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Genetic engineering of the porcine embryo

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ABSTRACT

The pig has become an increasingly important model organism in biomedical research. Due to similarities in size and physiology to humans, porcine disease models can bridge the gap between fundamental research and clinical studies. The development of CRISPR/CAS9 revolutionised genome engineering by enabling efficient targeting of specific sequences. However, compared to other species, *in vitro* production of viable embryos is difficult and remains a bottleneck for the creation of disease models in pigs.

This work describes the development of a robust *in vitro* system to generate and culture porcine embryos. First an efficient protocol for *in vitro* maturation of porcine oocytes was established. Sources of sperm suitable for *in vitro* fertilisation were identified and protocols for cryopreservation of porcine semen put in place. Conditions for *in vitro* fertilisation were refined and optimal sperm to oocyte ratios were determined for each boar individually. A high proportion of embryos developed to the blastocyst stage, and this was accompanied by a low level of polyspermic fertilisation, previously a major problem for porcine *in vitro* fertilisation.

CRISPR/Cas9 vectors targeting multiple different genes were designed and delivered into zygotes by intracytoplasmic microinjection. This approach resulted in efficient genome editing, as confirmed by a high ratio of modified blastocysts. *In vitro* derived embryos were surgically transferred into synchronised surrogate sows and viable genetically modified founder animals born. During the course of this project animal models for inflammatory bowel disease, thermogenesis, hepatitis research and xenotransplantation were generated. For a porcine model of pancreatic cancer, a Cre-driver-line was produced by intracytoplasmic microinjection of zygotes using transposon vectors. These models proved that a robust protocol for *in vitro* embryo production has been established, eliminating the need for *in vivo* embryo isolation, reducing the number of animals required, which is in accordance with the 3R principle. The quality of the embryos was sufficient to support development of viable offspring even after inactivation of single or multiple genes or addition of transgenes.

If even more complex genetic manipulations are required it might be advantageous to carry these out *in vitro* in cultured cells in order to verify the accuracy of genetic modification prior to the generation of the animal. The final part of this work therefore describes the establishment

of a handmade cloning system as a reliable alternative for traditional somatic cell nuclear transfer.

ZUSAMMENFASSUNG

Das Schwein erfreut sich als Modelorganismus in der biomedizinischen Forschung zunehmender Beliebtheit. Wegen seiner Ähnlichkeit zum Menschen in Bezug auf seine Physiologie und Größe stellt es ein ideales Bindeglied zwischen Studien in Mäusen und klinischen Studien dar. Die Entwicklung von CRISPR/CAS9 revolutionierte das Feld der Genomeditierung, indem es die effiziente Editierung spezifischer Gensequenzen ermöglichte. Im Vergleich zu anderen Spezies bleibt die *in vitro* Herstellung lebensfähiger Embryonen eine Engstelle bei der Erstellung von Krankheitsmodellen im Schwein.

Im Rahmen dieser Arbeit wurde ein *in vitro* Kultursystem für Schweineembryonen etabliert. Zuerst wurde ein effizientes Protokoll für die *in vitro* Reifung von Schweineeizellen optimiert. Für die *in vitro* Befruchtung wurde geeignetes Sperma identifiziert und Methoden für dessen Kryokonservierung eingerichtet. Bedingungen für die *in vitro* Befruchtung wurden verbessert und das optimale Verhältnis von Sperma zu Eizellen wurde für jeden Eber individuell ermittelt. Ein großer Anteil der Embryonen entwickelte sich bis zum Blastocystenstadium und niedrige Raten polyspermischer Befruchtung wurden bestätigt, was allgemein ein bedeutendes Problem der *in vitro* Befruchtung beim Schwein ist.

Intrazytoplasmatische Mikroinjektion wurde angewendet um CRISPR/CAS9 Vektoren, welche multiple, verschiedene Gensequenzen als Ziel hatten in Zygoten einzubringen. Die hohe Effizienz dieser Herangehensweise konnte durch den hohen Anteil an genetisch modifizierten Blastozysten, die in dieser Arbeit generiert wurden, bestätigt werden. *In vitro* produzierte Embryonen wurden auf synchronisierte Empfängertiere übertragen und genetisch modifizierte Gründertiere wurden geboren. Im Rahmen dieses Projektes wurden Tiermodelle für chronisch-entzündliche Darmerkrankungen, Thermogenese, Hepatitis-Forschung und Xenotransplantation generiert. Zur Erstellung eines Krankheitsmodells für Bauchspeicheldrüsenkrebs wurde durch intrazytoplasmatische Mikroinjektion von Transposon-Vektoren eine Schweinelinie mit pankreas-spezifischer Cre-Expression erstellt. Die Generierung dieser Tiermodelle zeigt, dass ein robustes Protokoll für die *in vitro* Embryo Produktion etabliert werden konnte. Dies verringert die Notwendigkeit zur Gewinnung von *in vivo* Embryos und reduziert in Einklang mit dem 3R Prinzip somit auch die benötigte Zahl an Versuchstieren. Die Qualität der *in vitro* produzierten Embryos war auch nach Inaktivierung einzelner oder mehrerer Gene sowie nach Einbringung von Transgenen hinreichend zur Generierung gesunder Nachkommen.

Für Anwendungen, die komplexere genetische Manipulationen erfordern ist es vorteilhaft, diese zuerst *in vitro* in der Zellkultur durchzuführen, um vor der Herstellung von Tieren die Genauigkeit der genetischen Modifikation sicherzustellen. Der letzte Teil dieser Arbeit beschreibt hierzu die Etablierung eines Handmade Cloning Systems, das eine zuverlässige Alternative zum herkömmlichen Kerntransfer darstellt.

1. INTRODUCTION

Mice are the most commonly used species in biomedical research, mostly because they are relatively inexpensive to house and techniques for their genetic modification are well established [1, 2]. Mouse studies have provided extensive insights into the underlying mechanisms of many human diseases but often they do not mimic human disease pathology or phenotypes accurately [3].

The "3R" principle demands replacement, reduction and refinement of animal experiments whenever possible [4] which means that the predictive value of data generated in animal experiments has to be maximised [5]. Regulatory agencies require preclinical studies in nonrodent species which makes large animal models of human diseases indispensable [6]. Similarities in organ anatomy, physiology, body size, diet and pathophysiology make pigs a useful model organism to gain insights into human diseases [7]. Surgical interventions and diagnostic procedures like imaging of vessels and organs can be carried out using standard equipment [8]. Public acceptance for the use of livestock in animal experiments is less controversial than for primate species or companion animals. Pigs are highly fertile and housing conditions including specific-pathogen-free (SPF) are well established [9, 10].

Genome engineering (GE) combined with sequencing of the whole porcine genome [11] has promised the generation of tailored porcine disease models for a variety of human conditions but the practical implementation remains challenging [12]. GE pigs that replicate human phenotypes and disease mechanisms functionally and on the molecular level have potential in translational medicine by "bridging the gap between bench and bedside" [13]. Porcine disease models have been generated for cancer research [14, 15], xenotransplantation [16, 17], diabetes [18], cystic fibrosis [19] and Duchene muscular dystrophy [20] but in the past the efficiency in generating these models has been low and restricted to a few groups worldwide.

Genome engineering also holds great promise for agriculture. It has the potential to revolutionise animal breeding [21], improve productivity, animal welfare, reduce use of

antibiotics in livestock production and protect the environment [22]. GE pigs resistant to porcine reproductive and respiratory syndrome (PRRSV) virus are exemplary [23].

1.1. The toolbox for genome engineering of livestock

1.1.1. Traditional methods for genome engineering of pigs

The first genetically modified pigs were created in 1985 by pronuclear DNA microinjection [24, 25]. Other methods for genome engineering of livestock include sperm-mediated gene transfer [26], viral vectors [27], somatic cell nuclear transfer (SCNT) [28, 29] and its close variation handmade cloning (HMC) [30].

1.1.1.1. Pronuclear DNA microinjection

Pronuclear DNA microinjection was the first, and for a while the most common method of generating genetically modified large animals [31]. Mice were the first species in which this method was successfully applied [32, 33] with pigs and other livestock animals following shortly after [24, 25]. Microinjected DNA can be integrated at the pronuclear stage but also in subsequent cell divisions [34] which leads to the generation of mosaic animals [35]. Other downsides are the need for expensive micromanipulation equipment and highly trained operators. In livestock species it is necessary to centrifuge the oocytes to visualise the pronuclei because of their pigmentation [36]. Perhaps the greatest drawback is however the low proportion of transgenic animals produced, about 3% in mice and lower in livestock [37] due to interspecies variation in DNA integration [38], and the lack of control over transgene integration. In its basic form pronuclear DNA microinjection (see Figure 1) results in the addition of transgenes at random locations in the host genome [39]. This leads to the 'position effect' in which the expression levels of integrated transgenes can differ widely under the influence of adjacent DNA sequences [40].

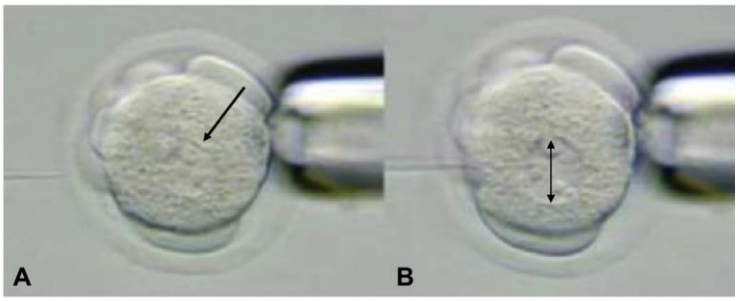


Figure 1: DNA microinjection into the male pronucleus of a one-cell mouse embryo A) The injection needle is inserted into the pronucleus (indicated by the arrow). B) Approximately 2pl of DNA is injected and the diameter of the pronucleus increases by about 50% under hydrostatic pressure. (adapted from DeMayo *et al.* [41]).

1.1.1.2. Sperm-mediated gene transfer

Sperm-mediated gene transfer (SMGT) was developed as a means of avoiding the need for embryo micromanipulation, embryo culture and embryo transfer (ET). The natural ability of sperm to transfer DNA into oocytes is employed to co-transfer exogenous DNA [42]. The procedure comprises of sperm collection, coincubation with DNA constructs and artificial insemination (AI) (illustrated in Figure 2). Following its first implementation in mice [43] there are several reports of transgenic livestock generated using this approach [26, 44]. However despite its simplicity, the successful implementation of SMGT has been limited to certain laboratories [45] rendering its validity questionable [46]. SMGT seems to work only with sperm samples from some donors for inexplicable reasons [47] which is a possible explanation for those mixed results. Another drawback is that that transgenes introduced this way are frequently fragmented [48].

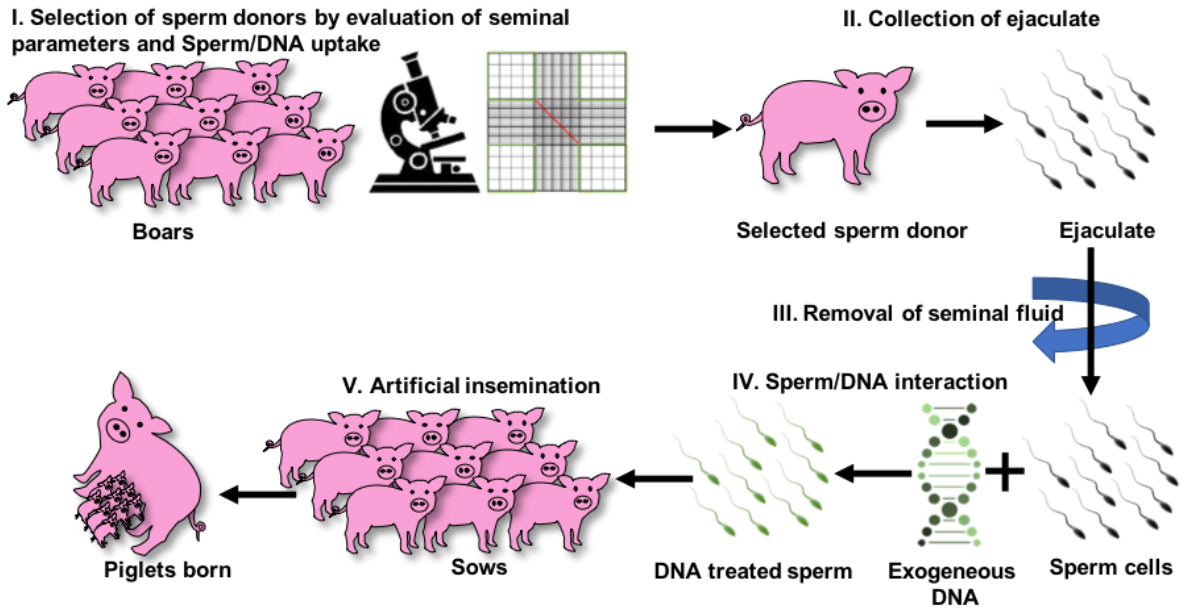


Figure 2: Sperm-mediated gene transfer. Sperm is collected from suitable donors and co-incubated with exogeneous DNA followed by artificial insemination. (adapted from Lavitrano *et al.[47]*).

Linker-based SMGT is a more recent approach in which uptake of DNA by sperm is facilitated through endocytosis of DNA-antibody complexes [49]. Another modification is intracytoplasmic sperm injection (ICSI) mediated gene transfer [50] which is claimed to be useful for transferring large transgenes [51] and eliminates problems associated with polyspermy in IVF. While ease of use would seem to make SMGT a superior method for the

generation of genetically modified livestock, issues of efficiency and reproducibility have prevented more widespread adoption [52].

1.1.1.3. Viral vectors

Lentiviruses are part of the family *Retroviridae* and possess the ability to infect cells and reverse transcribe their RNA to DNA. Viral DNA is randomly integrated into the host's genome and passed on to offspring through germline transmission. This ability can be utilised to transfer DNA sequences from one organism to another, a process termed transgenesis [53]. Following its initial use in mice [54] lentiviral gene transfer was successfully applied in porcine transgenesis [27, 55].

The advantages of lentiviral vectors include reportedly highly efficient transgenesis in livestock [56] and the ability to transduce non-dividing cells, which allows transduction of very early embryos thus reducing the likelihood of mosaicism [57]. Their disadvantages include the limited insert size (~ 5.2 kb) [58], multiple independent integration events resulting in transgene segregation in subsequent generations [59] and the possibility of vector recombination with a wild-type virus, leading to the production of infectious virions [60]. Another drawback of all viral vector systems is the time and labour-intensive preparation and concentration of viral particles.

Adenoviral vectors can infect a variety of different cell types, have a high infection efficiency and do not have to be integrated into the host genome [61]. This makes them especially suitable to deliver site-specific nucleases for genome engineering [62]. Delivery of targeting constructs via adenoviral vectors has been reported as an efficient means of producing gene targeted animals [63]. Drawbacks to adenoviral vectors are their relatively high immunogenicity and cytotoxicity [64].

Adeno associated viruses (AAVs) are a safe alternative for the delivery of RNA-guided endonucleases. They are not associated with any diseases in humans, rely on helper virus for replication and in vectors sequences for nearly all viral structural genes are removed [65]. The biggest drawback to AAVs is their small capacity of about 4.7 kb [58]. This is problematic when AAVs are to be used in combination with the clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein (Cas) system. The coding sequence

for the components of the CRISPR/Cas system plus the required promotor sequence exceeds 5kb [66]. This packaging limit can be bypassed with an innovative split-Cas9 system in which the N and C-terminal parts of Cas9 are fused to split-intein units that reconstitute the complete Cas9 protein upon co-expression [67].

1.1.1.4. Transposon-mediated transgenesis

Transposons or "jumping genes" are mobile genetic elements that are able to relocate within the genome [68]. Transposable elements (TE) make up a significant proportion of many species' genomes [69]. They can be categorised into class I or retrotransposons and class II or DNA transposons [70]. Class I transposons use a "copy and paste" mechanism based on reverse transcription to generate a copy of themself [71]. Class II transposons encode the protein transposase which recognises the inverted terminal repeat (ITR) sequences that flank a transposon, excises it from its current position in the genome and re-integrates the transposable element. This is termed a "cut and paste" mechanism (see Figure 3) [72]. Depending on the transposon type local hopping or a more random re-integration at "TTAA" sites occur.

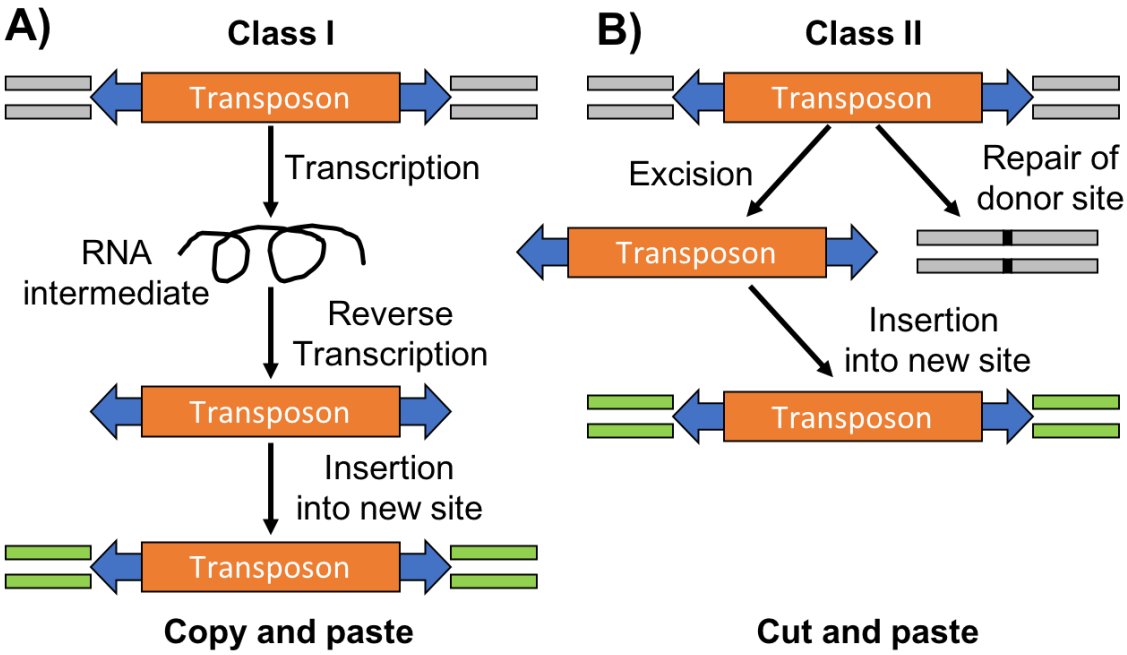


Figure 3: Mechanisms of transposition **A)** Class I Transposons rely on an RNA intermediate and reverse transcription. **B)** Class II transposons are excised by transposase and relocated by creating DSBs (adapted from Saha *et al.* [72]).

The class II transposon system has been adapted as a tool for the generation of transgenic animals by replacing the transposon gene with the gene of interest and providing the transposon activity via a second expression vector or as mRNA [73]. A number of transposon vectors have been developed. The most commonly used are PiggyBac (see Figure 4) and Sleeping Beauty transposon systems as these have the highest transposition activity in mammalian cells [74]. Transgenic pigs have been generated using both systems. [75, 76].

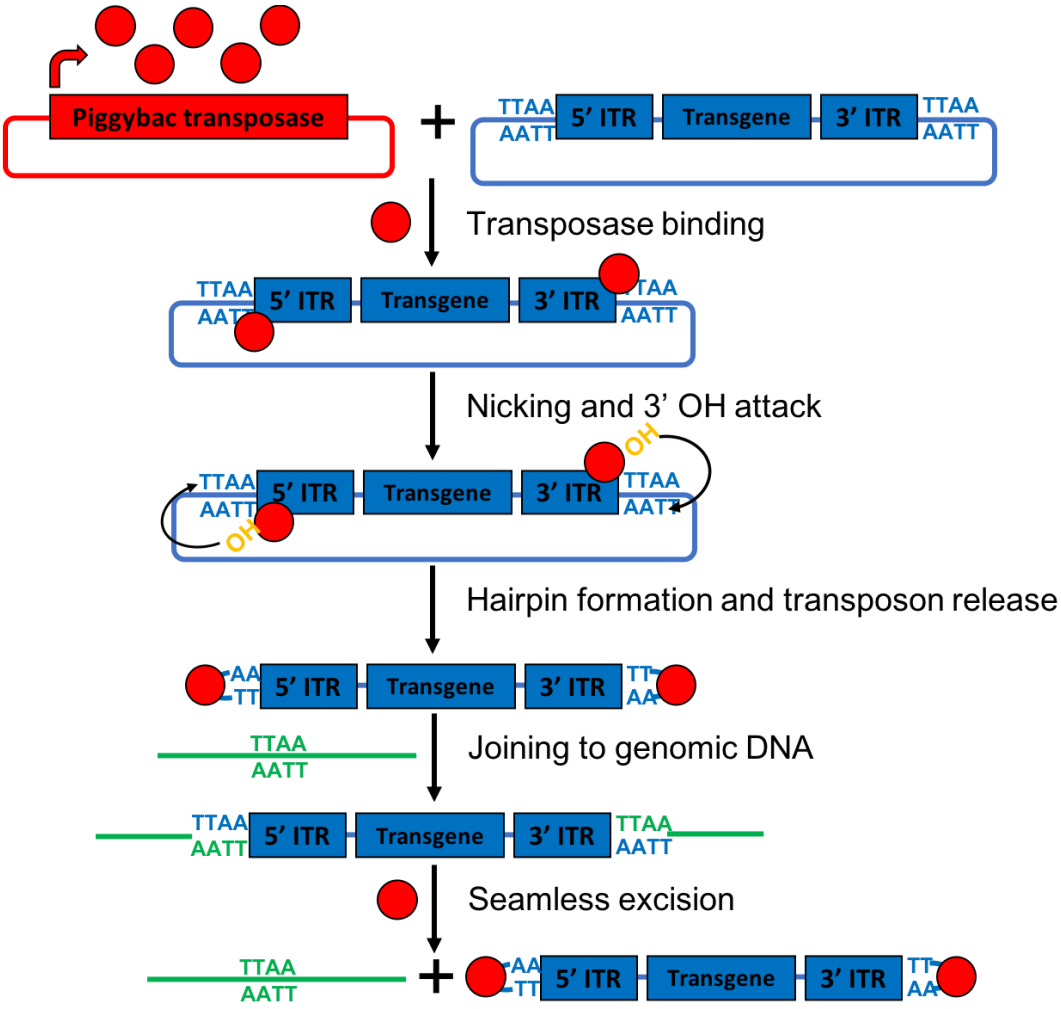


Figure 4: The mechanism of PiggyBac transposition. Transposase binds the PiggyBac ITRs. It nicks and attacks the TTAA ends leading to hairpin formation and transposon excision. The transposon is integrated into genomic DNA at TTAA sites (adapted from Woodard et al. [77]).

Transposon-mediated transgenesis facilitates efficient transgene insertion and stable expression compared to DNA microinjection [77]. Advantages over viral vectors are larger cargo size, ease of implementation and biosafety [78]. This approach only allows for random integration of transgenes and depends on microinjection as delivery method [78].

1.1.1.5. Somatic cell nuclear transfer

Traditionally genetically modified mice have been generated by pronuclear DNA microinjection [32], or by modification of embryonic stem cells (ESCs) - notably using HR to effect gene targeting [79]. The ability of ESCs to maintain pluripotency and a normal karyotype during long term culture [80] combined with the high frequency with which they support HR makes them a powerful tool [81]. Typically genetically modified ESCs are injected into a recipient blastocyst or aggregated with a precompaction stage embryo then transferred to a pseudo-pregnant female to gestate chimeric offspring [82]. Appropriate breeding results in mice carrying the desired genotype [83]. Totipotent porcine ESCs, capable of populating the germ line, are still unavailable but there are promising reports about pluripotent stem cells with enhanced differentiation potential [84].

The lack of livestock ES cells led to the development of somatic cell nuclear transfer (SCNT) which is currently the standard method to generate GE pigs [85]. In SCNT the desired modification is introduced into cultured primary somatic cells which are then placed in the perivitelline space of enucleated mature oocytes followed by fusion and embryo activation [86]. The reconstructed embryos can be transferred to a surrogate mother to generate 100% genetically modified offspring (see Figure 5). Work in mammals (sheep) was first restricted to the use of blastomeres from disaggregated early embryos [87], but successful nuclear transfer using cultured cells [88] including fetal and adult donor cells [89] opened the possibility of a practical alternative to ES cells. Nuclear transfer using somatic cells genetically modified in culture resulted in transgenic [90] and then the first gene-targeted sheep [91] and later pigs [28, 29, 92]. Today, SCNT using IVM oocytes [93] as recipient cytoplasts is used extensively in porcine genetic engineering [94].

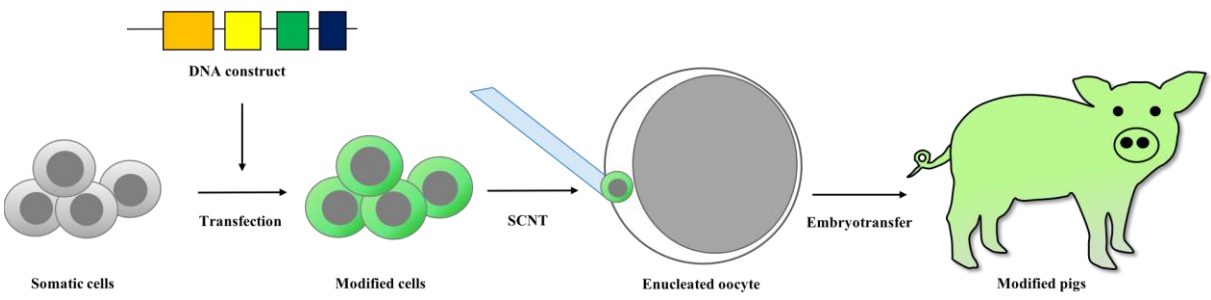


Figure 5: Somatic cell nuclear transfer. Somatic cells are transfected and selected for drug resistance and presence of the desired modification. Single cells are inserted into the perivitelline space of enucleated oocytes, followed by cell fusion and activation to generate reconstructed embryos for embryo transfer.

The advantages of SCNT include the ability to engineer DNA sequence replacement, deletion or addition via HR or genome editing. One also has the possibility to choose the sex of the resultant offspring [93] and mosaicism is unlikely to occur so all resulting offspring should carry the desired genetic modification [95]. This can be ensured by extensive selection and screening of the donor cells before producing embryos. Downsides to the method include the high level of skill required by the operator, the relatively low numbers of embryos that can be processed, the low efficiency in terms of the number of animals born per reconstructed embryo transferred [96], and the occurrence of health problems and high mortality in the resulting offspring due to deficient epigenetic reprogramming [97].

While the core SCNT procedure has undergone very few changes since it was first developed for mammals, progress in the enabling technologies such as IVM, IVC and oocyte activation have improved cloning efficiencies over time [86, 98].

1.1.1.6. Handmade cloning

Handmade cloning (HMC) is a micromanipulator-free alternative to traditional micromanipulator-based cloning (TC) [99]. The eponymous feature of this method is enucleation of oocytes with a handheld blade after partial zona pellucida (ZP) digestion. Two of the resulting cytoplasts are fused with a donor cell and activated. Culture to the blastocyst stage (see Figure 6) in a well of the well (WOW) system [100] is followed by transfer to a synchronised recipient [101]. This procedure was first performed in cattle [102, 103] and quickly adapted to porcine embryos [30] and used to produce GE pigs [104]. While porcine oocytes are more sensitive to manipulation than other livestock species [105] reported efficiencies of cloned pigs resulting from HMC are equal or higher than TC [106].

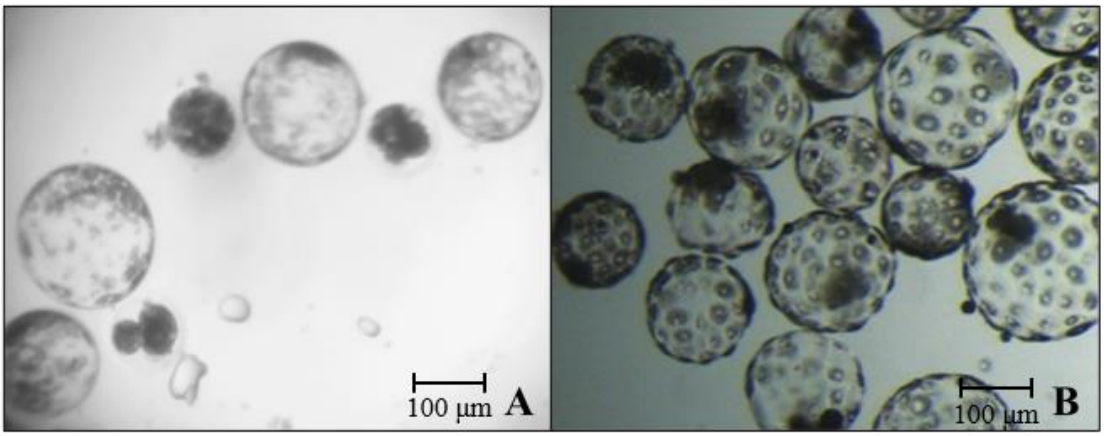


Figure 6: Blastocysts produced by HMC in comparison to IVF derived blastocysts. **A)** IVF-derived blastocysts with a clearly visible zona pellucida. **B)** HMC-derived blastocysts without zona pellucida (B adapted from Kragh *et al.* [107]).

Advantages of HMC include overall simplicity and less reliance on skilled personnel and precision equipment [108]. While there have been concerns regarding the three different origins of mitochondrial DNA, current evidence suggests no deleterious effects [109]. Embryos reconstructed by HMC are usually transferred to recipients at the blastocyst stage. Zona-free approaches require IVC of embryos to the blastocyst stage which is associated with reduced developmental potential [110]. This is reported to be offset by WOW culture systems and the positive effects of using two cytoplasts to counteract the loss of cytoplasm during enucleation [111] resulting in higher blastocyst quality [107].

There have been several published comparisons of TC versus HMC, the outcome of which are that efficiencies and resulting pregnancy rates are basically similar. [112-114].

1.1.2. CRISPRS/Cas9 mediated genome engineering directly in embryos

The emergence of highly specific endonuclease-based genome engineering technology (outlined in more detail in 1.3.) has expanded the toolbox for genetic modification of mammals [12]. Site-specific endonucleases can be delivered to early embryos by intracytoplasmic microinjection [89] or electroporation [115] to facilitate targeted genome engineering directly in zygotes. With this approach GE animals can be produced in one step [116] bypassing the need for SCNT [85].

1.1.2.1. Microinjection of site-specific endonucleases

Targeted genome engineering in one-cell embryos through delivery of site-specific endonucleases directly into zygotes was developed in mice [117]. Genome edited mice have been generated by microinjection of zygotes and embryos with zinc finger nucleases (ZFNs) [117], transcription activator-like effector nucleases (TALENs) [118] and CRISPR/Cas9 components [119]. Similar work has followed in pigs using ZFNs [120, 121] and TALENs [122], see also section 1.3. Since then CRISPR/Cas9 technology has facilitated the generation of a variety of porcine disease models by cytoplasmic injection of its components directly into zygotes [123-125]. The nuclear pore complex (NPC) catalyses active and passive transportation

of DNA, RNA, proteins and small molecules through the nuclear membrane [126]. Therefore, cytoplasmic microinjection of CRISPR/Cas9 components as DNA, RNA or protein molecules, are all considered practical options [119, 127]. This approach can be used to introduce indels [128], or together with single strand DNA templates to effect homologous sequence replacement in the genome of individual embryos [129]. Targeted multiplex genome engineering in one step is also possible by using multiple sgRNAs [119]. Another advantage of microinjection is the ability to introduce multiple different reagents like DNA vectors, guide RNAs and polypeptides at once [130] without constraints regarding type or size of the construct. Importantly, animals generated by microinjection of site-specific nucleases have not so far displayed any of the developmental defects [85] associated with SCNT [97].

This approach frequently causes mosaicism which arises from the ability of the CRISPR/Cas9 system to continuously edit cells at various stages of embryonic development [131, 132]. Mosaicism complicates genotype analysis and requires outcrossing of the mosaic founders to generate new genetically modified lines which is especially problematic in pigs due to the long generation interval [85].

Another limitation to this method is the difficulty of introducing modifications via homology directed repair (HDR) [129]. While pigs with targeted knock-ins have been created using this approach [133] large insertions remain challenging [134].

Genome engineering directly in porcine embryos is further limited by the need for large numbers of high-quality porcine zygotes, which is a problem due to the inefficiency of current porcine IVM and IVF systems [135], see also section 1.2. Researchers have therefore mainly used genome engineering in *in vivo* derived oocytes or zygotes with few exceptions [124, 136, 137].

1.1.2.2. Electroporation

Electroporation of zygotes is a high-throughput method of introducing CRISPR/Cas9 components into early embryos to produce genetically modified animals [115]. Electroporation was demonstrated to be a viable method for nucleic acid delivery to oocytes and zygotes in mice [138]. Initially this approach was limited by the need to remove the ZP, resulting in low

development and pregnancy rates [139]. Advances in electroporator technology facilitated ZP penetration which resulted in the generation of genetically modified rats by delivery of RNA guided endonucleases into early embryos via electroporation [140]. Application of poring pulses to create micro-holes in the ZP and oolemma followed by transfer pulses to deliver mRNA into the ooplasm [140] promotes high transfection efficiencies and embryo viability [141].

A recent publication reported the generation of a GE pig via electroporation of *in vitro* derived zygotes [142]. While the reported efficiencies are still very low, successful electroporation in embryos could offer a simpler alternative to microinjection [31].

1.2. *In vitro* production of porcine embryos

Production of GE pigs by direct manipulation of porcine zygotes requires a vast number of porcine embryos. The anatomy of the porcine genital tract makes it very difficult to carry out non-surgical ovum pick up from living animals, so i*n vivo* matured oocytes or zygotes can only be collected by flushing the oviducts of slaughtered donor sows, which is expensive and requires a large number of experimental animals [143]. Thus *in vitro* production (IVP) of porcine embryos using slaughterhouse-derived immature oocytes is the only practical means of providing a sufficient supply of porcine embryos while reducing the number of experimental animals in accordance with the "3R" principle. [144]

Assisted reproductive techniques (ART) that facilitate efficient *IVP* of embryos are highly developed in humans and many livestock species. In pigs however, *in vitro* embryo culture conditions are still considered suboptimal [145]. The high costs involved, and the relatively low financial value of individual pigs make such methods commercially unappealing for routine agricultural applications. However, pigs are playing an ever more important role in translational medicine [8, 12, 13], and ARTs are valuable tools for their use in biomedicine [146]. IVP of embryos comprises three steps: *In vitro* maturation (IVM) of oocytes, *in vitro* fertilisation (IVF) and *in vitro* culture (IVC). Spurred by the prospect of creating clinically relevant animal models for a wide spectrum of human conditions IVP systems for porcine embryos have been developed (see Table 1) but they are still considered inefficient compared to other species [147]. Problems like polyspermic fertilisation, insufficient cytoplasmic maturation of IVM oocytes and suboptimal culture conditions remain largely unsolved and result in reduced viability of IVP embryos [148]. Further optimisation is necessary to realise the full potential of this technology [94, 149].

Table 1: Milestones of porcine *in vitro* production. Modified from Grupen *et al.* [94].

Year	Details of manipulation / IVP procedure	Reference
1985	*In vivo* zygotes / transgene insertion by microinjection	Brem *et al.* [24] Hammer *et al.* [25]
1986	*In vivo* oocytes / IVF with fresh ejaculated sperm	Cheng *et al.* [150]
1988	*In vivo* oocytes / IVF with FT epididymal sperm	Nagai *et al.* [151]

1989	IVM oocytes / IVF with extended ejaculated sperm	Mattioli *et al.* [152]
	In vivo oocytes / NT using 4-cell stage blastomeres	Prather *et al.* [153]
	In vivo embryos / FT at the peri-hatching blastocyst stage	Hayashi *et al.* [154]
1993	IVM oocytes / IVF with fresh ejaculated sperm	Yoshida *et al.* [155]
1995	*In vivo* embryos / frozen-thawed at the 4-cell stage	Nagashima *et al.* [156]
1997	*In vivo* oocytes / IVF with sex-sorted sperm	Rath *et al.* [157]
1998	IVM oocytes / IVF with sex-sorted sperm	Abeydeera *et al.* [158]
2000	*In vivo* oocytes / SCNT	Onishi *et al.* [29] Polejaeva *et al.* [92]
	IVM oocytes / SCNT embryos	Betthauser *et al.* [28]
	In vivo oocytes / NT using 4-cell stage blastomeres	Li *et al.* [159]
2001	IVP embryos / IVC to the 2- to 4-cell and blastocyst stages	Marchal et al. [160]
	Somatic cell nuclear transfer (SCNT) embryos / GM donor cells	Park *et al.* [161]
2002	IVP embryos / IVC to the blastocyst stage	Kikuchi *et al.* [162]
	In vivo zygotes / IVC medium chemically defined	Yoshioka *et al.* [163]
	SCNT embryos / targeted GM donor cells	Dai *et al.* [164]
2003	IVM oocytes / IVF and IVC media chemically defined	Yoshioka *et al.* [165]
2006	SCNT embryos / IVC to the blastocyst stage	Lagutina *et al.* [166]
	SCNT embryos / FT at the blastocyst stage	Li *et al.* [167]
2007	IVP embryos / FT at the 4- to 8-cell stage	Nagashima *et al.* [168]
	SCNT embryos / handmade cloning / GM donor cells	Du *et al.* [30]
2009	IVP embryos/IVM, IVF and IVC media chemically defined	Akaki *et al.* [169]
	IVP zygotes / FT at the pronuclear stage	Somfai *et al.* [170]
	SCNT embryos / handmade cloning / GM donor cells	Kragh *et al.* [171]
2011	SCNT embryos / FT at the morula stage	Nakano *et al.* [172]
	SCNT embryos / handmade cloning / targeted GM donor cells	Luo *et al.* [173]
2012	IVP embryos / non-surgical embryo transfer	Yoshioka *et al.* [174]
	IVP embryos / FT at the morula stage	Maehara *et al.* [175]
2013	*In vivo* oocytes / intrafallopian insemination / GM donor sperm	Umeyama *et al.* [176]
2017	IVP embryos / triple cytokine supplemented (FLI)medium	Yuan *et al.* [177]
	Parthenogenesis / iPSC injection / human-pig chimeric embryo	Wu *et al.* [178]
2019	*In vivo* zygotes / non-surgical ovum pickup	Yoshioka *et al.* [179]

1.2.1. In vitro maturation

In female mammals, all oocytes ever produced (200.000 – 400.000) are arrested at the diplotene stage (prophase I) of meiosis I until sexual maturity [180]. Maturation describes a complex process during which oocytes undergo various cellular changes in which they gain the ability to be fertilised and proceed through embryogenesis (see Figure 7) [181]. As meiosis I resumes, one set of chromosomes is extruded forming the first polar body. The haploid secondary oocyte then advances to the metaphase of meiosis II where it is arrested once again until fertilisation.

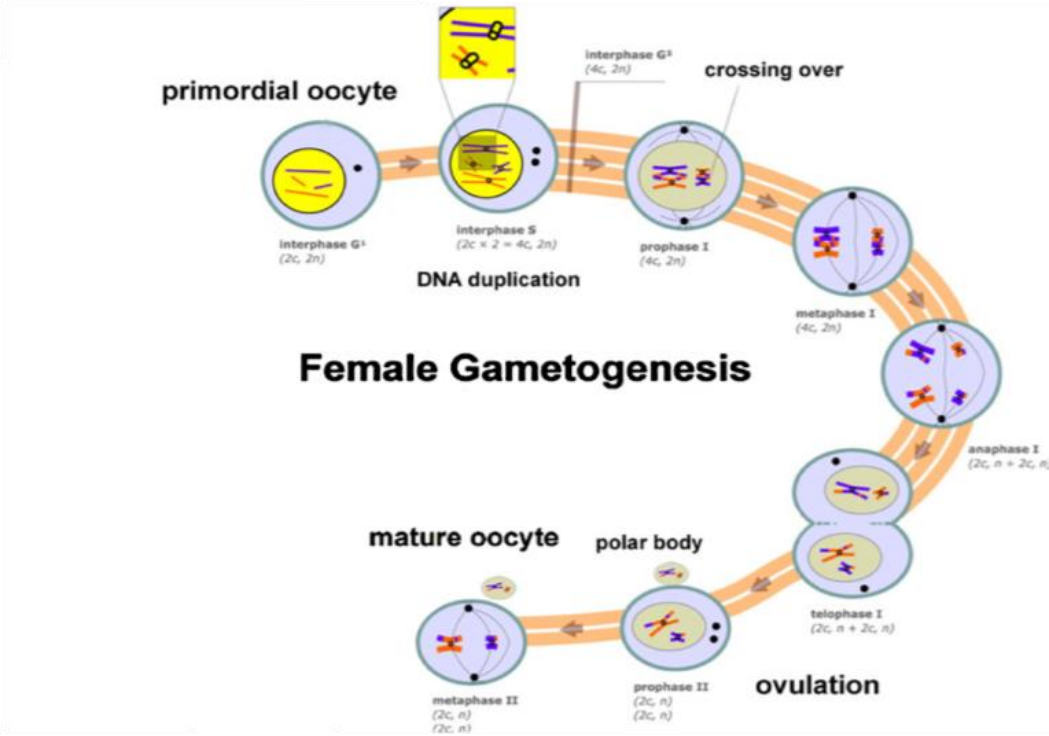


Figure 7: Female gametogenesis (adapted from Hill [182]). All oocytes are arrested at metaphase I of meiosis I. In the course of each cycle several oocytes complete meiosis I resulting in two haploid progeny cells. One of them develops into a secondary oocyte and the other into the first polar body. The secondary oocyte subsequently starts meiosis II and stays arrested at the metaphase of meiosis II until fertilisation.

Oocyte quality is the single most important readout determining the success of IVM [183]. Morphological features such as the presence of several compact layers of cumulus cells [184], cytoplasmic homogeneity [185] and large follicle size [186] strongly correlate with developmental competence. Selection of high-quality oocytes with such features is essential for the outcome of all IVM protocols [187, 188]. IVM oocytes suffer from several drawbacks compared to their *in vivo* derived counterparts. Their developmental competence is severely limited by their diminished ability to undergo monospermic fertilisation [94, 149]. The proportion of *in vitro* matured oocytes that can develop to blastocyst stage is also less than those obtained by ovum pickup [189]. Improving the quality and developmental competence of IVM oocytes are thus vital to realising the full potential of the pig as a model for translational research.

1.2.1.1. Cytoplasmic and nuclear maturation

The process of oocyte maturation can be divided into cytoplasmic and nuclear maturation. Modern IVM systems are effective at promoting nuclear maturation, which is characterised by

the resumption of meiosis and extrusion of the first polar body. However, cytoplasmic maturation distinguished by relocation of mitochondria, cortical granules and other cell organelles is still defective [190]. The poor developmental competence and high rate of polyspermy from IVF observed using IVM oocytes is commonly attributed to deficiencies in those cytoplasmic processes [191, 192].

1.2.1.2. Conditions for *in vitro* maturation

Modern IVM systems are typically based on the culture media formulations TCM-199 or NCSU-23 supplemented with hormones [94] that are designed to mimic *in vivo* conditions as closely as possible [183]. The reduced developmental potential of IVM oocytes has been identified as largely due to defective interactions between oocytes and cumulus cells due to suboptimal culture conditions [193]. Several media additives such as epidermal growth factor (EGF), cysteine, glutamine, sodium pyruvate and β mercaptoethanol promote better cytoplasmic maturation [194]. Supplementation with porcine follicular fluid (PFF) to protect oocytes from oxidative stress and enhance formation of male pronuclei [195, 196] has been standard practise for decades [197, 198]. However, PFF contains maturation inhibitors [199] and the mechanism how PFF affects maturation is unclear [200]. Furthermore, variation between batches of PFF make it difficult to standardise culture conditions. There have thus been efforts to replace PFF with chemically defined alternatives [177, 201]. Better understanding of the proteins and peptides contained in PFF and the mechanisms involved in oocyte maturation [202] has led to the development of chemically-defined maturation media [169]. This has improved reliability and also eliminated the risk of introducing contaminating pathogens from PFF and other biological fluids. Cytokine supplementation with fibroblast growth factor 2 (FGF2), leukaemia inhibitory factor (LIF) and insulin-like growth factor (IGF) facilitates more synchronised nuclear and cytoplasmic maturation [202]. This results in more efficient blastocyst production after IVF and higher mean litter size after ET [177]. Another approach is the addition of dibutyryl cyclic adenosine monophosphate (dbcAMP) during the first half of the maturation process. This reversibly inhibits meiosis, enhancing synchrony of cytoplasmic and nuclear maturation [203]. Further efforts to optimise IVM conditions include co-culture of oocytes with porcine oviduct epithelial cells during maturation [204, 205] and the use of medium conditioned by such co-culture [206].

1.2.2. *In vitro* fertilisation

IVF is a procedure whereby an egg is fertilized by sperm in a test tube or elsewhere outside the body to form a zygote [207]. In pigs, polyspermy and insufficient male pronucleus (MPN) formation have been the biggest hurdles in establishing efficient IVF protocols [149]. MPN formation could be greatly increased by supplementation of IVF media with cysteamine [208], cysteine and glutathione (GSH) [209]. Other attempts at improving MPN formation have included exposure of gametes to oviduct fluid [210], oviduct epithelial cells [211] or oviduct-specific glycoprotein [212].

Polyspermy is a multifactorial problem and therefore difficult to address directly [149, 213]. Rates of polyspermy in porcine IVF systems can reach up to 90% [214, 215]. The ratio of sperm to oocytes during fertilisation is closely related to the degree of polyspermy in IVF [216]. A high number of porcine spermatozoa is necessary to attain acceptable fertilisation rates *in vitro* compared to the amount that reaches the oviduct *in vivo* [217]. Experimenters are thus forced to compromise between optimal fertilisation and acceptable rates of polyspermy, because reducing the number of sperm cells also reduces the fertilisation rate [218]. Oocyte quality is another critical factor affecting polyspermy [219, 220]. Oocytes used for IVF are commonly recovered from prepubertal gilts because they are readily available from the slaughterhouse. However these have a poor ability to block polyspermy compared to oocytes from adult sows [160]. Other variables affecting oocyte quality are follicle size [186, 221], high temperatures resulting from processing of pig carcasses after slaughter [222] and seasonal infertility of pigs in summer [223]. Selection and preparation of sperm plays an important role in IVF success. Seminal plasma contains decapacitating factors that must be removed for fresh sperm IVF. This is usually conducted by simple centrifugation [151], but Percoll gradient centrifugation [224] [225] provides better rates of fertilisation [226] and blastocyst formation [227].

Frozen-thawed sperm drawn from the epididymis of “good freezer” boars [228] is reported as the most suitable choice for current IVF systems. It yields reproducible results [176, 229] while eliminating variability between batches of ejaculates [176]. The availability of good quality frozen sperm for IVF is however severely limited due to difficulties associated with cryopreservation. Pig sperm is more sensitive to oxidative stress, temperature fluctuation, osmolarity and pH-value than most other mammalian species [230, 231]. The membrane of porcine spermatozoa contains a high ratio of polyunsaturated to saturated fatty acids [232, 233]. This makes them more susceptible to cellular damage caused by the freeze-thaw process

compared to other species [234]. Moreover, differences between boars in maintaining fertility after cryopreservation [235] and even between ejaculates from the same boar [236] make the procedure unreliable and therefore commercially unappealing.

Efforts to reduce the incidence of polyspermy such as microchannel IVF [237], straw IVF [238], rolling culture systems [239] and modified swim-up method [240] all attempt to mimic *in vivo* selection of the most motile spermatozoa. Selection of sperm that quickly bind to zona pellucida (ZP) by shorter co-incubation has a similar effect [241] while minimizing detrimental effects caused by dying spermatozoa in IVF medium [242]. For optimal results, IVF parameters have to be optimised individually for each boar [235] and for fresh, frozen, ejaculated and epididymal sperm [243]. The latest innovations combine sperm selection methods with short co-incubation to reduce polyspermy (see Figure 8) [244].

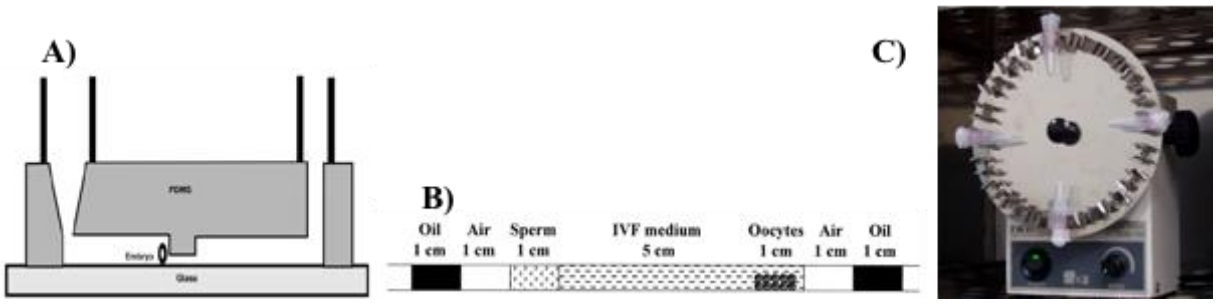


Figure 8: Methods to reduce polyspermy **A)** Microchannel IVF, **B)** straw IVF and **C)** rolling systems try to mimic *in vivo* conditions. They work by selecting the most motile spermatozoa (modified from Clark, Li, Kitaji *et al* [237-239]).

Detection of polyspermy is another persistent problem, because it does not reduce the embryos ability to develop to blastocyst, making this an unsuitable measure of monospermic fertilisation [245]. To do so, pronuclei can be visualized by aceto-orcein staining [246] or through polarization of lipid droplets by centrifugation (shown in Figure 9) [247].

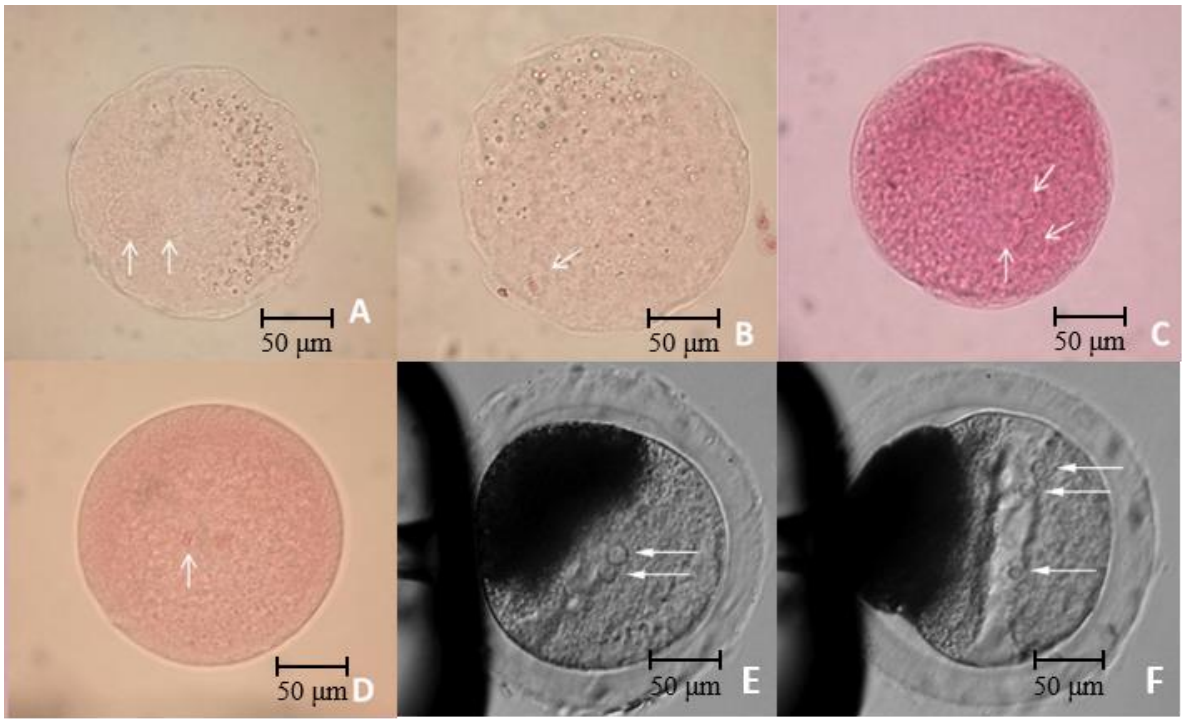


Figure 9: Visualization of pronuclei to detect polyspermic fertilisation. In illustrations **A-D** visualization of pronuclei is facilitated through aceto-orcein staining. **A)** porcine zygote with two pronuclei **B)** metaphase spindle **C)** porcine zygote with three pronuclei indicating polyspermy **D)** abnormal porcine oocyte. In illustration **E-F** pronuclei are made visible through centrifugation. This is necessary due to the high lipid content of porcine oocytes otherwise obstructing the view. Polarized lipid droplets form a dark matter visible to the left. **E)** porcine zygote with two pronuclei **F)** porcine zygote with three pronuclei (adapted and modified from Kurome *et al.* [50] and Gil *et al.* [247]).

1.2.3. *In vitro* culture

Current *in vitro* culture (IVC) systems for porcine zygotes are able to surpass the historically critical four-cell stage [248] and support embryonic development up to the blastocyst stage [135]. However, any period of IVC results in delayed embryo development [249] and lower cell counts in blastocysts [110].

To support optimal embryonic development, culture media are designed to mimic oviduct fluid composition [250]. Supplementation of media with oviduct fluid and co-culture with oviduct epithelial cells [251] has now been replaced by defined media to improve reproducibility and biosafety [252]. Comparative studies between Whitten's medium [253], NCSU-23-medium [251] and Beltsville embryo culture medium [254] proved that NCSU-23 medium is superior in facilitating blastocyst development [250, 251]. The inclusion of the amino acids glycine,

hypotaurine and taurine in NCSU-23 medium was found to especially benefit early embryo development [251, 255].

Porcine zygote medium 5 (PZM5) [163] was developed based on information regarding the concentrations of energy substrates [256, 257] and inorganic elements [258] in porcine oviducts. PZM5 has repeatedly been confirmed as the current medium of choice [165] for parthenogenetic [259], SCNT [260] and IVP embryos [94]. Recently, porcine blastocyst medium was shown to facilitate reliable hatching of blastocysts *in vitro* [261].

Results regarding other culture variables such as oxygen tension with reported optimal values between 5% [262] and 20% [263] or the physical culture environment, such as drop culture, IVF plates or well of the well (WOW) systems [101] have been inconsistent, making them difficult to optimise.

1.3. Precise genetic modification

Non-homologous end joining (NHEJ) in which double strand DNA breaks are re-ligated without the assistance of repair templates is the most common DNA repair pathway in mammalian cells [264]. This mechanism frequently results in insertions or deletions (indels) that can disrupt regulatory elements, or cause frameshift errors in coding regions, and so affect gene function [265].

HR is another natural DNA repair mechanism induced by DNA double strand breaks (DSB) in which homologous sequences are consulted to make accurate repair [266]. While HR is rarer than NHEJ [267] it can be utilized for targeted genome engineering by enabling recombination between the target site and exogenous DNA fragments (see Figure 10) [79]. This facilitates targeted transgene insertion [268] but results in low targeting efficiencies [269] of around one targeting event per 10^6 to 10^7 cells [270]. Precise transgene placement through site specific recombination [271] and HR [91] is preferable to random integration of transgenes which leads to varying expression levels [272], transgene segregation during breeding, and can impede functions of endogenous genes causing health problems [273].

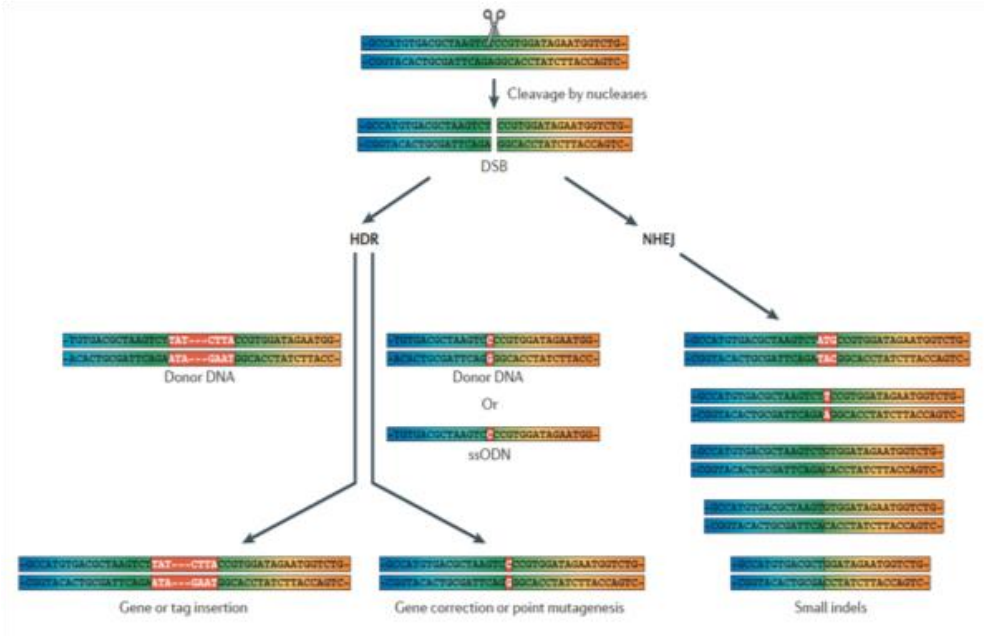


Figure 10: Repair of nuclease induced DSBs through HDR or NHEJ (adapted from Kim *et al.* [274]). Exogenous repair templates facilitate repair of nuclease induced DSBs via HDR thereby allowing for targeted modifications and transgene insertion. The NHEJ pathway frequently causes indel mutations.

Traditional gene targeting vectors comprising of selection cassette and transgene flanked by homologous arms can be utilized in genome engineering [164]. Strategies to improve targeting efficiency involve optimising length of homologous arms [275], gene trapping [276] and positive/negative selection [267].

The development of tailor-made highly specific endonucleases has facilitated the introduction of DSBs into unique sites within the host genome [277, 278] to trigger DNA repair mechanisms [279] thus enabling efficient genome engineering [280]. Categories of site-specific endonucleases include ZFNs [121], TALENs [122] and the CRISPR/Cas9 system [123].

1.3.1. Zinc finger nucleases

ZFNs are dimeric fusion proteins consisting of two DNA binding domains each connected to an unspecific DNA cleavage domain derived from the restriction enzyme FokI (see Figure 11) [281]. An active nuclease is formed through FokI dimerization when two monomers bind to their target sequence [282]. The resulting DSB induces endogenous DNA repair mechanisms, NHEJ and HR, therefore facilitating genome engineering [283]. Three to six zinc finger motifs each binding to a three base pair sequence provide specific recognition of 18 to 36 base pair (bp) target DNA sequences [284].

Successful genome engineering using ZFNs has been reported in a variety of species [120, 285, 286] but the application of ZFNs is limited by narrow design requirements that allow only one ZFN pair per 100bp. High targeting specificity can be achieved by employing multiple zinc fingers [287] and delivery to zygotes via microinjection is possible. However, ZFN design and production is labour intense [288] and unspecific interactions can cause high cytotoxicity [289].

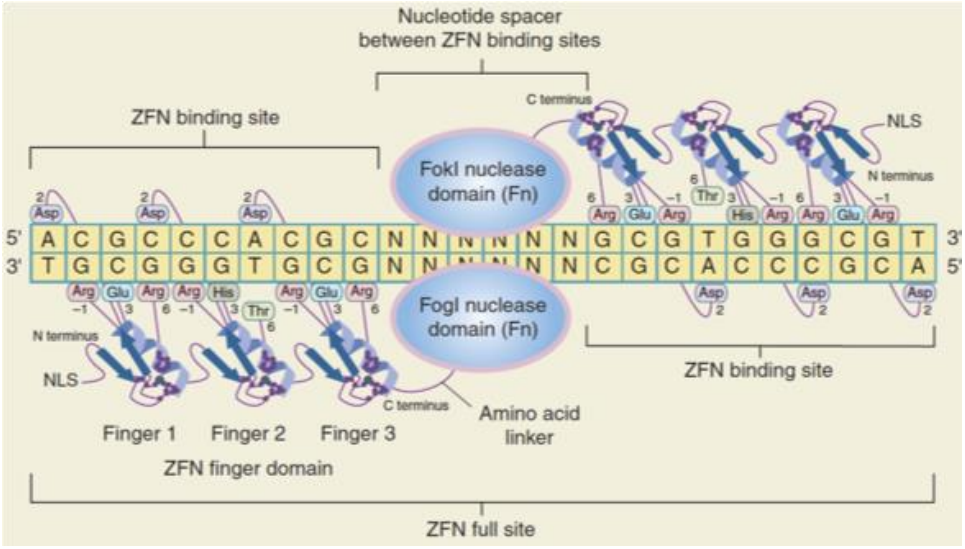


Figure 11: Zinc finger nuclease dimer binding to its target site. A dimer of ZFNs with three zinc fingers binding to a target site. The DNA recognition site is connected by a peptide link to the FokI derived DNA cleavage domain (adapted from Porteus *et al.* [290]).

1.3.2. TALENs

TALENs are artificial dimeric structures made of a TAL effector DNA binding domain derived from the bacterium Xanthomonas [291] fused to a FokI nuclease (see Figure 12) [288]. Tandem amino acid sequences each recognizing a single nucleotide facilitate sequence specific DNA binding. Base specificity is mediated by two amino acids termed the "repeat variable diresidues" [292]. Attachment of two TALEN monomers to their target sequence results in dimerisation of the FokI nuclease causing DSBs that can be repaired by HR or NHEJ, similar to ZFNs [280].

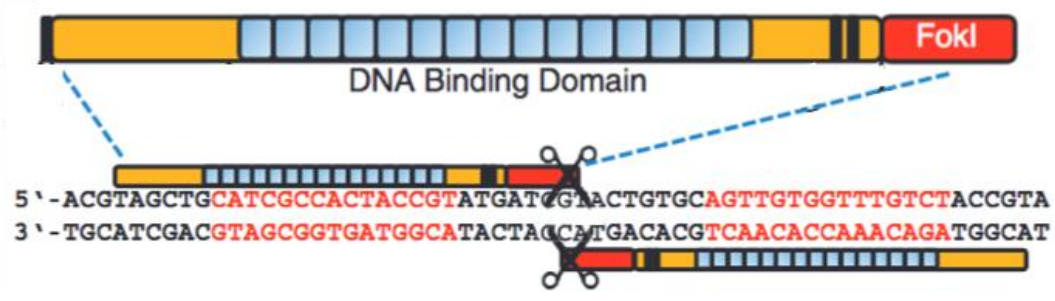


Figure 12: TALEN structure. Dimerization of two TALENs is necessary to enable FokI-mediated DNA cleavage. The target sequence is recognized by the TAL-effector DNA binding domain (adapted from Cermak *et al.* [292]).

Unlike ZFNs, TAL DNA binding domains can be artificially engineered to target any DNA sequence [40]. Due to their high targeting efficiency [293] TALENs have been used for genome

engineering in various livestock species [122]. The main disadvantage of TALENs lies in the complexity of designing DNA binding sequences for new targets [40].

1.3.3. CRISPR/CAS

The CRISPR/Cas system is part of the adaptive immune system in bacteria and archaea [294] that has been adapted for genome engineering [295]. CRISPR are repeating sequences intermediated by short protospacer segments containing genetic information originating from viruses or plasmids [296]. These sequences function as an immunological memory system in prokaryotes to combat viral infection [297]. Cas9 protein is an endonuclease guided by CRISPR RNA (crRNA) to induce DSBs in sequences complementary to spacer segments [298]. There are three types of CRISPR systems in prokaryotes [299]. CRISPR type II systems require only Cas9, crRNA and transactivating crRNA (tracrRNA) necessary for maturation of crRNA [300] to induce DSBs [301] whereas type I and III systems are more complex. Further simplification can be achieved by connecting the 3' end of crRNA to the 5' end of tracrRNA with a loop structure to form a synthetic single guide RNA (sgRNA) [302].

In contrast to ZFNs and TALENs, the CRISPR/Cas9 system can be adapted to recognize nearly any target sequence without protein engineering by using different sgRNAs [280]. Application of multiple sgRNAs enables targeting of multiple genetic loci [119]. Constraints are only imposed by the need for a "NGG" protospacer adjacent motif (PAM) sequence located 3' of the target sequence [298]. Plasmids coding for sgRNA, Cas9 protein and resistance markers facilitating selection of transfected cells [303] make this a very simple and versatile system (see Figure 13) [304].

Due to its high adaptability, usability, simple production and high efficiency CRISPR/Cas has become the preferred method for genome engineering [304, 305]. The CRISPR system has been used to generate GE plants [306], livestock [123] and humans [307]. The biggest downside to Cas9 and other site-specific nucleases are off-target effects, that is the induction of DSBs at unwanted locations [308].

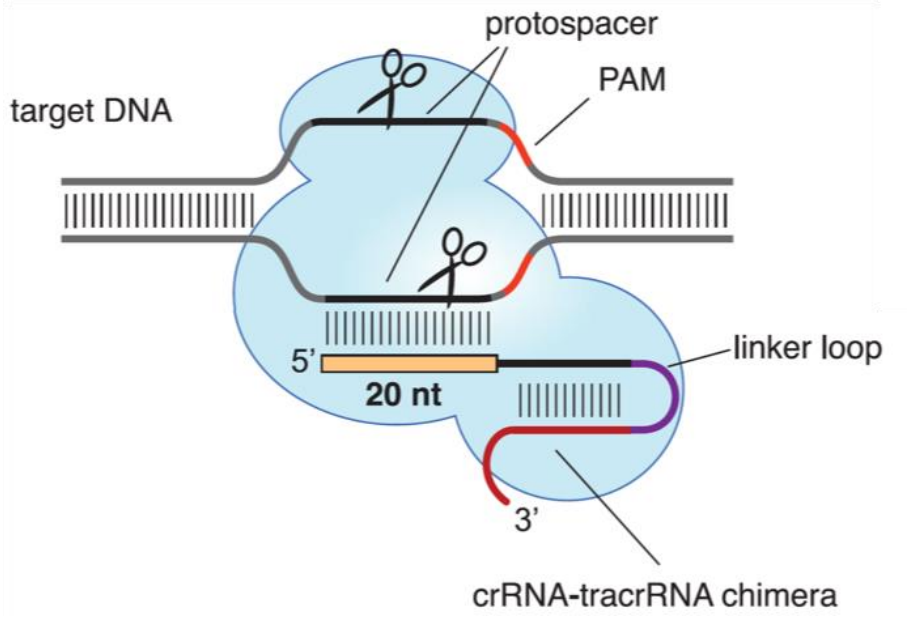


Figure 13: The CRISPR/Cas9 system as a tool for genome engineering. Natural CRISPR/Cas systems are guided to their target sequence by an RNA complex composed of crRNA and tracrRNA. For genome engineering purposes this complex has been replaced by an artificially engineered single guide RNA (sgRNA). This sgRNA has been generated by connecting the 3' end of crRNA with the 5' end of tracrRNA with a loop structure. Upon target size recognition two separate Cas9 domains cleave each DNA strand to make a DSB (adapted from Jinek *et al.* [302]).

CRISPR/Cas9 technology is rapidly advancing and many variants are being developed. Modifications to the CRISPR system include 'nickases' that cleave single strand breaks leading to HDR while reducing mutations in comparison to the original version [309]. So-called 'double nicking' approaches can create DSBs with high precision [310].

Another approach termed "base editing" converts specific bases into another without causing DSBs [311]. This is carried out by a deaminase enzyme fused to an inactive Cas9 protein used for DNA binding. However, this approach has been shown to cause frequent off-target mutations [312, 313].

'Prime editing' is a new approach that uses a catalytically inactive Cas9 connected to a reverse transcriptase enzyme. The target site plus the intended edit are both specified by a prime editing guide RNA at the same time. First reports claim higher efficiencies and reduced off-target effects compared to traditional Cas9 approaches [314].

1.4. Goals of the project

The pig is an important animal species in agriculture and biomedicine. Genome engineering provides new possibilities in both areas. It can be used to assess the function of genes, improve animal health and generate disease models or pigs for organ xenotransplantation. A reliable *in vitro* embryo production system based on slaughterhouse-derived ovaries is essential to minimise the required number of experimental animals. Protocols for the *in vitro* production of porcine embryos are however still suboptimal compared to other species.

The main goal of this project was to optimise the *in vitro* production of porcine embryos to facilitate the generation of genetically engineered pigs. This entails the identification of suitable sperm isolates, refinement of semen cryopreservation, establishment of a sperm bank and improvement of embryo culture conditions. Next the suitability of IVP embryos for the generation of transgenic, single or multiple genome edited and for a simplified cloning method was to be assessed.

A further objective of this work was the optimisation of genome engineering directly in porcine zygotes using CRISPR/Cas9 technology and subsequently proof that the resulting embryos are developmentally competent.

Direct manipulation of porcine zygotes is a powerful method for the inactivation of genes but its efficiency for more complex genome alterations is much lower. Somatic cell nuclear transfer is a more suitable tool for such applications because it allows pre-screening for the desired modification in cell culture. An additional goal was the implementation of handmade cloning as an efficient alternative to traditional cloning for this purpose.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals, buffers and solutions

Table 2: Chemicals, buffers and solutions

Name	Source
Acetic acid ($C_2H_4O_2$)	AppliChem, Darmstadt, GER
Agarose	Sigma-Aldrich, Steinheim, GER
Ammonium acetate ($C_2H_7NO_2$)	Sigma-Aldrich, Steinheim, GER
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Steinheim, GER
Amphotericin B	Sigma-Aldrich, Steinheim, GER
Biocoll	Biochrom, Berlin, GER
Bisbenzimide (Hoechst staining)	Sigma-Aldrich, Steinheim, GER
Bromphenol blue	Sigma-Aldrich, Steinheim, GER
BSA (fraction V)	Biomol, Hamburg, GER
Caffeine	Sigma-Aldrich, Steinheim, GER
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Steinheim, GER
Cetyltrimethylammonium ammonium bromide	Sigma-Aldrich, Steinheim, GER
Chloroform (99%)	Sigma-Aldrich, Steinheim, GER
CutSmart Buffer	New England Biolabs, Ipswich, USA
Cycloheximide	Sigma-Aldrich, Steinheim, GER
Cysteine ($C_3H_7NO_2S$)	Sigma-Aldrich, Steinheim, GER
Cytochalasin B	Sigma-Aldrich, Steinheim, GER
Cycloheximide	Sigma-Aldrich, Steinheim, GER
Demecolcine	Sigma-Aldrich, Steinheim, GER
Deoxynucleotide (dNTP) solution mix	New England Biolabs, Frankfurt, GER
DEPC-treated water (H_20)	Thermo Fisher, Waltham, MA, USA
Dimethyl-sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, GER
Dulbecco's phosphate buffered saline (dPBS)	Sigma-Aldrich, Steinheim, GER
Ethanol (EtOH) absolute	Fisher Scientific, Loughborough, GBR

Ethanol (EtOH) denatured	CLN GmbH, Niederhummel, GER
Ethylene diamine tetra-acetic acid (EDTA)	AppliChem, Darmstadt, GER
Foetal calf serum (FCS)	PAA laboratories, Pasching, Austria
Gel loading dye, purple (6x)	New England Biolabs, Frankfurt, GER
Glucose ($C_6H_{12}O_6$)	Sigma-Aldrich, Steinheim, GER
Glutamine	Invitrogen GmbH, Darmstadt, GER
Glycerol ($C_3H_8O_3$)	AppliChem, Darmstadt, GER
Glycine ($C_2H_5NO_2$)	Carl Roth, Karlsruhe, GER
Heparin sodium salt	Sigma-Aldrich, Steinheim, GER
HEPES buffer	Sigma-Aldrich, Steinheim, GER
KCl	Sigma-Aldrich, Steinheim, GER
Magnesium chloride ($MgCl_2$)	Carl Roth, Karlsruhe, GER
Methanol (CH_3OH)	Sigma-Aldrich, Steinheim, GER
$MgSO_4$	Sigma-Aldrich, Steinheim, GER
Mineral oil	Sigma-Aldrich, Steinheim, GER
Penicillin-Streptomycin	Sigma-Aldrich, Steinheim, GER
peqGREEN	VWR International, Ismaning, GER
Phenol red	Sigma-Aldrich, Steinheim, GER
Phenol-chloroform-alcohol	AppliChem, Darmstadt, GER
Polyvinyl alcohol (C_2H_4O)	Sigma-Aldrich, Steinheim, GER
Potassium chloride (KCL)	Carl Roth, Karlsruhe, GER
Potassium-bicarbonate (KHCO3)	Sigma-Aldrich, Steinheim, GER
Propanolol (C_3H_8O)	Fisher Scientific, Loughborough, GBR
Silicon grease	Obermeier, Bad Berleburg, GER
Sodium acetate (C2H3NaO2)	AppliChem, Darmstadt, GER
Sodium bicarbonate (NaHCO3)	Sigma-Aldrich, Steinheim, GER
Sodium chloride (NaCl)	Sigma-Aldrich, Steinheim, GER
Sodium hydroxide (NaOH)	Sigma-Aldrich, Steinheim, GER
Sodium pyruvate	Sigma-Aldrich, Steinheim, GER
Sorbitol	Sigma-Aldrich, Steinheim, GER
Sucrose ($C_{12}H_{22}O_{11}$)	Carl Roth, Karlsruhe, GER
Tris	AppliChem, Darmstadt, GER
Tris-HCL	Sigma-Aldrich, Steinheim, GER

Triton X100	Omnilab-Laborzentrum, Bremen, GER
Trypan blue	Thermo Fisher, Waltham, MA, USA
Trypsin	Sigma-Aldrich, Steinheim, GER
Tween 20	Sigma-Aldrich, Steinheim, GER

2.1.2. Enzymes and enzyme buffers

Table 3: Enzymes and enzyme buffers

Name	Source
5x Green GoTaq reaction buffer	Promega, Mannheim, GER
Bam-HF restriction enzyme	New England Biolabs, Ipswich, USA
DNA Polymerase I, Large Klenow Fragment	New England Biolabs, Ipswich, USA
GoTaq G2 DNA polymerase	Promega, Mannheim, GER
HindIII-HF restriction enzyme	New England Biolabs, Ipswich, USA
Hyaluronidase	Sigma-Aldrich, Steinheim, GER
Pronase	Sigma-Aldrich, Steinheim, GER
Proteinase K (20mg/ml)	Sigma-Aldrich, Steinheim, GER
Q5 high fidelity DNA polymerase	New England Biolabs, Ipswich, USA
Restriction endonucleases	New England Biolabs, Ipswich, USA
T4 DNA Ligase	New England Biolabs, Ipswich, USA

2.1.3. Kits

Table 4: Kits

Name	**Source**
DNeasy Blood and tissue kit	Quiagen GmbH, Hilden, GER
innuSPEED RNA kit	Analytik Jena AG, Jena, GER
Lipofectamine 2000	Jena Analytic, Jena, GER
MEGAclear kit	Ambion, Austin, TX, USA
MEGAshortscript T7 kit	Ambion, Austin, TX, USA
Mix2Seq kit	Eurofins, Ebersberg, GER
NucleoBond Xtra Midi kit	Macherey-Nagel, Düren, GER
PlateSeq DNA kit	Eurofins, Ebersberg, GER
Poly-A tailing kit	Ambion, Austin, TX, USA

SurePrep RNA/DNA/protein purification kit	Fisher Scientific, Hampton, NH, USA
Wizard SV gel and PCR clean-up system	Promega, Mannheim, GER

2.1.4. Cells

2.1.4.1. Bacteria

Table 5: Bacteria

Name	Genotype:	Source:
E. coli ElectroMAX DH10B	F-mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G	Thermo Fisher Scientific, Waltham, MA, USA

2.1.4.2. Eukaryotic cells

Table 6: Eukaryotic cells

Cell type	Genotype	Source
Porcine sperm	Wild type, TP53, KRAS, CD46, CD55, CD59, HO-1, GAL, CMAH, B4G, R26M	Bayerngenetik GmbH, Altenbach, GER; Chair of Livestock Biotechnology, TUM, Freising, GER;
Porcine foetal fibroblasts (several preparations)	Wild type	Chair of Livestock Biotechnology, TUM, Freising, GER
Porcine kidney fibroblasts (several preparations)	Wild type	Chair of Livestock Biotechnology, TUM, Freising, GER
Porcine oocytes	Wild type TP 53	Vion food GmbH, Landshut, GER

2.1.5. Oligonucleotides

2.1.5.1. Primers

Table 7: Primers and probes. All oligonucleotides were purchased from MWG Eurofins, Ebersberg, GER

Name	Sequence
B4G I1 F1	ACCAGACATCGTTCCCAGTG
B4G I2 R1	AACTGGCTGTAAAGTGGGCA
B4G Scr I2 F2	GAACCTTGCGGCCCTAAAAA

B4G Scr I3 R1	AGCTTCCGCTCCATCTCAGG
CMAH Scr E10 F2	TGCCGTAAACAAAGAGGGGATT
CMAH Scr E10 R2	TTGTCTGCTGGGTGGGATTC
F.GAPDH s.scrofa	TTCCACGGCACAGTCAAGGC
Gal Scr E7T56F	GCCAGTCACCACAAGCCATG
Gal Scr E7T56R	TGGCCCTGTGACACCATTCT
Gal Scr E8 T3 F	AAGACCATCGGGGAGCACAT
Gal Scr E8 T3 R	GGCTTTCATCATGCCACTCG
MHCI F1	CCAGTGGTCACATGAGGCTGC
MHCI R1	GCGCCCTCCTTACCCCATCT
pNCTP scr E2 F1	TGACCACCTGCTCCACCTTC
pNTCP scr E2 R1	CGCACATATTGTGGCCGTTT
R. GAPDH s.scrofa	GCAGGTCAGGTCCACAAC
TNF check_F2:	GGGTTTGGATTCCTGGATGC
TNF check_R2:	GCGGTTACAGACACAACTCC
TNF α F2	GGGTTTGGATTCCTGGATGC
TNF α R2	GCGGTTACAGACACAACTCC
UCP1_hs_5F	GGACTACTCCCAATCTGATGAGAAG
UCP1_sus_12R	GTTGTGAAGACCACTGCCCT

2.1.5.2. gRNA oligonucleotides

Table 8: gRNA oligonucleotides. All oligonucleotides were purchased from MWG Eurofins, Ebersberg, GER

Name	**Sequence**
B4GALNT2_E3T1 F	CACCGTGACGCCTTCGGGCATC
B4GALNT2_E3T1 R	AAACGATGCCCGAAGGCGTCAC
CMAH-E6-T3 F	GTCCTGCTTTTGCGCGAGGA
CMAH-E6-T3 R	TCCTCGCGCAAAAGCAGGAC
Gal-E8-T3-F	GACGAGTTCACCTACGAG
Gal-E8-T3-R	CTCGTAGGTGAACTCGTC
Nanos g7 F1	GACTACTTCAACCTGAGCC
Nanos g7 R1	GGCTCAGGTTGAAGTAGTC
Px-B4GNT2-E2-T1 F	CGATCCTCAAGATATCGA
Px-B4GNT2-E2-T1 R	TCGATATCTTGAGGATCGC

2.1.6. Nucleic acid ladders

Table 9: Nucleic acid ladders

Name	Source
1 kb DNA ladder	New England Biolabs, Frankfurt, GER
100 bp DNA ladder	New England Biolabs, Frankfurt, GER
2-log DNA ladder (0.1-10.0 kb)	New England Biolabs, Frankfurt, GER
Ribo Ruler high range RNA ladder	Thermo Scientific, Waltham, MA, USA

2.1.7. Molecular cloning vectors and DNA constructs

Table 10: Molecular cloning vectors and DNA constructs

Name	Specificity
pmaxGFP	Kan, maxGFP
PX330 3xKO	GGTA1, CMAH, B4GNT2
PX330 4xKO	GGTA1, CMAH, B4GNT2, B2M
PX330 hNTCP – guide 15	hNTCP – guide 15, Cas9
PX330 hNTCP– guide 16	hNTCP – guide 16, Cas9
PX330 hNTCP plasmid	Bla, hNTCP
PX330 NANOS – guide 1	NANOS2 – guide1, Cas9
PX330 NANOS – guide 2	NANOS2 – guide2, Cas9
PX330 NANOS – guide 3	NANOS2 – guide3, Cas9
PX330 NANOS – guide 4	NANOS2 – guide4, Cas9
PX330 TNF $^{\Delta ARE}$	TNF $^{\Delta ARE}$, Cas9
PX330 UCP1	UCP1, Cas9

2.1.8. Embryo culture media, supplements and reagents

Table 11: Embryo culture media, supplements and reagents

Name	Source
Amphotericin B	Sigma-Aldrich, Steinheim, GER
Androstar cryo plus sperm freezing medium	Minitube, Tiefenbach, GER
Androstar plus sperm dilution medium	Minitube, Tiefenbach, GER
BSA (fraction V)	Sigma-Aldrich, St. Louis, MO, USA

Ca-ionophore	Sigma-Aldrich, St. Louis, MO, USA
Cysteine	Sigma-Aldrich, St. Louis, MO, USA
D-glucose	Sigma-Aldrich, St. Louis, MO, USA
EDTA	Sigma-Aldrich, St. Louis, MO, USA
Egg yolk pasteurized	Minitube, Tiefenbach, GER
Epidermal growth factor (EGF)	Sigma-Aldrich, St. Louis, MO, USA
FBS Superior	Biochrom GmbH, Berlin, GER
Fibroblast growth factor (FGF)	Sigma-Aldrich, St. Louis, MO, USA
Glacial acetic acid	Sigma-Aldrich, Steinheim, GER
Hyaluronidase	Sigma-Aldrich, St. Louis, MO, USA
Insulin like growth factor (IGF)	Sigma-Aldrich, St. Louis, MO, USA
Intergonan (PMSG/ECG)	MSD-Tiergesundheit, Unterschleißheim, GER
Leukaemia inhibitory factor (LIF)	Sigma-Aldrich, St. Louis, MO, USA
Mannitol	Sigma-Aldrich, St. Louis, MO, USA
MgCl2	Sigma-Aldrich , St. Louis, MO, USA
MgSO4	Sigma-Aldrich, St. Louis, MO, USA
Mineral oil	Sigma-Aldrich, St. Louis, MO, USA
Ovogest (HCG)	MSD-Tiergesundheit, Unterschleißheim, GER
Penicillin/Streptomycin	Sigma-Aldrich, St. Louis, MO, USA
Phosphate-buffered saline (PBS)	Sigma-Aldrich, St. Louis, MO, USA
Phytohaemagglutinin	Sigma-Aldrich, St. Louis, MO, USA
Polyvinyl alcohol	Sigma-Aldrich, St. Louis, MO, USA
Porcine fertilisation medium (PFM)	Fujihira Industry, Tokyo, JAP
Porcine zygote medium 5 (PZM5)	Fujihira Industry, Tokyo, JAP
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA
Sodium pyruvate	Sigma-Aldrich, St. Louis, MO, USA
Sodium pyruvate	Sigma-Aldrich, St. Louis, MO, USA
Tissue culture medium 199	Sigma-Aldrich, Steinheim, GER
Tissue culture medium 199 hepes-modification	Sigma-Aldrich, Steinheim, GER

2.1.9. Bacterial culture media and supplements

Table 12: Bacterial culture media and supplements

Name	Source

Ampicillin (C16H19N3O4S)	Carl Roth, Karlsruhe, GER
Chloramphenicol (C11H12Cl2N2O5)	Sigma-Aldrich, Steinheim, GER
LB agar, Miller (Luria-Bertani)	Difco BD, Sparks, MD, USA
Luria Broth, Base, Miller	Difco BD, Sparks, MD, USA

2.1.10. Tissue culture media and supplements

Table 13: Tissue culture media and supplements

Name	**Source**
Accutase	Sigma-Aldrich, Steinheim, GER
Ala-Gln, 200mM	Sigma-Aldrich, Steinheim, GER
Amphotericin B	Sigma-Aldrich, Steinheim, GER
Blasticidin S	InvivoGen, San Diego, CA, USA
Cell culture water	Sigma-Aldrich, Steinheim, GER
DMSO	Sigma-Aldrich, Steinheim, GER
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, Steinheim, GER
Foetal calf serum	PAA Laboratories, Pasching, Austria
G418	Genaxxon Bioscience, Ulm, GER
GlutaMAX	Gibco, BRL, Paisley, UK
Hygromycin	AppliChem, Darmstadt, GER
Hypo-osmolar buffer	Eppendorf, Hamburg, GER
Lipofectamine 2000	Thermo Fisher Scientific, Waltham, MA, USA
MEM non-essential amino acids 100x	Sigma-Aldrich, Steinheim, GER
Opti-MEM	Gibco Life Technologies, Carlsbad, CA, USA
Penicillin/Streptomycin	Sigma-Aldrich, Steinheim, GER
Phosphate-buffered saline (PBS)	Sigma-Aldrich, Steinheim, GER
Puromycin	InvivoGen, San Diego, CA, USA
Sodium pyruvate solution, 100mM	Sigma-Aldrich, Steinheim, GER
Trypan blue	Gibco Life Technologies, Paisley, GBR
Trypsin-EDTA	PAA Laboratories, Pasching, Austria

2.1.11. Laboratory equipment

Table 14: Laboratory equipment

Name	Source
20G needle: Neolus	Becton and Dickinson Company, NJ, USA
7500 fast real-time PCR cycler	Life Technologies, Carlsbad, CA, USA
Aggregation needle DN-09/B	BLS Ltd. Budapest, Hungary
Accu-jet pro	Brand, Dietenhofen, GER
Blue light table	Serva, Heidelberg, GER
Bunsen burner “Gasprofi 2“akku	WLD-TEC GmbH, Arenshausen, GER
Camera AxioCam MR (Axiovision)	Carl Zeiss Jena GmbH, Jena, GER
Cell counting chamber: Neubauer improved	Brand GmbH, Wertheim, GER
Centrifuges „Sigma 3-16KL “„Sigma 1-15“ Sigma „1-15K “, „Sigma 4K15“	Sigma, Osterode, GER
Countess automatic cell counter	Invitrogen, Carlsbad, CA, USA
Digital microscope “M8”	PreciPoint, Freising, GER
Dry block heater PCH2	Grant Instruments, Royston, GBR
Dry block heater/cooler “PCH-2”	Grant instruments, Royston, GBR
Electrophoresis system (buffer, chamber, gel trays, combs)	Peqlab Biotechnologie, Erlangen, GER
Electroporation cuvettes	PEQLAB Biotechnologie GmbH, Erlangen, GER
Electroporator: BTX ECM 830 Electroporation generator	BTX, Holliston, MA, USA
Electroporator: Multiporator	Eppendorf, Hamburg, GER
FemtoJet Express	Eppendorf, Hamburg, GER
Freezer -20°C “GS 2481“	Liebherr, Bulle, SUI
Freezer -80°C “Forma 900 Series “	Thermo Fisher Scientific, Waltham, MA, USA
Fusion machine “BLS CF-150/C	BLS Ltd. Budapest, Hungary
Fusion chamber BTX microslide 0.5 mm, model 450	Thermo Fisher Scientific, Waltham, MA, USA
Gel documentation imaging system “Quantum ST5”	Vilber Lourmat, Eberhardzell, GER
Gel electrophoresis chamber + power adapter	Bio-Rad Laboratories GmbH, Munich, GER
Glasware	Marienfeld GmbH, Landa, GER
Heating plate HT 200	Minitube, Tiefenbach, GER
Heating plate HT50	Minitube, Tiefenbach, GER
Heating plate SC 300	Minitube, Tiefenbach, GER

Hera Safe clean bench	Heraeus Instruments, Hanau, GER
Ice machine	Manitowoc Ice, Manitowoc, WI, USA
Incubator (Heracell VioS 160i)	Thermo Fisher Scientific, Waltham, MA, USA
Incubator Steri-cycle CO2	Thermo Fisher Scientific, Waltham, MA, USA
Magnetic stirrer “AREC_X”, “AGE”	VELP Scientific, Usmate, ITA
Microblade “ESE 020”	Bioniche Animal Health, Clonee, Ireland
Microinjector: CellTram vario/air/pro	Eppendorf, Hamburg, GER
Microscope “Axiovert 40CLF”, “Axiovert 200M”, “Primo Star”	Carl Zeiss GmbH, Jena, GER
Microwave “MW17M70G-AU”	MDA Haushaltswaren, Barsbüttel, GER
Mini centrifuge “perfect spin mini”	Peqlab Biotechnologie, Erlangen, GER
Mr. Frosty freezing container	Thermo Fisher Scientific, Waltham, MA, USA
Nunc 4-well IVF plate	Thermo Fisher Scientific, Waltham, MA, USA
Orbital shaker	Thermo Fisher Scientific, Waltham, MA, USA
P97-micropipette puller	Sutter Instrument, CA, USA
PCR cycler “DNA Engine DYAD, PTC 0220”	Biorad Laboratories, Munich, GER
PCR cycler “peqStar”	Peqlab Biotechnologie, Erlangen, GER
Pipettes “Pipetman “2ul, 20ul, 1000ul”	Gilson, Middleton, WI, USA
Power supply “EPS 301”	Amersham Bioscience, Little Chalfont, UK
Power supply “peqPOWER”	Peqlab Biotechnologie, Erlangen, GER
Refrigerator “TSE1283”	Beko, Neu-Isenburg, GER
Rocker shaker “Unitwist 3-D”	Uniequip, Munich, GER
Safety cabinets HERA safe HSP	Heraeus Instruments, München, GER
Safety Workbench Hera safe class 2H	Heraeus Instruments, Munich, GER
Spectrophotometer: Bio photometer	Eppendorf, Hamburg, GER
Sperm filling machine: SFS	Minitube, Tiefenbach, GER
Sperm freezer: Ice Cube 14 S-A	Minitube, Tiefenbach, GER
Stereomicroscope Stemi 508	Carl Zeiss, Göttingen, Germany
Stereomicroscope Stemi 508	Carl Zeiss, Göttingen, Germany
Syringe filter (0.22 µm)	Berrytec, Grünwald, GER
Table centrifuge	Sigma-Aldrich GmbH, Steinheim, GER

Thermos container	Alfi GmbH, Wertheim, GER
Transfer man NK2 micromanipulator	Eppendorf, Hamburg, GER
Transportable incubator	Minitube, Tiefenbach, GER
Vacuum pump: Jun Air	Jun-Air, Redditch, UK
Vortex mixer “Vortex Genie 2”	Scientific industries, Bohemia, NY, USA
Water bath	Memmert, Schwabach, GER

2.1.12. Buffers and solutions

Table 15: Buffers and solutions

Type	Components	Amount
DNA miniprep solution I	$C_{12}H_{22}O_{11}$	1.7 g
	EDTA	2.9 g
	Tris	3.0 g
	H_2O	Fill up to 1 l
DNA miniprep solution II	NaOH	8.0 g
	SDS	10.0 g
	H_2O	Fill up to 1 l
DNA miniprep solution III	$C_2H_3NaO_2$	246.1 g
	H_2O	Fill up to 1 l
Electrophoresis buffer 10x	Tris	30 g
	$C_2H_5NO_2$	144 g
	SDS	10 g
	H_2O	Fill up to 1 l
SDS 10%	SDS	10 g
	H2O	Fill up to 100 ml
Sodium citrate buffer	$C_6H_5NaO_7$ x 2 H_2O	2.9 g
	H_2O	Fill up to 1 l
TAE 10x	Tris	242 g
	0.5 M EDTA	100 ml
	$C_2H_4O_2$	57.1 ml
	H_2O	Fill up to 5 l
TBE 10X	Tris	545 g
	H_3BO_3	275 g
	EDTA	39.2 g
	H_2O	Fill up to 5 l
TE buffer	Tris-HCl	158 mg
	EDTA	29 mg
	H_2O	Fill up to 100 ml
TTE buffer	Tris	242 mg
	Triton X 100	1 ml
	EDTA	584 mg
	H_2O	Fill up to 100 ml
Oocyte transportation solution	PBS	500 ml
	Penicillin/Streptomycin	5 ml
	Amphotericin B	5 ml

2.1.13. Handmade cloning stocks

Table 16: Handmade cloning stocks

Type	Component	Amount
Bovine fusion medium (cFM)	Stock A	2 ml
	Stock B	2 ml
	Mannitol-PVA-solution	196 ml
Cycloheximide stock	Cycloheximide	1 mg/ml
		25 µl aliquots
Cytochalasin B stock	Cytochalasin B	5 mg/ml
		5 µl aliquots
Hoechst stock	Hoechst stain	3 mg
	H_2O	3 ml
Hyaluronidase stock	Hyaluronidase	1 mg/ml
	TCM 199	500 µl aliquots
Mannitol-PVA-solution	Mannitol	10.93 g
	PVA	0.2 g
	H_2O	196 ml
Phytohaemagglutinin (PHA) stock	Phytohemagglutinin	5 mg/ml
	TCM 199	20 µl aliquots
Porcine fusion medium (pFM)	Same as cFM, but no stock A and B are added	
Pronase stock	Pronase	10 mg/ml
	T10	200 µl aliquots
Stock A	$MgSO_4$	25 mg (9.96 mM)
	H_2O	20 ml
Stock B	$CaCl_2$	14.7 mg (5.0 mM)
	H_2O	20 ml
T10	FCS	10 %
	TCM 199	2 ml aliquots
T2	FCS	2 %
	TCM 199	10 ml aliquots
T20	FCS	20 %
	TCM 199	2 ml aliquots

2.1.14. Consumables

Table 17: Consumables

Name	Source
Borosilicate glass with filament	Sutter Instruments, CA, USA
Carbon dioxide gas cylinders, 200 bar (CO_2)	Westfalen AG, Münster, GER
Cell culture flasks	Corning Inc., Corning, NY, USA
Cell culture plates	Corning Inc., Corning, NY, USA
CellStar tubes (15ml and 50 ml)	Greiner Bio-One, Frickenhausen, GER

Cloning rings	Brand, Wertheim, GER
Cover slips (24x60mm)	Menzel, Braunschweig, GER
Cryo tube vials	Nunc, Wiesbaden, GER
Cryo-vials	Corning Inc., Corning, NY, USA
Electroporation cuvettes (2mm/4mm)	Peqlab Biotechnology, Erlangen, GER
Filter pipette tips „Fisher brand Sure One “	Fisher Scientific, Hampton, NH, USA
Glass Pasteur pipettes	Brand, Wertheim, GER
IVF 4-well plates (nonunclon treated surface)	Fisher Scientific, Waltham, MA, USA
Micro loader Tip	Eppendorf, Hamburg, GER
Nitrogen gas cylinders, 200 bar (N2)	Westfalen AG, Münster, GER
Oxygen gas cylinders, 200 bar (O2)	Westfalen AG, Münster, GER
PCR tubes 0.2 ml 8-strip PCR tubes	Starlab, Hamburg, GER
Petri dishes	Greiner Bio-One, Frickenhausen, GER
Pipette tips	Brand, Wertheim, GER
Plastic pipettes „Costar Stripette“(1-50ml)	Corning Inc., Corning, NY, USA
Reaction tubes (5ml)	Starlab, Hamburg, GER
Reaction tubes, (1.5ml and 2ml)	Zefa Laborservice, Harthausen, GER
Sperm straws	Minitube, Tiefenbach, GER
Sterile filter 0.22µm	Berrytec, Grünwald, GER
Syringes	BD Bioscience, Le Pont De Claix, FRA
Tissue culture flasks (T25,75,150)	Corning Inc., Corning, NY, USA
Tissue culture plates (10cm, 6-, 12-, 24-well)	Corning Inc., Corning, NY, USA
Tubes (15ml)	Corning Inc., Corning, NY, USA
Tubes (50ml)	Corning Inc., Corning, NY, USA
Vacutip	Eppendorf, Hamburg, GER
White cap falcon	Greiner Bio-One, Frickenhausen, GER

2.1.15. Software and online tools

Table 18: Software and online tools

Name	Source
Benchling	https://www.benchling.com/

Chromatogram viewer" Finch TV"	Digital world biology LLC, CA, USA
Crispr design tool	http://crispor.tefor.net/crispor.py
Gel documentation software "Quantum ST5"	Vilber Lourmat, Eberhandzell, GER
Genome database "Ensembl"	https://www.ensembl.org/index.html
Microscope software "Axio Vision"	Carl Zeiss, Göttingen, Germany
Primer design tool "Primer3"	http://primer3.ut.ee/
Reverse Complement web tool	https://www.bioinformatics.org/sms/rev_comp.html
Sequence alignment tool "Clustal Omega"	https://www.ebi.ac.uk/Tools/msa/clustalo/
TIDE: Tracking of Indels by DEcomposition	https://tide.deskgen.com/
Uniprot	https://www.uniprot.org/
Vector design software "Everyvector"	http://www.everyvector.com/

2.1.16. Veterinarian medicinal products and equipment

Table 19: Veterinarian medicinal products and equipment

Name	**Sequence**
Altrenogest (Regumate®)	MSD-Tiergesundheit, Unterschleißheim, GER
Azaperone	Elanco GmbH, Bad Homburg, GER
Cauter	HBH Medizintechnik, Tuttlingen, GER
Cellulose swabs	B. Braun AG, Melsungen, GER
Disposable razor	B. Braun AG, Melsungen, GER
Disposable scalpels	Braun, Melsungen, GER
Intergonan (ECG / PMSG)	MSD-Tiergesundheit, Unterschleißheim, GER
Katheter Careflow 5F, 300mm	Merit Medical, Jordan, UT, USA
Ketanest	Elanco GmbH, Bad Homburg, GER
Needle holder, Matthieu, 20cm	Omega Medical, Winnenden, GER
Ovogest (HCG)	MSD-Tiergesundheit, Unterschleißheim, GER
Surgical drape	B. Braun AG, Melsungen, GER
Surgical gloves, Peha-taft latex	Omega Medical, Winnenden, GER
Surgical instruments	HBH Medizintechnik, Tuttlingen, GER

Surgicryl 910 HS 48, 5 (2), 90cm	Omega Medical, Winnenden, GER
Surgicryl Monofilament DS 24, 3.0 (2/0) 75cm	Omega Medical, Winnenden, GER
Syringes, (1ml,5ml,10ml,20ml)	B. Braun AG, Melsungen, GER

2.2. Methods

2.2.1. Embryology

2.2.1.1. Collection and transport of ovaries

Ovaries from prepubertal gilts were collected and transported to the laboratory at 38°C in phosphate buffered saline (PBS) supplemented with antibiotics and antimycotics. Transportation and handling times were kept as short as possible.

2.2.1.2. Oocyte collection and classification

Ovaries were rinsed several times with warm PBS supplemented with 1% Hexadecyltrimethylammonium bromide (CTAB). A second washing step was conducted utilizing only warm PBS solution to get rid of the detergent. The clean ovaries were placed in warm PBS and kept at 38°C during the puncturing process.

Follicles with a diameter of 3 to 6mm were punctured using a 10 ml syringe and a 18G needle (see Figure 14). Porcine follicular fluid (PFF) was extracted and stored at 38°C until further processing. PFF was removed from the falcon while cumulus oocyte complexes (COCs) sedimented at the bottom of the tube. Then 6-8ml of working medium (WM) supplemented with 1% Amphotericin B (Ampho B) and 1% Penicillin-Streptomycin were mixed with the sediment. Oocytes and working medium were transferred to a petri dish for collection. High quality oocytes with dark, evenly granulated cytoplasm and several compact layers of cumulus cells were identified under a stereomicroscope equipped with a heating plate.

Figure 14: Porcine ovaries; Antral follicles with a diameter of 3-6mm are best suited for oocyte aspiration.

Oocytes were rinsed twice in WM to get rid of cell detritus. For all transfer steps a mouth pipette and self-made glass capillaries of adequate diameter (approximately 300µm) were used to make the washing steps as efficient as possible. Glass capillaries were pulled by hand from sterilized Pasteur pipettes over the flame of a Bunsen burner.

2.2.1.3. In vitro maturation

For in vitro maturation oocytes were transferred to a triple gas incubator (5% O_2, 5% CO_2, 90% N_2, set up at 38,5°C humidified atmosphere). IVF was conducted in IVF four-well dishes containing 500 µl of maturation medium. Groups of 50 COCs were rinsed in maturation medium and transferred to a separate maturation well. After 45 hours successful maturation was confirmed by visual assessment of polar body extrusion from a sample group of ovaries. During the first half of the project maturation was carried out in NCSU-23 medium supplemented with hormones and PFF. Due to better reproducibility and maturation results this approach was later replaced by a chemically defined maturation medium.

Table 20: Composition of maturation media

NCSU-23 maturation medium	
Component	**Concentration**
$CaCl_2$	1.70 mM
Cysteine	0.6 mM
ECG	10 IU/ml
EGF	10 ng/ml
Glucose	5.55 Mm
HCG	10 IU/ml
Hypotaurine	5 mM
KCl	4.78 mM
KH_2PO_4	1.19mM
L-glutamine	1 mM
$MgSO_4$	1,19 mM
NaCl	108.73 mM

$NaHCO_3$	25.07 mM
Penicillin-G	65 mg/L
Porcine follicular fluid	10% v/v
Streptomycin sulphate	50 mg/L
Taurine	7.0 mM

Chemically defined TCM 199 based maturation medium (FLI-medium)

Component	**Concentration**
Cysteine	0.57mM
ECG	1 IU/ml
EGF	10ng/ml
FGF2	40ng/ml
Glucose	3.05mM
HCG	1 IU /ml
IGF1	20ng/ml
LIF	20ng/ml
PVA	0.1% w/v
Sodium pyruvate	0.91mM
TCM 199	-

2.2.1.4. In vitro fertilisation

After maturation all COCs were rinsed twice in working medium and once in equilibrated porcine fertilisation medium (PFM) then placed in 500 µl of PFM for 30 minutes. Frozen sperm was thawed, washed with prewarmed sperm diluent and centrifuged at 800G. Supernatant was discarded, and an identical washing step was repeated one more time. The resulting sperm pellet was dissolved in 500 µl of PFM and stored in the incubator until fertilisation. Post-thaw sperm quality was analysed regarding motility, morphology and sperm count. Sperm concentration was determined with a Neubauer improved counting chamber at a 1:50 dilution.

During IVF on average 7500 motile spermatozoa per oocyte were co-incubated with 50 cumulus oocyte complexes (COCs) for seven hours. The optimal sperm to oocyte ratio was determined for each boar individually. On average 375.000 live spermatozoa were added to

each IVF well with optimal individual numbers varying from 250.000 to 1.000.000 motile sperm cells per well. After IVF zygotes were denuded by gently pipetting them up and down in WM supplemented with hyaluronidase (1 mg/ml).

2.2.1.5. *In vitro* embryo culture

In vitro embryo culture (IVC) was carried out in a triple gas incubator (5% O_2, 5% CO_2, 90% N_2) set up at 38,5°C humidified atmosphere. Prior to culture all embryos were rinsed in working medium then in equilibrated culture medium to avoid contamination and transfer of media used in previous steps to the culture dish.

Zygotes and parthenogenetically activated embryos were cultured in 500 µl of PZM5 covered by mineral oil. Zona-free reconstructed embryos generated by handmade cloning were cultured in individual WOWs that were created with an aggregation needle in the culture dish. During the first half of this thesis all embryos were cultured in commercially available PZM 5 medium. To minimise variability between batches of IVC medium PZM3 was prepared in bulk, tested, aliquoted and frozen at -80°C. All further experiments were conducted using the same batch of culture medium previously proven to support embryonic development.

Table 21: Porcine zygote medium 3 (PZM3)

Component	Concentration
NaCl	108.00 mM
$NaHCO_3$	25.07 mM
KCl	10.00 mM
KH_2PO_4	0.35 mM
$MgSO_4$	0.40 mM
Ca-Lactate-$5H_2O$	2.00 mM
Na-pyruvate	0.2 mM
Myo-Inositol	2.78 mM
Phenol Red	0.27 mM
L-Glutamine	1.00 mM
Hypotaurine	5.00 mM
Gentamicin	0.04 g/L
BSA	3 g/L
NEAA100X	1% (v/v)

EAA50X	1% (v/v)

Adjust pH value to 7.2-7.4; osmolarity to 280 +/-8 mOsm, filter through 0.22 µm filter, freeze at -80°C.

2.2.1.6. Aceto-Orcein staining

Aceto-Orcein staining was conducted to determine optimal sperm to oocyte ratios during IVF experiments for each individual boar. Groups of five zygotes were denuded 10-18 hours after IVF (outlined in 5.1.2.4) and fixated on an object slide. The cover slip was glued to the object slide using fine stripes consisting of Vaseline, hair grease and hair wax (see Figure 15). The whole slide mounted with zygotes was submerged in methanol glacial acetic acid solution (3:1) for a minimum of 7 days at room temperature (RT).

Aceto-orcein staining solution was prepared by boiling 1g of orcein in 45ml of acetic acid followed by dilution with an equal amount of water. Zygotes were stained with this solution for 10 minutes then washed in a solution of glacial acetic acid, glycerol and MQ water (1:1:3). Analysis of cells was conducted under a phase contrast microscope. Zygotes were categorized as correctly fertilized if they showed two visible pronuclei. Oocytes without a visible pronucleus were classified as not fertilized while those with more than two pronuclei were graded as polyspermic. According to the results of this analysis sperm concentrations were adjusted for each individual boar to obtain the highest blastocyst development rate possible while minimising the rate of polyspermic fertilisation.

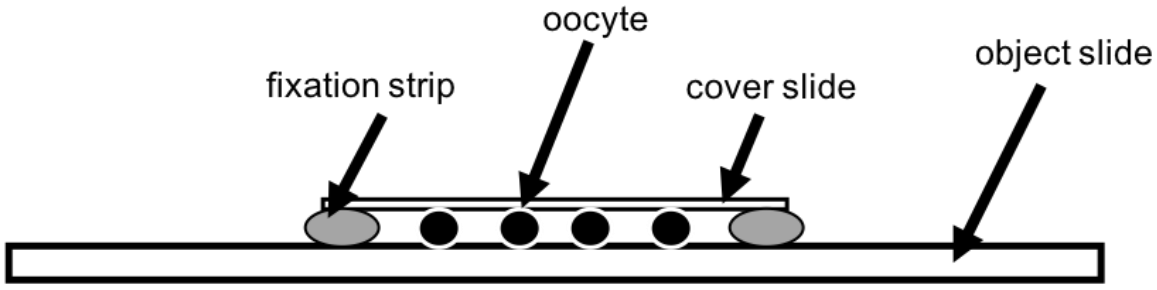


Figure 15: Fixation set-up for aceto-orcein staining of zygotes. Zygotes are positioned below a cover slip. Glue strips function as spacers to avoid squashing of the cells.

2.2.1.7. Microinjection

After IVF zygotes were visually examined and those extruding the first polar body were selected for microinjection. Approximately 20-30 zygotes at a time were transferred to a 4 µl droplet of working medium covered by mineral oil. Injection needles with a side filament were backfilled with gene targeting vectors dissolved in low tris EDTA buffer (10 mmol/L Tris-HCL, pH 7.6 and 0.25 mmol/L EDTA, pH 8.0) at a concentration of 5 ng/µl. Alternatively sgRNAs (prepared as outlined in 5.2.3.11) and Cas9 protein were delivered as RNA-protein-complexes (50ng/µl Cas9 protein, 100 ng/µl sgRNA). The components of the transposon system were delivered as PiggyBac transposon DNA vector (5ng/µl) plus PB Transposase mRNA (10ng/µl).

Zygotes, holding pipette and injection pipette were brought onto the same horizontal plane. Zygotes were fixed with the holding pipette positioning the polar body at the twelve or six o' clock orientation by carefully applying suction with a pulsed flow microinjector. The tip of the injection needle was opened by gently tapping it against the holding pipette (Vacutip). The injection pipette was gently inserted into the cytoplasm of each oocyte and approximately 10 pl of injection solution were delivered. Successful microinjection was visually confirmed by observing movement of the intracellular lipid droplets caused by the influx of injection solution. Injected zygotes were placed in the lower part of the droplet to separate them from non-injected ones (see Figure 16). Temperature was maintained at 38.5 °C during the whole microinjection procedure with a heating plate integrated into the microscope table.

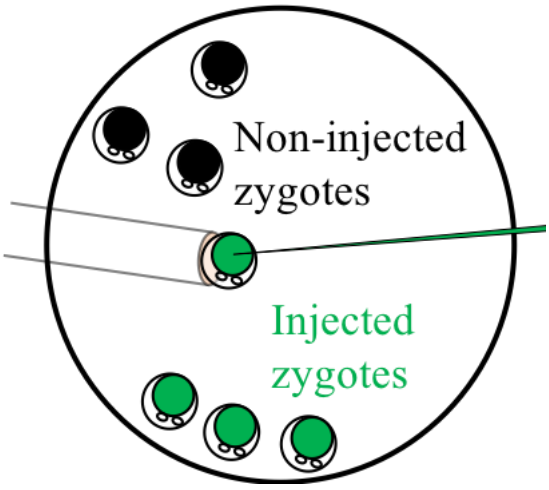


Figure 16: Micromanipulation drop. Non-injected zygotes were placed at the top of the drop. The injected ones were moved to the lower part of the drop to prevent them from mingling.

Injected zygotes were washed in equilibrated PZM then transferred to the triple gas incubator (5% O_2, 5% CO_2, 90% N_2, set up at 38,5°C humidified atmosphere). Groups of 50 zygotes were cultured in 500 µl of PZM covered by mineral oil. Zygotes destined for embryo transfer were cultured for 12-36 hours. Those that were subsequently used for DNA extraction to analyse of targeting efficiency were cultured to the blastocyst stage (six days).

2.2.1.8. Injection needle fabrication

Borosilicate glass needles suitable for microinjection were produced using a P-97 Flaming Brown micropipette puller with a through filament according to the manufacturers' instructions. A ramp test was performed to determine optimal melting temperatures for different strengths of borosilicate glass tubing. The diameter of holding pipettes was adjusted to 150 µm, an angle of 35° was given by hand with a blowtorch.

Table 22: Parameters for Flaming Brown micropipette puller

Parameter	Value
Heat	750
Pressure	500
Pull	72
Time	210
Velocity	42

2.2.1.9. DNA/RNA extraction from blastocysts

To analyse the efficiency of different targeting approaches DNA was extracted from blastocyst stage embryos using a protocol first described in [315]. Each individual embryo was washed twice in PBS then transferred to a PCR tube containing 10 µl of lysis buffer.

Table 23: Lysis buffer for DNA extraction

Component	Concentration
KCL	50 mM
$MgCl_2$	1.5 mM
Nonidet P-40	0.5% (w/v)
Proteinase K	100 µg/ml
Tris-Cl (pH 8.0)	10 mM
Tween-20	0.5% (v/v)

Incubation took place at 65°C for one hour followed by 95°C for ten minutes for inactivation of proteinase K. The lysate was then used as a template for PCR, agarose gel electrophoresis or DNA sequencing.

2.2.1.10. Parthenogenesis

For each experiment of in vitro embryo production, approximately 50 oocytes were parthenogenetically activated to provide a control group for IVF. Parthenogenesis followed by microinjection was also carried out to analyse targeting efficiency for new plasmids prior to using them for IVF. During the better part of this work parthenogenesis was carried out using chemical activation. Once the necessary equipment could be obtained it was replaced with electrical activation due to the higher efficiency of this method.

2.2.1.10.1. Chemical activation

Forty-five hours after starting maturation oocytes were denuded in WM supplemented with 1mg/ml hyaluronidase and rinsed twice in working medium. They were examined for evenly granulated cytoplasm and extrusion of the first polar body under a stereo microscope. Chemical activation was conducted by placing them in WM supplemented with 25 µm Ionomycin (calcium ionophore) for ten minutes. The oocytes were washed twice in WM and once in PZM5 then placed in the incubator in 500 µl of PZM5 supplemented with 5µg/ml of Cytochalasin for 3 hours. Afterwards they were rinsed twice in working medium and once in PZM 5. Subsequently they were cultured in vitro for 6 days to the blastocyst stage.

2.2.1.10.2. Electrical activation

For electrical activation oocytes were prepared as shown in 5.2.1.9.1. Then they were rinsed twice in activation medium and transferred to a fusion chamber (electrode distance 1.0mm) connected to a BTX electroporator. A single activating pulse (150V, 100µs) was applied then the identical procedure outlined for chemical activation was carried out.

Table 24: Activation medium

Component	Concentration
CaCl2	0.05 mM
H_2O	as necessary

Mannitol	280 mM
$MgSO_4$	0.1 mM
PVA	0.01 % w/v

Medium was sterile filtrated (22µm). PH was adjusted to 7.2-7.4 with NaOH. Osmolarity was adjusted to 300 Ω.

2.2.1.11. Synchronisation of recipients

Gilts aged six to seven months with a weight of 110-130kg were selected as recipients. Their oestrus cycle was synchronised by administering Altrenogest (Regumate ®) orally for 15 days followed by an injection of 750 IU ECG intramuscularly (i.m.) 24 hours after the last Altrenogest dispensation. Eighty hours after the administration of ECG, 750 IU of HCG was injected intramuscularly. Embry transfer was carried out one to two days after HCG administration.

2.2.1.12. Embryo transfer

Before surgery pigs were fasted for twelve hours. They were anaesthetized by intramuscular (i.m.) application of 5mg/kg bodyweight (BW) azaperone and 25mg/kg BW ketamine. Furthermore 0.4mg/kg BW of meloxicam and 15mg/kg BW were applied i.m. peri-operative. Recipients were elevated on a surgery table and fixed in a 30 ° head down position. The operating area was cleaned with warm water and soap, then disinfected with alcohol and iodine solution. A self-adhesive surgery drape was placed at the abdominal area and the skin incision was put at height of the second to last pair of teats at the Linea Alba. Fat and muscle tissue were separated bluntly, and one oviduct was placed on the surgical drape. Prior to the transfer of embryos, the correct ovulation state was controlled by visual assessment of preovulation follicles or fresh ovulation sites.

A sterile catheter was inserted as far as possible into the oviduct and 150-200 embryos were injected. The abdomen was closed in three layers using single stitches for peritoneum and muscle layers and running stitches for skin.

2.2.1.13. Flushing of *in vivo* zygotes

Flushing of *in vivo* zygotes was conducted for experiments requiring embryos with a specific genotype. Three to five prepubertal gilts were synchronized (outlined in 2.2.1.10) and artificially inseminated with sperm from GE boars 39 and 46 hours after HCG application. Seventeen hours after the second insemination donor gilts were euthanized. Their oviducts were flushed with warm PBS supplemented with 10% FCS and 1%Penicillin-Streptomycin.

2.2.1.14. Freezing of porcine semen

Sperm was kindly provided by Bayerngenetik GmbH and the Chair of molecular animal breeding and biotechnology (LMU). The sperm rich fraction of ejaculates from breeding boars was obtained using the gloved hand method. Sperm was diluted with Androstar® Plus sperm dilution medium and stored at 17°C overnight in a cooling centrifuge.

Sperm concentration was determined using a Neubauer counting chamber. Boar semen was centrifuged at 17°C, 800g for 20 minutes in 50ml centrifuge tubes. Supernatant was discarded and the sperm pellet was resuspended with Androstar® CryoPlus cooling extender prepared according to the manufacturer's instructions until 50% of the final intended volume was reached. Semen was placed in a cold room and slowly cooled for 1.5 hours. Temperature was monitored and upon reaching 5°C sperm concentration was adjusted to $1x10^9$ sperm cells/ml by adding the respective amount of Androstar® CryoPlus freezing extender necessary to reach the intended concentration.

Table 25: Androstar® CryoPlus cooling extender

Component	**Amount necessary for 970 ml**
Androstar® CryoPlus powder	84.9 g
H_20 bidistilled	770 ml
Pasteurized egg yolk	200 ml

Table 26: Composition of Androstar® CryoPlus freezing extender

Component	**Amount necessary for 500 ml**
Androstar® CryoPlus cooling extender	470 ml
Equex paste	5g
Glycerine	30 ml

A semiautomatic filling and sealing machine installed in a cold room at 4°C was used to fill the sperm into 0.5ml straws. All straws were sealed with small metal balls and handled with cold protection gloves to minimize temperature changes. The straws were transferred to a programmable freezing chamber (IceCube) and frozen by decreasing temperature at a rate of 30°C per minute. After completion of the freezing program all straws were transferred to a liquid nitrogen container for long term preservation.

Table 27: Boar semen freezing curve (adapted from IceCube user manual).

Step #	Temperature °C	Time elapsed (min)	Temperature change (°C)	Time required (min)	Temp decrease (°C/min)
1	4	0	-	-	-
2	1	1.5	-3	1.5	-2
3	-25	2.4	-26	0.9	-30
4	-140	6.2	-115	3.8	-30
5	-140	21.2	0	15	0

2.2.1.15. Handmade cloning

After maturation oocytes were denuded, examined for extrusion of the first polar body and moved to T2 (hepes-buffered M199, 2% FCS) drops in a bisection dish (60mm petri dish) while discarding dead and damaged oocytes. ZP digest was conducted by placing oocytes in pronase drops (3.3mg/ml) until deformation of the ZP could be observed. Then they were rinsed in a drop of T20 (hepes-buffered M199, 20% FCS) to deactivate the enzyme. Twenty oocytes at a time were transferred to a cytochalasin B drop (25µg/ml) aligning their polar bodies at the 12 o' clock position. Enucleation was performed by removing about one third of the ooplasm located around the polar body with a handheld microblade (see Figure 17). The resulting enucleated cytoplasts were washed in T2 and stored in fresh T2 drops until further processing.

Successful enucleation was confirmed by placing a sample of cytoplasts in Hoechst staining solution (10 µg/ml) for 10 minutes. All karyoplasts and incompletely enucleated cytoplasts were discarded.

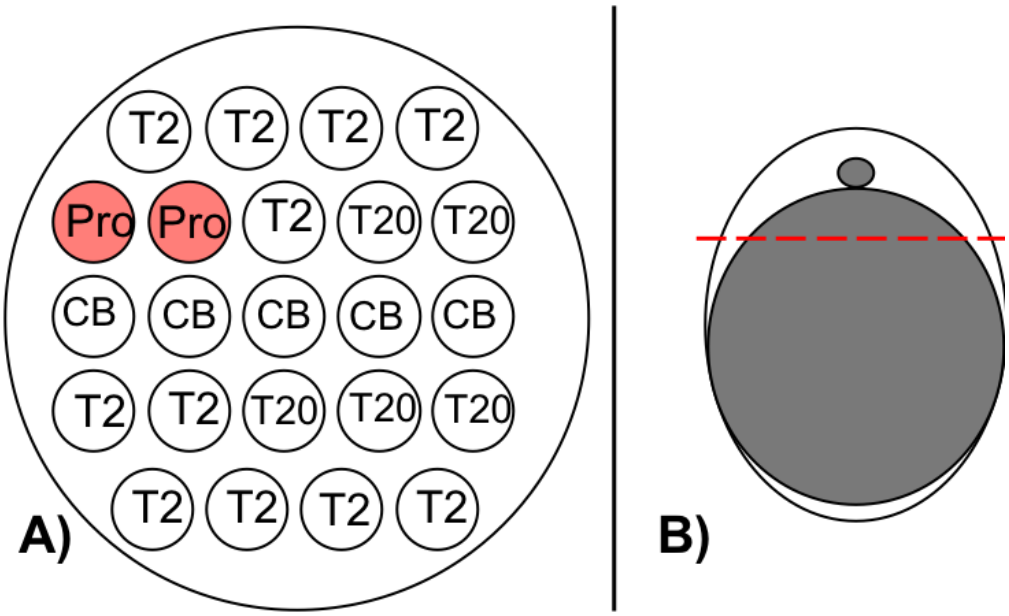


Figure 17: Enucleation of oocytes during handmade cloning. **A)** Bisection dish prepared for the enucleation of oocytes during HMC (20µl droplets in 60mm petri dish). CB = cytochalasin B in T2; Pro = pronase. **B)** The red line indicates where the bisection cut should be set thereby removing about one third of the ooplasm located around the polar body (adapted from Li et al. [316]).

Genetically modified donor cells were harvested with accutase and resuspended with 500µl T10 (hepes-buffered M199, 10% FCS). Half of the cytoplasts were transferred to a cell fusion dish and placed in T10 droplets. Five of them at a time were placed in PHA drops (phytohaemagglutinin 0.4mg/ml) to make their surface adhesive and one donor cell was attached to each cytoplast.

Five cytoplast-cell-complexes (CCCs) at a time were washed in porcine fusion medium, moved to the fusion chamber and aligned. Fusion was conducted by applying a single DC (direct current) pulse (100V, 9µs) to each CCC individually. CCCs were examined for successful fusion then covered and left on a warm heating plate for one hour to allow for reprogramming. Porcine fusion medium was then replaced with cow fusion medium (cFM) and the remaining half of complemental cytoplasts was moved to a T10 drop in the fusion dish (see Figure 18). Ten CCCs and their corresponding cytoplasts were moved to the fusion chamber, aligned and fused with a single DC pulse (85V, 80µs). The calcium ions present in cFM facilitate electrical activation in the same step.

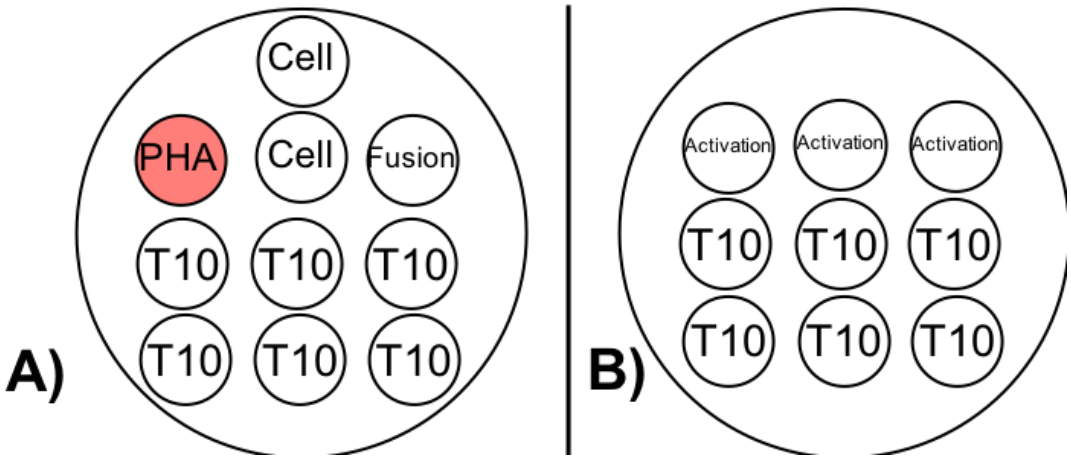


Figure 18: Fusion and activation of oocytes during handmade cloning. **A)** Cell fusion dish used in HMC (20µl droplets in 35mm petri dish). PHA = Phytohemagglutinin; Cell drops=T2 medium; Fusion = porcine fusion medium, later replaced with cow fusion medium for fusion of CCCs with cytoplasts. **B)** Cell activation dish used in HMC (20µl droplets in 35mm petri dish). Activation = activation medium (adapted from Li et al. [316]).

Reconstructed embryos were placed in the incubator for 4 hours in PZM5 supplemented with 5µg/ml Cytochalasin B and 10µg/ml Cycloheximide whereas defect ones were discarded. Then they were rinsed twice in culture medium and placed in individual WOWs. They were cultured for 6 days (38.5°C, 5% O_2, 5% CO_2, 90% N_2 in humidified atmosphere) and blastocyst formation was assessed.

Table 28: Composition of porcine fusion medium

Component	Concentration
H_2O	as necessary
Mannitol	3M
$MgSO_4$	0.1mM
Polyvinyl alcohol	0.1% w/v

Adjust pH to 7.4-8.8 with 0.5M Triz-base, adjust osmolarity to 280 Ω, sterile filter (22µm).

Table 29: Composition of activation medium (cFM)

Component	Concentration
$CaCl_2$	0.05mM
H_2O	as necessary
Mannitol	3M

$MgSO_4$	99.6nM
Polyvinyl alcohol	0.1% w/v

Adjust pH to 7.4-8.8 with 0.5M Triz-base, adjust osmolarity to 280 Ω, sterile filter (22μm).

2.2.2. Microbiology

2.2.2.1. Cultivation of bacteria

Bacteria were cultivated overnight at 37°C on agar plates or in LB-medium supplemented with 100 µg/ml ampicillin on an orbital shaker at 220 rpm.

2.2.2.2. Transformation of bacteria

Plasmid DNA was introduced into E. coli ElectroMAX DH10B bacteria by electroporation. 50 µl of bacteria were thawed on ice, mixed with 1-5 µl of ligation reaction and moved to a 2 mm electroporation cuvette. A single pulse (2500V, 5ms) was applied then bacteria were cultivated for 30 minutes in LB medium. Afterwards they were plated on LB plates supplemented with antibiotics selecting for the corresponding plasmid and incubated at 37°C overnight.

2.2.2.3. Cryopreservation of bacteria

To conserve plasmid bearing bacteria 0.5ml of overnight culture was mixed with an equal amount of 99% glycerol and stored at -80°C.

2.2.2.4. Isolation of plasmid DNA

Plasmid bearing bacteria from a glycerol stock were cultured overnight in 100ml of LB-medium as outlined in 5.2.2.1. Then the NucleoBond Xtra Midi Kit was carried out according to the manufacturer`s instructions. The resulting pellet was dissolved in low Tris EDTA buffer and used for microinjection.

2.2.3. Molecular biology

2.2.3.1. Measurement of DNA and RNA concentration

DNA and RNA concentrations were measured using the NanoDrop® Lite spectrophotometer according to the manufacturer‘s instructions.

2.2.3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify specific sequences from genomic DNA and plasmid DNA. Screening PCRs and the amplification of shorter sequences was carried out using GoTaq® Polymerase. When proofreading was required, or DNA was extracted from blastocysts yielding only low concentrations Q5® polymerase was used.

Table 30: PCR conditions for GoTaq® G2 and Q5 polymerase

GoTaq® G2 Polymerase

PCR mixture		**Cycling conditions**			
Component	**Concentration**	**Step**	**Temperature**	**Duration**	**Cycles**
DNA	10-250 ng	Initial Denaturation	98°C	2 min	1
5x buffer	1x	Denaturation	98°C	30 sec	35-40
dNTPs	200 µm each	Annealing	58-62°C	30 sec	
Primer F	0.2 µM	Extension	72°C	1 min/kb	1
Primer R	0.2 µM	Final extension	72°C	5 min	
Polymerase	0.03 U/µl	Storage	8°C	∞	
H20	Up to 25 µl				

Q5® Polymerase

PCR mix		**Cycling conditions**			
Component	**Concentration**	**Step**	**Temperature**	**Duration**	**Cycles**
DNA	1 pg-1 µg	Initial Denaturation	98°C	30 sec	1

5x buffer	1x	Denaturation	98°C	10 sec	35-40
dNTPs	200 µm each	Annealing	58-62°C	30 sec	
Primer F	0.5 µM	Extension	72°C	30 sec/kb	1
Primer R	0.5 µM	Final extension	72°C	2 min	
Polymerase	0.02 U/µl	Storage	8°C	∞	
H20	Up to 25 µl				

2.2.3.3. Colony PCR

After transformation E. coli colonies were screened for the intended plasmid via colony PCR. DNA templates for this PCR were generated by placing single bacterial clones from a LB plate in 30 µl TTE buffer and incubating this mix at 95°C for 5 min. Colony PCR was conducted using 2 µl of DNA solution with one primer designed to bind to the plasmid backbone and the second one binding to the plasmid insert.

2.2.3.4. Agarose gel electrophoresis

DNA fragments were analysed by agarose gel electrophoresis. DNA samples were loaded on gels prepared from 1xTBE or 1xTAE buffer and 1-2% agarose supplemented with 4 µl PeqGreen. DNA fragments were separated by size by applying 80-120V until adequate separation could be achieved (usually 1-5 hours). Subsequent analysis of DNA fragments was conducted under UV light (254-366nm) with the Quantum ST5 gel documentation system.

2.2.3.5. Restriction digest

Restriction digests were conducted to generate linearized plasmids for cloning and transfection or to confirm the correct length of plasmids.

Table 31: Conditions for restriction digest

Component	Amount
DNA	Linearization digest: 10-15 µg Analytical digest: 2-3 µg

10x NEB Buffer	5 µl
Digestive enzyme	3 U/ µg
H_20	up to 50 µl

Restriction digests were carried out at the optimal temperature for each respective enzyme according to the manufacturer's instructions. The solution was co-incubated with 2 µl of calf intestinal alkaline phosphatase at 37°C for at 30 minutes. This prevents re-ligation by stripping the vector backbone of its 5' phosphates.

2.2.3.6. Ligation

Ligation of vector backbones with DNA fragments was performed using T4 Ligase according to the manufacturer's protocol. Components of ligation mix were co-incubated for 2 hours at RT then left at 4°C overnight.

2.2.3.7. Blunting

When blunt ends were required for cloning, they were generated using DNA Polymerase I Large (Klenow) Fragment to remove 3' overhangs and fill in 5' overhangs. The reaction was carried out according to the manufacturer's protocol and dNTPs were supplemented to inhibit the polymerase's 3'-5' exonuclease activity.

Table 32: Conditions for blunting of DNA-fragments

Component	**Amount**
DNA	5 µg
10x NEBuffer	5 µl
dNTPs (2mM)	1.5 µl
Klenow enzyme	1U/ µg DNA
H_2O	up to 50 µl

All components were co-incubated for 15 minutes at 25 °C followed by the addition of 10 mM EDTA and an increase in temperature to 75 °C for 20 minutes to stop the reaction.

2.2.3.8. Isolation of DNA from agarose gels

After the separation of DNA fragments by gel electrophoresis, DNA bands were visualized under UV light and cut out with a scalpel blade. Purification of DNA was carried out by applying the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions.

2.2.3.9. Annealing of oligonucleotides

A dilution of 10ng/µl in TE buffer was prepared from two complementary single-stranded oligonucleotides. This solution was incubated at 100 °C for five minutes then left to cool to RT to facilitate double-strand formation.

2.2.3.10. Production of CRISPR/Cas9 vectors

Suitable guides with low predicted off-target binding were identified using an online CRISPR design tool (crispor.tefor.net). Oligonucleotides with the target guide sequence preceded by a single G necessary for U6 promoter transcription and overhangs corresponding to the BbsI digestion site were purchased from Eurofins Genomics (Ebersberg, GER). Hybridization of single-stranded oligonucleotides was conducted (outlined in 5.2.3.9) followed by ligation into the backbone of the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (see Figure 19). This vector was used for the transformation of bacterial cells (outlined in 5.2.2.2) and plasmid DNA was extracted (outlined in 5.2.2.4). In this work such vectors coding for gRNA and Cas9 protein at once were primarily used for microinjection.

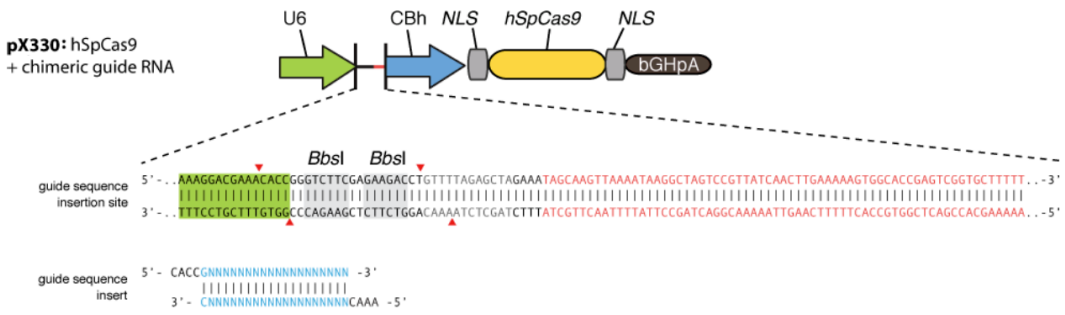


Figure 19: pX330-U6-Chimeric_BB-CBh-hSpCas9 vector. ▬ = U6 promoter; ▲= BbsI digestion sites; red sequence = tracr RNA; grey sequence = crRNA (adapted from Addgene [317]).

2.2.3.11. Generation of sgRNAs

Generation of sgRNAs by *in vitro* transcription of DNA templates was performed using the MEGAshortscript T7 kit according to the manufacturer's instructions. Polyadenylation and subsequent purification of the RNA transcript was conducted using the poly-A tailing kit and the MEGAclear kit as instructed by the manufacturer. In this work such sgRNAs were primarily used to generate RNA-protein (RNP) -complexes with Cas9 protein for microinjection.

2.2.3.12. Phenol-chloroform extraction

DNA extraction from mammalian tissue and from sperm was carried out via phenol-chloroform extraction. About 1g of tissue or sperm pellet was incubated in 1ml of lysis buffer overnight at 55° C.

Table 33: Lysis buffer for phenol-chloroform extraction

Component	**Concentration**
Tris-HCL	83 mM
SDS (sodium dodecyl sulphate)	0.8%
EDTA	0.2 M
NaCL	0.2 M
Proteinase K	100 µg/ml
H_20	-

Then an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) was incubated with the solution (10 minutes at RT) followed by centrifugation (10 minutes at 13.000g). The resulting supernatant was mixed with an equal volume of chloroform (99%) and centrifuged (10min, 17.000g). Supplementation of 10% v/v sodium acetate (5M) and 0.7% v/v isopropanol followed by thorough shaking resulted in DNA precipitation. Centrifugation (5 min, 13.000g) and rinsing of the pellet with 70% ethanol was followed by an identical centrifugation step. Finally, the resulting DNA pellet was air-dried and dissolved in TE buffer.

Purification of DNA fragments for *in vitro* transcription was conducted using a modified variation of phenol-chloroform extraction. Hereby the DNA containing supernatant was incubated (2 hours, -20°C) with 1/10 volume 5 M sodium acetate and two volumes of ethanol

(100%) after the first centrifugation step. The rest of the procedure was carried out as described above.

2.2.3.13. Sanger sequencing

DNA fragments were prepared for sequencing using the Mix2Seq kit according to the manufacturer's instructions. DNA sequencing was carried out by MWG Eurofins (Ebersberg, GER).

2.2.3.14. Evaluation of editing efficiency

The efficiency of genome engineering was assessed by quantifying the frequency of insertions and deletions (indels) in embryos, single cell clones or cell pools. After transfection or microinjection with CRISPR/Cas9 vectors DNA was extracted and used for PCR followed by sequencing. Monoallelic and biallelic frequency of indels was calculated by determining the ratio of edited cells in proportion to the total number of cells.

When analysing blastocysts or single cell clones sequencing data was analysed individually whereas the frequency of mutations in cell pools was assessed by "Tracking of Indels by Decomposition" (TIDE) analysis. This online tool (available at https://tide.deskgen.com/) facilitates determination of indel frequency and spectrum within a cell pool from sequencing data. Reliability of this data was confirmed by R_2 values above 0.9 indicating low negative interference caused by large deletions and sequencing noise.

2.2.4. Tissue culture

2.2.4.1. Cell isolation

Kidneys for the isolation of porcine kidney fibroblasts (PKDNFs) were obtained from a local abattoir or from pigs accommodated at the TUM experimental facility. Pieces of roughly 1 cm^3 were cut out, rinsed 3 times in ethanol (80%) and PBS respectively then minced and digested with collagenase (10 mg/ml) for 30 minutes at 37°C. After adding medium PKDNFs were centrifuged at 300x g for 5 minutes and distributed to three T-150 flasks. During the first week culture medium was supplemented with penicillin-streptomycin as well as amphotericin B and changed daily.

Porcine foetal fibroblasts were isolated upon ultrasonographic confirmation of pregnancy by euthanising the sow and extracting the foetuses from the uterus. Following removal of head and limbs about 1g of the remaining tissue was dissociated using the GentleMACS™ and the "Tissue Dissociation Kit 1" according to the manufacturer's instructions. All following steps were conducted as outlined above.

All tissue culture work was carried out in a class II laminar flow hood using sterilized materials.

2.2.4.2. Cell cultivation

Cells were cultivated in an incubator at 37°C, 5% CO_2 in humidified atmosphere. PKDNF and porcine foetal fibroblast culture was conducted in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1mM sodium pyruvate, 2mM Ala-Gln, 1x MEM non-essential amino acid solution (NEAA) and 10% FCS. Medium was changed every other day and cells were passaged upon reaching 80-90% confluency.

2.2.4.3. Freezing and thawing of cells

Cells were detached from the cell culture vessel using accutase and pelleted through centrifugation at 300x g for 5 minutes. The cell pellet was resuspended in 1 ml freezing medium consisting of 40% DMEM, 50% FCS and 10 % dimethyl sulfoxide (DMSO). This cell solution was pipetted into cryo-tubes, placed in Mr. Frosty® freezing containers and frozen at -80 °C. If cells had to be stored for long periods of time they were placed in liquid nitrogen.

Cells were thawed in a water bath at 37 °C until the medium became liquid again. The cell suspension was immediately diluted with 5 ml prewarmed medium and pelleted through centrifugation at 300x g for 5 minutes. The pellet was resuspended in prewarmed culture medium and transferred to the incubator for cultivation.

2.2.4.4. Counting of cells

Counting of cells was carried out using the Countess™ automated cell counter according to the manufacturer's instructions.

2.2.4.5. Transfection of cells

Transfection of PKDNF cells with DNA was carried out by lipofection or electroporation.

2.2.4.5.1. Lipofection

Prior to lipofection cells were cultivated to 50-70% confluency. The following day cells were rinsed with PBS and cultivated in 4 ml of Opti-MEM® in 10 cm cell culture dishes. Five µg DNA was dissolved in Opti-MEM® to a total volume of 300 µl and 6 µl Lipofectamine 2000 mixed with 294 µl Opti-MEM®. After 5 minutes of incubation at room temperature the Lipofectamine mix was gently added to the DNA solution and co-incubated for 25 minutes at room temperature. This compound solution was then trickled on the cells. After 6 hours of cultivation 8 ml of medium was added to the cell culture dish followed by overnight cultivation and a medium change the next day.

2.2.4.5.2. Electroporation

For electroporation cells were detached with accutase, counted and pelleted through centrifugation at 300x g for 5 minutes. Then $1x10^6$ cells were transferred to 400 µl hypo-osmolar buffer containing 5 µg of linearized plasmid DNA and transferred to an electroporation cuvette with a diameter of 4 mm. After five minutes of incubation at room temperature one pulse of 1200V was applied for 85µs. Following another five minutes of incubation at room temperature the cell suspension was transferred to a 10 cm dish with fresh medium. Medium was changed the following day.

2.2.4.6. Selection and isolation of single cell clones

Forty-eight hours after transfection cells were rinsed with PBS and selection medium supplemented with the appropriate antibiotic for the resistance cassette of the plasmid. During this project selection was carried out using Geneticin (G418), Puromycin and Hygromycin. Optimal concentrations for each antibiotic agent were determined in killing curve experiments. When single cell clones became visible, they were marked and picked using silicon grease and cloning rings. Each single cell clone was detached by gently pipetting accutase into the cloning ring. The resulting cell suspension was then transferred to 6-well plates for further expansion.

2.2.4.7. Isolation of genomic DNA

DNA was isolated from mammalian cells using QuickExtract DNA extraction solution. Cells were detached with accutase, pelleted and resuspended in 30 µl QuickExtract DNA extraction solution. This solution was incubated at 68 °C for 15 minutes followed by 8 minutes at 98 °C. If DNA of higher purity was required the SurePrep DNA purification kit was used according to the manufacturer's instructions.

2.2.4.8. Preparation of cells for handmade cloning

For handmade cloning cells were cultivated to 100% confluency and harvested with 0.05% trypsin. Then they were pelleted through centrifugation at 300x g for five minutes and resuspended in 500 µl M199 supplemented with 10 % FCS.

3. RESULTS

The goal of this thesis was to establish and improve methods for genome engineering in porcine embryos.

For this purpose, systems for *in vitro* production (IVP) of porcine embryos comprising *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC) were established. Techniques for the cryopreservation of boar sperm were optimised, used to freeze semen isolates suitable for IVF and build a sperm bank for genetically modified pig lines. Flushing of *in vivo* zygotes was standardised (addressed in 3.1).

CRISPR/Cas9 mediated genome engineering was performed directly in early stage embryos. Targeting vectors for the inactivation of the porcine NANOS2 gene were created. A variety of gRNAs with minimal predicted off-target effects were evaluated for their genome engineering efficiency. Those vectors and several others provided by colleagues were delivered to porcine zygotes by microinjection and assessed for embryotoxicity and editing efficiency. A transposon system was employed and the efficiency of this approach for transgenesis was evaluated. (outlined in 3.2).

Promising constructs were used to generate genetically modified embryos. Surgical embryo transfer was established and fourteen genetically modified pigs with four distinct genetic modifications were obtained (explained in 3.3).

Finally, handmade cloning was implemented to facilitate more complex genome alterations that require homology directed repair. These are inefficient and somatic cell nuclear transfer allows pre-screening for the desired modification in cell culture, which is not possible when performing the direct manipulation of porcine zygotes. Reconstructed embryos with the desired genotype were generated and cultured to the blastocyst stage (described in 3.4).

3.1. *In vitro* embryo production

The Chair of Livestock Biotechnology specialises in creating porcine disease models for a variety of human conditions and diseases. Reliable supply of *in vivo* derived porcine embryos in sufficient quantity for this purpose would entail immense financial costs and sacrifice of many donor animals. State of the art systems for *in vitro* production (IVP) of porcine embryos comprising *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC) were established during this work (see Figure 20). The optimised procedures for the IVP of porcine embryos described in detail in section 2.2.1. are the most important result of this thesis.

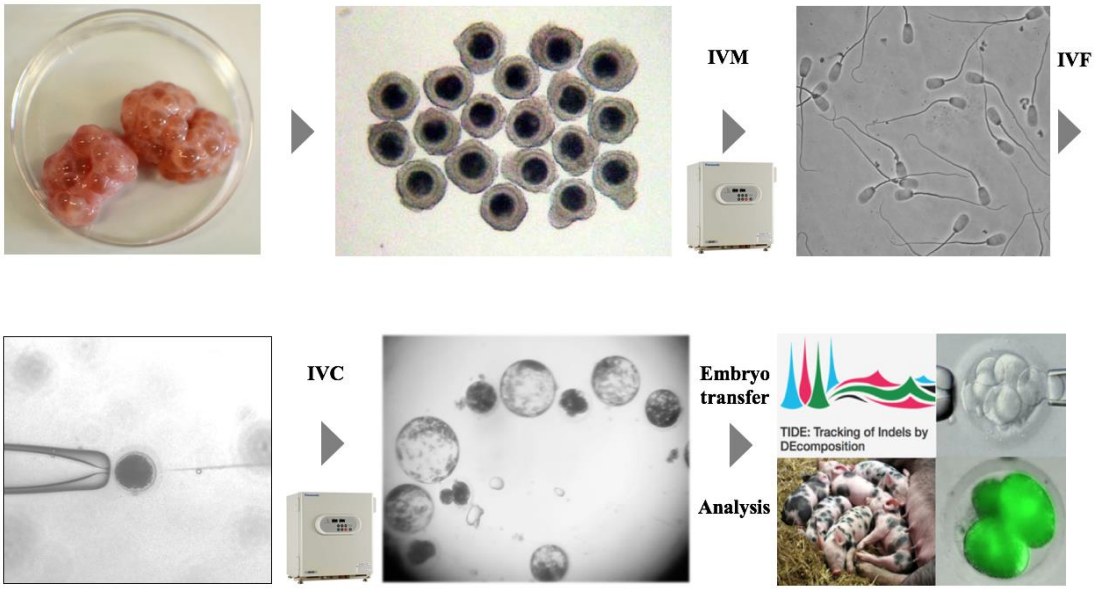


Figure 20: In vitro embryo production. Oocytes were isolated from porcine ovaries sourced from a local slaughterhouse. These were matured *in vitro* and served as cytoplast donors for handmade cloning or were in *vitro* fertilised followed by microinjection, *in vitro* culture and embryo transfer.

3.1.1. *In vitro* maturation

An efficient *in vitro* maturation system is the foundation of *in vitro* embryo production. IVF and handmade cloning require large quantities of mature oocytes, therefore both methods benefit from improved IVM outcomes. Porcine oocytes are highly sensitive regarding temperature, osmolarity, oxygen tension and medium composition. The objective was to optimise IVM conditions and thereby improve the quantity and quality of IVM oocytes measured by polar body extrusion, cleavage rate, blastocyst development and embryonic cell count.

NCSU23 based maturation medium was compared to chemically defined cytokine-enhanced maturation medium based on TCM199 (FLI-medium). FLI-medium attempts to approximately mimic the composition of porcine oviductal fluid through supplementation with FGF, LIF and IGF to promote more synchronous cytoplasmic and nuclear maturation. The composition of both media is outlined in table 20. An equal number (100-150) of high-quality cumulus oocyte complexes (COCs) with homogenous, dark, evenly granulated cytoplasm covered by multiple compact layers of cumulus cells (see Figure 21) was matured in each medium for 45 hours under otherwise identical conditions (outlined in 2.2.1.3).

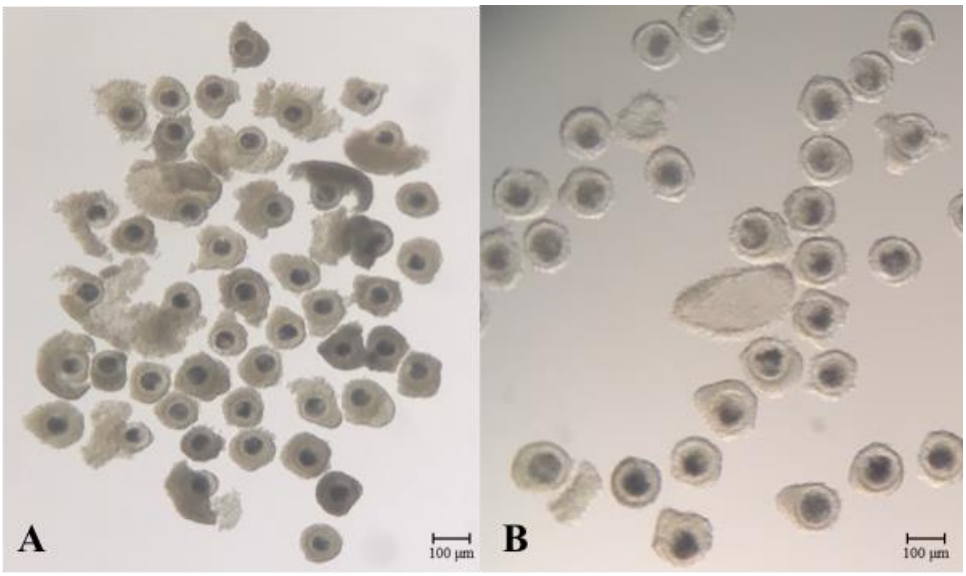


Figure 21: Cumulus oocyte complexes selected for IVM. A) High-quality COCs with homogenous, dark, evenly granulated cytoplasm covered by multiple compact layers of cumulus cells. B) Low-quality COCs sparsely covered by cumulus cells.

Maturation rate was analysed by visually determining the percentage of oocytes showing extrusion of the first polar body and cleavage rate (see Figure 22).

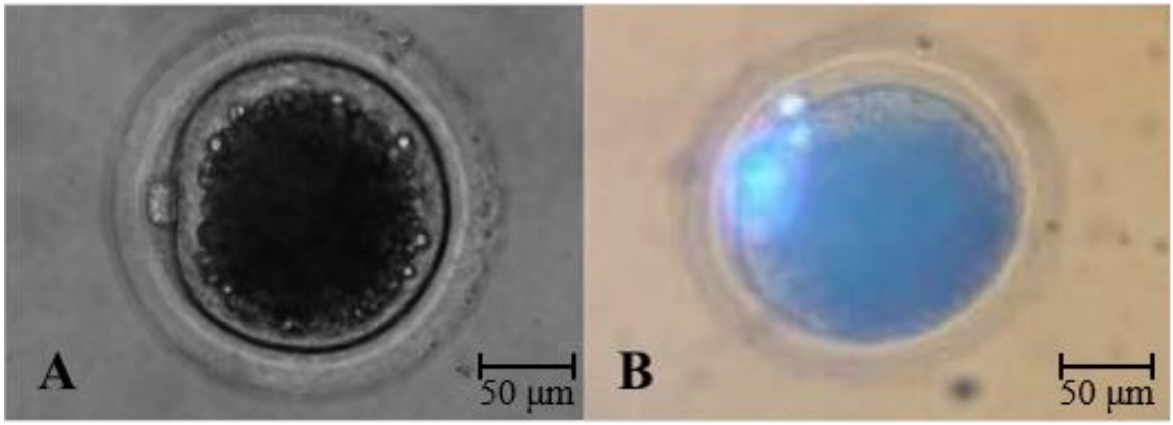


Figure 22: Extrusion of the first polar body. **A)** Porcine oocyte with polar body located at the 9 o' clock position. **B)** Hoechst staining of porcine oocyte with the polar body located at the 11 o' clock position and nucleus slightly below.

Oocytes matured in both media were parthenogenetically activated to induce embryonic development without fertilisation. This approach was chosen to exclude variability caused by sperm quality and IVF parameters. All embryos were cultured under identical conditions to compare their developmental competence. Embryonic cleavage and blastocyst development rates were evaluated. Five rounds of IVM were carried out with each maturation medium (see Table 35).

Table 34: Comparison of NCSU23 medium and chemically defined FLI-medium

Maturation medium	**Total (n)**	**Maturation (%)**	**Dead (%)**
NCSU 23	1473	1042 (70.7%)	172 (11.68%)
TCM 199 (FLI)	1296	1063 (82.02%)	130 (10.03%)
Parthenogenesis	**Total (n)**	**Cleavage (%)**	**Blastocyst (%)**
NCSU 23	1042	719 (69.00%)	**217 (20.83%)**
TCM 199 (FLI)	1063	753 (70.84%)	**378 (35.56%)**

Hoechst-staining was performed to determine the embryonic cell number on day 6 which is a widely used indicator of blastocyst quality (shown in Figure 23). *In vivo* derived blastocysts reach a cell number of approximately 100 cells at this point of embryonic development [318]. The average number of cells in blastocysts matured in NCSU 23 medium was 59.5±8.2 and 76.2±7.9 in FLI medium. Due to this data cytokine enhanced FLI maturation medium was used in all further IVM experiments consistently resulting in average maturation rates of 80-85%.

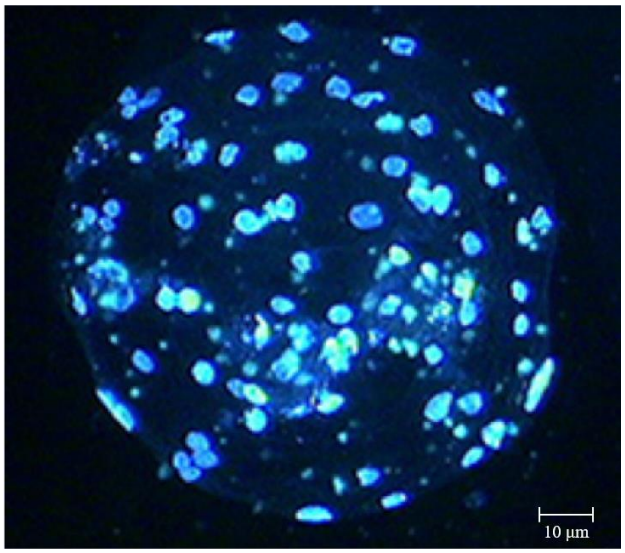


Figure 23: Parthenogenetically generated blastocyst stained with Hoechst.

Parthenogenesis was further used to analyse the editing efficiency and cytotoxicity for a variety of target genes. The efficiency of chemical and electrical parthenogenetic activation of porcine embryos (outlined in 2.2.1.9.2) was compared over ten experiments. The average blastocyst rate resulting from electrical activation was 50% (see Figure 24) compared to 36% after chemical activation. Thus, electrical activation was used for all further parthenogenesis experiments.

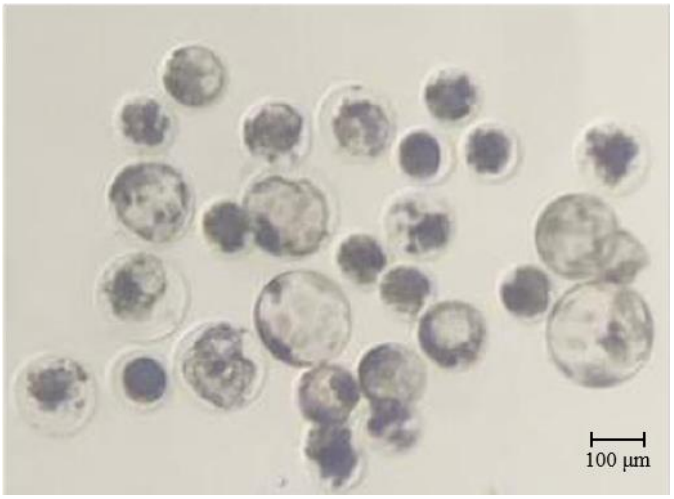


Figure 24: Blastocyst development rate of 50% after electrical parthenogenesis.

3.1.2. Cryopreservation of porcine sperm

High-quality frozen sperm facilitates consistent IVF outcomes over multiple experiments by eliminating inter-ejaculate variability. The high sensitivity of porcine spermatozoa to oxidative stress, temperature fluctuations, osmolarity and pH-value however makes their cryopreservation challenging [231]. The objective was to optimise the freezing of porcine sperm and compare the post-thaw survival rate of in-house sperm from GE boars and commercial wildtype sperm.

During this project a system for the cryopreservation and storage of boar sperm was established. Utilising the protocol outlined in 2.2.1.13 ejaculates from five breeding boars and ten GE pig lines were frozen and a sperm bank was established (see Table 39).

Table 35: Sperm bank. Semen from GE pig lines is numerically labelled, samples from GE boars with the respective names.

Boar:	**Genotype:**	**Motility:**
#123, #710	KRAS $^{G12D/WT}$	30%, 40%
#227	APC $^{1311/WT}$, KRAS $^{G12D/WT}$	50 %
#278, #3	TP 53 $^{R167H/R167H}$	60%, 40%
#598	APC $^{1311/WT}$	60%
#662, #750, #908	humanised IgH and IgK	40%, 40%, 50%
#1530	TNF $^{\Delta ARE/WT}$	30%
#760	R26-mT, reporter	10%
#869	humanised CD46, CD55, CD59, HO1, A20; GGTA1 $^{+/-}$	50%
#87	GGTA1$^{-/-}$, CMAH$^{-/-}$, B4GNT2$^{-/-}$	40%
#10261 (2x)	GGTA1$^{-/-}$, CMAH$^{-/-}$, B4GNT2$^{-/-}$, B2M$^{-/-}$	40%, 50%
Fadros	wildtype	40%
Fossil	wildtype	30%
Uteus	wildtype	40%

Wadtbandt	wildtype	40%
Wal	wildtype	30%

Cryopreservation decreased past-thaw sperm motility on average by 30% ranging from 25-50%. The average post-thaw motility rate between all samples was 42%. Similar post-thaw survival rates of at least 30% could be obtained for semen from wildtype boars and semen from GE boars except boar #760. Only ejaculates of subpar quality could be obtained from this subfertile boar expressing red fluorescent protein. Three sows were artificially inseminated with cryopreserved sperm from boar #10261 resulting in three pregnancies.

3.1.3. *In vitro* fertilisation

The inefficiency of IVF due to polyspermy and insufficient male pronucleus formation is the biggest hurdle for the IVP of porcine embryos [149]. The objective was to reduce the rate of polyspermic fertilisation in porcine IVF and make the generation of GE pigs more efficient. First, a working IVF system was established using proven sperm. Then semen isolates from breeding boars were assessed for IVF suitability and the sperm to oocyte ratio was individually optimised.

3.1.3.1. Establishment of a working IVF system

To supply the necessary number of embryos required to produce GE pigs by microinjection an efficient IVF system needed to be established as part of this project. For this purpose, boar semen repeatedly proven to generate blastocysts in IVF was kindly provided by Dr. Mayuko Kurome (Chair for Molecular Animal Breeding and Biotechnology, LMU). Five IVF experiments were conducted (as outlined in 2.2.1.4) adding $1x10^6$ spermatozoa to each IVF well. Average blastocyst formation rates of 21.3% could be obtained (see Table 36 and Figure 25).

Table 36: Blastocyst development rates using sperm provided by Dr. Mayuko Kurome.

Oocytes (n)	Blastocysts (%)
195	52 (26.7%)
243	66 (27.1%)
198	48 (24.2%)
207	24 (11.6%)
189	30 (15.9%)
Total: 1032	Total: **220 (21.3%)**

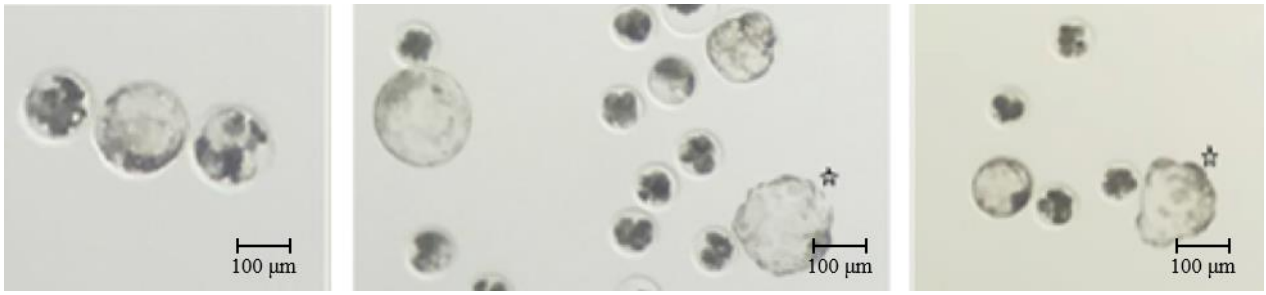


Figure 25: Porcine Blastocysts produced by IVF; hatching blastocysts are marked with an asterisk.

3.1.3.2. Identification of suitable sperm isolates for IVF

Under 3.1.3.1. the basic protocol for IVF was established. The next step was to identify eligible sperm donors because only semen from a minority of boars is suitable for IVF after cryopreservation [151]. The goal was to identify such sperm isolates and assess *in vitro* blastocyst development. High quality ejaculates from eighteen breeding boars and three GE boars present at the animal facility were collected. Eight samples were frozen in house, thirteen could be obtained frozen. At least three rounds of IVF were carried out for sperm from each individual boar. In total 21 different sperm isolates were analysed for their IVF suitability (shown in Table 37).

Motility rates after thawing were vastly different for all sperm samples. The initial sperm to oocyte ratio was set at 7500 motile spermatozoa per oocyte to make results more comparable. This was known to be a reasonable baseline from previous experiments.

Table 37: IVF suitability of 21 different boars. The first three numerically labelled sperm isolates are from boars present at the TUM facility. The 18 animals identified by their names originate from a breeding company.

Boar	Total oocytes (n)	Total Blastocysts (%)
260	390	42 (11%)
420	300	14 (5%)
869	395	3 (1%)
Cadura	250	41 (16%)
Cor	320	36 (11%)
Fadros	700	**167 (24%)**
Fossil	300	6 (2%)
Icerico	250	1 (0%)
Igelspitz	250	7 (3%)
Madura	250	2 (1%)
Maswald	200	1 (0%)
Mozzi	250	8 (3%)
Orlaki	500	40 (8%)
Pablura	300	26 (9%)
Ryder	250	1 (0%)
Uteus	300	27 (9%)

Wadtbandt	300	22 (7%)
Wadtlise	300	6 (2%)
Wadtpill	300	12 (4%)
Wadttext	300	9 (3%)
Wal	200	4 (2%)

With a total number of 167 blastocysts produced from 700 oocytes (24%) Fadros showed the highest performance in IVF. Consequently, semen from this boar was used for all further experiments. The quality of Fadros' sperm could later be confirmed by the generation of twenty-nine healthy piglets through IVF (outlined in 3.3).

3.1.3.3. Optimisation of sperm to oocyte ratio

A high sperm to oocyte ratio increases the fertilisation rate in IVF but also raises the degree of polyspermy [216]. The objective was to optimise the sperm to oocyte ratio to avoid polyspermy while maintaining high fertilisation rates and blastocyst development.

Several IVF experiments using three different sperm donors and different sperm concentrations were conducted for this purpose (see Table 38). Blastocyst development occurs at a normal rate in polyspermic embryos which makes it an unsuitable parameter for measuring monospermic fertilisation [245]. The rate of polyspermic fertilisation was therefore evaluated by performing aceto-orcein staining (outlined in 2.2.1.5). An example is shown in Figure 26.

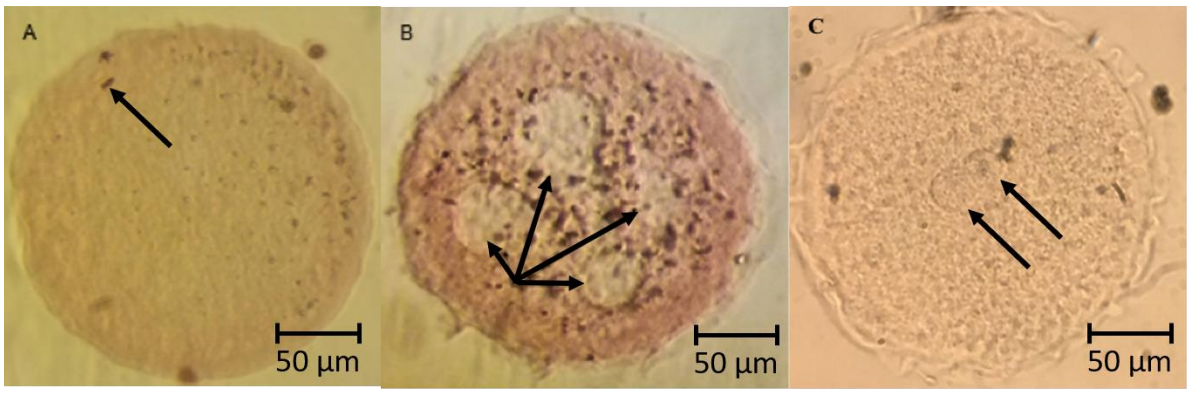


Figure 26: Zygotes in different fertilisation states. **A)** Unfertilized oocyte characterised by metaphase plate (indicated by the arrow) **B)** Polyspermic fertilisation indicated by more than two pronuclei **C)** Monospermic fertilisation indicated by exactly two visible pronuclei. Arrows indicate the positions of pronuclei.

Again, the most promising results were obtained for Fadros sperm for which monospermic fertilisation rates of 55-60% could be confirmed repeatedly when using a concentration of 20.000 spermatozoa per oocyte (see Table 38). The overall fertilisation rate was 80% and polyspermy could be limited to 23% of all oocytes. Higher sperm to oocyte ratios led to better fertilisation rates but also caused a disproportionate increase in polyspermy. Considering the average motility rate of 30% after thawing for this sperm this corresponds to a ratio of 6.000 motile spermatozoa per oocyte.

Table 38: Comparison of monospermic fertilisation rates from three different boars by aceto-orcein staining. Optimal sperm concentration is indicated by a high percentage of oocytes with two pronuclei.

Boar	**Sperm/oocyte**	**Monospermy %**	**Polyspermy %**	**Fertilised %**
869	25.000	5.2 %	0 %	5.2 %
869	38.000	0 %	0 %	0 %
869	51.000	0 %	6.67 %	6.67 %
Uteus	25.000	0 %	0 %	0 %
Uteus	50.000	8 %	4 %	12 %
Uteus	100.000	0 %	30 %	30 %
Fadros	10.000	45 %	18 %	63 %
Fadros	**20.000**	**57 %**	**23 %**	**80 %**
Fadros	50.000	38 %	47 %	85 %

In summary, an efficient IVF system was established, suitable sperm isolates were identified and the sperm to oocyte ratio was optimised. Monospermic fertilisation rates of 57% were achieved while polyspermy could be reduced to 23%. All further IVF experiments were conducted following this protocol.

3.1.4. Flushing of *in vivo* zygotes

The quality of *in vitro* produced porcine embryos is inferior to that of their *in vivo* derived counterparts [149]. Furthermore, only wild type oocytes can be extracted from slaughterhouse-derived ovaries. Flushing of *in vivo* zygotes is an effective method to obtain high-quality porcine zygotes from GE pig lines if the necessary number of experimental animals is available. Further genome alterations can then be performed on this genetic background.

Two $TP53^{R167H/R167H}$ gilts were super ovulated, artificially inseminated twice with sperm from a $TP53^{R167H/WT}$ boar and euthanised 17 hours after the second AI (protocol outlined in 2.2.1.12). Flushing of *in vivo* zygotes was performed yielding 35 one-cell-stage zygotes. To confirm their developmental competence all zygotes were cultured *in vitro* for six days resulting in 16 blastocysts (see Figure 27). As expected, the rate of blastocyst development for *in vivo* derived zygotes (45.7%) was much higher compared to the best result from *in vitro* generated zygotes (24%).

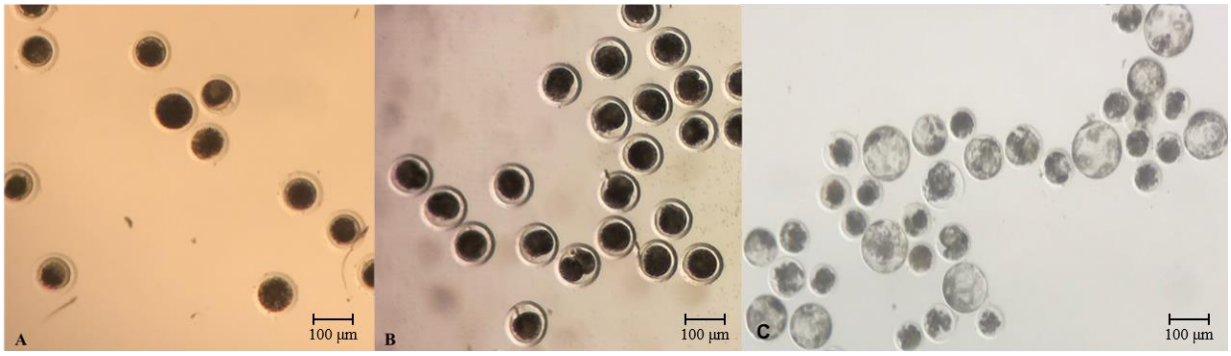


Figure 27: Morphological comparison of *in vivo* and *in vitro* generated zygotes. **A)** In vitro generated zygotes; two polar bodies are visible (top right). **B)** *In vivo* zygotes; Their greater size, bigger perivitelline space and dark, even cytoplasm indicates their high quality. **C)** Blastocyst development after IVC of *in vivo* zygotes.

This experiment shows that *in vivo* zygotes could be extracted and successfully cultured to the blastocyst stage *in vitro* with high efficiency. This method facilitates future projects that require high quality embryos from GE pig lines to introduce additional modifications.

3.2. Genome engineering directly in porcine embryos

The objective of this part of the project was to use IVP porcine embryos for genome engineering to generate new animal models. This method facilitates introduction of indels, or together with single strand DNA templates to effect homologous sequence replacement through direct manipulation of individual embryos. In combination with an efficient IVP system for porcine embryos this approach can "fast-track" the generation of GE pigs for biomedical applications.

3.2.1. Viability of IVP embryos after microinjection

Microinjection facilitates delivery of transgenes, donor DNA and genome engineering components but also causes cellular damage which adversely impacts embryo development [319]. The average survival and blastocyst development rate after microinjection for *in vitro* derived porcine zygotes is reported at 40-60% and 5-25% respectively [320]. The goal was to optimise different technical parameters to improve the viability of IVP embryos after microinjection. A variety of different needle types, shapes and diameters, injection volumes and pressures were tested resulting in the protocol described in 2.2.1.6.

To visualize successful delivery of the injection solution eGFP mRNA was injected into the cytoplasm of parthenogenetically activated porcine embryos. Twenty-four hours after injection 72% of all zygotes showed green fluorescence (see Figure 28), 15% were dead.

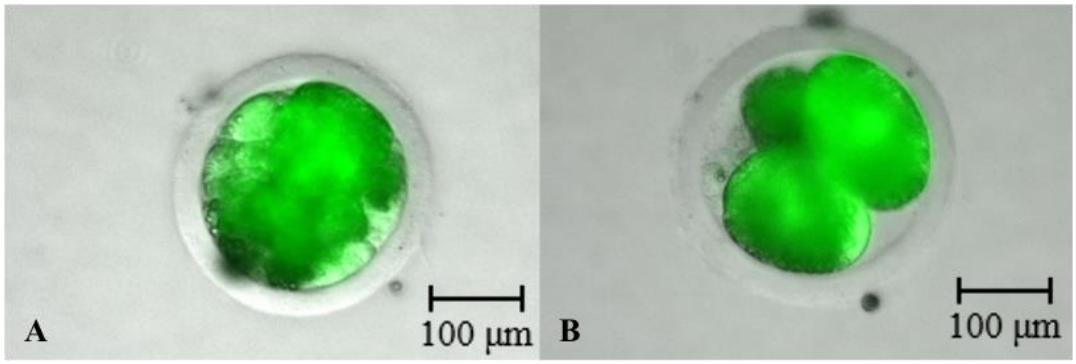


Figure 28: Green fluorescent porcine zygotes generated through microinjection of eGFP mRNA. **A)** One-cell-stage; **B)** Cleaved oocyte 24 hours after eGFP injection.

Cleavage occurred in 55% of the oocytes showing eGFP expression and 22% developed to the blastocyst stage. The control group that had undergone parthenogenetic activation without

microinjection showed cleavage rates of 70.84% and 35.56% blastocyst development. These numbers indicate a 15.84% decrease in cleavage and 13.56% reduction in blastocyst development after microinjection. An identical experiment was conducted injecting porcine zygotes created by IVF resulting in blastocyst development rates of 14%. In the non-injected control group 24% of all embryos developed to the blastocyst stage.

Overall, the optimised protocol for microinjection still had a negative impact on embryo development but the outcomes compare favourably to the literature and facilitate the use of IVP embryos for genome engineering.

3.2.2. Genome engineering in IVP embryos

The efficiency of genome engineering and embryotoxicity was assessed for a variety of target genes and applications. DNA expression vectors are normally injected into one of the pronuclei but this is difficult in pigs due to the pigmentation of porcine oocytes. Here we explored if the cytoplasmic injection of DNA expression vectors is a suitable method for GE.

First, a DNA GE vector containing an expression cassette for both Cas9 and sgRNA was microinjected into the cytoplasm of *in vitro* derived porcine zygotes. The efficiency of this approach for the insertion of DNA fragments via homologous recombination was explored. Then the precise excision of a DNA fragment using two gRNAs and simultaneous GE of multiple target genes were tested. A transposon system was employed and the efficiency of this approach for transgenesis was evaluated.

3.2.2.1. NANOS2

NANOS2 plays a key role in the sexual differentiation of germ cells. Male animals with a homozygous knockout of this gene have intact testis completely lacking germ cells while females carrying the same modification have a normal phenotype. Such males could therefore be ideal recipients for the transfer of spermatogonial stem cells to enhance the reproductive potential of GE boars or valuable breeding animals [321].

GE of the NANOS2 gene was used extensively to establish and improve technical aspects of micromanipulation, DNA isolation protocols from porcine blastocysts and evaluation of genome editing events.

3.2.2.1.1. Comparison of NANOS2 guide RNAs

Four different gRNAs for the NANOS2 gene (NANOS2 G1-4) with minimal predicted off-target activity were identified and cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (see figure 29).

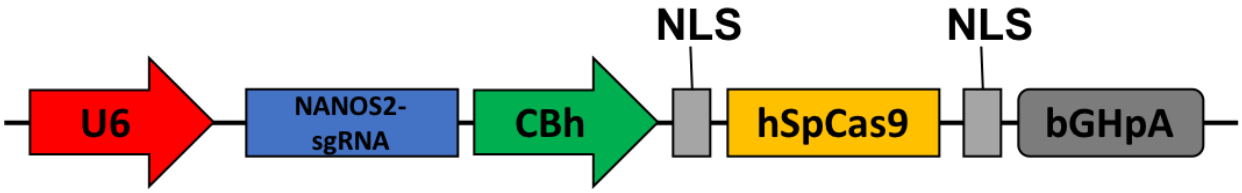
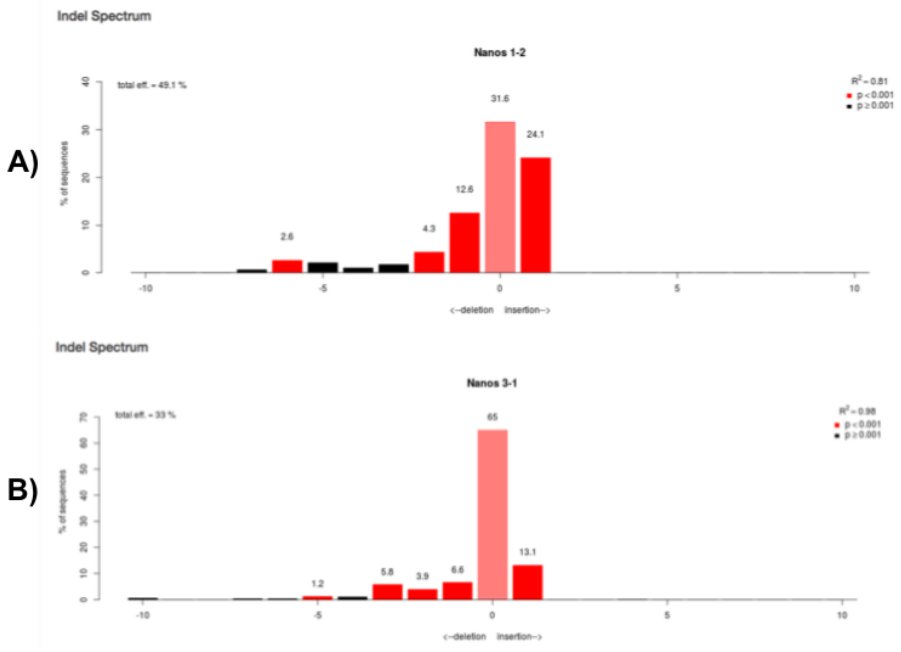


Figure 29: Structure of pX330-U6-Chimeric_BB-CBh-hSpCas9-NANOS2. It contains the CBh promoter and hSpCas9 gene flanked by two nuclear localization signals (NLS), followed by a bGH-poly-A terminator sequence. The sgRNA sequence includes an 18 bp gRNA homologous to the target sequence in exon 1 of the NANOS2 gene followed by a gRNA scaffold driven by the U6 promotor.

Porcine kidney fibroblasts (PKDNFs) (isolate 250515) were transfected with each of the four vectors (as outlined in 2.2.4.5). DNA was isolated from the pool of transfected cells and PCR amplification was performed across the target sites. DNA sequencing was conducted and the frequency of indel mutations determined by TIDE analysis (see figure 30).



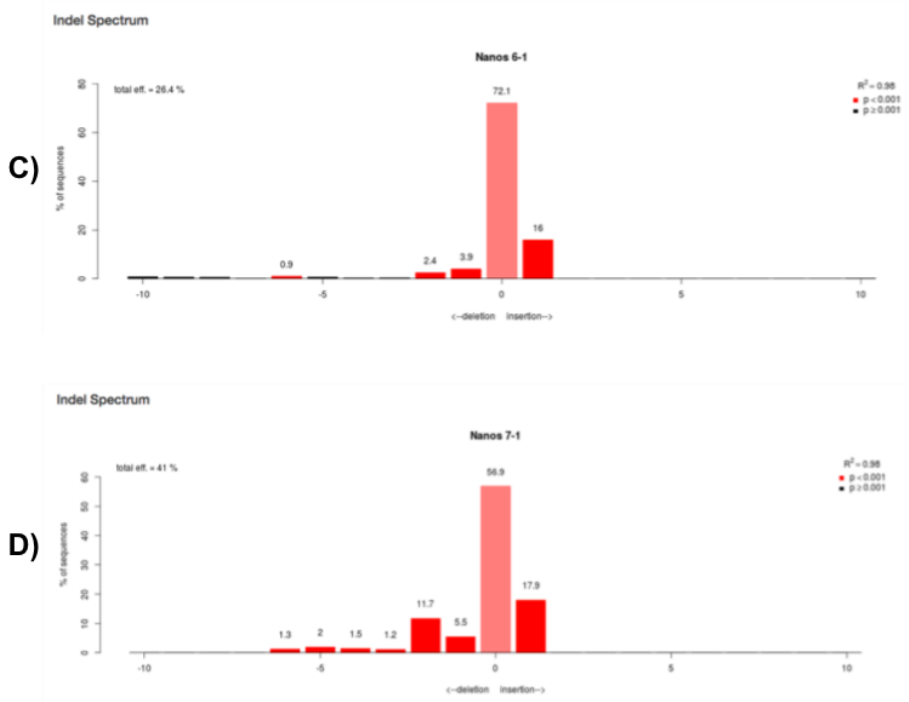


Figure 30: Comparison of frequency and spectrum of indels at the NANOS2 target site by TIDE analysis. **A)** NANOS2 G1 (49,1%), **B)** NANOS2 G2 (33%), **C)** NANOS2 G3 (26,4%), **D)** NANOS2 G4 (41%).

Out of the tested gRNAs NANOS2 G1 reached the highest on-target cleavage efficiency of 49,1% and was therefore used for GE in porcine embryos.

3.2.2.1.2. Detection of NANOS2 GE in porcine blastocysts

Most protocols for the isolation of DNA from blastocysts are optimised for mice. Porcine IVP blastocysts contain only 50-75 cells and store large amounts of RNAs, proteins and especially lipids. This reduces the quality of extracted DNA and makes downstream assays such as PCR less efficient [322]. The objective was to identify suitable protocols for the isolation of DNA from porcine blastocysts and assess if the injection of DNA GE vectors into the cytoplasm of in vitro derived porcine zygotes results in efficient genome editing.

Two-hundred porcine oocytes were microinjected with the NANOS2G1 targeting vector (5gn/µl) followed by parthenogenesis. Fifty-two embryos (26%) developed to the blastocyst stage. Methods for the extraction of DNA from porcine blastocysts based on freeze-drying [323], proteinase K digest [324], chemical lysis [315] and commercial DNA isolation kits were compared. DNA of sufficient quality and quantity could only be obtained from 10 blastocysts using the DNA isolation protocol described by Li *et al* which was used for all further experiments [315].

PCR amplification was performed across the target site. DNA sequencing revealed indel mutations in 7/10 blastocysts (70%). These results were confirmed by GE of 100 IVF zygotes resulting in twelve blastocysts (12%), eight of which carried indel mutations at the target site (66.6%).

In summary, an efficient protocol for the isolation of DNA from porcine blastocysts was established and successful editing of the NANOS2 gene by microinjection of a DNA GE vector into the cytoplasm of porcine IVP zygotes was confirmed.

3.2.2.2. Reactivation of the porcine UCP1 gene

The next question was whether microinjection of a CRISPR/Cas9 vector plus DNA donor template into the cytoplasm of porcine zygotes leads to the insertion of the DNA fragment via homologous recombination.

Uncoupling protein one (UCP1) is an ion exchanger in the internal mitochondrial membrane of brown fat tissue. This transmembrane protein can uncouple fuel oxidation in the respiratory chain from ATP synthesis to produce heat. UCP1 is present in many mammals including humans but it is not functional in pigs due to the deletion of several exons [325].

The objective of this experiment was to generate pigs with functional UCP1 by inserting the coding part of human UCP1 into the porcine genome. The important role this protein plays in energy metabolism could give new insights about obesity and type II diabetes. These pigs could also be used to increase animal welfare and reduce the energy expenditure and cost of meat production used to warm piglets.

Five ng/µl of UCP1 targeting vector and 7.5 ng/ µl of the complementary ssDNA template (1.5kb) including the coding part of human UCP1 (both provided by Guanglin Niu) were microinjected into 160 *in vitro* produced zygotes. Here, only seven blastocysts could be generated (4.4%) and DNA was extracted, followed by PCR amplification across the target site (see Figure 31). Successful integration of human UCP1 could be verified in four out of seven blastocysts (57%).

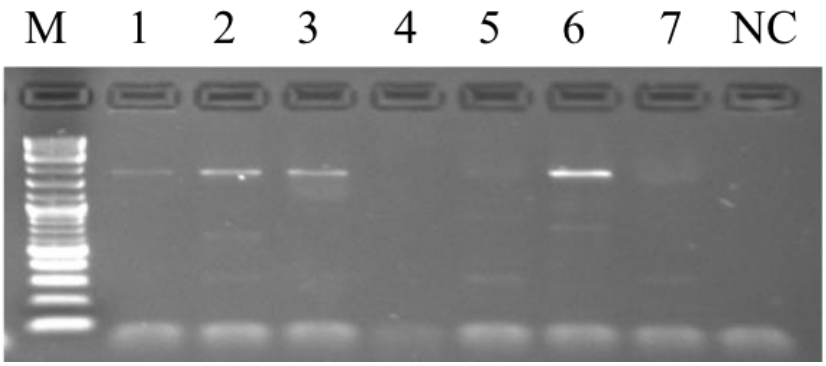


Figure 31: PCR analysis of blastocysts injected with UCP1 targeting vector plus complementary template coding for human UCP1; NC = water control.

Three-hundred *in vitro* generated zygotes were microinjected with the same targeting vector plus complementary ssDNA template. All zygotes were transferred to a surrogate pig, but no pregnancy could be established.

Overall, the successful integration of the donor DNA at the target site could be confirmed *in vitro* but no GE pigs could be generated.

3.2.2.3. Precise excision of the ΔARE element from the TNFα gene

The objective for this project was to explore if the injection of a DNA vector coding for two gRNAs simultaneously into the cytoplasm of porcine zygotes is an efficient method for the excision of a DNA fragment. Here, the objective was to excise the AU-rich elements ($TNF^{\Delta ARE}$) from the tumour necrosis factor-α (TNF-α) gene. In mice this modification is associated with systemically elevated TNF-alpha levels and inflammation in the terminal ileum [326].

The GE vector (generated by Alessandro Grodziecki) was injected into 250 *in vitro* generated porcine zygotes over the course of two experiments. In total 5 blastocyst were obtained and used for DNA isolation followed by DNA sequencing. In three of them (60%) a precise excision of the $TNF^{\Delta ARE}$ sequence could be detected (see Figure 32).

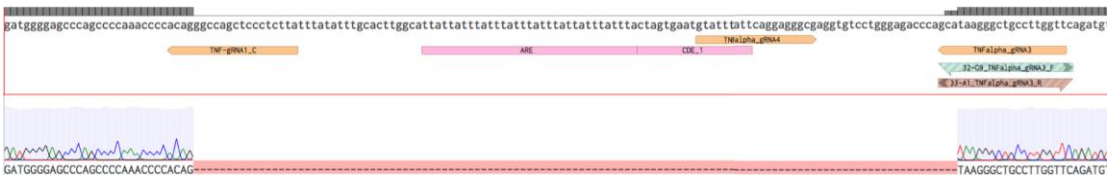


Figure 32: Blastocyst with a precise excision of the $TNF^{\Delta ARE}$ sequence.

In summary, the $TNF^{\Delta ARE}$ sequence was successfully excised using a GE vector that codes for two gRNAs simultaneously.

3.2.3. Simultaneous genome editing of CMAH and B4GALNT2

The goal was to explore whether the injection of a DNA vector into the cytoplasm of porcine zygotes is a suitable method to simultaneously edit multiple target genes. The removal of xenoreactive antigens through inactivation of porcine genes can minimise the rejection of pig organs by human recipients in xenotransplantation. CMAH and B4GALNT2 are genes coding for major xenogeneic antigens. During this thesis a targeting vector aimed at the inactivation of these two genes (provided by Beate Rieblinger) was tested in porcine embryos.

The plasmid was microinjected into 373 *in vitro* produced zygotes. Seventy-three zygotes were cultured *in vitro* for six days and five blastocysts (6.8%) could be obtained compared to twelve out of 88 (13.6%) blastocysts in the non-injected control group. The remaining 300 injected zygotes were transferred into the oviduct of a synchronised recipient, but no pregnancy could be established.

DNA isolation from the injected blastocysts and analysis by PCR amplification followed by DNA sequencing of the target sequences of the CMAH and B4GALNT2 genes was conducted by Thomas Winogrodzki (master student). Homozygous knockouts of CMAH and B4GALNT2 could be verified in one (20%) blastocyst whereas heterozygous knockouts of CMAH and B4GALNT2 could be observed in three (60%) blastocysts (see Table 40).

Table 39: DNA sequencing results from blastocysts microinjected with CMAH- B4GALNT2-double knockout vector.

Blastocyst	CMAH	B4GNT2
1	+1 Heterozygous T-insert	Wildtype
2	+1 Heterozygous T-insert	Multiple mutations
3	+1 Homozygous T-insert	Multiple mutations
4	+1 Heterozygous T-insert	Heterozygous T-deletion
5	Homozygous T-insert	Homozygous T-insertion

In brief, two target genes could simultaneously be inactivated by microinjection of a GE vector into the cytoplasm of *in vitro* derived porcine zygotes.

3.2.3.1. Transposon mediated transgenesis via cytoplasmic injection of embryos

The objective for this project was to explore if the injection of a PiggyBac (PB) transposon DNA vector plus PB Transposase mRNA into the cytoplasm of porcine zygotes is an efficient method for transgenesis. PB Transposase recognises the inverted terminal repeats (ITRs) of the transposon vector excises it from the plasmid backbone and randomly integrates the vector containing the gene of interest into the genome at "TTAA" sites via a cut and paste mechanism [78].

Here the gene of interest was the codon-improved Cre recombinase (iCre) driven by the pancreas-specific mouse pancreas duodenum homeobox-1 (mPdx1) promoter for the activation of conditional oncogenic mutations (see 3.3.5).

The transposon plasmid and transposase-mCherry mRNA (both generated by Daniela Kalla, see Figure 33) were injected into 100 *in vitro* generated porcine zygotes followed by parthenogenetic activation.

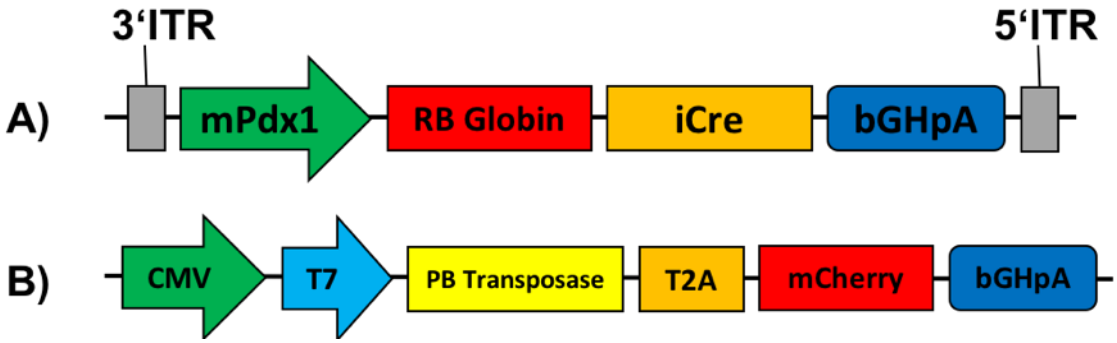


Figure 33: Structure of the components of the transposon system **A)** MPdx1-iCre Transposon DNA vector. It contains the mPdx1 promoter, rabbit beta globin intron, iCre and bGH-poly-A flanked by two ITRs. **B)** Structure of PB transposase expression vector. It contains the CMV promoter, T7 promoter, PB Transposase, a T2A, the mCherry sequence and bGH-poly-A.

Upon translation of the injected mRNA the polypeptide is broken apart at the T2A site via ribosome skipping into PB Transposase and mCherry [327]. Twenty-four hours after microinjection the oocytes were assessed for expression of mCherry and PB Transposase indicated by red fluorescence (see Figure 34).

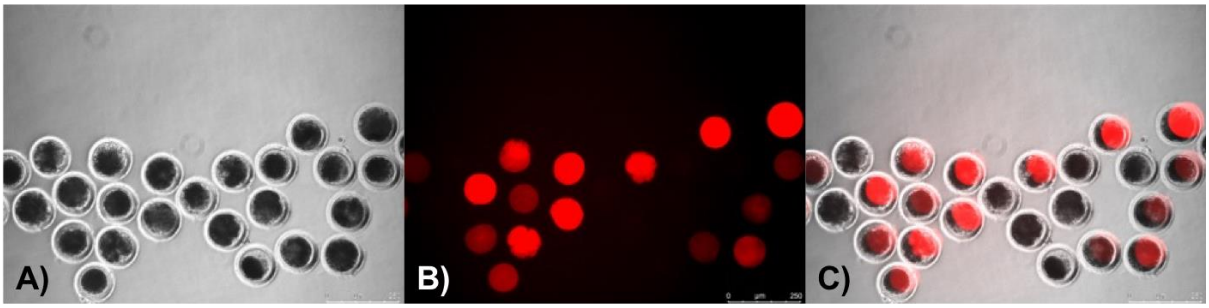


Figure 34: Oocytes 24 hours after microinjection with transposon plasmid and transposase mRNA. Red fluorescence indicates the expression of mCherry and PB Transposase. **A)** Bright field: The oocytes have not undergone cleavage but their membranes are intact. **B)** Dark field, fluorescence imaging: MCherry fluorescence is clearly visible. **C)** Overlay.

After six days of *in vitro* culture 42/100 (42%) oocytes developed to the blastocyst stage compared to 25/50 (50%) in the non-injected control group. DNA isolation followed by PCR amplification across the target site revealed the mPdx1-iCre sequence in 18/42 (43%) blastocysts (see Figure 35).

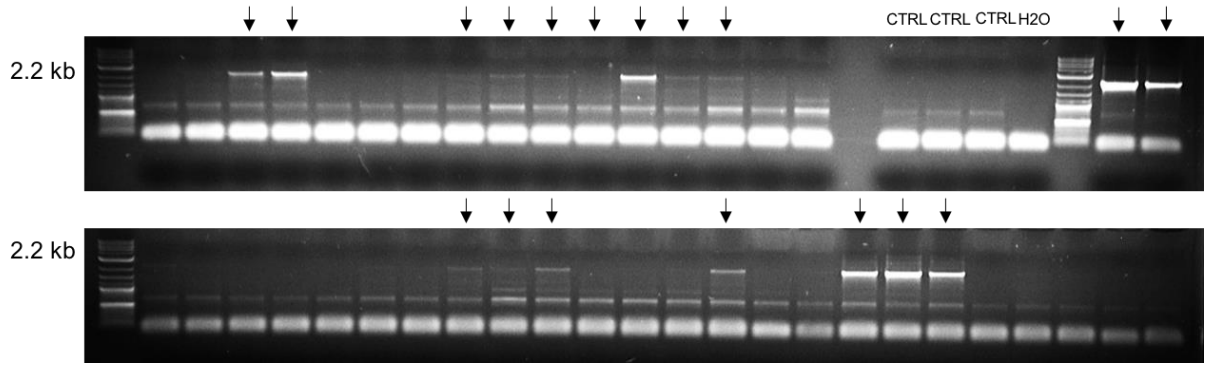


Figure 35: PCR analysis of blastocysts injected with mPdx1-iCre transposon plasmid plus PB Transposase mRNA. Black arrows indicate the positive samples; CTRL = non-injected blastocysts; H2O = water control.

In Summary, the mPdx1-iCre sequence was successfully ascertained after cytoplasmic injection of a DNA transposon plasmid plus Transposase mRNA.

3.3. Generation of porcine models for biomedicine

The generation of porcine disease models was the main objective of this project. All previously described methods, such as *in vitro* production of porcine embryos, cryopreservation of sperm, genome engineering of porcine zygotes by microinjection and embryo transfer were optimised to facilitate this goal.

3.3.1. Embryo transfer

During this thesis endoscopic and surgical embryo transfer (ET) were carried out. Training for endoscopic ET was obtained from Dr. Barbara Kessler. Surgical ET was established as an alternative for endoscopic ET (see Figure 36). Both techniques facilitate transfer of one to two-cell stage embryos to the oviduct. Surgical ET also allows for bicornual transfer of zona-free blastocysts directly to the uterus. This is necessary for HMC because zona-free embryos must be cultured to the blastocyst stage *in vitro* to be able to survive *in vivo*.

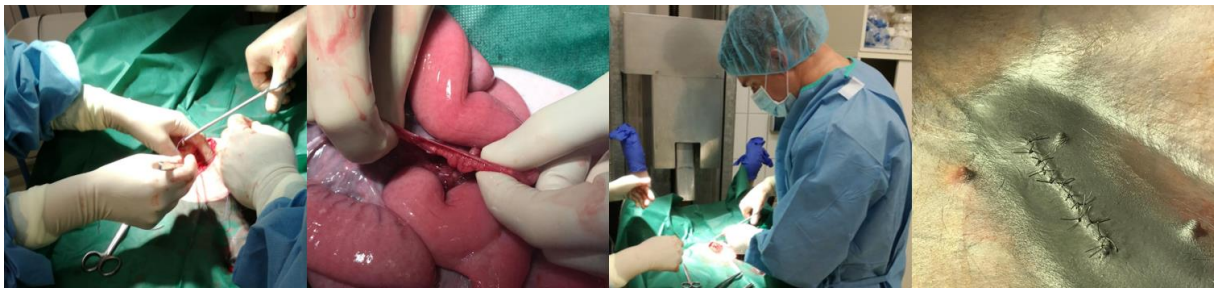


Figure 36: Surgical embryo transfer. The recipient pig was fixated on the surgery table. The abdomen was opened, and embryos were transferred directly into the oviduct with a sterile catheter. The surgery wound was stitched in three layers and aluminium spray was applied.

Vectors that were previously tested in *in vitro* generated and cultured embryos (outlined in 3.3) were used to generate genetically modified embryos. In total twenty-two ETs were carried out and eleven pregnancies were established. Twelve of them were performed endoscopically by Dr. Barbara Kessler (Chair for Molecular Animal Breeding and Biotechnology) and ten surgically by me (as outlined in 2.2.1.11). Five out of ten surgical ETs and six out of twelve endoscopic ETs resulted in pregnancies yielding an equal pregnancy rate of 50% for both methods. One surgical ET experiment was discontinued due to postoperative complications and the pig was euthanised. Three pregnancies were confirmed by sonography on day 21 but due to resorption of the embryos they were not carried to full term. Five pregnancies resulted in the birth of 29 piglets with fourteen of them carrying four different genetic modifications. One

pregnancy with 14 piglets was terminated to isolate foetal fibroblasts. Two pregnancies are still ongoing at the time of writing. The average litter size was 5.8 which increases to 7.2 if the terminated pregnancy with 14 piglets is considered.

3.3.2. Porcine model for Crohn's Disease

In this project pigs with an excision of the tumour necrosis factor (TNF) AU-rich elements ($TNF^{\Delta ARE}$) sequence were generated as a potential model for human Crohn 's Disease, uveitis and rheumatoid arthritis.

The plasmid containing the Cas9 expression vector and two gRNAs to excise the $TNF^{\Delta ARE}$ sequence was microinjected into 1178 *in vitro* generated porcine zygotes over the course of three experiments. Five embryo transfers were carried out resulting in two pregnancies. From those two pregnancies seven piglets with an excision of the $TNF^{\Delta ARE}$ sequence could be obtained (see Figure 37). The degree of mosaicism in the animals generated during this thesis has not been thoroughly analysed at the time of writing but preliminary data showed signs of mosaicism in three pigs.

Figure 37: $TNF^{\Delta ARE}$ knockout piglets

The intended modifications were confirmed by PCR amplification and DNA sequencing of the target region (data sample from 2 pigs shown in Figure 38).

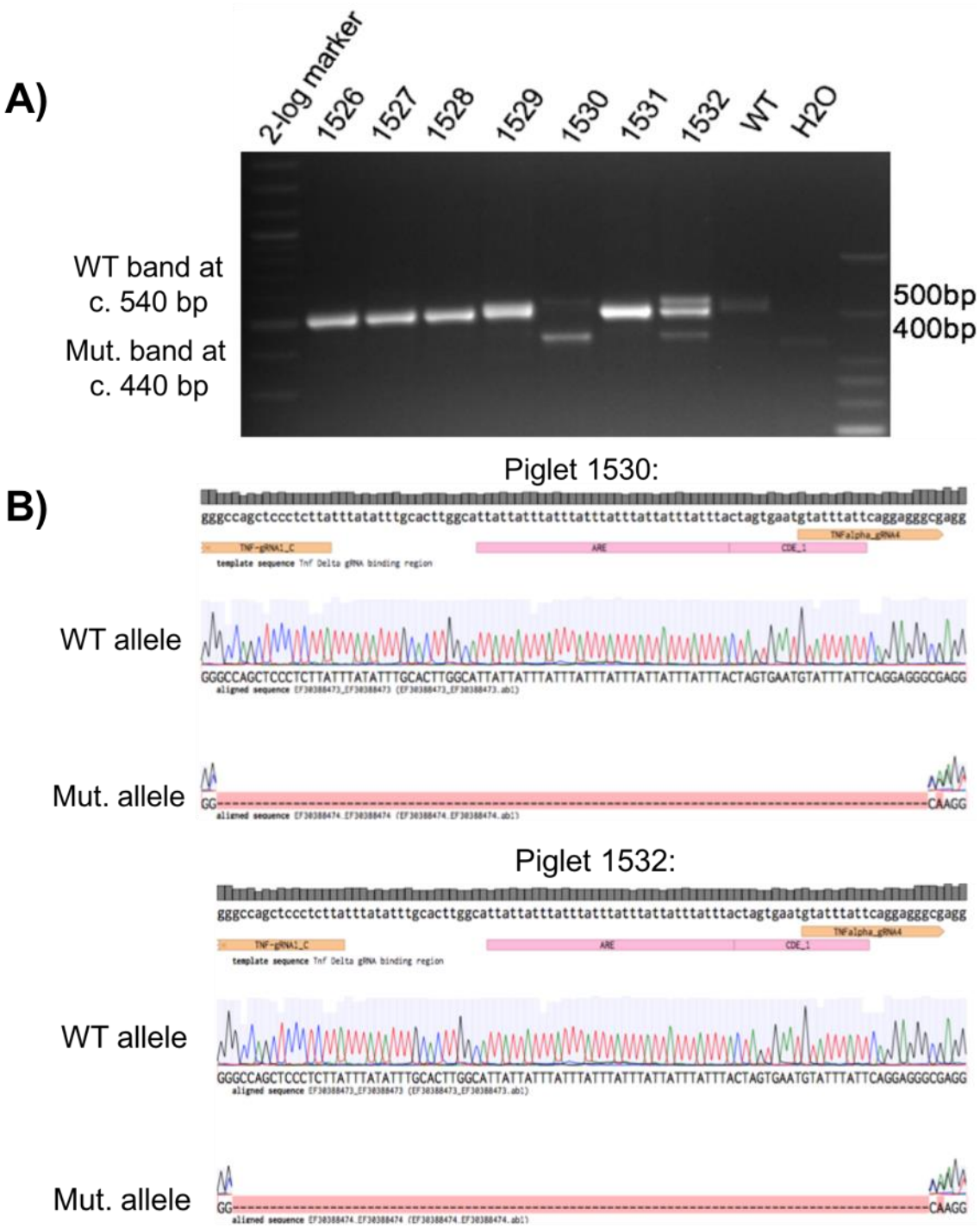


Figure 38: Data sample from two TNF$^{\Delta ARE}$ pigs. **A)** PCR amplification of the target site reveals mutant bands at 440bp for pigs #1530 and #1532. **B)** DNA sequencing of the target region confirms the excision of the TNF$^{\Delta ARE}$ sequence.

Overall, seven pigs with an excision of the TNF$^{\Delta ARE}$ sequence were generated in this project. It remains to be seen if these animals develop a disease phenotype with age.

3.3.3. Preliminary work towards a porcine Hepatitis model

The objective for this project was to generate GE pigs susceptible to hepatitis B virus (HBV) infection. Similar to the editing experiment of the UCP1 gene this project requires the targeted insertion of a DNA sequence via homologous recombination but here the sequence is much shorter (20bp compared to 1.5 kb).

Viral hepatitis is a major global health problem causing approximately 880.000 deaths per year worldwide [328]. HBV binds to the natrium-taurocholate co-transporting polypeptide (NTCP) which is a bile acid transporter encoded by the SLC10A1 gene [329]. HBV infections are limited to great apes (Hominidae) and humans due to interspecies variations in the amino acid sequence of the NTCP receptor. The NTCP amino acid sequence has been modified in mice to match the human equivalent but these animals were not susceptible to HBV infection. Porcine hepatocytes expressing the human NTCP receptor however, have been shown to enable productive HBV infections which could make NTCP humanized pigs a suitable animal model for HBV research [330].

The targeting vector and sgRNA required for the generation of a possible model for human Hepatitis were produced by Dr. Konrad Fischer. In five experiments this targeting vector plus ssDNA donor template was injected into 1672 *in vitro* generated porcine zygotes. Five embryo transfers were carried out resulting in two pregnancies from which in total five piglets were obtained. In two piglets (40%) indel mutations in the porcine NTCP gene could be identified by PCR amplification and DNA sequencing (see Figure 39). The human NTCP sequence could not be ascertained in any of those pigs.

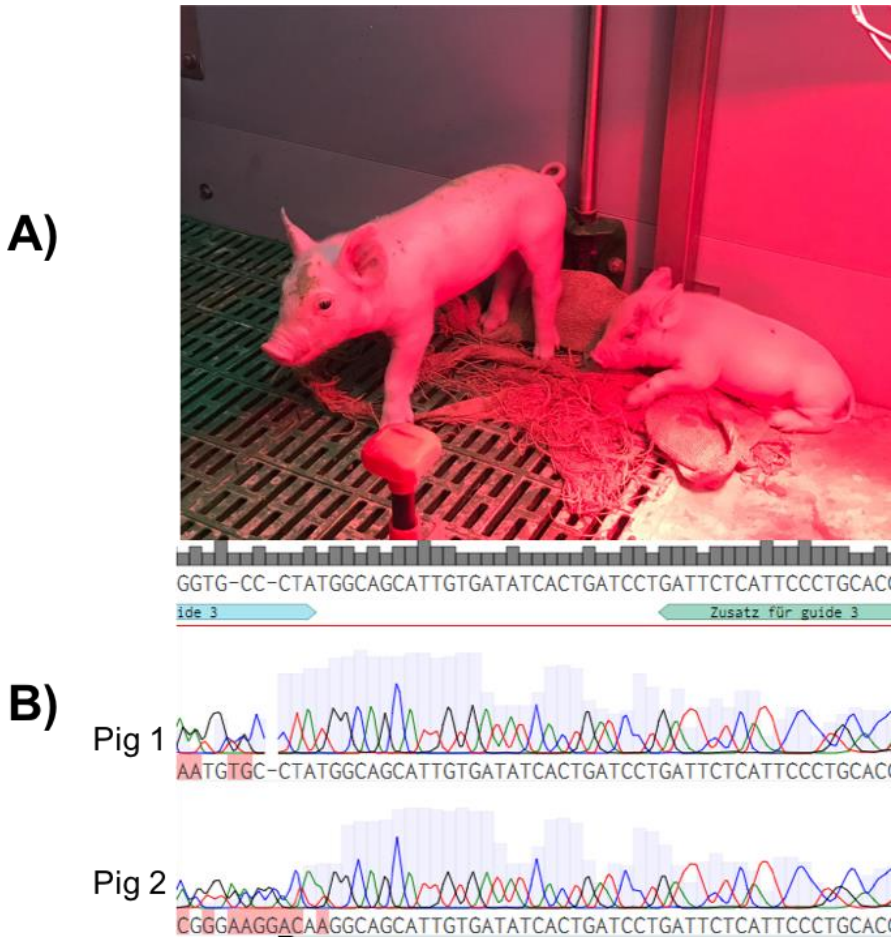


Figure 39: A) Two pigs with indel mutations in the porcine NTCP gene. **B)** DNA Sequencing across the target site reveals indel mutations in the porcine NTCP gene but the intended humanisation of the NTCP gene could not be achieved.

In summary, two pigs with indel mutations in the NTCP gene could be generated but the integration of the human NTCP sequence was not successful.

3.3.4. Simultaneous GE of multiple genes relevant for xenotransplantation

The objective for this project was to evaluated if quadruple knockout pigs can be generated for xenotransplantation, and if so with which efficiency. The gene editing vector tested in 3.3.2.4 aimed to knockout two genes that synthesize xenogeneic glycosylation patterns (CMAH and B4GALNT2). The fourfold knockout vector used for this experiment (created by Beate Rieblinger) targets the GGTA1 and SLA class I genes in addition the other two genes (GGTA1/CMAH/B4GALNT2/SLA class I).

The vector was microinjected into 200 *in vitro* produced zygotes. Those zygotes were transferred to a synchronized recipient alongside 200 injected with the $TNF^{\Delta ARE}$ vector resulting in a pregnancy. Seven piglets were born, two of them with an excision of the $TNF^{\Delta ARE}$ sequence

(see 3.3.2.) and one of them with homozygous knockouts of the GGTA1 and B4GALNT2 genes (see Figure 40). No indel mutations could be found in the CMAH and SLA class I genes.

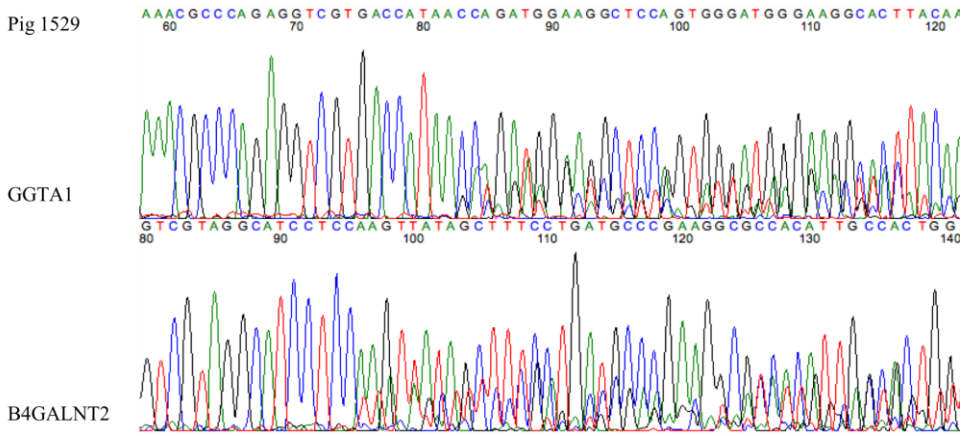


Figure 40: Sequencing of the GGTA1 B4GALNT2 knockout pig #1529.

In summary, a pig with homozygous knockouts of the GGTA1 and B4GALNT2 genes could be generated but the other two target genes remained unmodified.

3.3.5. Porcine model for pancreatic cancer

The overarching goal of this project is the generation of pigs predisposed to pancreatic cancer by conditional, tissue-specific activation of oncogenic mutations. For this purpose, a pig line carrying oncogenic $KRAS^{G12D}$ and $TP53^{R167H}$ mutations silenced by a "stop" cassette had been previously generated [331, 332]. Here the objective was to generate a pig line expressing Cre-recombinase specifically in the pancreas to activate these conditional mutations by crossbreeding. For this purpose, the mPdx1 promoter was used to direct Cre expression to the developing pancreas.

The mPdx1-iCre transposon vector and PB transposase/mCherry mRNA (see 3.2.2.5.) was injected into 1227 *in vitro* generated porcine zygotes over the course of three experiments. Five embryo transfers were carried out resulting in two pregnancies. One pregnancy was confirmed sonographically on day 21 but was not carried to full term due to resorption of the embryos. From the other pregnancy ten piglets were obtained, three of them carrying the desired mPdx1-iCre sequence (see Figure 41).

Figure 41: A) PCR amplification reveals mutant bands at 2226bp for pigs #2017, 2022 and 2024 (indicated by the pink arrows). **B)** Three transgenic pigs carrying the mPdx1-iCre sequence. **C)** DNA sequencing of the target region confirms the integration of the mPdx1-iCre sequence (data sample from pig #2017).

In summary, three transgenic mPdx1-iCre pigs were generated. It remains to be seen if these animals develop a disease phenotype after crossbreeding with the $KRAS^{G12D}$ and $TP53^{R167H}$ line.

3.4. Handmade cloning

Previous experiments have shown that the integration of DNA fragments using GE-vectors and DNA donor templates is inefficient. These more complex genome alterations are better carried out in somatic cells because this approach enables pre-selection for the desired modification. Modified donor cells can then be used for somatic cell nuclear transfer to generate GE pigs. Therefore, the next goal of this thesis was to establish handmade cloning to facilitate projects that require homologous recombination or editing of multiple genes simultaneously. Initial instructions for HMC were given by Prof. Lin (Department of biomedicine, Aarhus University).

The first objective was to generate cytoplasts and test their viability after enucleation. Mature oocytes were enucleated with a handheld blade after zona pellucida removal (as outlined in 2.2.1.14). Successful enucleation and generation of the resulting zona free cytoplasts was confirmed by Hoechst staining (see Figure 42).

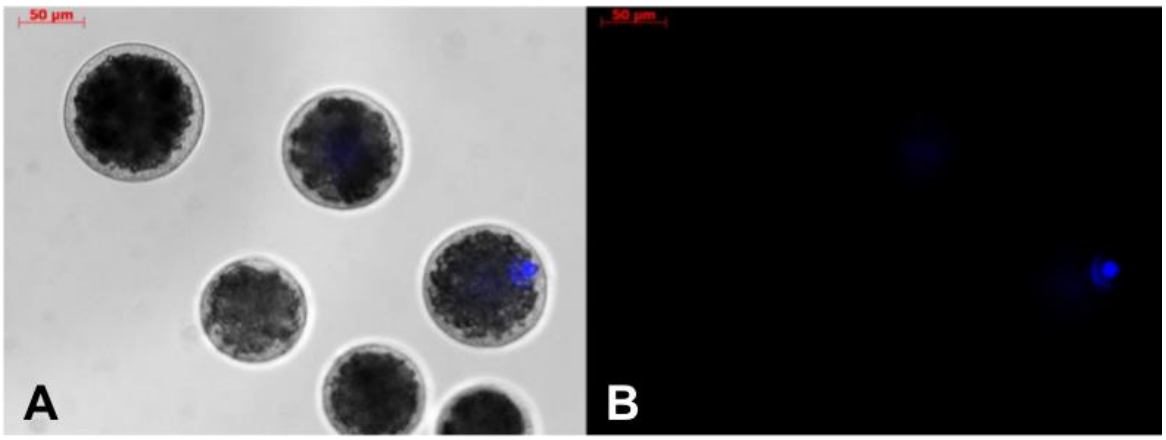


Figure 42: Hoechst-staining of zona-free cytoplasts generated through enucleation of mature oocytes. **A)** Bright field, **B)** Dark field; Insufficient enucleation is indicated by blue fluorescence (oocyte to the right).

The next objective was to generate reconstructed embryos and assess their developmental potential. Cytoplasts were fused with porcine kidney fibroblasts in two steps to generate eight reconstructed embryos. They were cultured *in vitro* for six days in a well-of-the-well system. Two blastocysts were generated (25%) and four embryos developed to the morula stage (50%) (see Figure 43).

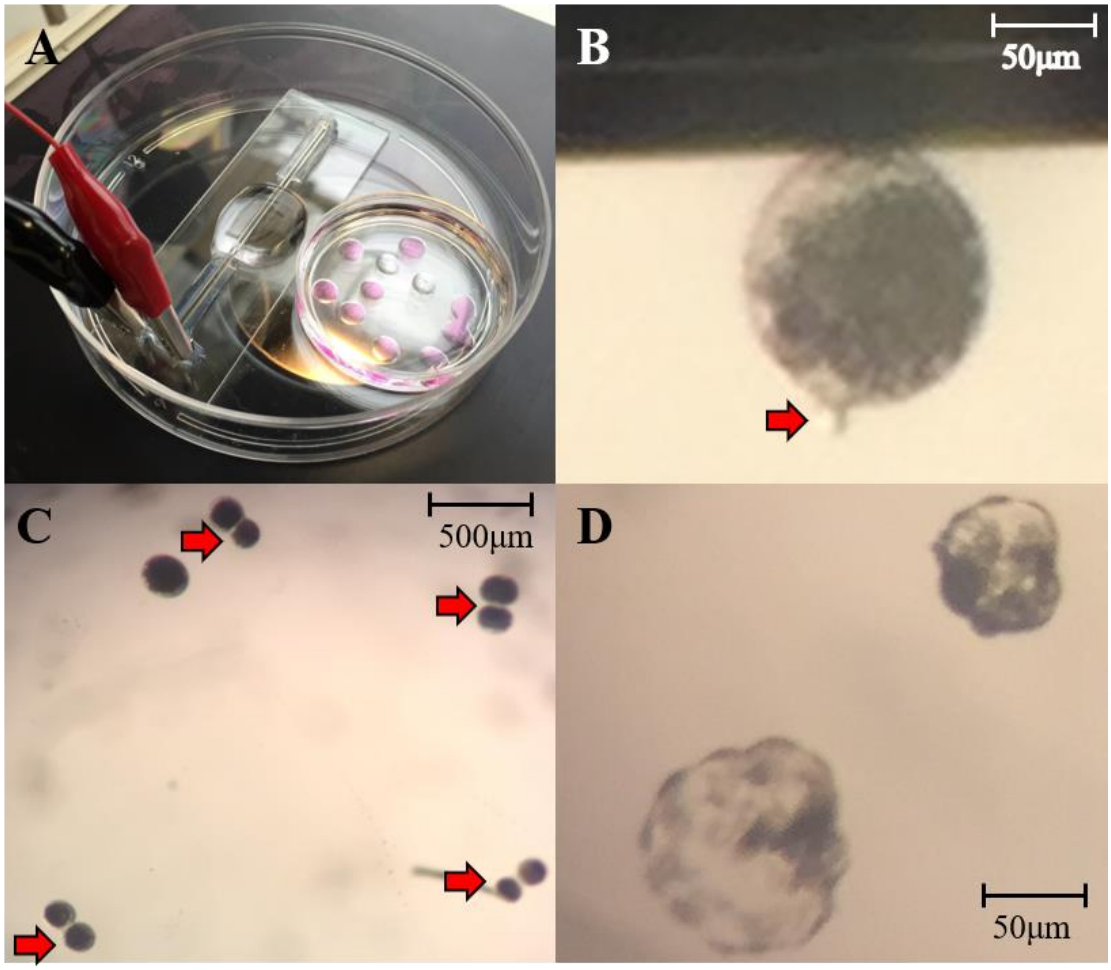


Figure 43: Fusion steps during handmade cloning procedure resulting in blastocysts after six days of *in vitro* culture **A)** Fusion chamber used for both fusion steps in HMC. **B)** Somatic cell fused to cytoplast (indicated by red arrow). **C)** Reconstructed embryos after fusion with second cytoplast (indicated by red arrows) **D)** Blastocysts developed from reconstructed HMC embryos after six days of in vitro culture.

Preliminary, somatic cell nuclear transfer was successfully conducted using the handmade cloning technique. Twenty-five percent of reconstructed embryos developed to the blastocyst stage but this number of is insufficient for embryo transfer.

4. DISCUSSION

The objective of this work was to optimise the *in vitro* production of porcine zygotes and establish a system for direct manipulation of embryos to "fast-track" the generation of porcine models for biomedicine.

Section 4.1 addresses the optimisation of the IVP system for porcine embryos including *in vitro* maturation, *in vitro* fertilisation, *in vitro* culture and the cryopreservation of boar sperm. CRISPR/Cas9 mediated genome engineering directly in early embryos and transgenesis using a transposon system is examined in segment 4.2. The generation of thirteen GE pigs with four distinct genotypes is debated in passage 4.3. Finally, the establishment of HMC as an alternative to TC is discussed is section 4.4

4.1. In vitro embryo production

In vitro embryo production is a multistage process that requires proper interaction of a variety of techniques including IVM, sperm preparation, IVF and IVC of embryos [218] which are discussed in this section. The quality of embryos measured by embryonic cell count, maturation rates and blastocyst development generated with the IVP system described here could be improved markedly over the course of the project.

4.1.1. *In vitro* maturation

An efficient *in vitro* maturation system is essential to supply an adequate number of mature oocytes for SCNT and IVF [85]. The maturation protocol that was established and optimised in this thesis reliably promotes maturation rates above 80% which matches or slightly exceeds rates described in most publications [144, 149]. The average blastocyst development rate of 50% after electrical activation further exemplifies the high developmental potential of oocytes generated with this IVM system. The close proximity of a slaughterhouse decreased transportation times for ovaries to less than 30 minutes which is known to positively influence oocyte quality [333].

Maturation media are usually supplemented with EGF to support maturation [334] but analysis of follicular fluid provided evidence that several other growth factors are required to adequately support oocyte maturation [335]. Meiotic arrest of oocytes in the follicle is mediated by high levels of cAMP [336]. A sudden decrease in cAMP levels induced by removal of oocytes from their follicular environment causes asynchronous cytoplasmic and nuclear maturation and impairs embryonic development [202]. FGF, IGF and LIF are all cytokines that are known to be present in porcine follicular fluid (PFF) [335, 337]. Together they effectively regulate cAMP levels leading to more synchronous cytoplasmic and nuclear maturation [202].

This effect can be replicated by including PFF in maturation media for its growth factor content but PFF also contains potent maturation inhibitors such as hypoxanthine [199]. Chemically defined media as used here eliminate the potential for transmission of pathogens that might be present in biological fluids [338]. They also lead to higher reproducibility by eliminating biological variables, such as the quality of PFF and FCS [169, 339].

Nuclear maturation rates consistently exceeding 80% and a 15% higher blastocyst rate after parthenogenesis compared to the PFF supplemented medium show the effectiveness of the chemically defined, cytokine enhanced approach. The increased embryonic cell count, comparatively high rate of monospermic fertilisation and high consistency support the validity of this IVM system. Downsides are its slightly higher complexity and costs.

In summary, the IVM system established during this thesis consistently yielded mature oocytes of high quality. Similar efficiencies are described for other cytokine-supplemented IVM media [177]. Performance metrics regarding embryo quality compare favourably to most other IVM outcomes described in the literature [135, 169, 181, 201].

4.1.2. *In vitro* fertilisation

IVF remains a limiting factor for the IVP of porcine embryos due to the unsolved issue of polyspermy [149]. Polyspermy is a complex problem to solve because it is influenced by many different variables, such as gamete coincubation times [242], supplementation of media with different molecules [212], sperm quality [340] and sperm to oocyte ratio [341].

All IVF parameters were optimised exclusively for frozen-thawed sperm to standardize for differences between boars and ejaculates from the same boar [236]. The starting point for all experiments was a proven IVF protocol utilising sperm provided by Dr. Mayuko Kurome (Chair for Molecular Animal Breeding and Biotechnology, LMU). The next step was the identification of suitable sperm donors to further improve IVF outcomes.

Ejaculates from 21 different boars were analysed for their IVF suitability. Blastocysts could be produced with most sperm samples but IVF performance was vastly different among boars which is also described in the literature [235, 342]. Many publications suggest that only sperm from a minority of boars is suitable for IVF after cryopreservation [151, 343]. The data generated during this thesis supports this assumption even though exclusively high-quality ejaculates from highly fertile breeding boars were used for IVF.

Blastocyst formation rates of on average 24% using sperm from the highest performing boar (Fadros) are in line with reports from other IVF laboratories using *in vitro* derived oocytes [149, 165]. While there are publications reporting much higher blastocyst development rates these numbers are usually achieved using *in vivo* derived oocytes [125] or associated with rates of polyspermy reaching up to 90% [214, 215]. Reports of high blastocyst development rates calculated based on pre-selected subgroups of mature or fertilised oocytes should be seen in perspective [344].

Blastocyst development rates are only relevant in the context of monospermic fertilisation which is a more suitable parameter to measure the validity of an IVF system [245]. The degree of polyspermy in IVF is closely tied to the ratio of sperm to oocytes during fertilisation [216]. Individual optimisation of IVF parameters resulted in monospermic fertilisation rates of 57% accompanied by 23% of polyspermy which is consistent with the 50-60% efficiency reported for modern IVF systems [218]. The slightly higher fertilisation rates that were observed when adding more spermatozoa during IVF came with a strong increase in polyspermy (results are shown in table 24). This confirms the prevailing assumption that a compromise has to be made between optimal fertilisation and acceptable rates of polyspermy [218].

Oocyte quality is another critical factor affecting polyspermy [219, 220]. Here, a chemically defined, cytokine supplemented maturation medium was used to improve the quality of IVM oocytes (discussed in 4.1.1). Higher rates of monospermic fertilisation can be achieved using

oocytes from adult sows [160] or *in vivo* derived zygotes [247] but the ability of *in vitro* matured oocytes to block polyspermy remains low [345]. The IVF protocol used here foregoes removal of cumulus cell. This reduces polyspermy by posing an additional barrier mimicking *in vivo* selection of the most motile spermatozoa [346].

In vivo fertilisation takes place in oviductal fluid (OF) which makes it a logical supplement for IVM media [347]. Its chemical composition and beneficial effects on monospermic fertilisation are dependent on the phase of the oestrous cycle upon collection [348, 349]. This variability in composition is further increased because the presence of spermatozoa [350], oocytes, or a combination of both [351] which leads to large alterations in the oviduct proteome. While OF supplementation has positive effects on IVF outcomes when using fresh sperm [352] detrimental effects have been reported for frozen-thawed sperm [348]. These findings demonstrate OFs potential as a supplement for IVF media, but they also highlight its high complexity and variability. Sanitary certified OF, classified for oestrous cycle and biological activity is an auspicious additive that could reduce the incidence of polyspermy [218].

A 3-dimensional IVF system within an organ-on-a-chip system [353] is another promising concept to improve the quality of *in vitro* derived porcine embryos. Until such options become commercially available a combination of sperm selection methods with short co-incubation times [244] is a practical approach to further optimise the IVF system discussed here.

In summary, an IVF system was established and optimised reaching 57% efficiency measured by the rate of monospermic fertilisation. While polyspermy remains an unsolved problem, it was minimised by optimising the sperm to oocyte for each boar and improving the quality of *in vitro* matured oocytes. The generation of 29 pigs using zygotes derived from this IVF system (discussed in 4.3) during the first year of implementation further supports its validity.

4.1.3. Cryopreservation of boar sperm

Standardised, efficient *in vitro* production of porcine embryos requires high-quality frozen sperm suitable for IVF [176, 229] to eliminate inter-ejaculate variability and improve IVF consistency [176]. However, only sperm from a minority of "good freezer" boars [228] is suitable for IVF after cryopreservation [151, 343]. The high sensitivity of pig sperm to oxidative stress, temperature fluctuation, osmolarity and pH-value [230, 231] make its cryopreservation challenging and commercially unappealing [236]. Therefore, good quality frozen sperm for IVF is scarce. A growing demand for such sperm due to the increasing popularity of porcine disease models in combination with improved cryopreservation methods might help to alleviate this shortage in the future.

During this project cryopreservation of sperm was performed using a controlled-rate freezer. This method minimises ice crystal formation which improves sperm survival rates [354]. The resulting average post-thaw motility rate of 42% lies right in the middle of the broad 20-60% range described by literature [228, 355]. Some ejaculates were already characterised by low motility prior to cryopreservation which has a negative impact on post-thaw survival rates [356]. The large variability in sperm function after freezing can be explained by male-to-male variability [235] and has even been described between ejaculates from the same boar [236]. Artificial insemination of three sows with cryopreserved sperm from boar #10261 resulted in three pregnancies. This suggests that the quality of this batch of frozen semen is sufficient for the re-derivation of GE pig lines.

Post-thaw motility is a suitable parameter to predict boar sperm fertilisation competence during artificial insemination (AI) [357] but there is only a weak correlation to the rate of monospermic fertilisation in IVF [236, 356]. Therefore, it is an inadequate indicator for the suitability of cryo-conserved boar sperm for IVF which can only be assessed by measuring the rate of monospermic fertilisation [358].

The same cryopreservation protocol was used to build a sperm bank (shown in table 39) for GE pig lines to prevent the loss of genetic information due to infection or injury-related death of valuable boars. A plausible threat is African Swine Fever Virus which is present in both neighbour countries France and Poland at the time of writing [359, 360]. Frozen sperm proven suitable for IVF can be shipped to other laboratories together with the optimised protocols to

facilitate consistent IVF experiments there. Moreover, frozen sperm from GE boars can be delivered to other research institutes and used for crossbreeding via AI.

In summary, the sperm freezing system established during this thesis promotes post thaw motility rates similar to other publications [228, 355]. It was successfully used to freeze sperm from breeding boars for IVF and establish a sperm bank for GE pig lines.

4.2. Genome engineering in IVP embryos

Microinjection has been a foundational technology in manipulating mammalian genomes since the creation of one of the first genetically modified animals in 1974 but it only allowed for random transgene addition [33]. The emergence of nuclease-based genome engineering technology has expanded the potential of microinjection by contributing the ability to efficiently introduce targeted modifications during embryogenesis [361]. Here, genome engineering was performed directly in early embryos by delivering several GE vectors and the constituents of a transposon system to porcine zygotes via microinjection.

Microinjection facilitates efficient delivery of genome engineering components but it also has a negative impact on embryo development [319]. A decrease of 15.84% in cleavage rate and 10% in blastocyst development rate was observed in microinjected IVF embryos compared to the non-injected control group. Similar adverse effects were seen in parthenogenetic embryos which can be explained through the cellular damage caused by microinjection itself [362]. EGFP mRNA was used to visualize successful delivery of the injection solution. Therefore, the cytotoxicity and immunogenicity of GFP is another possible explanation [363].

Besides its adverse effect on embryo development microinjection of site specific nucleases also frequently leads to mosaicism which is especially problematic in pigs due to the long generation interval [85]. In rodents the incidence of mosaicism lies between 20-70% [364, 365] whereas the rate of mosaicism in pigs is reported at approximately 10-20% [124, 136]. Differences in embryo development, timing of microinjection and varying efficiencies of the genome engineering components used in each study are plausible explanations for this disparity [366].

The degree of mosaicism in pigs created during this project has not been thoroughly analysed at the time of writing but preliminary data revealed mosaicism in at least four pigs. Increasing the concentrations of CRISPR components reduces mosaicism but it diminishes embryonic viability at the same time [367]. Another approach is to make Cas9 protein less persistent by tagging it with an ubiquitin-proteasome degradation signal [368] or to use multiple sgRNAs targeting the same gene [369]. Injection of Cas9 protein instead of plasmids or Cas9 RNA was shown to reduce mosaicism [370]. Timing of the microinjection is another critical factor. Here, microinjection was performed right after the IVF protocol which takes 7 hours to complete.

This is before the time frame of pronucleus formation at 12-15h after fertilisation where genome replication reportedly takes place [371].

In this thesis GE was performed using DNA GE vectors coding for the specific sgRNA and Cas9 protein. Other possible options include delivery of the CRISPR/Cas9 components as Cas9 protein or mRNA together with the sgRNA. These approaches don't require transcription and translation to facilitate genome engineering which can reduce the rate of mosaicism [127, 372]. They also eliminate the risk for unwanted integration of the DNA vector into the host genome but are more labour intense to prepare [303].

A concern regarding CRISPR/Cas9 technology in general is the potential for off-target cleavage [373]. Such unintended DSBs occur at sites that differ by up to 5 bases from the target sequence and can result in adverse phenotypic consequences [374]. Detection of off-target mutations is hampered by the limited usefulness of in *silico tools* that predict possible off-target sites based on their similarity to the target sequence [375]. Whole genome sequencing and another approach termed genome-wide off-target analysis by two-cell embryo injection report the frequency of off-target effects caused by the CRISPR/Cas9 system to be relatively low [312, 376]. However, there are strategies to further improve the specificity of genome engineering such as pairing two Nickases [310], "Base Editing" [311] and "Prime Editing" which uses a catalytically inactive Cas9 connected to a reverse transcriptase enzyme [314].

During *in vitro* testing of NANOS2 guide RNAs indel mutations in 70% of all parthenogenetic and 66.6% of IVF embryos and a blastocyst rate of 26% and 12% respectively was obtained. This data shows the potential of this approach to efficiently knock out specific genes but it also highlights the detrimental effects microinjection and potentially cytotoxic GE vectors have on embryo development. Most publications describe knockout efficiencies in the 60 - 70% range [123, 125], similar to what we observed here while some report efficiencies up to 100% [137]. Variations in the effectiveness of CRISPR/Cas9 mediated genome engineering when targeting different genomic loci caused by chromatin state and secondary structure of gRNAs are plausible explanations for this variance [377].

In vitro testing of the porcine UCP1 guide RNAs plus donor DNA for the human UCP1 gene resulted in correct integration of the human sequence in 57% of the blastocysts. This shows that targeted knock-ins are possible with this approach which is also supported by literature [133].

GE knock-in pigs have been generated using CRISPR/Cas9 plus DNA donor templates but not many groups have been able to replicate this feat [378]. Targeting of embryos through HDR [129], especially the introduction of large insertions remains challenging [134]. This is exasperated by the high cytotoxicity of double-stranded DNA donor templates [379] which could explain the low blastocyst rate of 4.4% and futile attempts at establish a pregnancy. Single-stranded donor templates can be a solution for this problem because they are less cytotoxic [379]. HDR efficiency could further be increased by inhibiting the more frequent non-homologous end joining (NHEJ) pathway [380] or by taking advantage of the open chromatin structure during G2 phase by performing microinjection at the two-cell stage [381].

Several groups have reported targeted multiplex genome engineering directly in early embryos by using multiple sgRNAs [119, 125]. Here, this strategy was tested in vitro and then applied to generate a pig carrying multiple knockouts from *in vitro* derived oocytes (discussed in 4.3). However, the intended homozygous modifications could only be verified in two (20%) embryos. The rest carried no modifications which highlights the limitations of this approach.

Pronuclear microinjection, SCNT using transgenic donor cells, ESC mediated gene transfer or viral-based based approaches are the predominant methods for the generation of transgenic animals [382]. However, pronuclear microinjection and SCNT show low efficiencies in livestock and viral transgenesis is hampered by biosafety considerations and limited transgene size [13, 22, 57]. True porcine ESCs that meet the strict array of criteria for pluripotency have not yet been established [383]. However, the recent derivation of porcine expanded potential stem cells (EPSCs) that express key pluripotency genes, differentiate to all three germ layers in chimeras and produce germ cell-like cells *in vitro* is promising [384].

Transposon systems have the ability to efficiently integrate large transgenes into a host genome but unlike lentiviruses they are not capable of traversing the cell membrane [78]. Here, cytoplasmic injection of a PiggyBac (PB) transposon DNA vector plus PB Transposase mRNA into porcine zygotes was evaluated as a method for transgenesis. A similar experiment was conducted by Li *et al.* using a single DNA vector containing all the transpositional elements necessary for transgenesis [75]. We observed blastocyst development of 42% in the injected group compared to 50% in the non-injected control group which compares favourably to the 12-27% range reported by Li *et al.* [75]. However, the proportion of transgenic embryos was lower here (43%) compared to the other study (53%) which might be explained by differences

in plasmid concentration. Both experiments lead to the conclusion that microinjecting the components of a transposon system into the cytoplasm of porcine zygotes is an efficient method for the generation of transgenic porcine embryos.

In summary, microinjection of DNA GE vectors into the cytoplasm of *in vitro* derived porcine zygotes is a suitable and effective method for the generation of GE embryos. By combining this method with a transposon system even large transgenes can be efficiently introduced into the host genome. The biggest limitations to this approach are the difficulty of introducing large, targeted insertions via HDR [134], mosaicism [85], the potential for off-target mutations [373] and the requirement for large numbers of high-quality zygotes [135]. Due to its simplicity and efficiency genome engineering directly in porcine embryos is a potent addition to the toolbox for the generation of GE pigs despite these drawbacks.

4.3. Generation of porcine models for biomedicine

In this thesis *in vitro* embryo production was combined with cytoplasmic microinjection of GE vectors and the components of a transposon system to “fast-track” the generation of porcine models for biomedical research.

Twenty-two embryo transfers were carried out with eleven of them resulting in pregnancies (50.0%). This is comparable to the 50-80% range reported by literature for *in vivo* derived zygotes [214, 385]. However, reports of genetically modified pigs generated from entirely *in vitro* derived pig embryos are a more suitable reference group. To date, there have been very few such publications but they report similar efficiencies as observed here [124, 162, 165]. Embryo quality is important but the selection of recipients also has a major impact on pregnancy rates [386]. Prepubertal gilts were used as recipients during this thesis but use of sows is preferable due to better endocrine and uterine development [386]. Fourteen out of twenty-nine pigs (45%) generated in this project carried genetic modifications. This proportion is similar to other reports using microinjection [123, 125].

The average number of piglets obtained from each pregnancy (5.8) is higher than commonly reported for similar IVF procedures (3.8) but the litter size previously reported for cytokine supplemented maturation media (8.6) could not be replicated [177]. This number is highly influenced by the genetic modification, number of embryos transferred, breed and age of surrogate pigs [97]. Porkers were used for most embryo transfers which might have negatively affected pregnancy rates [387] due to their comparably lower fertility compared to German Landrace or other breeds selected for fertility [388]. Other factors that adversely influence fertility such as high temperature, infectious diseases [389] and low quality of feed [390] were controlled and can therefore be ruled out. Here foetal resorption was observed in three out of eleven pregnancies but this is a common physiological occurrence in pigs, especially during the early stages of pregnancy [391].

The pregnancy rates for surgical and endoscopic embryo transfer were very similar. This was expected because both methods are reportedly equally efficient [392, 393]. The endoscopic procedure is less invasive [86, 394] but is less suitable for bicornual transfer of zona-free embryos generated by HMC [105].

Animals generated by microinjection of site-specific nucleases don't have the characteristic developmental defects [85] caused by deficient epigenetic reprogramming frequently observed in animals generated by SCNT [395]. None of the 29 pigs that were generated showed obvious developmental abnormalities at birth other than the phenotype caused by the intended genetic modification.

Recently a new method termed genome editing via oviductal nucleic acids delivery (GONAD) has been shown to facilitate *in vivo* genome editing of preimplantation embryos in mice [396]. This approach still has low efficiencies and high rates of mosaicism [397]. However, if those problems can be overcome GONAD has the potential to be the simplest method of gene delivery to embryos, eliminating isolation, handling, culture, manipulation of embryos and embryo transfer. This would be especially beneficial in species such as pigs where those steps are difficult [31].

4.3.1. Porcine model for Crohn's Disease

In this thesis seven pigs with an excision of the $TNF^{\Delta ARE}$ sequence were generated. Due to their systemically elevated TNF-alpha levels these pigs are a potential model for Crohn's Disease, uveitis or rheumatoid arthritis [326].

This project required the excision of a single DNA sequence using two gRNAs which can be efficiently performed using CRISPR/Cas9 technology [398]. Here this was achieved by microinjection of a GE vector coding for two gRNAs simultaneously into the cytoplasm of porcine zygotes. Similar excisions directly in porcine zygotes have been reported by other groups but all of then used RNA-protein complexes for this task [123, 137]. Furthermore, with few exceptions all of them were conducted using *in vivo* derived oocytes [85, 124].

The observations made here are in accordance with the consensus in the literature that genome engineering by injection of DNA into the cytoplasm of porcine IVP embryos is an effective method for the excision of DNA fragments in pigs that avoids many of the drawbacks of nuclear transfer [12, 85, 218, 399].

4.3.2. Porcine Hepatitis model

Two pigs with indel mutations in the NTCP gene were generated in this project but the original goal of replacing the porcine with the human NTCP sequence could not be met. The pigs generated here are of little utility to study HBF infection but they could be useful to study the function of the NTCP receptor.

CRISPR-based strategies have been used to create targeted insertions via one-step delivery directly to zygotes but overall this strategy is inefficient [129]. Especially the targeted introduction of large insertions is difficult [134]. A limited number of knock-in mice has been created with this approach [1, 400] but publications in pigs are scarce [133]. The concentration of CRISPR/Cas9 components influences insertion efficiency but other parameters are largely unknown [401]. A targeting strategy that combines CRISPR RNP complexes with long (~1600 bp) ssDNA donor templates was shown to increase the efficiency of targeted DNA cassette insertions in mouse zygotes [402].

The cytotoxicity of single-stranded DNA templates as used here is lower than that of double-stranded DNA donor templates [379] but it could still have affected litter size as only three piglets were born. This is consistent with the comparably low number of blastocysts that were obtained during *in vitro* testing of the NTCP and UCP1 targeting vectors plus DNA donor templates.

In summary, the targeted insertion of DNA fragments by homologous recombination directly in zygotes is possible but inefficient. Therefore, introducing the desired insertion into somatic cells followed by SCNT remains a more efficient approach to produce transgenic pigs.

4.3.3. Simultaneous GE of multiple genes relevant for xenotransplantation

One GGTA1/B4GALNT2-double knockout pig was generated during this thesis but no indel mutations in the CMAH and SLA class I genes could be observed. Editing of multiple genes was successful but the goal of producing pigs in which all four xenoreactive antigen genes had been inactivated could not be met.

CRISPR/Cas9 technology can be used to edit multiple genes simultaneously by encoding multiple guide sequences into a single CRISPR array [403]. This approach has been

implemented directly in zygotes to generate multi-knockout mice [119] and rabbits [404] but not in pigs. These results show that pigs with multiple different modifications can be generated in one step but not all target genes could be inactivated here. Parallel experiments in which porcine somatic cells were edited, selected for the inactivation of all four genes and then used for SCNT led to the production of viable pigs [405].

Targeting of multiple genes directly in zygotes is challenging because every single incidence of genome editing is a separate stochastic event. Donor cells for SCNT can be submitted to antibiotic selection and screened to make sure they carry all intended modifications simultaneously which is difficult in early embryos [303]. Pre-implantation embryo biopsies could be used to detect CRISPR/Cas9 induced mutations and select only correctly edited embryos [406]. However, the procedure is labour and time intensive which makes screening large numbers of embryos impractical.

Selection of efficient gRNAs is especially important when targeting multiple genes to maximise the probability of all modifications occurring in the same cell. Targeting efficiency and the frequency of off-target cleavage is influenced by the length of the gRNA sequence [407, 408]. The gRNAs used here had previously been tested and applied in cell culture followed by SCNT to generate pigs carrying all four desired knockouts simultaneously [405].

In summary, these observations indicate that knocking out multiple genes simultaneously directly in porcine zygotes is possible but challenging. Production of donor cells carrying intended modification followed by SCNT remains the method of choice to generate pigs with multiple genetic modifications.

4.3.4. Porcine model for pancreatic cancer

Three transgenic mPdx1-iCre pigs were generated in this project. The generation of transgenic pigs by cytoplasmic microinjection of transposons has been reported by several groups but all of them used *in vivo* derived porcine zygotes [75, 76, 409]. There have been previous attempts to apply this approach to *in vitro* derived porcine embryos but both publications conclude that the quality of *in vitro* derived porcine embryos is insufficient [75, 409]. Here, the components of the transposon system were injected into *in vitro* derived zygotes and ten piglets could be obtained from one pregnancy, three of them transgenic. This compares favourably with

pronuclear DNA microinjection where the proportion of transgenic animals remains below 1% for livestock species [37]. A higher concentration of transposon vector or transposase mRNA might increase the proportion of transgenic animals but this also reduces embryo viability [75].

It remains to be seen if the mPdx1-iCre pigs actually express Cre-recombinase specifically in the pancreas and if they develop a disease phenotype after crossbreeding with the $KRAS^{G12D}$ and $TP53^{R167H}$ lines.

In summary, cytoplasmic injection of transposons is an efficient method for the generation of transgenic pigs from *in vitro* derived porcine zygotes.

4.4. Handmade cloning

The final objective of this thesis was to establish handmade cloning as an alternative to traditional cloning (TC). The only change in mammalian SCNT technology since it was first published in 1984 [87] is the use of somatic cells instead of blastomeres as donor cells [89]. HMC technology is a radical simplification of SCNT that requires only minimal equipment in form of a stereomicroscope and a fusion machine. This greatly reduces the required investment to transform an IVF laboratory into a cloning facility.

HMC is in theory a simple, easy-to-learn and time efficient technique which is crucial as time spent outside the incubator adversely affects embryo quality [108]. An experienced operator can produce 30-50 transferrable blastocysts per workday [410]. This is enough for one embryo transfer into pigs but reaching this level of productivity requires several months of intensive practice [107]. Besides experience in handling porcine embryos the first reconstructed embryos could be produced within three to four hours of HMC but speed should improve with practise.

The relevant performance variables of HMC match or exceed those of TC. Pregnancy rates of ~ 50% have been reported using cloned, zona-free embryos in pigs [166], cattle [411], horses [412] and mice [413]. Zona-free embryos overcome problems related to hatching which favourably impacts litter size [410]. The greatest litter (ten piglets) and highest number of pigs per transferred embryo (22%) from SCNT have been generated by HMC. Sample size is too low to draw definitive conclusions but pregnancy and farrowing rates are at least identical with those reported after TC [30]. HMC has potential for automation using microchannel technology which could enable large-scale standardised production of cloned embryos [108].

One disadvantage of HMC is the tendency of zona-free embryos to attach to each other. Their separation is time intensive and can result in losses but with proper handling this problem can be minimised [410]. Removal of the zona pellucida increases the potential for disease transmission but the zona is not intact in TC either which equalises this theoretical risk for both approaches. HMC introduces mitochondria from three different animals into one individual but no experimental or practical disadvantages of this heteroplasmy have been reported so far [109, 414].

HMC requires more oocytes than TC as two cytoplasts are required for every single reconstructed embryo. This inefficiency is more than compensated for by the positive effect of the bigger volume of cytoplasm on the efficiency of all further steps like enucleation, fusion and blastocyst development [415]. In fact, several reports suggest that the quality of cloned embryos and cloning efficiency is better in HMC compared to TC [98, 416]. A reliable IVM system such as the one optimised during this thesis makes the higher requirement for oocytes even less of a practical consideration.

In summary, HMC is a simple and efficient alternative to TC that decreases costs while possibly increasing productivity. Reconstructed embryos were successfully generated during this thesis. Their number is still insufficient for embryo transfer but with additional practice the generation of adequate numbers of embryos is highly feasible.

5. CONCLUSION AND OUTLOOK

As part of this thesis systems for porcine embryo IVP and direct manipulation of porcine zygotes were optimised. The focus of this work was to overcome the inefficiency of porcine IVF to facilitate the generation of porcine models for biomedicine.

Genome engineering is essential to realise the full potential of pigs, both as livestock and as animal models for biomedicine. SCNT and direct manipulation of zygotes are the prevalent methods for the generation of GE pigs. CRISPR/Cas9 mediated genome engineering directly in early embryos is a convenient and efficient method that excels at introducing indels via NHEJ. Cytoplasmic injection of transposons is an efficient method for transgenesis. SCNT and its simpler version HMC facilitate pre-screening of donor cells for the intended modification which makes them a more suitable alternative for targeting several genes simultaneously or introducing DNA fragments into the genome via HDR. The individual strengths and weaknesses of these approaches complement each other well and together they provide an efficient toolbox for the generation of GE pigs. The $TNF^{\Delta ARE}$ pigs generated during this thesis will find application as a potential disease model for Crohn‘s Disease. This line will be bred with the mutant APC^{1311} line available at our chair to investigate the interaction between inflammation and colorectal cancer. The transgenic mPdx1-iCre pigs will be crossbred with the $KRAS^{G12D}$ and $TP53^{R167H}$ line to generate a potential porcine model for pancreatic cancer.

Polyspermy remains an unsolved problem but optimised IVM protocols, sperm selection and optimisation of sperm to oocyte ratios can greatly reduce its incidence. *In vitro* production of porcine embryos and cryopreservation of sperm will continue to be improved. This increases the efficiency of both SCNT and genome engineering in zygotes thereby benefiting agriculture and biomedical research. Despite all progress the problem of polyspermy in IVF could remain the limiting factor for the generation of GE pigs for the foreseeable future. Establishment of porcine pluripotent stem cells would be a big step to make the production of GE pigs more efficient. Electroporation of porcine zygotes could render microinjection obsolete and make high-throughput genome engineering in livestock a reality. Ultimately, the IVP of embryos could be replaced altogether by *in vivo* electroporation of porcine zygotes directly in the maternal oviduct.

6. ABBREVIATIONS

∞	infinitely
AAV	Adeno associated viruses
AI	Artificial insemination
Ala	Alanine
Ampho B	Amphotericin B
ART	Assisted reproductive techniques
Bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
CCCs	Cytoplasm-cell-complexes
cFM	Bovine fusion medium
COCs	Cumulus oocyte complexes
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR/CAS 9	Clustered regularly interspaced short palindromic repeats / Cas9
crRNA	CRISPR-RNA
D-PBS	Dulbecco's phosphate buffered saline
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
DSB	Double-strand break
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EPSC	Expanded potential stem cells
ESC	Embryonic stem cell
ET	Embryo transfer
EtOH	Ethanol
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FLI	FGF2 LIF IGF
GE	Genome engineered

Gln	Glutamine
GSH	Glutathione
HBV	Hepatitis B virus
HDR	Homology-directed repair
HMC	Handmade cloning
HR	Homologous recombination
HR	Homologous recombination
iCre	Codon-improved Cre recombinase
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
ITR	Inverted terminal repeat
IVC	*In vitro* culture
IVF	In vitro fertilisation
IVP	*In vitro* production
LIF	Leukaemia inhibitory factor
MII-phase	Metaphase II
MPdx1	mouse pancreas duodenum homeobox-1
MPN	Male pronucleus formation
mRNA	Messenger ribonucleic acid
NEAA	Non-essential amino acid
NHEJ	Non-homologous end joining
NPC	Nuclear pore complex
NTCP	Natrium-taurocholate co-transporting polypeptide
OF	Oviductal fluid
PB	PiggyBac
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFF	Porcine follicular fluid
pFM	Porcine fusion medium
PFM	Porcine fertilisation medium
PHA	Phytohemagglutinin
PKDNF	Porcine kidney fibroblasts
Pro	Pronase
PVA	Polyvinyl alcohol
PZM5	Porcine zygote medium 5
RNP	RNA-protein complexes

RT	Room temperature
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SMGT	Sperm mediated gene transfer
SPF	Specific-pathogen-free
SSC	Spermatogonial stem cell
ssODN	Single stranded oligonucleotide
T10	TCM 199, hepes modification supplemented with 10% FCS
T2	TCM 199, hepes modification supplemented with 2% FCS
T20	TCM 199, hepes modification supplemented with 20% FCS
TAE	Tris-acetate-EDTA-buffer
TALEN	Transcription activator-like effector nuclease
TBE	Tris-borate-EDTA-buffer
TC	Traditional micromanipulator-based cloning
TCM 199	Tissue culture medium 199
TE	Transposable element
tracrRNA	Trans-activating CRISPR RNA
WM	Working medium
ZFN	Zinc finger nuclease
ZP	Zona pellucida

7. LIST OF TABLES

8. LIST OF FIGURES

9. LITERATURE

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