9, a tool to efficiently increase the development of recombinant African swine fever viruses. Scientific Rep 8(1):3154. https://doi.org/10.1038/s41598-018-21575-8

Brem G, Brenig B, Goodman HM, Selden RC, Graf F, Kruff B, Springman K, Hondele J, Meyer J, Winnacker EL, Kräußlich H (1985) Production of transgenic mice, rabbits and pigs by microinjection into pronuclei. Reprod Domest Anim 20(4):251–252. https://doi.org/10.1111/j.1439-0531.1985.tb00423.x

Brinkman EK, Kousholt AN, Harmsen T, Leemans C, Chen T, Jonkers J, van Steensel B (2018) Easy quantification of template-directed CRISPR/Cas9 editing. Nucleic Acids Res 46(10):e58. https://doi.org/10.1093/nar/gky164

Bui HT, Van Thuan N, Wakayama T, Miyano T (2006) Chromatin remodeling in somatic cells injected into mature pig oocytes. Reproduction 131(6):1037–1049. https://doi.org/10.1530/rep.1.00897

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(2):e1006206. https://doi.org/10.1371/journal.ppat.1006206

Canovas S, Ivanova E, Romar R, Garcia-Martinez S, Soriano-Ubeda C, Garcia-Vazquez FA, Saadeh H, Andrews S, Kelsey G, Coy P (2017) DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids. eLife 6:e23670. doi:https://doi.org/10.7554/eLife.23670

Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CB, Fahrenkrug SC (2012) Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A 109(43):17382–17387. https://doi.org/10.1073/pnas.1211446109

Chen F, Wang Y, Yuan Y, Zhang W, Ren Z, Jin Y, Liu X, Xiong Q, Chen Q, Zhang M, Li X, Zhao L, Li Z, Wu Z, Zhang Y, Hu F, Huang J, Li R, Dai Y (2015) Generation of B cell- deficient pigs by highly efficient CRISPR/Cas9-mediated gene targeting. J Genet Genom Yi chuan xue bao 42(8):437–444. https://doi.org/10.1016/j.jgg.2015.05.002

Chen S, Lee B, Lee AY, Modzelewski AJ, He L (2016) Highly Efficient Mouse Genome Editing by CRISPR Ribonucleoprotein Electroporation of Zygotes. J Biol Chem 291 (28):14457- 14467. https://doi.org/10.1074/jbc.M116.733154

Chen B, Gu P, Jia J, Liu W, Liu Y, Liu W, Xu T, Lin X, Lin T, Liu Y, Chen H, Xu M, Yuan J, Zhang J, Zhang Y, Xiao D, Gu W (2019a) Optimization strategy for generating gene- edited tibet minipigs by synchronized oestrus and cytoplasmic microinjection. Int J Biol Sci 15(12):2719–2732. https://doi.org/10.7150/ijbs.35930

Chen J, Wang H, Bai J, Liu W, Liu X, Yu D, Feng T, Sun Z, Zhang L, Ma L, Hu Y, Zou Y, Tan T, Zhong J, Hu M, Bai X, Pan D, Xing Y, Zhao Y, Tian K, Hu X, Li N (2019b) Generation of Pigs Resistant to Highly Pathogenic-Porcine Reproductive and Respiratory Syndrome Virus through Gene Editing of CD163. Int J Biol Sci 15(2):481–492. https://doi.org/10.7150/ijbs.25862

Chen J, An B, Yu B, Peng X, Yuan H, Yang Q, Chen X, Yu T, Wang L, Zhang X, Wang H, Zou X, Pang D, Ouyang H, Tang X (2020) CRISPR/Cas9-mediated knockin of human factor IX into swine factor IX locus effectively alleviates bleeding in hemophilia B pigs. Haematologica. https://doi.org/10.3324/haematol.2019.224063

Cho B, Kim SJ, Lee EJ, Ahn SM, Lee JS, Ji DY, Lee K, Kang JT (2018) Generation of insulin- deficient piglets by disrupting INS gene using CRISPR/Cas9 system. Transgenic Res 27(3):289–300. https://doi.org/10.1007/s11248-018-0074-1
Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186(2):757–761. https://doi.org/10.1534/genetics.110.120717
Chuang CK, Chen CH, Huang CL, Su YH, Peng SH, Lin TY, Tai HC, Yang TS, Tu CF (2017) Generation of GGTA1 mutant pigs by direct pronuclear microinjection of CRISPR/Cas9 plasmid vectors. Anim Biotechnol 28(3):174–181. https://doi.org/10.1080/10495398.2016.1246453
Clark JL (2015) Killing the enviropigs. J Anim Ethics 5(1):20–30
Clifford H (2014) AquAdvantage® Salmon - a pioneering application of biotechnology in aquaculture. BMC Proc 8(S4):O31. https://doi.org/10.1186/1753-6561-8-s4-o31
Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823. https://doi.org/10.1126/science.1231143
Crociara P, Chieppa MN, Vallino Costassa E, Berrone E, Gallo M, Lo Faro M, Pintore MD, Iulini B, D'Angelo A, Perona G, Botter A, Formicola D, Rainoldi A, Paulis M, Vezzoni P, Meli F, Peverali FA, Bendotti C, Trolese MC, Pasetto L, Bonetto V, Lazzari G, Duchi R, Perota A, Lagutina I, Quadalti C, Gennero MS, Dezzutto D, Desiato R, Boido M, Ghibaudi M, Valentini MC, Caramelli M, Galli C, Casalone C, Corona C (2019) Motor neuron degeneration, severe myopathy and TDP-43 increase in a transgenic pig model of SOD1- linked familiar ALS. Neurobiol Dis 124:263–275. https://doi.org/10.1016/j.nbd.2018.11.021
Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002) Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 20(3):251–255. https://doi.org/10.1038/nbt0302-251
Dang-Nguyen TQ, Nguyen HT, Somfai T, Wells D, Men NT, Viet-Linh N, Noguchi J, Kaneko H, Kikuchi K, Nagai T (2018) Sucrose assists selection of high-quality oocytes in pigs. Anim Sci J Nihon chikusan Gakkaiho 89(6):880–887. https://doi.org/10.1111/asj.13015
Das S, Koyano-Nakagawa N, Gafni O, Maeng G, Singh BN, Rasmussen T, Pan X, Choi KD, Mickelson D, Gong W, Pota P, Weaver CV, Kren S, Hanna JH, Yannopoulos D, Garry MG, Garry DJ (2020) Generation of human endothelium in pig embryos deficient in ETV2. Nat Biotechnol 38(3):297–302. https://doi.org/10.1038/s41587-019-0373-y
Dehairs J, Talebi A, Cherifi Y, Swinnen JV (2016) CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing. Scientific reports 6(1):28973. https://doi.org/10.1038/srep28973
Diao YF, Naruse KJ, Han RX, Li XX, Oqani RK, Lin T, Jin DI (2013) Treatment of fetal fibroblasts with DNA methylation inhibitors and/or histone deacetylase inhibitors improves the development of porcine nuclear transfer-derived embryos. Anim Reprod Sci 141(3-4):164–171. https://doi.org/10.1016/j.anireprosci.2013.08.008
Dorado B, Ploen GG, Barettino A, Macias A, Gonzalo P, Andres-Manzano MJ, Gonzalez-Gomez C, Galan-Arriola C, Alfonso JM, Lobo M, Lopez-Martin GJ, Molina A, Sanchez-Sanchez R, Gadea J, Sanchez-Gonzalez J, Liu Y, Callesen H, Filgueiras-Rama D, Ibanez B, Sorensen CB, Andres V (2019) Generation and characterization of a novel knockin minipig model of Hutchinson-Gilford progeria syndrome. Cell Discov 5(1):16. https://doi.org/10.1038/s41421-019-0084-z
Du Y, Kragh PM, Zhang Y, Li J, Schmidt M, Bogh IB, Zhang X, Purup S, Jorgensen AL, Pedersen AM, Villemoes K, Yang H, Bolund L, Vajta G (2007) Piglets born from handmade cloning, an innovative cloning method without micromanipulation. Theriogenology 68(8):1104–1110. https://doi.org/10.1016/j.theriogenology.2007.07.021
Dziuk P (1985) Effect of migration, distribution and spacing of pig embryos on pregnancy and fetal survival. J Reprod Fertil Suppl 33:57–63
Eid A, Alshareef S, Mahfouz MM (2018) CRISPR base editors: genome editing without double-stranded breaks. Biochem J 475(11):1955–1964. https://doi.org/10.1042/bcj20170793

Elkhadragy L, Regan MR, W MT, Goli KD, Patel S, Garcia K, Stewart M, Schook LB, Gaba RC, Schachtschneider KM (2021) Generation of genetically tailored porcine liver cancer cells by CRISPR/Cas9 editing. Biotechniques 70(1):37–48. https://doi.org/10.2144/btn-2020-0119

EMA (2001) Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products. European Medicines Agency (EMA), London

EMA (2006) Guideline on environmental risk assessments for medicinal products consisting of, or containing, genetically modified organisms (GMOs). European Medicines Agency (EMA), London

EMA (2009) Guideline on xenogeneic cell-based medicinal products. European Medicines Agency (EMA), London

EMA (2013) Guideline on Quality of biological active substances produced by transgene expression in animals. European Medicines Agency (EMA), London

EMA (2015) Kanuma sebelipase alfa European Medicines Agency (EMA)

EMA (2019) ATryn. Withdrawal of the marketing authorisation in the European Union EMA (2020) Ruconest conestat alfa European Medicines Agency (EMA)

Fajrial AK, He QQ, Wirusanti NI, Slansky JE, Ding X (2020) A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing. Theranostics 10(12):5532–5549. https://doi.org/10.7150/thno.43465

FAO (2020) Food outlook biannual report on global food markets – June 2020. Food Outlook, Rome. https://doi.org/10.4060/ca9509en

FDA (2017) Guidance for industry regulation of intentionally altered genomic DNA in animals

FDA (2020) GalSafe pig. NADA 141-542. US Food & Drug Administration.

Feng J, Yang F (2019) The Regulation of Genetically Modified Food in China. Biotechnol Law Report 38(5):289–293. https://doi.org/10.1089/blr.2019.29137.jf

Fischer K, Rieblinger B, Hein R, Sfriso R, Zuber J, Fischer A, Klinger B, Liang W, Flisikowski K, Kurome M, Zakhartchenko V, Kessler B, Wolf E, Rieben R, Schwinzer R, Kind A, Schnieke A (2020) Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALNT2. Xenotransplantation 27(1):e12560. https://doi.org/10.1111/xen.12560

Forsberg CW, Meidinger RG, Liu M, Cottrill M, Golovan S, Phillips JP (2013) Integration, stability and expression of the E. coli phytase transgene in the Cassie line of Yorkshire Enviropig. Transgenic Res 22(2):379–389. https://doi.org/10.1007/s11248-012-9646-7

Forsberg CW, Meidinger RG, Ajakaiye A, Murray D, Fan MZ, Mandell IB, Phillips JP (2014a) Comparative carcass and tissue nutrient composition of transgenic Yorkshire pigs expressing phytase in the saliva and conventional Yorkshire pigs. J Anim Sci 92 (10):4417–4439. https://doi.org/10.2527/jas.2014-7780

Forsberg CW, Meidinger RG, Murray D, Keirstead ND, Hayes MA, Fan MZ, Ganeshapillai J, Monteiro MA, Golovan SP, Phillips JP (2014b) Phytase properties and locations in tissues of transgenic pigs secreting phytase in the saliva. J Anim Sci 92(8):3375–3387. https://doi.org/10.2527/jas.2014-7782

Fu R, Fang M, Xu K, Ren J, Zou J, Su L, Chen X, An P, Yu D, Ka M, Hai T, Li Z, Li W, Yang Y, Zhou Q, Hu Z (2020) Generation of GGTA1−/−β2M−/−CIITA−/− Pigs Using CRISPR/Cas9 technology to alleviate xenogeneic immune reactions. Transplantation 104(8):1566–1573. https://doi.org/10.1097/tp.0000000000003205

Gadea J, Garcia Vazquez F (2010) Applications of transgenic pigs in biomedicine and animal production. Itea-Informacion Tecnica Economica Agraria 106(1):30–45

Gadea J, Garcia-Vazquez FA, Hachem A, Bassett A, Romero-Aguirregomezcorta J, Canovas S, Romar R, Parrington J (2018) Generation of TPC2 knock out pig embryos by CRISPR- Cas technology. Reprod Domest Anim 53:87–88

Gao QS, Xuan MF, Luo ZB, Paek HJ, Kang JD, Yin XJ (2019) Hairless-knockout piglets generated using the clustered regularly interspaced short palindromic repeat/CRISPR- associated-9 exhibit abnormalities in the skin and thymus. Exp Anim 68(4):519–529. https://doi.org/10.1538/expanim.19-0018

Garcia-Vazquez FA, Ruiz S, Matas C, Izquierdo-Rico MJ, Grullon LA, De Ondiz A, Vieira L, Aviles-Lopez K, Gutierrez-Adan A, Gadea J (2010) Production of transgenic piglets using ICSI-sperm-mediated gene transfer in combination with recombinase RecA. Reproduction 140(2):259–272. https://doi.org/10.1530/REP-10-0129

Garcia-Vazquez FA, Hernandez-Caravaca I, Martin M, Gomez E, Rodriguez A, Sanchez-Sanchez R, Gadea J (2011) Two cases of reciprocal chromosomal translocation (4; 7)(p+; q-) (2; 8)(q-; q+) in piglets produced by ICSI. Reprod Domes Anim 46(4):728–730. https://doi.org/10.1111/j.1439-0531.2010.01707.x

Giuffra E, Kijas JM, Amarger V, Carlborg O, Jeon JT, Andersson L (2000) The origin of the domestic pig: independent domestication and subsequent introgression. Genetics 154(4):1785–1791

Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard JW, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19(8):741–745. https://doi.org/10.1038/90788

Golovan SP, Hakimov HA, Verschoor CP, Walters S, Gadish M, Elsik C, Schenkel F, Chiu DK, Forsberg CW (2008) Analysis of Sus scrofa liver proteome and identification of proteins differentially expressed between genders, and conventional and genetically enhanced lines. Comparative biochemistry and physiology Part D, Genomics & proteomics 3(3):234–242. https://doi.org/10.1016/j.cbd.2008.05.001

Gordon JW, Ruddle FH (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. Science 214(4526):1244–1246. https://doi.org/10.1126/science.6272397

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A 77(12):7380–7384. https://doi.org/10.1073/pnas.77.12.7380

Guo C, Wang M, Zhu Z, He S, Liu H, Liu X, Shi X, Tang T, Yu P, Zeng J, Yang L, Cao Y, Chen Y, Liu X, He Z (2019a) Highly Efficient Generation of Pigs Harboring a Partial Deletion of the CD163 SRCR5 Domain, Which Are Fully Resistant to Porcine Reproductive and Respiratory Syndrome Virus 2 Infection. Front Immunol 10 (1846):1846. doi:https://doi.org/10.3389/fimmu.2019.01846

Guo Z, Lv L, Lui D, Fu B (2019b) Delayed activation methods and development of pig somatic cell nuclear transfer embryos: a meta-analysis. Large Anim Rev 25(2):69–73

Hai T, Teng F, Guo R, Li W, Zhou Q (2014) One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res 24(3):372–375. https://doi.org/10.1038/cr.2014.11

Hai T, Guo W, Yao J, Cao C, Luo A, Qi M, Wang X, Wang X, Huang J, Zhang Y, Zhang H, Wang D, Shang H, Hong Q, Zhang R, Jia Q, Zheng Q, Qin G, Li Y, Zhang T, Jin W, Chen ZY, Wang H, Zhou Q, Meng A, Wei H, Yang S, Zhao J (2017) Creation of miniature pig model of human Waardenburg syndrome type 2A by ENU mutagenesis. Hum Genet 136(11-12):1463–1475. https://doi.org/10.1007/s00439-017-1851-2

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315(6021):680–683. https://doi.org/10.1038/315680a0

Han X, Gao Y, Li G, Xiong Y, Zhao C, Ruan J, Ma Y, Li X, Li C, Zhao S, Xie S (2020) Enhancing the antibacterial activities of sow milk via site-specific knock-in of a lactoferrin gene in pigs using CRISPR/Cas9 technology. Cell Biosci 10(1):133. https://doi.org/10.1186/s13578-020-00496-y

Hatada L (2017) Genome editing in animals, vol 1630. Methods in molecular biology. Springer New York, New York. https://doi.org/10.1007/978-1-4939-7128-2

Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, Zhang L, Meng X, Gregory PD, Schwinzer R, Cost GJ, Niemann H (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc Natl Acad Sci U S A 108(29):12013–12017. https://doi.org/10.1073/pnas.1106422108

He Q, Yu D, Bao M, Korensky G, Chen J, Shin M, Kim J, Park M, Qin P, Du K (2020) High-throughput and all-solution phase African Swine Fever Virus (ASFV) detection using CRISPR-Cas12a and fluorescence based point-of-care system. Biosens Bioelectron 154:112068. https://doi.org/10.1016/j.bios.2020.112068

Hein R, Sake HJ, Pokoyski C, Hundrieser J, Brinkmann A, Baars W, Nowak-Imialek M, Lucas-Hahn A, Figueiredo C, Schuberth HJ, Niemann H, Petersen B, Schwinzer R (2020) Triple (GGTA1, CMAH, B2M) modified pigs expressing an SLA class I(low) phenotype- Effects on immune status and susceptibility to human immune responses. Am J Transplant 20(4):988–998. https://doi.org/10.1111/ajt.15710

Hinrichs A, Kessler B, Kurome M, Blutke A, Kemter E, Bernau M, Scholz AM, Rathkolb B, Renner S, Bultmann S, Leonhardt H, de Angelis MH, Nagashima H, Hoeflich A, Blum WF, Bidlingmaier M, Wanke R, Dahlhoff M, Wolf E (2018) Growth hormone receptor- deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver. Molecular metabolism 11:113–128. https://doi.org/10.1016/j.molmet.2018.03.006

Hinrichs A, Riedel EO, Klymiuk N, Blutke A, Kemter E, Langin M, Dahlhoff M, Kessler B, Kurome M, Zakhartchenko V, Jemiller EM, Ayares D, Bidlingmaier M, Flenkenthaler F, Hrabe de Angelis M, Arnold GJ, Reichart B, Frohlich T, Wolf E (2020) Growth hormone receptor knockout to reduce the size of donor pigs for preclinical xenotransplantation studies. Xenotransplantation n/a (n/a):e12664. https://doi.org/10.1111/xen.12664

Hirata M, Tanihara F, Wittayarat M, Hirano T, Nguyen NT, Le QA, Namula Z, Nii M, Otoi T (2019) Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes. Vitro Cell Dev Biol Anim 55(4):237–242. https://doi.org/10.1007/s11626-019-00338-3

Hirata M, Wittayarat M, Tanihara F, Sato Y, Namula Z, Le QA, Lin Q, Takebayashi K, Otoi T (2020) One-step genome editing of porcine zygotes through the electroporation of a CRISPR/Cas9 system with two guide RNAs. In Vitro Cell Dev Biol Anim 56 (8):614–621. https://doi.org/10.1007/s11626-020-00507-9

Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29(8):731–734. https://doi.org/10.1038/nbt.1927

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4(11):1054–1060. https://doi.org/10.1038/sj.embor.embor7400007

Hooper M, Hardy K, Handyside A, Hunter S, Monk M (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326(6110):292–295. https://doi.org/10.1038/326292a0

Hornak M, Hulinska P, Musilova P, Kubickova S, Rubes J (2009) Investigation of chromosome aneuploidies in early porcine embryos using comparative genomic hybridization. Cytogenet Genome Res 126(1-2):210–216. https://doi.org/10.1159/000245922

Hryhorowicz M, Lipinski D, Hryhorowicz S, Nowak-Terpilowska A, Ryczek N, Zeyland J (2020) Application of genetically engineered pigs in biomedical research. Genes (Basel) 11(6):670. https://doi.org/10.3390/genes11060670

Hu S, Qiao J, Fu Q, Chen C, Ni W, Wujiafu S, Ma S, Zhang H, Sheng J, Wang P, Wang D, Huang J, Cao L, Ouyang H (2015) Transgenic shRNA pigs reduce susceptibility to foot and mouth disease virus infection. eLife 4:e06951. https://doi.org/10.7554/eLife.06951

Huan YJ, Zhu J, Xie BT, Wang JY, Liu SC, Zhou Y, Kong QR, He HB, Liu ZH (2013) Treating cloned embryos, but not donor cells, with 5-aza-2′-deoxycytidine enhances the developmental competence of porcine cloned embryos. J Reprod Dev 59(5):442–449. https://doi.org/10.1262/jrd.2013-026

Huang L, Hua Z, Xiao H, Cheng Y, Xu K, Gao Q, Xia Y, Liu Y, Zhang X, Zheng X, Mu Y, Li K (2017) CRISPR/Cas9-mediated ApoE -/- and LDLR -/- double gene knockout in pigs elevates serum LDL-C and TC levels. Oncotarget 8(23)

Huang J, Wang A, Huang C, Sun Y, Song B, Zhou R, Li L (2020a) Generation of marker-free pbd-2 knock-in pigs using the CRISPR/Cas9 and Cre/loxP systems. Genes (Basel) 11(8):1–14. https://doi.org/10.3390/genes11080951

Huang X, Zou X, Xu Z, Tang F, Shi J, Zheng E, Liu D, Moisyadi S, Urschitz J, Wu Z, Li Z (2020b) Efficient deletion of LoxP-flanked selectable marker genes from the genome of transgenic pigs by an engineered Cre recombinase. Transgenic Res 29(3):307–319. https://doi.org/10.1007/s11248-020-00200-3

Hubner A, Petersen B, Keil GM, Niemann H, Mettenleiter TC, Fuchs W (2018) Efficient inhibition of African swine fever virus replication by CRISPR/Cas9 targeting of the viral p30 gene (CP204L). Scientific Rep 8(1):1449. https://doi.org/10.1038/s41598-018-19626-1

Hunter RHF, Polge C, Rowson LE (1967) The recovery, transfer and survival of blastocysts in pigs. J Reprod Fertil 14(3):501–502

Hwang SU, Eun K, Yoon JD, Kim H, Hyun SH (2018) Production of transgenic pigs using a pGFAP-CreER(T2)/EGFP (LoxP) inducible system for central nervous system disease models. J Vet Sci 19(3):434–445. https://doi.org/10.4142/jvs.2018.19.3.434

Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, Prather RS (2004) In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61(6):1125–1135. https://doi.org/10.1016/j.theriogenology.2003.06.006

Jeong YS, Yeo S, Park JS, Lee KK, Kang YK (2007) Gradual development of a genome-wide H3-K9 trimethylation pattern in paternally derived pig pronucleus. Dev Dyn 236(6):1509–1516. https://doi.org/10.1002/dvdy.21150

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821. https://doi.org/10.1126/science.1225829

Kang JT, Ryu J, Cho B, Lee EJ, Yun YJ, Ahn S, Lee J, Ji DY, Lee K, Park KW (2016) Generation of RUNX3 knockout pigs using CRISPR/Cas9-mediated gene targeting. Reprod Domes Anim 51(6):970–978. https://doi.org/10.1111/rda.12775

Kang J-D, Kim S, Zhu H-Y, Jin L, Guo Q, Li X-C, Zhang Y-C, Xing X-X, Xuan M-F, Zhang G-L, Luo Q-R, Kim YS, Cui C-D, Li W-X, Cui Z-Y, Kim J-S, Yin X-J (2017) Generation of cloned adult muscular pigs with myostatin gene mutation by genetic engineering. RSC Adv 7(21):12541–12549. https://doi.org/10.1039/c6ra28579a

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T (2002) Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol Reprod 66(4):1033–1041

Kim YB, Komor AC, Levy JM, Packer MS, Zhao KT, Liu DR (2017) Increasing the genome- targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat Biotechnol 35(4):371–376. https://doi.org/10.1038/nbt.3803

Koblan LW, Doman JL, Wilson C, Levy JM, Tay T, Newby GA, Maianti JP, Raguram A, Liu DR (2018) Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. Nat Biotechnol 36(9):843–846. https://doi.org/10.1038/nbt.4172

Koketsu Y, Tani S, Iida R (2017) Factors for improving reproductive performance of sows and herd productivity in commercial breeding herds. Porcine Health Manag 3:1. https://doi.org/10.1186/s40813-016-0049-7

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533(7603):420–424. https://doi.org/10.1038/nature17946

Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW, Kim YB, Badran AH, Liu DR (2017) Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. Sci Adv 3 (8):eaao4774. https://doi.org/10.1126/sciadv.aao4774

Koppes EA, Redel BK, Johnson MA, Skvorak KJ, Ghaloul-Gonzalez L, Yates ME, Lewis DW, Gollin SM, Wu YL, Christ SE, Yerle M, Leshinski A, Spate LD, Benne JA, Murphy SL, Samuel MS, Walters EM, Hansen SA, Wells KD, Lichter-Konecki U, Wagner RA, Newsome JT, Dobrowolski SF, Vockley J, Prather RS, Nicholls RD (2020) A porcine model of phenylketonuria generated by CRISPR/Cas9 genome editing. JCI Insight 5(20). https://doi.org/10.1172/jci.insight.141523

Kumar BM, Maeng GH, Lee YM, Lee JH, Jeon BG, Ock SA, Kang T, Rho GJ (2013) Epigenetic modification of fetal fibroblasts improves developmental competency and gene expression in porcine cloned embryos. Vet Res Commun 37(1):19–28. https://doi.org/10.1007/s11259-012-9542-x

Kurome M, Kessler B, Wuensch A, Nagashima H, Wolf E (2015) Nuclear transfer and transgenesis in the pig. In: N., H., A. (eds) Nuclear reprogramming. Methods in molecular biology, vol 1222. Methods and protocols. Humana Press, New York, pp 37–59. https://doi.org/10.1007/978-1-4939-1594-1_4

Kurtz S, Lucas-Hahn A, Schlegelberger B, Gohring G, Niemann H, Mettenleiter TC, Petersen B (2021) Knockout of the HMG domain of the porcine SRY gene causes sex reversal in gene-edited pigs. Proc Natl Acad Sci U S A 118(2). https://doi.org/10.1073/pnas.2008743118

Kwon J, Namgoong S, Kim NH (2015) CRISPR/Cas9 as tool for functional study of genes involved in preimplantation embryo development. PLoS One 10(3):e0120501. https://doi.org/10.1371/journal.pone.0120501

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002) Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295(5557):1089–1092. https://doi.org/10.1126/science.1068228

Lai S, Wei S, Zhao B, Ouyang Z, Zhang Q, Fan N, Liu Z, Zhao Y, Yan Q, Zhou X, Li L, Xin J, Zeng Y, Lai L, Zou Q (2016) Generation of knock-in pigs carrying Oct4-tdTomato reporter through CRISPR/Cas9-mediated genome engineering. PLoS One 11 (1):e0146562. https://doi.org/10.1371/journal.pone.0146562

Lamas-Toranzo I, Guerrero-Sanchez J, Miralles-Bover H, Alegre-Cid G, Pericuesta E, Bermejo-Alvarez P (2017) CRISPR is knocking on barn door. Reprod Domes Anim 52 Suppl 4:39–47. https://doi.org/10.1111/rda.13047

Lamas-Toranzo I, Fonseca Balvis N, Querejeta-Fernandez A, Izquierdo-Rico MJ, Gonzalez-Brusi L, Lorenzo PL, Garcia-Rebollar P, Aviles M, Bermejo-Alvarez P (2019a) ZP4 confers structural properties to the zona pellucida essential for embryo development. eLife 8:e48904. doi:https://doi.org/10.7554/eLife.48904

Lamas-Toranzo I, Galiano-Cogolludo B, Cornudella-Ardiaca F, Cobos-Figueroa J, Ousinde O, Bermejo-Alvarez P (2019b) Strategies to reduce genetic mosaicism following CRISPR- mediated genome edition in bovine embryos. Scientific reports 9(1):14900. https://doi.org/10.1038/s41598-019-51366-8

Lassnig C, Müller M (2015) Disease-resistant transgenic animals. In: Meyers (ed) Encyclopedia of sustainability science and technology. Springer New York, New York, pp 1–17. https://doi.org/10.1007/978-1-4939-2493-6_10-3

Lavitrano M, Forni M, Varzi V, Pucci L, Bacci ML, Di Stefano C, Fioretti D, Zoraqi G, Moioli B, Rossi M, Lazzereschi D, Stoppacciaro A, Seren E, Alfani D, Cortesini R, Frati L (1997) Sperm-mediated gene transfer: production of pigs transgenic for a human regulator of complement activation. Transplant Proc 29(8):3508–3509. https://doi.org/10.1016/s0041-1345(97)00998-6

Le QA, Hirata M, Nguyen NT, Takebayashi K, Wittayarat M, Sato Y, Namula Z, Nii M, Tanihara F, Otoi T (2020) Effects of electroporation treatment using different concentrations of Cas9 protein with gRNA targeting Myostatin (MSTN) genes on the development and gene editing of porcine zygotes. Anim Sci J Nihon chikusan Gakkaiho 91 (1):e13386. https://doi.org/10.1111/asj.13386

Lee K, Prather RS (2014) Cloning pigs by somatic cell nuclear transfer. In: Cibelli, Gurdon, Wilmut et al. (eds) Principles of cloning, 2nd edn. Academic, San Diego, pp 245–254. https://doi.org/10.1016/B978-0-12-386541-0.00019-9

Legge M (1995) Oocyte and zygote zona pellucida permeability to macromolecules. J Exp Zool 271(2):145–150. https://doi.org/10.1002/jez.1402710210

Li J, Rieke A, Day BN, Prather RS (1996) Technical note: porcine non-surgical embryo transfer. J Anim Sci 74 (9):2263–2268. https://doi.org/10.2527/1996.7492263x

Li L, Pang D, Wang T, Li Z, Chen L, Zhang M, Song N, Nie D, Chen Z, Lai L, Ouyang H (2009) Production of a reporter transgenic pig for monitoring Cre recombinase activity. Biochem Biophys Res Commun 382(2):232–235. https://doi.org/10.1016/j.bbrc.2009.02.146

Li S, Flisikowska T, Kurome M, Zakhartchenko V, Kessler B, Saur D, Kind A, Wolf E, Flisikowski K, Schnieke A (2014) Dual fluorescent reporter pig for Cre recombination: transgene placement at the ROSA26 locus. PLoS One 9(7):e102455. https://doi.org/10.1371/journal.pone.0102455

Li P, Estrada JL, Burlak C, Montgomery J, Butler JR, Santos RM, Wang ZY, Paris LL, Blankenship RL, Downey SM, Tector M, Tector AJ (2015) Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. Xenotransplantation 22(1):20–31. https://doi.org/10.1111/xen.12131

Li G, Zhang X, Zhong C, Mo J, Quan R, Yang J, Liu D, Li Z, Yang H, Wu Z (2017a) Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells. Scientific Rep 7(1):8943. https://doi.org/10.1038/s41598-017-09306-x

Li Z, Yang HY, Wang Y, Zhang ML, Liu XR, Xiong Q, Zhang LN, Jin Y, Mou LS, Liu Y, Li RF, Rao Y, Dai YF (2017b) Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting. Journal of biomedical research 31 (5):445- 452. https://doi.org/10.7555/JBR.31.20170026

Li M, Ouyang H, Yuan H, Li J, Xie Z, Wang K, Yu T, Liu M, Chen X, Tang X, Jiao H, Pang D (2018) Site-Specific Fat-1 Knock-In Enables Significant Decrease of n-6PUFAs/n- 3PUFAs Ratio in Pigs. G3 (Bethesda, Md) 8(5):1747–1754. https://doi.org/10.1534/g3.118.200114

Li L, Meng H, Zou Q, Zhang J, Cai L, Yang B, Weng J, Lai L, Yang H, Gao Y (2019a) Establishment of gene-edited pigs expressing human blood-coagulation factor VII and albumin for bioartificial liver use. J Gastroenterol Hepatol 34(10):1851–1859. https://doi.org/10.1111/jgh.14666

Li R, Miao J, Wang Z (2019b) Production of genetically engineered porcine embryos by handmade cloning. Methods Mol Biol 1874:347–360. https://doi.org/10.1007/978-1-4939-8831-0_20

Li G, Zhang X, Wang H, Mo J, Zhong C, Shi J, Zhou R, Li Z, Yang H, Wu Z, Liu D (2020a) CRISPR/Cas9-Mediated Integration of Large Transgene into Pig CEP112 Locus. G3 (Bethesda, Md) 10(2):467–473. https://doi.org/10.1534/g3.119.400810

Li R, Zeng W, Ma M, Wei Z, Liu H, Liu X, Wang M, Shi X, Zeng J, Yang L, Mo D, Liu X, Chen Y, He Z (2020b) Precise editing of myostatin signal peptide by CRISPR/Cas9 increases the muscle mass of Liang Guang Small Spotted pigs. Transgenic Res 29 (1):149–163. https://doi.org/10.1007/s11248-020-00188-w

Li W, Shi L, Zhuang Z, Wu H, Lian M, Chen Y, Li L, Ge W, Jin Q, Zhang Q, Zhao Y, Liu Z, Ouyang Z, Ye Y, Li Y, Wang H, Liao Y, Quan L, Xiao L, Lai L, Meng G, Wang K (2020c) Engineered Pigs Carrying a Gain-of-Function NLRP3 Homozygous Mutation Can Survive to Adulthood and Accurately Recapitulate Human Systemic Spontaneous Inflammatory Responses. J Immunol 205(9):2532–2544. https://doi.org/10.4049/jimmunol.1901468

Liu L, Lee C, Moor RM (1996) DNA synthesis, microtubule and nuclear dynamics in porcine parthenotes. Zygote 4(2):139–144. https://doi.org/10.1017/s0967199400003014

Liu L, Liu Y, Gao F, Song G, Wen J, Guan J, Yin Y, Ma X, Tang B, Li Z (2012) Embryonic development and gene expression of porcine SCNT embryos treated with sodium butyrate. J Exp Zool B Mol Dev Evol 318(3):224–234. https://doi.org/10.1002/jez.b.22440

Liu T, Dou H, Xiang X, Li L, Li Y, Lin L, Pang X, Zhang Y, Chen Y, Luan J, Xu Y, Yang Z, Yang W, Liu H, Li F, Wang H, Yang H, Bolund L, Vajta G, Du Y (2015) Factors determining the efficiency of porcine somatic cell nuclear transfer: data analysis with over 200,000 reconstructed embryos. Cell Reprogram 17(6):463–471. https://doi.org/10.1089/cell.2015.0037

Liu Y, Lucas-Hahn A, Petersen B, Li R, Hermann D, Hassel P, Ziegler M, Larsen K, Niemann H, Callesen H (2017) Developmental competence and epigenetic profile of porcine embryos produced by two different cloning methods. Cell Reprogram 19(3):171–179. https://doi.org/10.1089/cell.2016.0055

Liu X, Liu H, Wang M, Li R, Zeng J, Mo D, Cong P, Liu X, Chen Y, He Z (2019) Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs. Transgenic Res 28(1):141–150. https://doi.org/10.1007/s11248-018-0107-9

Luo W, Li Z, Huang Y, Han Y, Yao C, Duan X, Ouyang H, Li L (2014a) Generation of AQP2- Cre transgenic mini-pigs specifically expressing Cre recombinase in kidney collecting duct cells. Transgenic Res 23(2):365–375. https://doi.org/10.1007/s11248-013-9774-8

Luo W, Li Z, Li P, Huang Y, Han Y, Yao C, Zhang Z, Yan H, Pang D, Ouyang H, Li L (2014b) Expression of Cre recombinase in alveolar epithelial cells of the AQP2-Cre transgenic mini-pigs. Cell Physiol Biochem 34(5):1597–1613. https://doi.org/10.1159/000366363

Luo L, Wang S, Zhu L, Fan B, Liu T, Wang L, Zhao P, Dang Y, Sun P, Chen J, Zhang Y, Chang X, Yu Z, Wang H, Guo R, Li B, Zhang K (2019) Aminopeptidase N-null neonatal piglets are protected from transmissible gastroenteritis virus but not porcine epidemic diarrhea virus. Scientific Rep 9(1):13186. https://doi.org/10.1038/s41598-019-49838-y

Lv L, Lu X, Feng T, Rehman S, Sun J, Wu Z, Shi D, Liu Q, Cui K (2020) Valproic acid enhances in vitro developmental competence of porcine handmade cloned embryos. Livestock Science 233:103957. https://doi.org/10.1016/j.livsci.2020.103957

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA- guided human genome engineering via Cas9. Science 339(6121):823–826. https://doi.org/10.1126/science.1232033

Mao J, Ajakaiye A, Lan Y, Olk DC, Ceballos M, Zhang T, Fan MZ, Forsberg CW (2008) Chemical structures of manure from conventional and phytase transgenic pigs investigated by advanced solid-state NMR spectroscopy. J Agric Food Chem 56(6):2131–2138. https://doi.org/10.1021/jf071588x

Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P (2001) Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 56(1):17–29. https://doi.org/10.1016/s0093-691x(01)00539-8

Martinez EA, Martinez CA, Cambra JM, Maside C, Lucas X, Vazquez JL, Vazquez JM, Roca J, Rodriguez-Martinez H, Gil MA, Parrilla I, Cuello C (2019) Achievements and future perspectives of embryo transfer technology in pigs. Reprod Domes Anim 54 Suppl 4(S4):4–13. https://doi.org/10.1111/rda.13465

Martinez CA, Cambra JM, Gil MA, Parrilla I, Alvarez-Rodriguez M, Rodriguez-Martinez H, Cuello C, Martinez EA (2020) Seminal Plasma Induces Overexpression of Genes Associated with Embryo Development and Implantation in Day-6 Porcine Blastocysts. Int J Mol Sci 21 (10):3662. https://doi.org/10.3390/ijms21103662

McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405(6790):1066–1069. https://doi.org/10.1038/35016604

Mehravar M, Shirazi A, Nazari M, Banan M (2019) Mosaicism in CRISPR/Cas9-mediated genome editing. Dev Biol 445(2):156–162. https://doi.org/10.1016/j.ydbio.2018.10.008

Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25(7):778–785. https://doi.org/10.1038/nbt1319

Miyoshi K, Kawaguchi H, Maeda K, Sato M, Akioka K, Noguchi M, Horiuchi M, Tanimoto A (2016) Birth of cloned microminipigs derived from somatic cell nuclear transfer embryos that have been transiently treated with valproic acid. Cell Reprogram 18(6):390–400. https://doi.org/10.1089/cell.2016.0025

Mojica FJM, Díez-Villaseñor C, Soria E, Juez G (2000) Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. vol 36. https://doi.org/10.1046/j.1365-2958.2000.01838.x

Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 60(2):174–182. https://doi.org/10.1007/s00239-004-0046-3

Moore FE, Reyon D, Sander JD, Martinez SA, Blackburn JS, Khayter C, Ramirez CL, Joung JK, Langenau DM (2012) Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). PLoS One 7(5):e37877. https://doi.org/10.1371/journal.pone.0037877

Murray JD, Maga EA (2016) Genetically engineered livestock for agriculture: a generation after the first transgenic animal research conference. Transgenic Res 25(3):321–327. https://doi.org/10.1007/s11248-016-9927-7

Murray D, Meidinger RG, Golovan SP, Phillips JP, O'Halloran IP, Fan MZ, Hacker RR, Forsberg CW (2007) Transgene and mitochondrial DNA are indicators of efficient composting of transgenic pig carcasses. Bioresource technology 98(9):1795–1804. https://doi.org/10.1016/j.biortech.2006.06.029

Naeimi Kararoudi M, Hejazi SS, Elmas E, Hellstrom M, Naeimi Kararoudi M, Padma AM, Lee D, Dolatshad H (2018) Clustered regularly interspaced short palindromic repeats/Cas9 gene editing technique in xenotransplantation. Front Immunol 9:1711. https://doi.org/10.3389/fimmu.2018.01711

Navarro-Serna S, Romar R, Dehesa-Etxebeste M, Lopes JS, Lopez de Munain A, Gadea J (2019) First production of Calpain3 KO pig embryo by CRISPR/Cas9 technology for human disease modelling: efficiency comparison between electroporation and intracytoplasmic microinjection. In: 35rd Scientific meeting Association of Embryo Technology in Europe (AETE). Murcia (Spain). 13-14th September 2019

Navarro-Serna S, Vilarino M, Park I, Gadea J, Ross PJ (2020) Livestock gene editing by one- step embryo manipulation. J Equine Vet Sci 89:103025. https://doi.org/10.1016/j.jevs.2020.103025

Navarro-Serna S, Hachem A, Canha-Gouveia A, Hanbashi A, Garrappa G, Lopes JS, Paris-Oller E, Sarrias-Gil L, Flores-Flores C, Bassett A, Sanchez R, Bermejo-Alvarez P, Matas C, Romar R, Parrington J, Gadea J (2021) Generation of nonmosaic, two-pore channel 2 Biallelic knockout pigs in one generation by CRISPR-Cas9 microinjection before oocyte insemination. CRISPR J 4(1):132–146. https://doi.org/10.1089/crispr.2020.0078

Niemann H, Petersen B (2016) The production of multi-transgenic pigs: update and perspectives for xenotransplantation. Transgenic Res 25(3):361–374. https://doi.org/10.1007/s11248-016-9934-8

Nishio K, Tanihara F, Nguyen TV, Kunihara T, Nii M, Hirata M, Takemoto T, Otoi T (2018) Effects of voltage strength during electroporation on the development and quality of in vitro-produced porcine embryos. Reprod Domes Anim 53 (2):313–318. https://doi.org/10.1111/rda.13106

Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, Lesha E, Wang G, Luo Y, Qing Y, Jiao D, Zhao H, Zhou X, Wang S, Wei H, Güell M, Church GM, Yang L (2017) Inactivation of porcine endogenous retrovirus in pigs using CRISPR- Cas9. Science 357(6357):1303–1307. https://doi.org/10.1126/science.aan4187

Niu D, Ma X, Yuan T, Niu Y, Xu Y, Sun Z, Ping Y, Li W, Zhang J, Wang T, Church GM (2021) Porcine genome engineering for xenotransplantation. Adv Drug Delivery Rev 168:229–245. https://doi.org/10.1016/j.addr.2020.04.001

Novoselova TA, Meuwissen MPM, Jongbloed AW, Huirne RBM (2013) Expected economic performance of genetic modification in pork production. NJAS Wageningen J Life Sci 64-65:9–15. https://doi.org/10.1016/j.njas.2012.03.002

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC (2000) Pig cloning by microinjection of fetal fibroblast nuclei. Science 289(5482):1188–1190. https://doi.org/10.1126/science.289.5482.1188

Ostedgaard LS, Price MP, Whitworth KM, Abou Alaiwa MH, Fischer AJ, Warrier A, Samuel M, Spate LD, Allen PD, Hilkin BM, Romano Ibarra GS, Ortiz Bezara ME, Goodell BJ, Mather SE, Powers LS, Stroik MR, Gansemer ND, Hippee CE, Zarei K, Goeken JA, Businga TR, Hoffman EA, Meyerholz DK, Prather RS, Stoltz DA, Welsh MJ (2020) Lack of airway submucosal glands impairs respiratory host defenses. eLife 9:e59653. doi:https://doi.org/10.7554/eLife.59653

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300(5893):611–615. https://doi.org/10.1038/300611a0

Paris-Oller E, Navarro-Serna S, Soriano-Ubeda C, Lopes JS, Matas C, Ruiz S, Latorre R, Lopez-Albors O, Romar R, Canovas S, Coy P (2021) Reproductive fluids, used for the in vitro production of pig embryos, result in healthy offspring and avoid aberrant placental expression of PEG3 and LUM. J Anim Sci Biotechnol 12(1):32. https://doi.org/10.1186/s40104-020-00544-0

Park KW, Cheong HT, Lai LX, Im GS, Kuhholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS (2001) Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Animal Biotechnology 12(2):173–181

Park SJ, Park HJ, Koo OJ, Choi WJ, Moon JH, Kwon DK, Kang JT, Kim S, Choi JY, Jang G, Lee BC (2012) Oxamflatin improves developmental competence of porcine somatic cell nuclear transfer embryos. Cell Reprogram 14(5):398–406. https://doi.org/10.1089/cell.2012.0007

Park KE, Park CH, Powell A, Martin J, Donovan DM, Telugu BP (2016) Targeted gene knockin in porcine somatic cells using CRISPR/Cas ribonucleoproteins. Int J Mol Sci 17(6):810–810. https://doi.org/10.3390/ijms17060810

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 Rep 7:42458. https://doi.org/10.1038/srep42458

Peng J, Wang Y, Jiang J, Zhou X, Song L, Wang L, Ding C, Qin J, Liu L, Wang W, Liu J, Huang X, Wei H, Zhang P (2015) Production of human albumin in pigs through CRISPR/Cas9-mediated knockin of human cDNA into swine albumin locus in the zygotes. Scientific Rep 5:16705. https://doi.org/10.1038/srep16705

Perleberg C, Kind A, Schnieke A (2018) Genetically engineered pigs as models for human disease. Dis Models Mechan 11 (1):dmm030783. https://doi.org/10.1242/dmm.030783

Petersen B (2017) Basics of genome editing technology and its application in livestock species. Reprod Domes Anim 52 Suppl 3:4-13. doi:10.1111/rda.13012

Petersen B, Frenzel A, Lucas-Hahn A, Herrmann D, Hassel P, Klein S, Ziegler M, Hadeler KG, Niemann H (2016) Efficient production of biallelic GGTA1 knockout pigs by cytoplasmic microinjection of CRISPR/Cas9 into zygotes. Xenotransplantation 23(5):338–346. https://doi.org/10.1111/xen.12258

Petters RM, Shuman RM, Johnson BH, Mettus RV (1987) Gene transfer in swine embryos by injection of cells infected with retrovirus vectors. J Exp Zool 242(1):85–88. https://doi.org/10.1002/jez.1402420111

Pfeiffer CA, Meyer AE, Brooks KE, Chen PR, Milano-Foster J, Spate LD, Benne JA, Cecil RF, Samuel MS, Ciernia LA, Spinka CM, Smith MF, Wells KD, Spencer TE, Prather RS, Geisert RD (2020) Ablation of conceptus PTGS2 expression does not alter early conceptus development and establishment of pregnancy in the pigdagger. Biol Reprod 102(2):475–488. https://doi.org/10.1093/biolre/ioz192

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407(6800):86–90. https://doi.org/10.1038/35024082

Prather RS, Sims MM, First NL (1989) Nuclear transplantation in early pig embryos. Biol Reprod 41(3):414–418. https://doi.org/10.1095/biolreprod41.3.414

Prather RS, Rowland RR, Ewen C, Trible B, Kerrigan M, Bawa B, Teson JM, Mao J, Lee K, Samuel MS, Whitworth KM, Murphy CN, Egen T, Green JA (2013) An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. J Virol 87(17):9538–9546. https://doi.org/10.1128/JVI.00177-13

Ramlee MK, Yan T, Cheung AMS, Chuah CTH, Li S (2015) High-throughput genotyping of CRISPR/Cas9-mediated mutants using fluorescent PCR-capillary gel electrophoresis. Scientific Rep 5:15587. doi:https://doi.org/10.1038/srep15587., https://www.nature.com/articles/srep15587#supplementary-information

Ramlee MK, Wang J, Cheung AMS, Li S (2017) Using a Fluorescent PCR-capillary Gel Electrophoresis Technique to Genotype CRISPR/Cas9-mediated Knockout Mutants in a High-throughput Format. J Vis Exp 122. https://doi.org/10.3791/55586

Redel BK, Spate LD, Lee K, Mao J, Whitworth KM, Prather RS (2016) Glycine supplementation in vitro enhances porcine preimplantation embryo cell number and decreases apoptosis but does not lead to live births. Mol Reprod Dev 83(3):246–258. https://doi.org/10.1002/mrd.22618

Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323(6087):445–448. https://doi.org/10.1038/323445a0

Rocadembosch J, Amador J, Bernaus J, Font J, Fraile LJ (2016) Production parameters and pig production cost: temporal evolution 2010-2014. Porcine Health Manag 2:11. https://doi.org/10.1186/s40813-016-0027-0

Romar R, Canovas S, Matas C, Gadea J, Coy P (2019) Pig in vitro fertilization: Where are we and where do we go? Theriogenology 137:113–121. https://doi.org/10.1016/j.theriogenology.2019.05.045

Sake HJ, Frenzel A, Lucas-Hahn A, Nowak-Imialek M, Hassel P, Hadeler KG, Hermann D, Becker R, Eylers H, Hein R, Baars W, Brinkmann A, Schwinzer R, Niemann H, Petersen B (2019) Possible detrimental effects of beta-2-microglobulin knockout in pigs. Xenotransplantation 26(6):e12525. https://doi.org/10.1111/xen.12525

Sakurai T, Watanabe S, Kamiyoshi A, Sato M, Shindo T (2014) A single blastocyst assay optimized for detecting CRISPR/Cas9 system-induced indel mutations in mice. BMC Biotechnol 14(1):69. https://doi.org/10.1186/1472-6750-14-69

Sato M, Miyoshi K, Nagao Y, Nishi Y, Ohtsuka M, Nakamura S, Sakurai T, Watanabe S (2014) The combinational use of CRISPR/Cas9-based gene editing and targeted toxin technology enables efficient biallelic knockout of the alpha-1,3-galactosyltransferase gene in porcine embryonic fibroblasts. Xenotransplantation 21(3):291–300. https://doi.org/10.1111/xen.12089

Sato M, Kosuke M, Koriyama M, Inada E, Saitoh I, Ohtsuka M, Nakamura S, Sakurai T, Watanabe S, Miyoshi K (2018) Timing of CRISPR/Cas9-related mRNA microinjection after activation as an important factor affecting genome editing efficiency in porcine oocytes. Theriogenology 108:29–38. https://doi.org/10.1016/j.theriogenology.2017.11.030

Schachtschneider KM, Schwind RM, Darfour-Oduro KA, De AK, Rund LA, Singh K, Principe DR, Guzman G, Ray CE, Jr., Ozer H, Gaba RC, Schook LB (2017) A validated, transitional and translational porcine model of hepatocellular carcinoma. Oncotarget 8 (38):63620-63634. https://doi.org/10.18632/oncotarget.18872

Schook LB, Collares TV, Hu W, Liang Y, Rodrigues FM, Rund LA, Schachtschneider KM, Seixas FK, Singh K, Wells KD, Walters EM, Prather RS, Counter CM (2015) A genetic porcine model of cancer. PLoS One 10(7):e0128864. https://doi.org/10.1371/journal.pone.0128864

Schook LB, Rund L, Begnini KR, Remiao MH, Seixas FK, Collares T (2016) Emerging technologies to create inducible and genetically defined porcine cancer models. Front Genet 7:28. https://doi.org/10.3389/fgene.2016.00028

Sheets TP, Park CH, Park KE, Powell A, Donovan DM, Telugu BP (2016) Somatic cell nuclear transfer followed by CRIPSR/Cas9 microinjection results in highly efficient genome editing in cloned pigs. Int J Mol Sci 17(12):2031. https://doi.org/10.3390/ijms17122031

Sheets TP, Park KE, Park CH, Swift SM, Powell A, Donovan DM, Telugu BP (2018) Targeted mutation of NGN3 gene disrupts pancreatic endocrine cell development in pigs. Scientific Rep 8(1):3582. https://doi.org/10.1038/s41598-018-22050-0

Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X, Skarnes WC (2014) Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 11(4):399–402. https://doi.org/10.1038/nmeth.2857

Shi J, Zhou R, Luo L, Mai R, Zeng H, He X, Liu D, Zeng F, Cai G, Ji H, Tang F, Wang Q, Wu Z, Li Z (2015) Influence of embryo handling and transfer method on pig cloning efficiency. Anim Reprod Sci 154:121–127. https://doi.org/10.1016/j.anireprosci.2015.01.006

Shi X, Tang T, Lin Q, Liu H, Qin Y, Liang X, Cong P, Mo D, Liu X, Chen Y, He Z (2020) Efficient generation of bone morphogenetic protein 15-edited Yorkshire pigs using CRISPR/Cas9dagger. Biol Reprod 103(5):1054–1068. https://doi.org/10.1093/biolre/ioaa138

Smedley D, Salimova E, Rosenthal N (2011) Cre recombinase resources for conditional mouse mutagenesis. Methods 53(4):411–416. https://doi.org/10.1016/j.ymeth.2010.12.027

Somfai T, Yoshioka K, Tanihara F, Kaneko H, Noguchi J, Kashiwazaki N, Nagai T, Kikuchi K (2014) Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production. PLoS One 9(5):e97731. https://doi.org/10.1371/journal.pone.0097731

Song Y, Lai L, Li L, Huang Y, Wang A, Tang X, Pang D, Li Z, Ouyang H (2016) Germ cell- specific expression of Cre recombinase using the VASA promoter in the pig. FEBS open bio 6(1):50–55. https://doi.org/10.1002/2211-5463.12005

Soppe JA, Lebbink RJ (2017) Antiviral goes viral: harnessing CRISPR/Cas9 to combat viruses in humans. Trends Microbiol 25(10):833–850. https://doi.org/10.1016/j.tim.2017.04.005

Sperandio S, Lulli V, Bacci ML, Forni M, Maione B, Spadafora C, Lavitrano M (1996) Sperm mediated gene transfer in bovine and swine species. Anim Biotech 7:59–77

Su X, Chen W, Cai Q, Liang P, Chen Y, Cong P, Huang J (2020) Effective generation of maternal genome point mutated porcine embryos by injection of cytosine base editor into germinal vesicle oocytes. Sci China Life Sci 63(7):996–1005. https://doi.org/10.1007/s11427-019-1611-1

Sun JM, Cui KQ, Li ZP, Lu XR, Xu ZF, Liu QY, Huang B, Shi DS (2017) Suberoylanilide hydroxamic acid, a novel histone deacetylase inhibitor, improves the development and acetylation level of miniature porcine handmade cloning embryos. Reprod Domes Anim 52(5):763–774. https://doi.org/10.1111/rda.12977

Tajima S, Uchikura K, Kurita T, Kikuchi K (2020) Insemination of recipient sows improves the survival to term of vitrified and warmed porcine expanded blastocysts transferred non- surgically. Anim Sci J Nihon chikusan Gakkaiho 91 (1):e13453. https://doi.org/10.1111/asj.13453

Tanihara F, Takemoto T, Kitagawa E, Rao S, Do LT, Onishi A, Yamashita Y, Kosugi C, Suzuki H, Sembon S, Suzuki S, Nakai M, Hashimoto M, Yasue A, Matsuhisa M, Noji S, Fujimura T, Fuchimoto D, Otoi T (2016) Somatic cell reprogramming-free generation of genetically modified pigs. Sci Adv 2(9):e1600803. https://doi.org/10.1126/sciadv.1600803

Tanihara F, Hirata M, Nguyen NT, Le QA, Hirano T, Takemoto T, Nakai M, Fuchimoto DI, Otoi T (2018) Generation of a TP53-modified porcine cancer model by CRISPR/Cas9- mediated gene modification in porcine zygotes via electroporation. PLoS One 13(10):e0206360. https://doi.org/10.1371/journal.pone.0206360

Tanihara F, Hirata M, Nguyen NT, Le QA, Hirano T, Takemoto T, Nakai M, Fuchimoto DI, Otoi T (2019a) Generation of PDX-1 mutant porcine blastocysts by introducing CRISPR/Cas9-system into porcine zygotes via electroporation. Anim Sci J Nihon chikusan Gakkaiho 90(1):55–61. https://doi.org/10.1111/asj.13129

Tanihara F, Hirata M, Nguyen NT, Le QA, Wittayarat M, Fahrudin M, Hirano T, Otoi T (2019b) Generation of CD163-edited pig via electroporation of the CRISPR/Cas9 system into porcine in vitro-fertilized zygotes. Anim Biotechnol:1–8. doi:https://doi.org/10.1080/10495398.2019.1668801

Tanihara F, Hirata M, Nguyen NT, Sawamoto O, Kikuchi T, Doi M, Otoi T (2020a) Efficient generation of GGTA1-deficient pigs by electroporation of the CRISPR/Cas9 system into in vitro-fertilized zygotes. BMC Biotechnol 20(1):40. https://doi.org/10.1186/s12896-020-00638-7

Tanihara F, Hirata M, Thi Nguyen N, Anh Le Q, Hirano T, Otoi T (2020b) Generation of viable PDX1 gene-edited founder pigs as providers of nonmosaics. Mol Reprod Dev 87 (4):471–481. doi:https://doi.org/10.1002/mrd.23335

Tanihara F, Hirata M, Nguyen NT, Sawamoto O, Kikuchi T, Otoi T (2021) One-step generation of multiple gene-edited pigs by electroporation of the CRISPR/Cas9 system into zygotes to reduce xenoantigen biosynthesis. Intl J Mol Sci 22(5):2249

Tao L, Yang M, Wang X, Zhang Z, Wu Z, Tian J, An L, Wang S (2016) Efficient biallelic mutation in porcine parthenotes using a CRISPR-Cas9 system. Biochem Biophys Res Commun 476(4):225–229. https://doi.org/10.1016/j.bbrc.2016.05.100

Taweechaipaisankul A, Kim GA, Jin JX, Lee S, Qasim M, Kim EH, Lee BC (2019) Enhancement of epigenetic reprogramming status of porcine cloned embryos with zebularine, a DNA methyltransferase inhibitor. Mol Reprod Dev 86(8):1013–1022. https://doi.org/10.1002/mrd.23178

Tu Z, Yang W, Yan S, Guo X, Li XJ (2015) CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. Mol Neurodegener 10(1):35. https://doi.org/10.1186/s13024-015-0031-x

Tu Z, Yang W, Yan S, Yin A, Gao J, Liu X, Zheng Y, Zheng J, Li Z, Yang S, Li S, Guo X, Li XJ (2017) Promoting Cas9 degradation reduces mosaic mutations in non-human primate embryos. Scientific reports 7(1):42081. https://doi.org/10.1038/srep42081

Ulloa Ullo CM, Yoshizawa M, Komoriya E, Mitsui A, Nagai T, Kikuchi K (2008) The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in in vitro produced porcine embryos. J Reprod Dev 54(1):22–29. https://doi.org/10.1262/jrd.19102

Vajta G, Zhang Y, Machaty Z (2007) Somatic cell nuclear transfer in pigs: recent achievements and future possibilities. Reprod Fertil Dev 19(2):403–423. https://doi.org/10.1071/rd06089

Van Eenennaam AL, Silva FF, Trott JF, Zilberman D (2020) Genetic engineering of livestock: the opportunity cost of regulatory delay. Ann Rev Anim Biosci 9 (1):null. https://doi.org/10.1146/annurev-animal-061220-023052

Vanamee ES, Santagata S, Aggarwal AK (2001) FokI requires two specific DNA sites for cleavage. J Mol Biol 309(1):69–78. https://doi.org/10.1006/jmbi.2001.4635

Wall RJ, Pursel VG, Hammer RE, Brinster RL (1985) Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol Reprod 32(3):645–651. https://doi.org/10.1095/biolreprod32.3.645

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 Rep 5(1):16623. https://doi.org/10.1038/srep16623

Wang X, Zhou J, Cao C, Huang J, Hai T, Wang Y, Zheng Q, Zhang H, Qin G, Miao X, Wang H, Cao S, Zhou Q, Zhao J (2015b) Efficient CRISPR/Cas9-mediated biallelic gene disruption and site-specific knockin after rapid selection of highly active sgRNAs in pigs. Scientific reports 5:13348. https://doi.org/10.1038/srep13348

Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, Wang J, Zhou J, Hu B, Kang N, Gao J, Yu L, Huang X, Wei H (2015c) Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Scientific reports 5(1):8256. https://doi.org/10.1038/srep08256

Wang X, Cao C, Huang J, Yao J, Hai T, Zheng Q, Wang X, Zhang H, Qin G, Cheng J, Wang Y, Yuan Z, Zhou Q, Wang H, Zhao J (2016) One-step generation of triple gene-targeted pigs using CRISPR/Cas9 system. Scientific reports 6(1):20620. https://doi.org/10.1038/srep20620

Wang HX, Li M, Lee CM, Chakraborty S, Kim HW, Bao G, Leong KW (2017a) CRISPR/Cas9-Based Genome Editing for Disease Modeling and Therapy: Challenges and Opportunities for Nonviral Delivery. Chem Rev 117(15):9874–9906. https://doi.org/10.1021/acs.chemrev.6b00799

Wang K, Tang X, Xie Z, Zou X, Li M, Yuan H, Guo N, Ouyang H, Jiao H, Pang D (2017b) CRISPR/Cas9-mediated knockout of myostatin in Chinese indigenous Erhualian pigs. Transgenic Res. https://doi.org/10.1007/s11248-017-0044-z

Wang H, Shen L, Chen J, Liu X, Tan T, Hu Y, Bai X, Li Y, Tian K, Li N, Hu X (2019a) Deletion of CD163 Exon 7 Confers Resistance to Highly Pathogenic Porcine Reproductive and Respiratory Viruses on Pigs. Int J Biol Sci 15(9):1993–2005. https://doi.org/10.7150/ijbs.34269

Wang J, Liu M, Zhao L, Li Y, Zhang M, Jin Y, Xiong Q, Liu X, Zhang L, Jiang H, Chen Q, Wang C, You Z, Yang H, Cao C, Dai Y, Li R (2019b) Disabling of nephrogenesis in porcine embryos via CRISPR/Cas9-mediated SIX1 and SIX4 gene targeting. Xenotransplantation 26(3):e12484. https://doi.org/10.1111/xen.12484

Wang H, Li G, Zhong C, Mo J, Sun Y, Shi J, Zhou R, Li Z, Wu Z, Liu D, Zhang X (2020) Generation of Multi-Transgenic Pigs Using PiggyBac Transposons Co-expressing Pectinase, Xylanase, Cellulase, beta-1.3-1.4-Glucanase and Phytase. Front Genet 11 (1521):597841. https://doi.org/10.3389/fgene.2020.597841

Wasmer M (2019) Roads forward for European GMO policy-uncertainties in wake of ECJ judgment have to be mitigated by regulatory reform. Front Bioeng Biotechnol 7 (JUN):132. doi:https://doi.org/10.3389/fbioe.2019.00132

Wells KD, Prather RS (2017) Genome-editing technologies to improve research, reproduction, and production in pigs. Mol Reprod Dev 84(9):1012–1017. https://doi.org/10.1002/mrd.22812

Whitelaw CB, Sang HM (2005) Disease-resistant genetically modified animals. Rev Sci Tech 24(1):275–283

Whitelaw CB, Sheets TP, Lillico SG, Telugu BP (2016) Engineering large animal models of human disease. J Pathol 238(2):247–256. https://doi.org/10.1002/path.4648

Whitworth KM, Prather RS (2017) Gene editing as applied to prevention of reproductive porcine reproductive and respiratory syndrome. Mol Reprod Dev 84(9):926–933. https://doi.org/10.1002/mrd.22811

Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, Manandhar G, Sutovsky M, Green JA, Sutovsky P, Prather RS (2009) Method of oocyte activation affects cloning efficiency in pigs. Mol Reprod Dev 76(5):490–500. https://doi.org/10.1002/mrd.20987

Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O'Gorman C, Walters EM, Murphy CN, Driver J, Mileham A, McLaren D, Wells KD, Prather RS (2014) Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod 91(3):78. https://doi.org/10.1095/biolreprod.114.121723

Whitworth KM, Rowland RR, Ewen CL, Trible BR, Kerrigan MA, Cino-Ozuna AG, Samuel MS, Lightner JE, McLaren DG, Mileham AJ, Wells KD, Prather RS (2016) Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol 34(1):20–22. https://doi.org/10.1038/nbt.3434

Whitworth KM, Benne JA, Spate LD, Murphy SL, Samuel MS, Murphy CN, Richt JA, Walters E, Prather RS, Wells KD (2017) Zygote injection of CRISPR/Cas9 RNA successfully modifies the target gene without delaying blastocyst development or altering the sex ratio in pigs. Transgenic Res 26(1):97–107. https://doi.org/10.1007/s11248-016-9989-6

Whitworth KM, Cecil R, Benne JA, Redel BK, Spate LD, Samuel MS, Prather RS, Wells KD (2018) Zygote injection of RNA encoding Cre recombinase results in efficient removal of LoxP flanked neomycin cassettes in pigs. Transgenic Res 27(2):167–178. https://doi.org/10.1007/s11248-018-0064-3

Whitworth KM, Rowland RRR, Petrovan V, Sheahan M, Cino-Ozuna AG, Fang Y, Hesse R, Mileham A, Samuel MS, Wells KD, Prather RS (2019) Resistance to coronavirus infection in amino peptidase N-deficient pigs. Transgenic Res 28(1):21–32. https://doi.org/10.1007/s11248-018-0100-3

Whyte JJ, Meyer AE, Spate LD, Benne JA, Cecil R, Samuel MS, Murphy CN, Prather RS, Geisert RD (2018) Inactivation of porcine interleukin-1beta results in failure of rapid conceptus elongation. Proc Natl Acad Sci U S A 115(2):307–312. https://doi.org/10.1073/pnas.1718004115

Wolfe SA, Nekludova L, Pabo CO (2000) DNA recognition by Cys2His2 zinc finger proteins. Annu Rev Biophys Biomol Struct. 29. https://doi.org/10.1146/annurev.biophys.29.1.183

Wu J, Vilarino M, Suzuki K, Okamura D, Bogliotti YS, Park I, Rowe J, McNabb B, Ross PJ, Belmonte JCI (2017) CRISPR-Cas9 mediated one-step disabling of pancreatogenesis in pigs. Scientific reports 7(1):10487. https://doi.org/10.1038/s41598-017-08596-5

Wu H, Liu Q, Shi H, Xie J, Zhang Q, Ouyang Z, Li N, Yang Y, Liu Z, Zhao Y, Lai C, Ruan D, Peng J, Ge W, Chen F, Fan N, Jin Q, Liang Y, Lan T, Yang X, Wang X, Lei Z, Doevendans PA, Sluijter JPG, Wang K, Li X, Lai L (2018) Engineering CRISPR/Cpf1 with tRNA promotes genome editing capability in mammalian systems. Cell Mol Life Sci 75(19):3593–3607. https://doi.org/10.1007/s00018-018-2810-3

Xie Z, Pang D, Wang K, Li M, Guo N, Yuan H, Li J, Zou X, Jiao H, Ouyang H, Li Z, Tang X (2017) Optimization of a CRISPR/Cas9-mediated Knock-in strategy at the Porcine Rosa26 locus in porcine foetal fibroblasts. Scientific reports 7(1):3036. https://doi.org/10.1038/s41598-017-02785-y

Xie Z, Pang D, Yuan H, Jiao H, Lu C, Wang K, Yang Q, Li M, Chen X, Yu T, Chen X, Dai Z, Peng Y, Tang X, Li Z, Wang T, Guo H, Li L, Tu C, Lai L, Ouyang H (2018) Genetically modified pigs are protected from classical swine fever virus. PLoS pathogens 14(12):e1007193. https://doi.org/10.1371/journal.ppat.1007193

Xie J, Ge W, Li N, Liu Q, Chen F, Yang X, Huang X, Ouyang Z, Zhang Q, Zhao Y, Liu Z, Gou S, Wu H, Lai C, Fan N, Jin Q, Shi H, Liang Y, Lan T, Quan L, Li X, Wang K, Lai L (2019) Efficient base editing for multiple genes and loci in pigs using base editors. Nat Commun 10(1):2852. https://doi.org/10.1038/s41467-019-10421-8

Xie F, Zhou X, Lin T, Wang L, Liu C, Luo X, Luo L, Chen H, Guo K, Wei H, Wang Y (2020a) Production of gene-edited pigs harboring orthologous human mutations via double cutting by CRISPR/Cas9 with long single-stranded DNAs as homology-directed repair templates by zygote injection. Transgenic Res 29(5-6):587–598. https://doi.org/10.1007/s11248-020-00218-7

Xie Z, Jiao H, Xiao H, Jiang Y, Liu Z, Qi C, Zhao D, Jiao S, Yu T, Tang X, Pang D, Ouyang H (2020b) Generation of pRSAD2 gene knock-in pig via CRISPR/Cas9 technology. Antiviral Res 174:104696. https://doi.org/10.1016/j.antiviral.2019.104696

Xu K, Zhou Y, Mu Y, Liu Z, Hou S, Xiong Y, Fang L, Ge C, Wei Y, Zhang X, Xu C, Che J, Fan Z, Xiang G, Guo J, Shang H, Li H, Xiao S, Li J, Li K (2020) CD163 and pAPN double- knockout pigs are resistant to PRRSV and TGEV and exhibit decreased susceptibility to PDCoV while maintaining normal production performance. eLife 9:e57132. doi:https://doi.org/10.7554/eLife.57132

Yamashita S, Kogasaka Y, Hiradate Y, Tanemura K, Sendai Y (2020) Suppression of mosaic mutation by co-delivery of CRISPR associated protein 9 and three-prime repair exonuclease 2 into porcine zygotes via electroporation. J Reprod Dev 66(1):41–48. https://doi.org/10.1262/jrd.2019-088

Yang H, Wu Z (2018) Genome Editing of Pigs for Agriculture and Biomedicine. Front Genet 9:360. https://doi.org/10.3389/fgene.2018.00360

Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA, Church G (2015) Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science 350(6264):1101–1104. https://doi.org/10.1126/science.aad1191

Yang H, Zhang J, Zhang X, Shi J, Pan Y, Zhou R, Li G, Li Z, Cai G, Wu Z (2018) CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus. Antiviral Res 151:63–70. https://doi.org/10.1016/j.antiviral.2018.01.004

Yao J, Huang J, Zhao J (2016) Genome editing revolutionize the creation of genetically modified pigs for modeling human diseases. Hum Genet 135(9):1093–1105. https://doi.org/10.1007/s00439-016-1710-6

Yao J, Zeng H, Zhang M, Wei Q, Wang Y, Yang H, Lu Y, Li R, Xiong Q, Zhang L, Chen Z, Xing G, Cao X, Dai Y (2019) OSBPL2-disrupted pigs recapitulate dual features of human hearing loss and hypercholesterolaemia. J Genetics Genom Yi chuan xue bao 46(8):379–387. https://doi.org/10.1016/j.jgg.2019.06.006

Yin Y, Hao H, Xu X, Shen L, Wu W, Zhang J, Li Q (2019) Generation of an MC3R knock-out pig by CRSPR/Cas9 combined with somatic cell nuclear transfer (SCNT) technology. Lipids in health and disease 18(1):122. https://doi.org/10.1186/s12944-019-1073-9

Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 66(1):112–119. https://doi.org/10.1095/biolreprod66.1.112

Yoshioka K, Suzuki C, Itoh S, Kikuchi K, Iwamura S, Rodriguez-Martinez H (2003) Production of piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during in vitro fertilization. Biol Reprod 69(6):2092–2099. https://doi.org/10.1095/biolreprod.103.020081

Yoshioka K, Noguchi M, Suzuki C (2012) Production of piglets from in vitro-produced embryos following non-surgical transfer. Anim Reprod Sci 131(1-2):23–29. https://doi.org/10.1016/j.anireprosci.2012.01.018

Youngs CR (2001) Factors influencing the success of embryo transfer in the pig. Theriogenology 56(8):1311–1320. https://doi.org/10.1016/s0093-691x(01)00632-x

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. Int J Mol Sci 17(10). https://doi.org/10.3390/ijms17101668

Yuan Y, Spate LD, Redel BK, Tian Y, Zhou J, Prather RS, Roberts RM (2017) Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation. Proc Natl Acad Sci U S A 114(29):E5796–e5804. https://doi.org/10.1073/pnas.1703998114

Yuan H, Yu T, Wang L, Yang L, Zhang Y, Liu H, Li M, Tang X, Liu Z, Li Z, Lu C, Chen X, Pang D, Ouyang H (2020) Efficient base editing by RNA-guided cytidine base editors (CBEs) in pigs. Cell Mol Life Sci 77(4):719–733. https://doi.org/10.1007/s00018-019-03205-2

Yuk IH, Zhang JD, Ebeling M, Berrera M, Gomez N, Werz S, Meiringer C, Shao Z, Swanberg JC, Lee KH, Luo J, Szperalski B (2014) Effects of copper on CHO cells: insights from gene expression analyses. Biotechnol Prog 30(2):429–442. https://doi.org/10.1002/btpr.1868

Zettler S, Renner S, Kemter E, Hinrichs A, Klymiuk N, Backman M, Riedel EO, Mueller C, Streckel E, Braun-Reichhart C, Martins AS, Kurome M, Kessler B, Zakhartchenko V, Flenkenthaler F, Arnold GJ, Frohlich T, Blum H, Blutke A, Wanke R, Wolf E (2020) A decade of experience with genetically tailored pig models for diabetes and metabolic research. Anim Reprod 17(3):e20200064. https://doi.org/10.1590/1984-3143-AR2020-0064

Zhai Y, Li W, Zhang Z, Cao Y, Wang Z, Zhang S, Li Z (2018) Epigenetic states of donor cells significantly affect the development of somatic cell nuclear transfer (SCNT) embryos in pigs. Mol Reprod Dev 85(1):26–37. https://doi.org/10.1002/mrd.22935

Zhang R, Wang Y, Chen L, Wang R, Li C, Li X, Fang B, Ren X, Ruan M, Liu J, Xiong Q, Zhang L, Jin Y, Zhang M, Liu X, Li L, Chen Q, Pan D, Li R, Cooper DKC, Yang H, Dai Y (2018a) Reducing immunoreactivity of porcine bioprosthetic heart valves by genetically- deleting three major glycan antigens, GGTA1/beta4GalNT2/CMAH. Acta Biomater 72:196–205. https://doi.org/10.1016/j.actbio.2018.03.055

Zhang X, Li Z, Yang H, Liu D, Cai G, Li G, Mo J, Wang D, Zhong C, Wang H, Sun Y, Shi J, Zheng E, Meng F, Zhang M, He X, Zhou R, Zhang J, Huang M, Zhang R, Li N, Fan M, Yang J, Wu Z (2018b) Novel transgenic pigs with enhanced growth and reduced environmental impact. eLife:7. https://doi.org/10.7554/eLife.34286

Zhang B, Wang C, Zhang Y, Jiang Y, Qin Y, Pang D, Zhang G, Liu H, Xie Z, Yuan H, Ouyang H, Wang J, Tang X (2020) A CRISPR-engineered swine model of COL2A1 deficiency recapitulates altered early skeletal developmental defects in humans. Bone 137:115450. https://doi.org/10.1016/j.bone.2020.115450

Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS (2009) Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod 81(3):525–530. https://doi.org/10.1095/biolreprod.109.077016

Zheng Q, Lin J, Huang J, Zhang H, Zhang R, Zhang X, Cao C, Hambly C, Qin G, Yao J, Song R, Jia Q, Wang X, Li Y, Zhang N, Piao Z, Ye R, Speakman JR, Wang H, Zhou Q, Wang Y, Jin W, Zhao J (2017) Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. Proc Natl Acad Sci U S A 114(45):E9474–E9482. https://doi.org/10.1073/pnas.1707853114

Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, Zhao Y, Zhao B, Liu Z, Lai S, Yi X, Guo L, Esteban MA, Zeng Y, Yang H, Lai L (2015) Generation of CRISPR/Cas9-mediated gene- targeted pigs via somatic cell nuclear transfer. Cell Mol Life Sci 72(6):1175–1184. https://doi.org/10.1007/s00018-014-1744-7

Zhou X, Wang L, Du Y, Xie F, Li L, Liu Y, Liu C, Wang S, Zhang S, Huang X, Wang Y, Wei H (2016) Efficient Generation of Gene-Modified Pigs Harboring Precise Orthologous Human Mutation via CRISPR/Cas9-Induced Homology-Directed Repair in Zygotes. Hum Mutat 37(1):110–118. https://doi.org/10.1002/humu.22913

Zhu XX, Zhong YZ, Ge YW, Lu KH, Lu SS (2018) CRISPR/Cas9-mediated generation of Guangxi Bama minipigs harboring three mutations in alpha-synuclein causing Parkinson's disease. Scientific Rep 8(1):12420. https://doi.org/10.1038/s41598-018-30436-3

Zhu X, Wei Y, Zhan Q, Yan A, Feng J, Liu L, Tang D (2020a) CRISPR/Cas9-Mediated Biallelic Knockout of IRX3 Reduces the Production and Survival of Somatic Cell-Cloned Bama Minipigs. Animals (Basel) 10(3):501–501. https://doi.org/10.3390/ani10030501

Zhu XX, Zhan QM, Wei YY, Yan AF, Feng J, Liu L, Lu SS, Tang DS (2020b) CRISPR/Cas9- mediated MSTN disruption accelerates the growth of Chinese Bama pigs. Reprod Domes Anim 55(10):1314–1327. https://doi.org/10.1111/rda.13775

Zou Y, Li Z, Zou Y, Hao H, Li N, Li Q (2018) An FBXO40 knockout generated by CRISPR/Cas9 causes muscle hypertrophy in pigs without detectable pathological effects. Biochem Biophys Res Commun 498(4):940–945. https://doi.org/10.1016/j.bbrc.2018.03.085

Zou X, Ouyang H, Yu T, Chen X, Pang D, Tang X, Chen C (2019) Preparation of a new type 2 diabetic miniature pig model via the CRISPR/Cas9 system. Cell Death Dis 10(11):823. https://doi.org/10.1038/s41419-019-2056-5

Zuo E, Cai YJ, Li K, Wei Y, Wang BA, Sun Y, Liu Z, Liu J, Hu X, Wei W, Huo X, Shi L, Tang C, Liang D, Wang Y, Nie YH, Zhang CC, Yao X, Wang X, Zhou C, Ying W, Wang Q, Chen RC, Shen Q, Xu GL, Li J, Sun Q, Xiong ZQ, Yang H (2017) One-step generation of complete gene knockout mice and monkeys by CRISPR/Cas9-mediated gene editing with multiple sgRNAs. Cell Res 27(7):933–945. https://doi.org/10.1038/cr.2017.81