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Genome editing in livestock-optimisation of porcine embryo culture condition for direct zygotic manipulation

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Abstract

Genome editing tools based on the transient activity of programmable nucleases like zinc finger nucleases (ZNF), Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein-9 (Cas 9) promise to immensely increase the efficiency and, in parallel, to reduce the costs for the generation of genetically engineered pigs. However, the progress has been relatively slow. A persistent bottleneck for porcine genome editing is the lack of reliable supply of competent porcine embryos, which are suitable for direct micromanipulation experiments. In this study an efficient and easily reproducible *in vitro* culture system for porcine embryos meant for direct zygote microinjection was established. An *in vitro* maturation system that support very high rate of nuclear maturation (86.48%) was optimised. The developmental competence of those *in vitro* matured oocytes was corroborated by high rate (83%) of parthenogenetic activation. A relatively high *In vitro* fertilisation rate (80.2%) was achieved. However, the incidence of polyspermy was also high. By injecting CRISPR/Cas 9 targeting the exon 10 region of *CMAH* and exon 8 of *GGTA* 1gene into IVF produced embryos successful gene editing was achieved. The results indicated 64% homozygous knockout and 14% heterozygous knockout for *CMAH*, while for *GGTA*1 30% knockouts were homozygous and 10% were heterozygous. 20% of the embryos were double knockouts. 79.5% of the injected zero stage embryos developed up to the morula stage.

With the objective of further improving the selection of good quality porcine oocytes for *In vitro* embryo production, the usefulness of a novel dye lissamine green B (LB) for non-invasive prediction of maturational competence of porcine oocytes was

evaluated. It was seen that LB staining can give valuable information about the quality of oocytes in terms of maturational ability, developmental potential and extent of DNA damage.

In vitro production of embryo is heavily dependent on supply of slaughterhouse ovaries. From these slaughterhouse obtained ovaries, for *in vitro* culture, porcine oocytes are isolated from a cohort of dominant and subordinate follicles, most commonly by aspiration. As a result, it leads to a batch-to-batch variability during *in vitro* embryo production. At present there is no way of identifying those follicles with the best quality oocytes. Therefore, in order to improve the overall success rate of porcine IVF, it is very important to develop newer methods to characterise the porcine ovarian follicle used for oocyte collection. With this in mind, a novel combination of clearing and imaging of porcine ovarian follicles with single plane illumination microscopy (SPIM) was developed. Using the method, follicles of all developmental stages were identified and their diameters were determined, ranging from small primordial follicles of 70 µm up to 2.5 mm graafian follicles. Continuing with the same objective, supravital dye brilliant cresyl blue (BCB) was explored as a nontoxic contrast medium for imaging porcine ovarian follicle with multi spectral optoacoustic tomography (MSOT). The results have provided the proof of principle that BCB can be used as a contrast medium and when coupled with MSOT a really simple, gentle and very efficient imaging method can be developed.

Although, the gene targeted embryos, produced during the experiment, were not subsequently used for embryo transfer the results of this exploratory study showed that if transferred into surrogate mother there is the possibility of producing transgenic animal using the current protocol. With minor optimisation of current protocol a robust system for porcine genome editing can be developed.

Zusammenfassung

Werkzeuge für das Genome Editing, die auf der transienten Aktivität von programmierbaren Nukleasen wie zinc finger nuclease (ZNF), Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein-9(Cas 9) basieren, haben das Potential, zum einen enorm die Effizienz zu steigern, mit der genetisch veränderte Schweine hergestellt werden können, und zum anderen gleichzeitig die dafür nötigen Kosten zu senken. Allerdings machen diese Technologien nur langsam Fortschritte; eine der Engpässe ist dabei der Mangel an verlässlichen Quellen für kompetente Schweineembryonen, die für direkte Mikromanipulations-Experimente geeignet sind. Im Rahmen der vorliegenden Studie wurd ein effizientes und leicht reproduzierbares In Vitro-Kultivierungssystem für Schweineembryonen, die für direkte Zygoten-Mikroinjektion bestimmt sind, etabliert. Ein IVM-System, welches sehr hohe Raten an Kernreifung (86.48%) ermöglicht, wurde optimiert. Die Entwicklungsfähigkeit dieser in vitro gereiften Oozyten wurde durch hohe Raten an parthogener Aktivität (83%) bestätigt. Ebenso wurde eine relative hohe Rate an in vitro-Befruchtung beobachtet (80.2%). Allerdings trat auch gehäuft Polyspermie auf. Indem CRISPR/Cas9, welche auf Exon 10 von *CMAH* und Exon 8 von *GGTA1* gerichtet waren, in IVF produzierte Embryonen injiziert wurden, wurde erfolgreich Gene Targeting erreicht. Die Ergebnisse deuten 64% an homozytogen Knock-Outs und 14% an heterozygoten Knock-Outs für *CMAH* Exon 10 an. Für *GGTA1* Exon 8 waren 30% der Knock-outs homozygot und 10% heterozygot. 20% der Embryos trugen einen doppelten Knock-Out. 79.5% der injizierten Embryos im Nullstadium entwickelten sich bis zum Morula-Stadium.

Mit dem Ziel, die Selektion von guten Schweine-Oozyten für die in vitro Embryoproduktion weiter voranzutreiben wurde der Nutzen des neuen Farbstoffs

Lissamine Green B (LB) für die nicht-invasive Vorhersage der Entwicklungskompetenz von Schweine-Oozyten beurteilt. Es wurde gezeigt, dass LB-Färbung nützliche Informationen über die Qualität der Oozyten im Hinblick auf Maturationsfähigkeit, Entwicklungspotential und Umfang der DNA-Schädigung liefern kann.

In vitro Herstellung von Embryonen hängt stark vom Nachschub an Eierstöcken aus dem Schlachthaus ab. Von den Eierstöcken, die vom Schlachthaus kommen, werden für die *in vitro* Kultivierung Schweine-Oozyten aus einer Kohorte von Haupt- und Nebenfollikeln isoliert, zumeist mittels Aspiration. Dies führt im Ergebnis zu einer Variabilität bei der *in vitro* Embryonenherstellung zwischen den einzelnen Chargen. Zum gegenwärtigen Zeitpunkt gibt es keine Möglichkeit, die Follikel mit den Oozyten der höchsten Qualität zu identifizieren. Um die Erfolgsrate für porcine IVF insgesamt zu verbessern, ist es deswegen von herausragender Wichtigkeit, neue Methoden für die Charakterisierung von porcinen Eierstockfollikeln, die für die Oozyten-Gewinnung verwendet werden, zu entwickeln.

Mit diesem Ziel vor Augen wurde eine neuartige Kombination zur Sortierung und zum Imaging von porcinen Eierstockfollikeln mit Hilfe von Single Plane Illumination-Mikroskopie (SPIM) entwickelt. Mithilfe dieser Methode wurde Follikel aller Entwicklungsstufen identifiziert und deren Durchmesser bestimmt, wobei dieser von 70µm bei kleinen Urfollikeln bis hin zu 2.5 mm bei Graaf'schen Follikeln reichte. Immer noch mit demselben Ziel wurde der supravitale Farbstoff Brilliant Cresylblau (BCB) als nichttoxisches Kontrastmittel für das Imaging von porcinen Eierstockfollikeln mit Hilfe von multispektraler optoakustischer Tomographie (MSOT) getestet. Die Ergebnisse erbringen den Nachweis, dass BCB als Kontrastmittel eingesetzt werden kann und dass bei gleichzeitiger Verwendung von MSOT eine sehr einfache, schonende und äußerst effiziente Imaging-Methode entwickelt werden kann.

Obwohl die genetisch modifizierten Embryonen, die während des Experiments produziert wurden, nicht weiter für den Embryotransfer genutzt wurden, zeigen die Ergebnisse dieser abklärenden Studie, dass es bei Transfer in eine Leihmutter die Möglichkeit gibt, mit Hilfe des jetzigen Protokolls transgene Tiere zu produzieren. Mit nur geringer Optimierung des jetzigen Protokolls kann somit ein robustes System für porcines Genome Editing entwickelt werden.

Chapter I

Introduction

General introduction

Genome editing directly in mammalian embryos is a powerful new technique that promises to radically streamline the genetic modification of large animal species such as pigs, and so provide exciting new resources for biomedical research (Meyer *et al*., 2011; Flisikowska *et al*., 2013). The genetically engineered animals such as transgenic (Tg) and knockout (KO) have been widely used for analysis of *in vivo* gene function and for models of human diseases. The recent advances in Tg and KO methods based on genome editing technologies [TALEN (transcription activator like effector nucleases), Zinc Finger Nuclease, CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat)] promise efficient and robust creation of such animals. In particular, the CRISPR/Cas9 system (Ran *et al*., 2013) is a significant advance that facilitates *in vivo* genome editing. Genome editing directly in mammalian embryos by CRISPR/Cas9 system is now being widely used as an alternative to traditional embryonic stem cell targeting-based KO production as ES cells are yet not available for most of the species. Recent reports have shown that direct zygote microinjection of CRISPR/Cas9 system can very efficiently (nearly 100% efficiency) generate genetically modified animals in one step (Whitworth *et al*., 2015; Hai *et al*., 2014). The CRISPR system can also be multiplexed to simultaneously mutate several genes (Platt *et al*., 2014). However, the tremendous potential of one-step production of KO large animals is yet to be realised because of the lack of a reliable supply of developmentally competent embryos. In case of large animals, developing *in vitro* produced (IVP) embryos have many bottlenecks including *in vitro* maturation (IVM) and the culture systems available are still unacceptably inefficient, especially in species such as pig (Grupen, 2014). Since the first reported successful birth

of a piglet derived by *in vitro* fertilisation (IVF) of porcine oocytes flushed from the reproductive tract almost three decades ago (Cheng *et al*.,1986), there have been numerous reports of live births in pigs by IVF (Grupen 2014), but obtaining *in vivo* matured oocytes still remains very inefficient, costly and time consuming (Nakai *et al*., 2003). Porcine IVP has not been fully developed because of several problems associated with different techniques. *In vitro* matured porcine oocytes have problems at several stages in the process. A good example of the problems encountered in growing pig embryos in culture is provided by the fact that only ~25% of in-vitro-produced (IVP) putative zygotes develop to the blastocyst stage (Stokes *et al*., 2005). The IVM oocytes have reduced ability to be fertilised *in vitro*, to block polyspermic penetration, to undergo normal pronuclear formation upon sperm penetration and culture conditions are not well suited for supporting embryo development beyond the initial cleavage divisions. Moreover, unlike cattle and other large animals, non-surgical ovum pick up is not practical in pigs. The major reason of this restriction is the anatomy of the porcine genital tract. Therefore, embryo collection can be performed *in vitro* by flushing the genital tract after slaughter, but this technique has the limit to use donor sows only once. Realisation of the unlimited potential of pig as a model animal in large animal transgenic research will only be possible if the problem of reliable supply of developmentally competent porcine embryos can be addressed effectively.

The following sections consider the current status and problems associated with porcine embryo culture. This is followed by a review of direct zygote microinjection, and different types of customisable nucleases and their application in the genetic modification of large animals.

1 Porcine embryo production

1.1 *In vitro* production of porcine embryo

In vitro embryo production (IVP) is a form of assisted reproductive technology (ART) that is used to increase the reproductive potential and the rate of genetic improvement of domestic animals. It is used to obtain farm animal embryos *in vitro* for commercial production, or for basic research, as well as for infertility treatment. In addition to increasing genetic gain IVP utilises the maximum potential of the ovarian pool of immature oocytes, thereby reducing reproductive wastage. Oocytes could technically be recovered *post mortem* from valuable females and subjected to IVM and IVF for the purpose of genetic rescue (Galli *et al*., 2003). By applying this technology to immature oocytes recovered from juvenile females, an increase in the rate of genetic gain could also be achieved which significantly reduces the generation interval. This technology has also been harnessed to increase the yield of embryos obtainable from genetically superior females (Cognié *et al*., 2004). In domestic animals, IVP is a methodology comprising of multiple steps namely: (a) *In vitro* maturation (IVM) of oocytes aspirated directly from ovarian follicles, (b) *In vitro* fertilisation (IVF) or co-incubation of capacitated spermatozoa with *in vitro* matured oocytes and (c) *In vitro* culture (IVC) of zygotes.

Porcine embryos are an excellent carrier to introduce a novel or altered genetic inheritance into individuals and herds. Since the first somatic cell nuclear transfer (SCNT) pigs were born, dozens of reports have described the production of transgenic cloned pigs, and literally hundreds of GM pigs have now been generated for xenotransplantation studies and to model human diseases (Kues and Niemann, 2004). The use of genetically engineered pigs for augmenting animal production also holds

great promise; however, these benefits are yet to be fulfilled. The need for good quality oocytes and embryos for GE pig production has driven much of the work on improving the effectiveness of porcine embryo IVP procedures (Gil *et al*., 2010). In addition, new gene editing tools, such as ZFNs, TALENs and CRISPR/Cas9, have facilitated precise manipulation of the pig genome. However, the problem of polyspermic fertilisation has still not been adequately resolved, and *in vitro* embryo culture conditions are still considered to be relatively suboptimal. The improvement in the techniques of porcine embryo production is expected to result in further increases in the efficiency of pig genome editing. The following timeline illustrates the relatively slow but continuing progress of porcine embryo production technology.

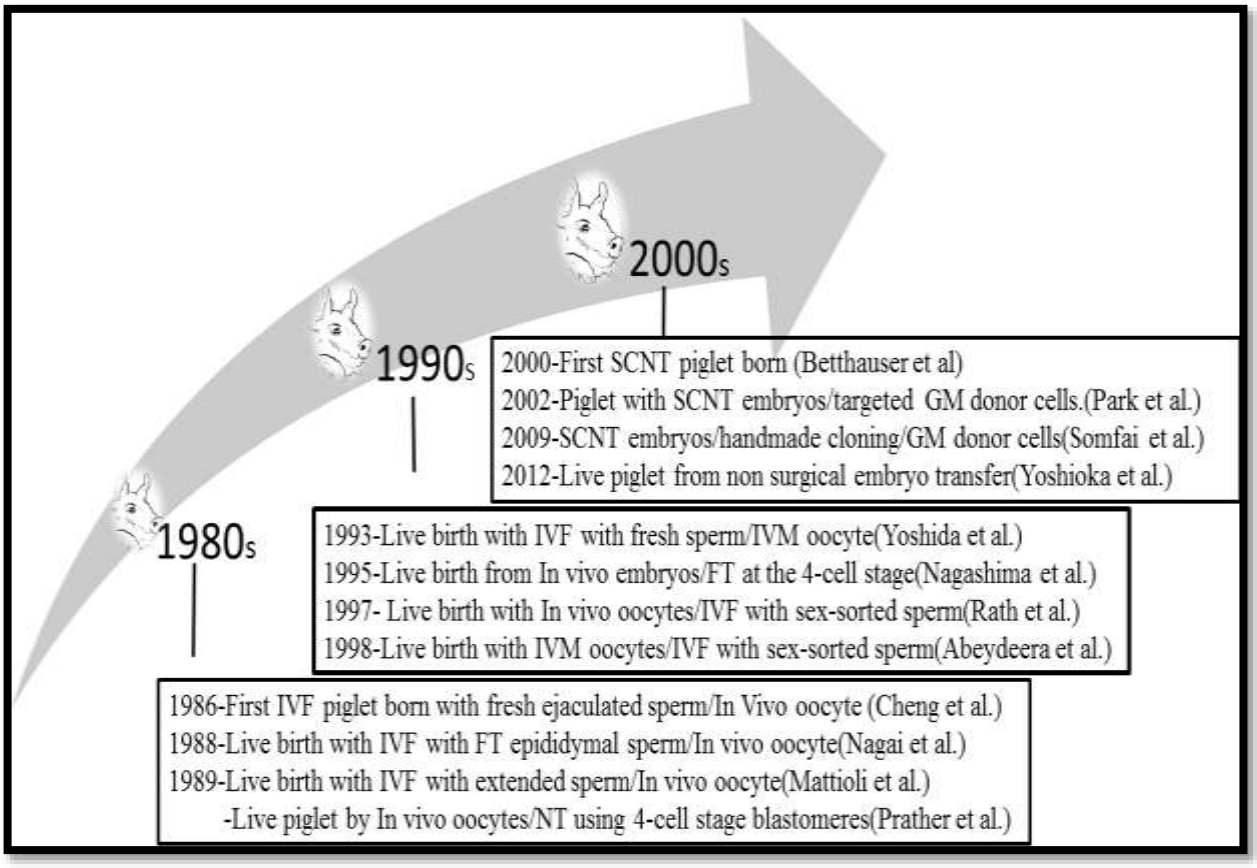

Fig 1.A time line of development of *in vitro* embryo production in porcine through the most significant milestones (1986-2012).

1.1.1 *In vitro* Maturation (IVM) of porcine oocytes

IVM refers to the maturation of immature oocytes in culture after their recovery from small antral follicles at the stage prior to selection and dominance. It is a lengthy process during which oocytes undergo transitions in nuclear status (Figure 2) and attains the competence to be fertilised and undergo embryogenesis (Hardy *et al*., 2000). Properties associated with each meiotic stage include cell size, permeability of the plasma membrane, presence or absence of a nuclear membrane, presence or absence of a spindle and chromosome configuration (Gosden and Bownes, 1995). The majorities of the oocytes in the ovary is not growing and are small and immature. At regular intervals, a number of them start to grow and mature, but of this cohort, only one gets ovulated, while the rest undergo atresia.

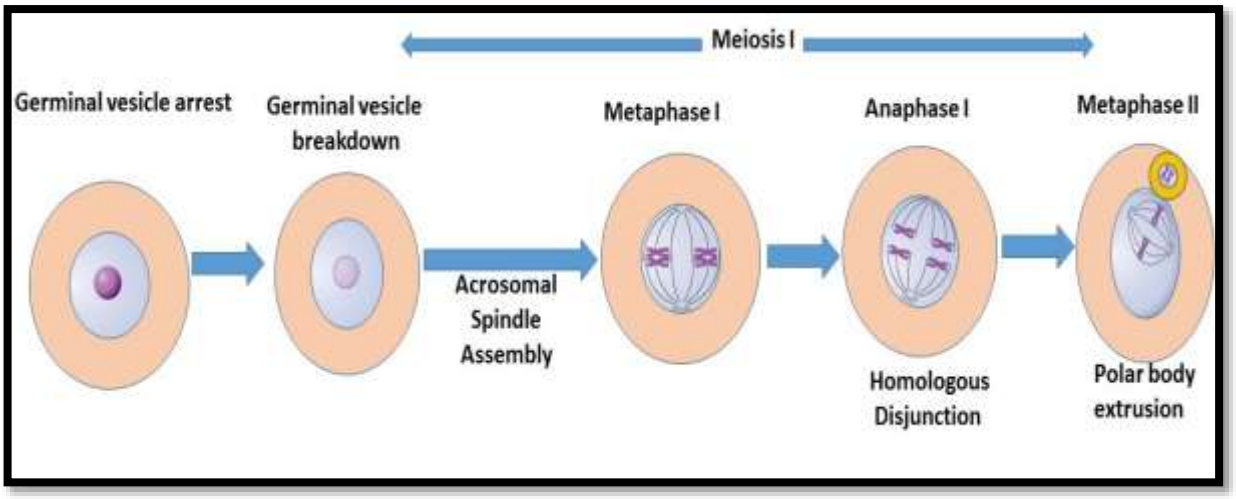

Fig 2. Nuclear changes during IVM. Mammalian oocytes arrested at the dictyate stage of prophase I are identifiable by the presence of a germinal vesicle (GV). Resumption of meiosis I is marked by GV breakdown (GVBD) following which bivalents are brought to alignment at the spindle equator by metaphase I. Anaphase I then begins when chromosomes segregate between the secondary oocyte and the polar body. Following first polar body extrusion (PBE), oocytes progress directly into meiosis II where they are arrested for a second time at metaphase II.

A large number of matured oocytes are needed when IVF is performed for generation of porcine zygote. It is important to be able to use IVM oocytes obtained

from slaughterhouse materials because of the cost and time required in the preparation of *in vivo* matured oocytes is prohibitive (Nakai *et al*., 2003). As mentioned earlier the procedure of ovum pick up is not technically feasible in pig. The most important problem associated with slaughterhouse ovaries is the inability to control batch-to-batch variation. Temperature is a fundamental variable in the cellular environment and porcine oocyte quality is known to be highly susceptible to temperature stress. Oocytes, especially porcine, are sensitive to transient temperature shock brought about by heating *in vivo* during slaughter (Yuge *et al*., 2003) or by cooling *in vitro* of slaughter-house-derived tissue (Tong *et al*., 2004). These stresses result in poor maturation and subsequent embryo development *in vitro*. However, if IVM materials are to be used, then success rates need to be improved by modification of the procedure. *In vitro* matured porcine oocytes have problems at several stages in the process. They have reduced ability to be fertilised *in vitro*, to block polyspermy, to undergo normal pronuclear formation upon sperm penetration (Abeydeera, 2002) and culture conditions are suboptimal for supporting embryo development beyond the initial cleavage divisions (Grupen 2014). Therefore, the most crucial factor for successful IVM of porcine oocytes is standardizing the culture condition that facilitates nuclear maturation proceeding to metaphase II stage. Evidence suggests that reduced ability of *in vitro* matured porcine oocyte to be fertilised by IVF is due to faulty interactions between the culture medium, cumulus cells and oocyte cytoplasm during IVM (Mattioli *et al*., 1988).

1.1.2 Nuclear and cytoplasmic maturation

In general, the oocyte maturation process can be broadly divided into two parts, nuclear and cytoplasmic maturation. Nuclear maturation is a term that refers to the resumption of meiosis and progression to the M II stage. Cytoplasmic maturation refers to other maturational events not directly related to meiotic processes but to other events

that prepare the oocyte for fertilisation and preimplantation development. In addition, relocation of cytoplasmic organelles, such as mitochondria and cortical granules (CG), also takes place during oocyte maturation (Moor *et al*., 1990). Although nuclear maturation seems to be completely established during IVM, the maturation of the cytoplasm is still defective. This is responsible, at least in part, for the frequent occurrence of polyspermy and the low developmental rates after IVF of IVM oocytes. The IVM conditions could cause incomplete movement of mitochondria to the inner cytoplasm of the oocytes and thus affect cytoplasmic maturation (Sun *et al*., 2001). Intracellular glutathione (GSH) content and the ability of cytoplasm to decondense the sperm nucleus or to induce male pronucleus formation have been used as indicators of cytoplasmic maturation. Cytoplasmic maturation and subsequent development of pig oocytes can be improved by the addition of cysteine, cysteamine, epidermal growth factor, glutamine, β mercaptoethanol, 9-cis retinoic acid and hormones (Grupen *et al*. 1995; Abeydeera *et al*. 1998; Day *et al*. 2000).

1.1.3 Culture medium for IVM

The most common media used for porcine maturation culture are NCSU-23, NCSU37 and TCM-199. Most of these maturation mediums are supplemented with porcine follicular fluid (pFF). The beneficial effect of the addition of pFF as the only protein supplement has been demonstrated in several studies (Yoshida *et al*., 1992; Vatzias and Hagen, 2004; Algriany *et al*., 2004). It was also hypothesised that pFF is critical in protecting oocytes from oxidative stress. However, the exact role of pFF in the maturation medium remains unclear (Cruz *et al*., 2014). Moreover, it is nearly impossible to control the batch-to-batch variability between the pFF.

1.1.4 Quality control of porcine oocytes

As with many mammalian species, porcine COCs are usually graded by visual assessment of morphological features such as the thickness and compactness of the cumulus investment, ooplasm homogeneity (Garg *et al.,* 2012) and the size of follicles (Hendriksen *et al.,* 2000, Hyttel *et al.,* 1997) or oocytes (Coticchio *et al*., 2004). Based on light microscopy, mammalian COCs surrounded by several layers of cumulus cells and with evenly granulated ooplasm have higher developmental competence in vitro than oocytes with irregularly granulated ooplasm and fewer cumulus layers (Sirrad *et al.,* 2006, Miyano *et al.,* 2007, Wang *et al.,*1997). Many other different selection criteria have also been reported (Gandhi *et al.,* 2001, Galeati *et al.,* 1991). However visual observation provides only limited information about its functional competence. Alternative methods of analysing cumulus cells are available, including evaluation of telomere length (Lee *et al*., 2001), cumulus cell apoptosis (Corn *et al*., 2005, Assou *et al.,*2010) and gene expression profiling using microarray analysis (Ruppert-Lingham *et al*., 2006), but are too time consuming and expensive for routine laboratory use. The cumulus layer is undoubtedly vital for oocyte development, for example it plays an important role in the distribution of cortical granules, and thus the ability to undergo sperm penetration (Cheng *et al.,* 2013). Propidium iodide staining has also been used as a non-invasive method of determining cumulus cell integrity (Uchikura *et al.,* 2011), but this requires fluorescent microscopy, which may not be available for some researchers and may be potentially mutagenic. Since oocyte grading based on morphological evaluations are subjective, and categorisation standards vary among investigators standard assays for quality control are needed for better standardisation of porcine embryo culture.

1.1.5 Selection of developmentally competent porcine oocytes

Another critical point of the maturation culture is the selection of the oocytes. Immature oocytes are generally recovered from ovaries of slaughtered animals, which results in a mixture of oocytes at different stages. Reports have shown that oocytes from large follicles (more than 5 mm in diameter) have more ability to develop into embryos than oocytes from small follicles (<3 mm in diameter) (Marchal *et al*., 2002a). However, in terms of efficiency, the diameter of follicles is difficult to control. Furthermore, after aspiration of the follicles, the oocytes are commonly selected using various criteria such as their morphology, including the numbers of cumulus cell layers and evaluation of the granulation of the cytoplasm. Those morphological evaluations are subjective, and categorisation standards vary among investigators. As an alternative, exposure of the oocytes to brilliant cresyl blue (BCB) has been suggested to select developmentally competent oocytes, according to differing colour (Roca *et al*., 1998; Wongsrikeao *et al*., 2006; Ishizaki *et al*., 2009). The BCB test permits assessment of the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesised in growing oocytes, but with decreased activity in oocytes that have finished their growth phase. BCB is a blue compound giving a blue colour to the cytoplasm of oocytes that have finished their growth, because G6PD activity decreases (Karami Shabankareh *et al*., 2014).

1.1.6 Porcine embryo culture medium

It has been demonstrated that during IVF, sperm penetration as well as polyspermic fertilisation begins at 2 h post insemination with frozen-thawed spermatozoa (Marchal *et al*., 2002b) and／or with fresh semen. Sperm penetration quickly induces the resumption of meiosis and the cortical reaction that blocks further

penetration of surplus spermatozoa (polyspermy). It has been previously demonstrated that any period of *in vitro* culture reduces the pig embryo's developmental potential particularly during the first 48 h after fertilisation (Beebe, 2013). Therefore, micro manipulated embryos are normally transferred at the one cell stage to minimise this effect. Hence, there is an unmet need for a pig embryo culture medium that minimises this loss of developmental potential and produces *in vitro* embryos of high developmental competence. Pig embryos can develop from the zygote to the blastocyst stage *in vitro*. Several media, such as modified Whitten medium (Beckmann and Day, 1993), North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993), modified Chatot, Ziomek, Bavister medium and Beltsville embryo culture medium (BECM)-3 (Dobrinsky *et al*., 1996), are available for the successful culture of embryos to the blastocyst stage. Out of all these media NCSU-23 was the first embryo culture media developed to specifically meet the metabolic and nutrient needs of the pig embryo was (Beebe, 2013). Although embryos cultured in NCSU-23 develop well up to the blastocyst stage they have a lower cell number (28 vs. 59) and embryos develop at rate that is a full cleavage division behind *in vivo* controls (Gajda, 2009). NCSU-23 benefits the embryonic protein metabolism. However, the lower incorporation of methionine into *in vitro* developed compared to *in vivo* derived blastocysts demonstrate that cultivation over a longer period has yet to be substantially improved (Yoshioka *et al*., 2003). But none of these above mentioned media were chemically defined which led to the common problem of lack of reproducibility. In 2002 Yoshioka *et al*., reported Porcine Zygote Medium (PZM) a chemically defined medium. A chemically defined medium eliminates undefined factors present in biological materials such as serum or serum albumin. Application of a chemically defined medium to IVP of embryos has certain advantages, for instance, it facilitates the analysis of the physical action of

substances on the development of preimplantation embryos, improves reliability of formulations, yields a higher reproducibility of results, and ensures biosafety of culture media by elimination of protein preparations, which may be contaminated with pathogens (Bavister, 1995). A successful porcine *in vitro* culture system depends on the interaction of several critical factors, including the culture medium and the quality of oocytes, as shown in fig 3.

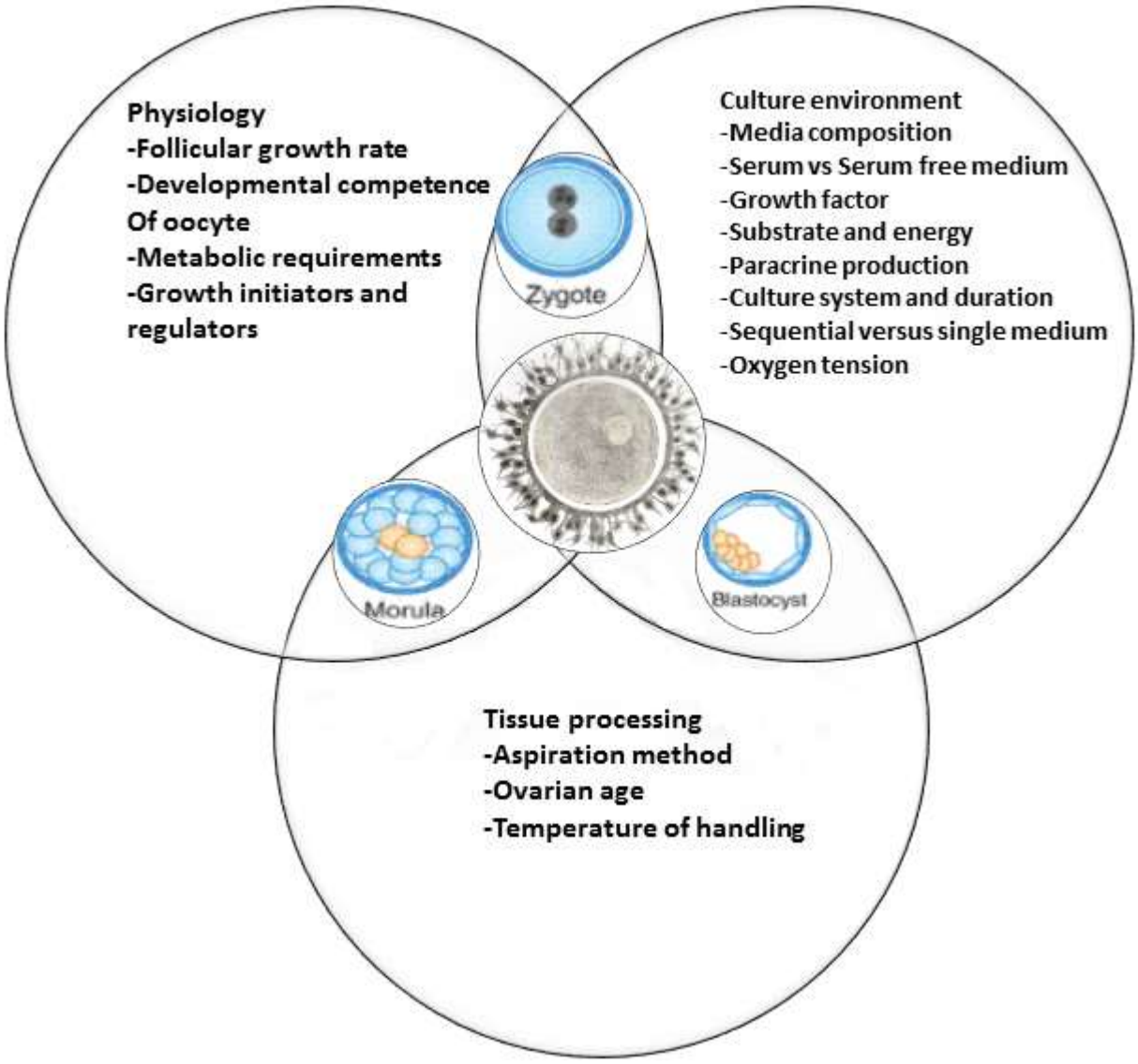

Fig 3. Summary of the critical determinants of the success of *in vitro* growth and maturation systems for mammalian oocytes. Adapted from H M Picton *et al*. Reproduction 2008; 136: 703-715.

1.1.7 Polyspermy and related problems in porcine IVF

Pig embryo production via IVM-IVF techniques has been hampered by two major problems, poor male pronucleus (MPN) formation and polyspermy (Abeydeera, 2002). Various refinements to IVM culture techniques, such as addition of follicular fluid, co-culture with follicular somatic cells, limited exposure to gonadotropins, and supplementation of EGF or cysteine, significantly improved cytoplasmic maturation as evidenced by higher MPN formation after sperm penetration (Abeydeera, 2001). Increased MPN formation caused by cysteine supplementation has been reported, and it is correlated with higher concentrations of intracellular GSH in matured oocytes (Yoshida, 1993). A similar effect on intracellular GSH level was also observed when oocytes were co-cultured with follicular somatic cells (Abeydeera *et al*., 1998). Synthesis of GSH during oocyte maturation is known to be a prerequisite for hamster sperm nuclear chromatin decondensation and successful MPN formation (Perreault *et al*., 1988). It is therefore, reasonable to assume that MPN formation is compromised in oocytes with lower levels of intracellular GSH.

Polyspermy is a persistent problem of IVF (Brüssow *et al*., 2000). The incidence of polyspermic penetration of porcine oocytes ranges between 13 and 90% (Niwa, 1993). Usually, a relatively high number of spermatozoa is added to fertilisation media to maintain capacitation and a high number of spermatozoa per oocyte is associated with high incident of polyspermic penetration. One possibility to reduce the occurrence of polyspermic penetration is to minimise the number of spermatozoa per oocyte. However a low rate of polyspermic is accompanied by reduced penetration rate (Gajda, 2009). It has been reported that the high level of polyspermy in pig oocytes inseminated *in vitro* is not attributable to delayed or incomplete cortical granule exocytosis but more likely to a delayed zona reaction and／or multiple sperm entry (Wang *et al*., 1999). This

failure may be due to inherent deficiencies in the isolated oocytes themselves and/or suboptimal IVM culture systems (Abeydeera, 2002). Typically in standard IVF systems, a large number of spermatozoa are present at the site of fertilisation, often resulting in high number of spermatozoa penetrating the oocyte simultaneously. Therefore, suboptimal IVF conditions are one of the reasons for polyspermic penetration.

Another problem with porcine IVP is non-availability of commercial frozen sperm doses due to the difficulty in boar sperm cryopreservation. Pig sperm is more sensitive to changes in osmotic balance, low temperature exposure, cryoprotectant intoxication and oxidative stress. Although moderate and varied success has been achieved when using frozen-thawed boar sperm for IVF (Nagai *et al*., 1988; Cordova *et al*., 1997; Daigneault *et al*., 2014), the average IVF success and embryonic development rates with porcine cryopreserved semen are lower than other domestic species, such as cattle (Lim *et al*., 2007). The reason for the low adoption of frozen semen for IVF is likely the same as the cause for low adoption of frozen semen for AI, the reduced fertility caused by cryopreservation (Rath *et al*., 2009; Didion *et al*., 2013; Estrada *et al*., 2014). Furthermore, the efficacy of porcine IVF with frozen-thawed semen is often hindered by the large male-to-male variability in the success of maintaining sperm function following cryopreservation (Gil *et al*., 2008). The difference in IVF success is notable even between ejaculates from the same boar (Daigneault *et al*., 2014).

1.1.8 Need for improved imaging tools for follicular characterisation

As previously mentioned in this thesis *in vitro* production of embryo is heavily dependent on a supply of slaughterhouse ovaries. Typically a mammalian ovary (including porcine species) contains about 4 to 5 million primordial follicles at 18 - 22 weeks post-conception. The supply of follicles decreases slightly before birth and keeps

on depleting progressively throughout the lifespan of the animal. During the follicular phase, under hormonal influence a small cohort of follicles (approximately 10–14) begins to develop. One member of this group is physiologically selected to ovulate in uniparous mammals, exhibiting greatly increased hormonal activity and increasing its growth the others, lacking hormonal support, undergo atresia (Wallace and Kelsey, 2010). However, for *in vitro* culture porcine oocytes are isolated from a cohort of dominant and subordinate follicles, most commonly by aspiration. Follicles of size roughly 3-6 mm are chosen for aspiration simply based on the visual observation. The developmental competence of the *in vitro* isolated oocytes varies according to the developmental stage of the ovarian follicle. As a result it leads to a batch-to-batch variability during *in vitro* embryo production. At present, there is no available technique to identify those follicles with the best quality oocytes.

The size of ovarian follicle, the size of the developing oocyte within, and the thickness of the follicular wall are all important folliculometric parameters for selection of superior quality porcine ovaries for oocyte aspiration. With the commonly used ultrasound scans, the growing follicle appears as a black bubble on the ultrasound image without much folliculometric information. In ultrasound, most of the folliculometric information including follicular wall thickness is determined by manual or semi-automated segmentation. At present relatively low success rate of IVF may be attributed to the poor quality of retrieved oocytes, due to the improper timing of oocyte retrieval. Selection of developmentally competent oocytes for in vitro culture can significantly improve the outcome of IVF. There is a need for better imaging techniques that can simultaneously deliver anatomical, functional and molecular information with both high resolution and penetration capabilities.

1.1.9 Future direction

Since the first reported birth of a SCNT cloned pig, dozens of reports have described the production of transgenic pigs, and literally hundreds of genetically modified pigs have now been generated for xenotransplantation research and as models of various human diseases. Despite the progress in IVP of pig embryos, the *in vitro* embryo culture conditions are still considered to be relatively suboptimal. The methodologies involved with IVM–IVF–IVC need to be improved to overcome polyspermy as well as to enhance oocyte maturation and embryo development. Many problems associated with porcine embryo IVP have been addressed systematically, resulting in enhanced rate of oocyte nuclear and cytoplasmic maturation, improved pronuclear formation rates, acceptable blastocyst formation rates, and the development of effective cryopreservation protocols. The increase in overall efficiency of these basic technologies combined with advances in somatic cell nuclear transfer and freezing procedures will further allow the establishment of a gene bank of genetically modified pigs.

1.2 Technologies for transgenic animal production

The production of transgenic animals, in general, is a complex procedure and gene delivery into an oocyte or an embryo has to overcome the presence of zona pellucida, a glycoprotein layer surrounding the plasma membrane of an oocyte that functions as a barrier to exogenous materials. Therefore, a successful gene delivery procedure into an oocyte or an embryo should minimise the potential inhibitory response of the zona pellucida. Despite increasingly sophisticated technologies, the efficiency of the process remains disproportionately low compared to the labour inputs and costs. To date, the most widely used methods for the production of transgenic animals are (i) nuclear transfer using genetically modified embryonic or somatic donor cells (somatic cell nuclear transfer, SCNT) (ii) embryonic stem (ES) cell transfer, (iii) sperm-mediated gene transfer (SMGT) (iv) retrovirus mediated gene transfer (v) Direct microinjection of exogenous nucleic acid, transposomes etc. into the pronucleus of fertilised oocytes (DNA pronuclear microinjection, DNA-PMI).

1.2.1Somatic cell nuclear transfer

Among the above-mentioned techniques SCNT has been most extensively used in porcine transgenesis. Somatic cell nuclear transfer is a technique in which the nucleus (DNA) of a somatic cell is transferred into an enucleated metaphase-II oocyte for the generation of a new individual, genetically identical to the somatic cell donor. The success of cloning an entire animal, Dolly, from a differentiated adult mammary epithelial cell (Wilmut *et al*., 1997) has revolutionised developmental biology. It demonstrated that genes inactivated during tissue differentiation can be completely re-activated by a process called nuclear reprogramming: the reversion of a differentiated nucleus back to a totipotent status (Tian *et al*., 2003). SCNT may be used to generate

multiple copies of genetically elite farm animals, to produce transgenic animals for pharmaceutical protein production or xenotransplantation (Stice *et al*., 1998). However, SCNT has been inefficient in all species in which live clones have been produced. One of the most important problem that is stifling the enormous potential of SCNT is low efficiency and high incidence of developmental abnormalities (Young *et al*., 1998; Garry *et al*., 1996). The developmental defects encompass higher rates of pregnancy loss, prolonged gestation, higher birth weight, higher rates of peri- and post-natal mortality and specific adult phenotypes (Hill *et al*., 1999; De Sousa *et al*., 2001). The incidence of these anomalies varies according to the species, genotype, donor cell status, or specific aspects of the nuclear transfer or cultural protocols used and it is not clear which of them could be eliminated by technical improvements. It has been postulated that low cloning efficiency may be largely attributed to the incomplete reprogramming of epigenetic signals (Rideout *et al*., 2001; Bourchis *et al*., 2001).

1.2.2 SMGT

The SMGT method is based on the spontaneous ability of sperm cells to bind and internalise exogenous DNA and to carry it to oocyte during fertilisation, producing genetically modified animals with high efficiency (Lavitrano *et al*., 1989). SMGT is an alternative since the sperm itself is a natural vector carrying genetic information into the oocyte. SMGT has proved to be a useful method for generating transgenic embryos and animals in a considerable number of species. However issues still remain to be addressed regarding its repeatability, efficiency between different species, and the understanding of the underlying molecular basis of its action. Such issues will be important to resolve if this approach is to be employed for the generation of transgenic animals on a routine basis (Parrington *et al*., 2011). As already mentioned in the introduction porcine IVF suffer from the problem of polyspermy. Therefore, the

polyspermy issue has to be resolved effectively in order to exploit the benefits offered by SMGT in pig.

1.2.3 Retrovirus-mediated gene transfer

Starting from mid-1970s attempts were made to use retroviruses to infect murine embryos (Jaenisch *et al*., 1975, 1976) to produce genetically modified mice. In practice, preimplantation embryos or oocytes (Chan *et al*., 1998) can be exposed *in vitro* to concentrated virus solutions or incubated over a single layer of virus-producing cells. After infection, the retrovirus produces a DNA copy of its RNA genome using the viral enzyme reverse transcriptase. The DNA copy of the viral genome, or provirus, integrates randomly into the host cell genome. Embryos infected with viruses are then transferred back to recipient females to complete gestation. In the near future, application of the new-generation lentiviral vectors such as human immunodeficiency virus (HIV), whose transduction efficiency is increasingly promising, is likely improve the efficiency of the production of transgenic animals, including transgenic pigs (Dull *et al*., 1998; Reichenbach *et al.*, 2010). However, this may be possible only when researchers have developed commercially available vectors using safer types of lentiviruses, which enables their use in laboratories with lower security levels (Jura *et al*., 2009).

1.2.4 Embryonic stem cell transfer

ES cells have been extensively used to transfer genetic modifications from cultured cells to whole animals, principally mice. ES cells carrying engineered alterations such as gene-targeted modifications generated by homologous recombination *in vitro* can be isolated and incorporated into a host embryo by either injection into the blastocoel cavity of a blastocyst stage embryo, or aggregated with a pre-compaction embryo. These are then transferred into a foster mother to generate

chimeric animals, which after appropriate breeding produce offspring with the modified genotype (Capecchi, 1989; Koller and Smithies, 1992). This approach is however not available for porcine transgenesis as fully functional ES cells or an equivalent are not available in pigs or other large mammals (Jura *et al*., 2009). Recently published report of successful derivation of porcine IPS cells (Wei *et al.*, 2015; Zhang *et al.*, 2015) have given rise to the hope of using this approach as a viable alternative in pig transgenesis too.

1.2.5 Electroporation

At present, considering the availability and efficiency to deliver foreign genetic materials including DNA and RNA into zygotes, another method of delivery is electroporation (Joo *et al*., 2014). Briefly, the embryos are kept in a solution having the gene of interest. The solution and the cells are exposed to a very short duration of a high voltage electrical pulse. The pulse causes a temporary breakdown of the cell membrane. This technology has been utilized in attempts to produce transgenic animals by electroporation of DNA into sperm, which then carry the exogenous DNA to the egg at fertilization (Gagné *et al*., 1991). This method has great potential either alone or in combination with others to efficiently transfer genes for the production of transgenic individuals. However, the disadvantages of electroporation include physical damage to the plasma membrane and low efficiency of gene delivery into zygotes (Robl *et al*., 2007). Recently there have been some reports of direct zygote electroporation protocols that bring high efficiency, high throughput genome engineering in animal models within closer reach (Kaneko *et al*., 2014; Hashimoto & Take Moto, 2015; Quinn *et al*., 2015). However, these protocols fail to address the most significant bottleneck of transgenic animal production, i.e. *in vitro* embryo production.

1.2.6 Nucleic acid (DNA/RNA) microinjection

Nucleic acid microinjection is a popular and direct method of creating transgenic animals. By this method, a very fine glass pipette is used to manually inject exogenous nucleic acid into the eggs. In 1974, Jaenisch and Mintz microinjected the simian virus 40 (SV40) viral DNA into explanted mouse blastocysts and generated the first transgenic mice. Later, Gordon *et al*. (1980) microinjected a recombinant plasmid composed of segments of herpes simplex virus and simian virus 40 viral DNA into pronucleus of fertilised mouse oocytes and also obtained the transgenic mice. Its efficiency is limited and lower for rabbits, rats, and pigs than for mice. It is very low in ruminants. This difference does not seem to be because of microinjection problems but instead to the natural capacity of the different genomes to integrate foreign DNA (Houdebine, 2007). It is considered as a standard technique for delivery of nucleic acids (NAs; transgenes or genome editing tools such as CRISPR/Cas9) into embryos, for creating genetically modified organisms. Generally, the creation of genetically engineered animals involves modifying their genomes at early embryonic stages. This complex process consists of three major and absolutely critical steps (depicted in fig 4) i) isolation of zygotes from super-ovulated females, ii) delivery of nucleic acids (NAs) into the isolated zygotes and iii) subsequent embryo transfer into pseudo-pregnant animals to produce viable offspring.

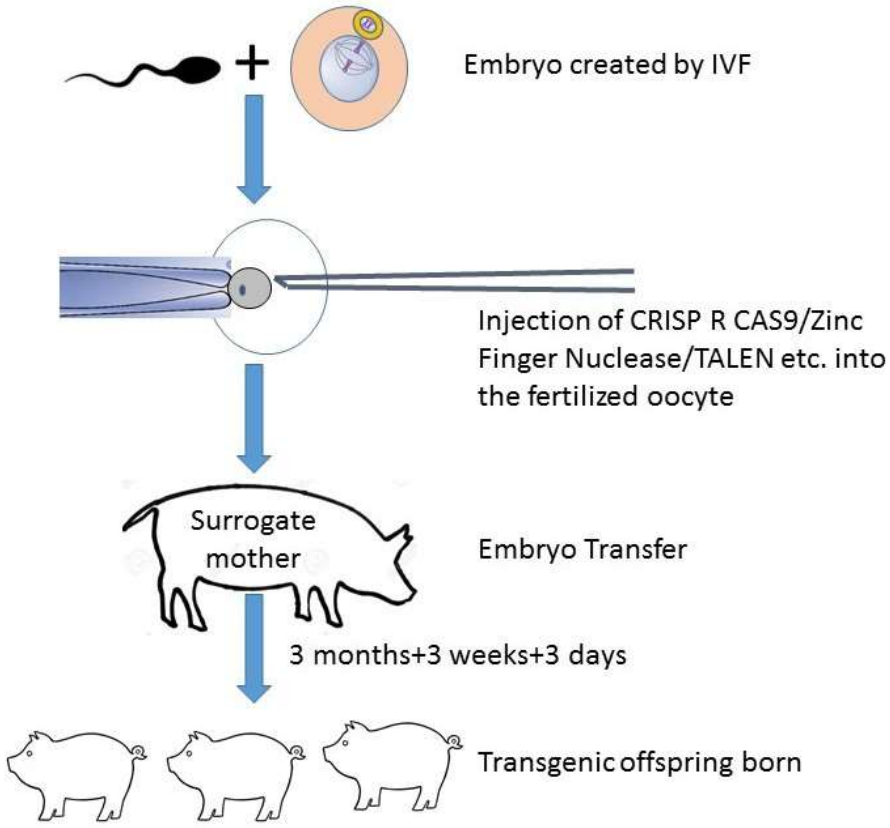

Fig 4 Generation of transgenic pigs direct zygotic microinjection.

The technique of zygote microinjection generally demands the use of micromanipulators and a microinjection apparatus to inject into embryos a solution containing recombinant DNA. Linearised DNA constructs are more promptly incorporated into the host genome. The presence of foreign plasmid or vector sequences can adversely affect expression of the integrated transgene. Much precaution must be taken when dealing with large DNA constructs, such as those derived from bacterial or yeast artificial chromosomes, to avoid shearing during the process of microinjection. Gene constructs when microinjected, generally integrate randomly into the genome of the embryo, but typically only in a single chromosomal location (Dunn *et al*., 2005). After gene transfer, pregnant recipients will follow normal

gestation and deliver young at term. Advantages and disadvantages of microinjection are listed in the Table 1.

Sl No.	**Advantages of Microinjection**
1	Ease of generating transgenic animals from viable microinjected embryos transferred to recipient females.
2	A lack of constraint on the size or type of DNA constructs used.
3	The stability of the transgene as it is transmitted from generation to generation.
	Disadvantages of Microinjection
1	The random and potentially significant influence that the site of integration can exert on transgene expression (positional effects).
2	The potential for undesired insertional mutagenesis.
3	The time required in developing the necessary micromanipulation and microinjection skill sets.
4	Process is expensive

One tremendous advantage of microinjection, when compared to other methods of introducing material into zygote, such as electroporation or transfection, is the ability to be quantitative and to precisely control and quantify the volume of the injection. Perhaps the most powerful aspect of microinjection is the ability to introduce several types of reagents into zygote simultaneously, including DNA constructs, antibodies short interfering RNAs (siRNAs), and peptides etc. (Rose, 2007). No other techniques available provide these capabilities.

In pigs, transgenesis efficiency using standard DNA microinjection, which continues to be the leading technique in this species, does not exceed 2% in terms of

the number of zygotes subjected to microinjection (Jura *et al*., 2009). However, this bleak picture seems to be changing dramatically with the improvement of customisable endonuclease like ZFN, TALEN and CRISPR/ Cas 9. Recent reports have shown that direct zygote microinjection of CRISPR/Cas9 system can very efficiently (nearly 100% efficiency) generate transgenic animals in one step (Whitworth *et al*., 2015; Hai *et al*., 2014). With the continuing improvement of porcine *in vitro* embryo production, the microinjection-mediated approach is likely to play a very in generation of transgenic animals in future.

1.3 Targeted genome engineering using designer nucleases

The recent development of nuclease-based genome editing technologies capable of directing double strand breaks to a specific target sequence provides a new tool for reverse genetics. These targetable DNA cleavage reagents can be engineered to recognise and cleave a precise DNA sequence within a genome. The induced double stand breaks are a target for repair by the error prone non-homologous end-joining repair pathway, resulting in targeted mutation in a proportion of events. Unlike transgenesis, genome editing does not require a transferred DNA transgene and no genetic mark beyond that of the mutation is introduced into the genome (Lillico *et al*., 2013). Studies in the last five years have demonstrated the great potential of genome editing technologies based on programmable nucleases such as meganucleases, zinc finger nucleases (ZNF), transcription activator–like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease (Cas9) in targeted genome engineering (Cox *et al*., 2015). These advances are already broadening the ability to elucidate the contribution of genetics to disease by facilitating the creation of more accurate cellular and animal models of pathological processes. A particularly tantalising application of programmable nucleases is the potential to directly correct genetic mutations in affected tissues and cells to treat diseases that are refractory to traditional therapies.

1.3.1 Genome editing technologies

Gene targeting is a method to repair or inactivate any desired gene of interest. Gene targeting strategies use the introduction of a double-stranded break (DSB) into a genomic locus to enhance the efficiency of recombination with an exogenously introduced homologous DNA "repair template". Programmable nucleases enable

precise genome editing by introducing DNA double-strand breaks (DSBs) at specific genomic loci (Harrison *et al*., 2014). DSBs subsequently recruit endogenous repair machinery for either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) to the DSB site to mediate genome editing (Rouet *et al*., 1994). In NHEJ, DSBs are repaired by simple ligation of the free ends (Fig 5). Due to the error-prone nature of this mechanism, small insertions or deletions (indels) may occur, which can result in a frameshift and thus a knockout of the gene of interest. This process is also termed directed mutagenesis. When an exogenous DNA donor containing homologous regions is supplied, DSBs will stimulate the HDR pathway, which will lead to the targeted

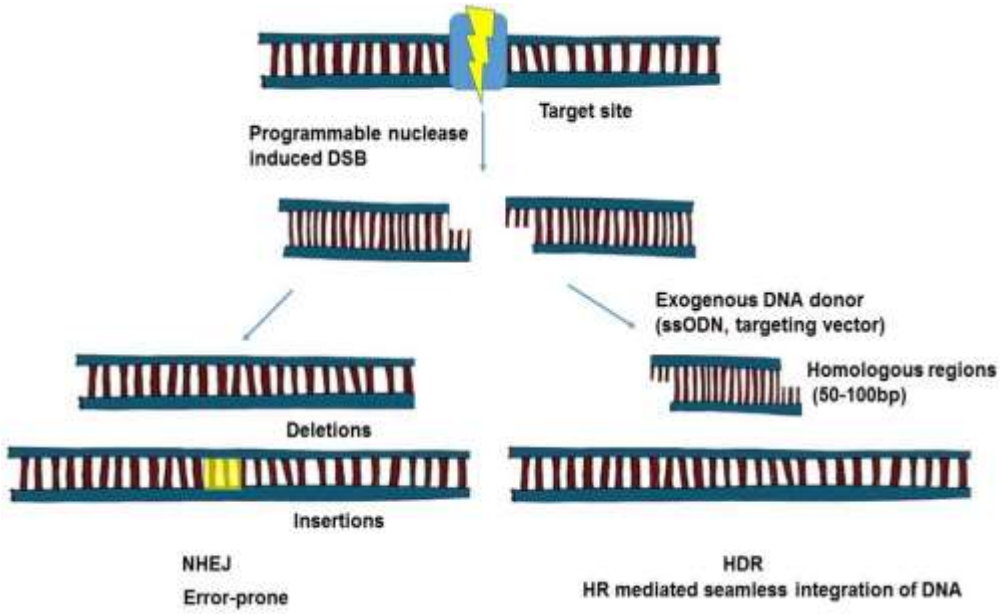

Fig 5 Mechanism of DSB repair. DSBs are repaired by endogenous repair machinery either by non-homologous end-joining (NHEJ) or homology-directed repair (HDR)

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incorporation of the exogenous DNA. Consequently, programmable nucleases offer four editing possibilities: simple NHEJ-mediated knockouts; induction of larger deletions or inversions by simultaneous introduction of two (pairs) of CENs; introduction of a defined mutation or corrected allele via HR; and gene addition via either pathway.

1.3.2 Genome editing with customisable nucleases

To date, four major classes of nucleases—meganucleases and their derivatives (Boissel *et al*., 2014), zinc finger nucleases (ZFNs) (Bibikova *et al*., 2002), transcription activator–like effector nucleases (TALENs) (Boch *et al*., 2009) and CRISPR-associated nuclease Cas9 (Cong *et al*., 2013) —have been developed to enable site-specific genome editing (Table 3). These nuclease systems can be broadly classified into two categories based on their mode of DNA recognition: ZFNs, TALENs and meganucleases achieve specific DNA binding via protein DNA interactions, whereas Cas9 is targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions.

Table 2 Comparison of different customisable endonuclease based strategies adopted from Cox *et al*. 2015.

Parameter	Zinc finger nuclease	TALEN	Cas 9	Meganuclease
Recognition site	Typically 9-18 bp per ZFN monomer,18-36 bp per ZFN pair	Typically 14-20 bp per TALEN monomer,28-40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp proto spacer adjacent motif (PAM) for Streptococcus pyogenes Cas9)	up to 44 bp for double nicking Between 14 and 40 bp
Specificity	Small no's of positional mismatches tolerated	Small numbers of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated	Small numbers of positional mismatches tolerated
Targeting constraints	Difficulty to target non G rich sequences	5´targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM	Targeting novel sequences often result in low efficiency.
Ease of engineering	Difficult; may require substantial protein engineering	Moderate requires complex molecular cloning methods	Easily retargeted using standard cloning procedure and oligo synthesis	Difficult; may require substantial protein engineering
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic	Unknown; protein derived from Xanthamonas sp.	Unknown; protein derived from various bacterial species.	Unknown; meganuclease may be derived from many organisms, including eukaryotes
Ease of ex-vivo delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of in-vivo delivery	Relatively easy as small size of ZFN expression Cassettes allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs leading to unwanted recombination events when packaged into lentiviral vectors	Moderate the commonly used Cas9 from S. pyogenes is large and may impose packaging problems for viral vectors like AAV, but smaller orthologs exist	Relatively easy as small size of meganucleases allows use in a variety of viral vectors.
Ease of multiplexing	Low	Low	High	Low

1.3.3 Zinc Finger Nucleases

ZFNs have been used to precisely modify the genomes of many species as well as primary human cells, including pluripotent stem cells. Zinc finger nucleases (ZFNs) are synthetic proteins consisting of an engineered zinc finger DNA-binding domain fused to the cleavage domain of the FokI restriction endonuclease. ZFNs can be used to induce double-stranded breaks (DSBs) in specific DNA sequences and thereby promote site-specific homologous recombination and targeted manipulation of genomic loci in a variety of different cell types (Miller *et al*., 2007). ZFNs consist of a DNA-binding zinc finger domain (composed of three to six fingers) covalently linked to the non-specific DNA cleavage domain of the bacterial FokI restriction endonuclease (Fig 6, left panel). ZFNs can bind as dimers to their target DNA sites, with each monomer using its zinc finger domain to recognise a "half-site" (Fig 6, right panel). Dimerisation of ZFNs is mediated by the FokI cleavage domain, which cleaves within a five or six base pair "spacer" sequence that separates the two inverted "half sites" (Figure 6, right). Importantly, because the DNA-binding specificities of zinc finger domains can in

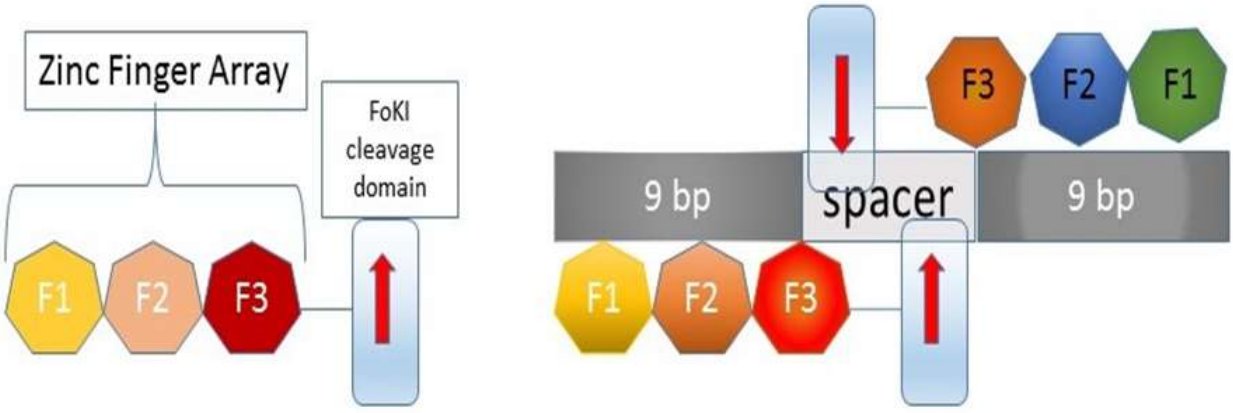


Fig 6 Schematic of a zinc finger nuclease. Left, the structure of a 3 finger ZFN. Right, a ZFN dimer binding the cleavage site. The spacer sequence shown is typically 5-7 bp long; the red arrows show the cleavage point on the DNA.

principle be re-engineered using one of various methods, customised ZFNs can theoretically be constructed to target nearly any gene sequence. Creation of a unique break at a predetermined site in the host genome can be used to inactivate genes by introduction of small deletions and insertions, or to catalyse homology-directed repair and hence specific gene modifications. The ability to alter the sequence or structure of any gene of interest would be enormously useful for biological research and molecular therapeutics. However, the widespread use of ZFNs has been hindered by the complexity of generating domains that bind DNA with sufficient affinity and specificity.

1.3.4 TAL nuclease

TALENs offer a useful new functional equivalent. Fusing TAL effector DNA binding domains to a Fok1 nuclease domain generates TALENs. In contrast to ZFNs, the recognition code of transcription activator-like effector proteins enables the construction of new DNA-binding domains with unprecedented freedom. The TAL DNA recognition code is based on four variants of 34-residue peptide elements (TAL repeats) that each mediate binding to one of the four DNA nucleotides. TAL repeats can be combined in the appropriate order and fused with a FokI nuclease domain to generate a nuclease. TALENs have been used to inactivate genes in mammalian cell lines, mouse, rat, and pig embryos (Mussolino and Cathomen, 2012) and to catalyse generate targeted modifications (Wefers *et al*., 2013).

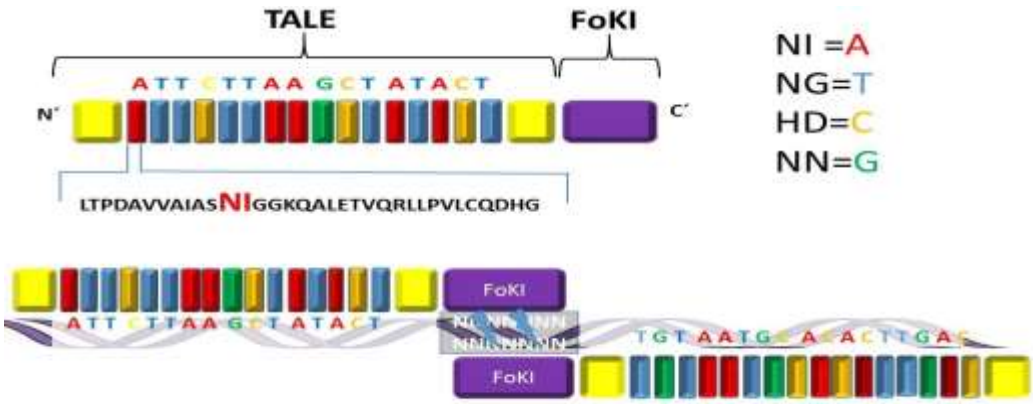


Fig 7 Schematic overview of TALEN structures. Binding to the target site and cleavage after TALEN dimerisation. RVD (repeat-variable di-residue) determines DNA targeting; FokI: FokI catalytic domain.

1.3.5 Cas9: an RNA-guided nuclease for genome editing

The advent of genome editing, particularly the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) locus and the CRISPR-associated protein (Cas) system (Ran *et al*., 2013), is a significant advance that facilitates genome editing including many important procedures such as mutation of genes in a particular tissue or organ *in vivo*. “Knockin” mouse lines have been generated that express Cas9 nuclease constitutively from a transgene placed at the permissive Rosa26 locus (Platt *et al*., 2014). Moreover, appropriate guide RNAs can be delivered locally into a particular organ of these animals via electroporation or adeno-associated virus (AAV) vector. The CRISPR system can also be multiplexed, multiple guide RNAs have been introduced into the lungs of Cas9 mice to simultaneously mutate several genes associated with lung cancer (Platt *et al*., 2014). The CRISPR/Cas9 system has been adapted from prokaryotes where it is used as a defence mechanism (Wiedenheft *et al*., 2011). Genome editing directly in mammalian embryos by CRISPR/Cas9 zygotes is now being widely used as an alternative to traditional embryonic stem cell targeting-based KO

production Recent reports have shown that direct zygote microinjection of CRISPR/Cas9 can very efficiently (nearly 100% efficiency) generate genome modified animal in one step (Whitworth *et al*., 2014; Hai *et al*., 2014).

1.3.6 Applications of CRISPR/Cas9 as a Genome-editing and Genome Targeting Tool

In nature, the Cas9 system requires three components, an RNA (~20 bases) that contains a region that is complementary to the target sequence (crRNA), an RNA that contains a region complementary to the crRNA (tracrRNA), and Cas9, the enzymatic protein component in this complex. A single guide RNA (gRNA) can be constructed to serve the roles of the base-paired crRNA and tracrRNA. The gRNA/protein complex can scan the genome and catalyse a double strand break at regions that are complementary to the crRNA/ gRNA (Hsu *et al*., 2014). Following its initial demonstration in 2012, the CRISPR/Cas9 system has been successfully used to target important genes in many cell lines and organisms, including human (Mali *et al*., 2013), bacteria (Fabre *et al*., 2014), zebrafish (Hwang *et al*., 2013), C. elegans (Dickinson *et al*., 2013), Drosophila (Gratz *et al*., 2014), monkeys (Niu *et al*., 2014), rabbits (Yang *et al*., 2014), pigs (Ruan *et al*., 2015), rats (Ma *et al*., 2014) and mice (Mashiko *et al*., 2014). Several groups have now taken advantage of this method to introduce single point mutations (deletions or insertions) in a particular target gene, via a single gRNA (Nishimasu *et al*., 2014). Using a pair of gRNA-directed Cas9 nucleases instead, it is also possible to induce large deletions or genomic rearrangements, such as inversions or translocations (Gratz *et al*., 2014). A recent exciting development is the use of the dCas9 version of the CRISPR/Cas9 system to target protein domains for transcriptional regulation (Perez-Pinera *et al*., 2013), epigenetic modification (Hu *et al*., 2014), and microscopic visualisation of specific genome loci (Chen *et al*., 2013).

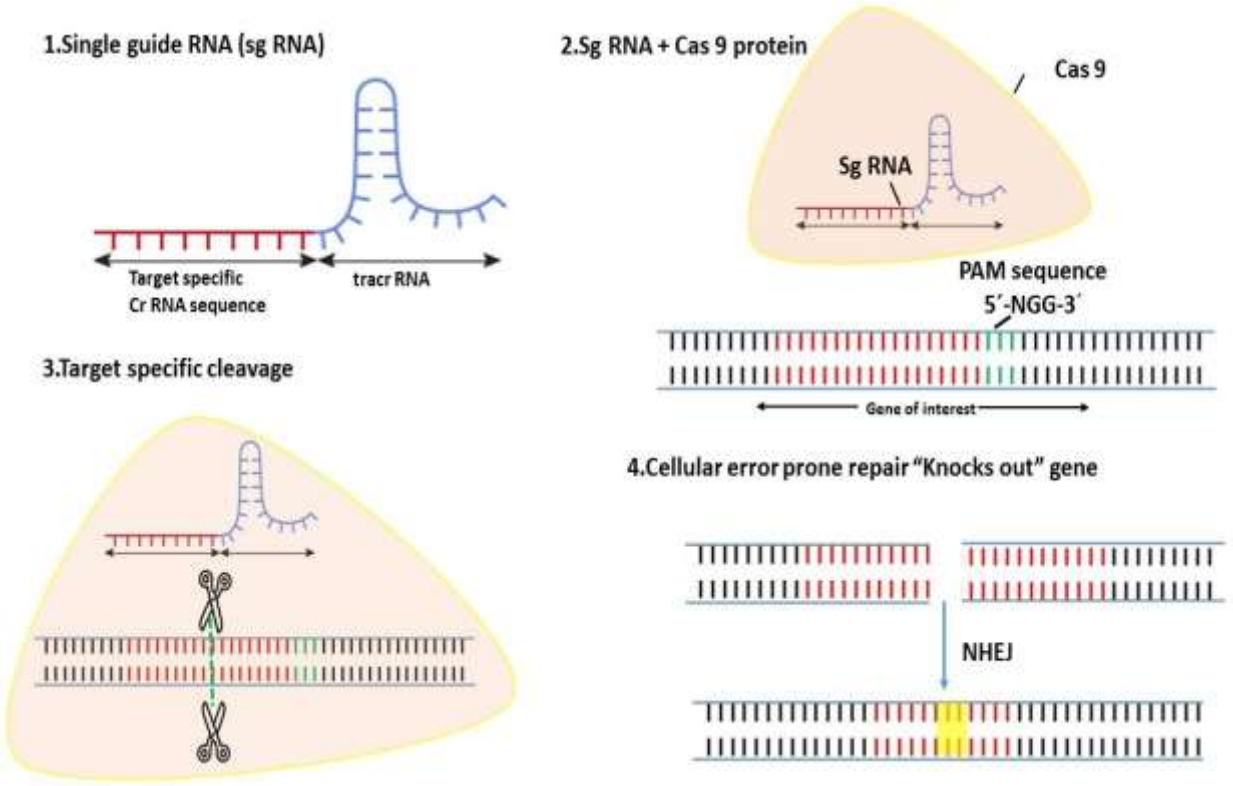


Fig 8 Schematic overview of sgRNA and Cas9 mediated cleavage. (1-2) In sgRNAs, crRNA (red) and tracrRNA (blue) are fused together and connected by a loop. (3-4) Upon recognition of a PAM (green)at the non-complimentary strand, Cas9 starts interrogating the adjacent sites for crRNA complementarity; after binding and formation of an R-loop, DNA is cleaved by the two nuclease domains of Cas9.

The CRISPR/Cas9 system requires only the redesign of the crRNA to change target specificity. This contrasts with other genome editing tools, including zinc finger and TALENs, where redesign of the protein-DNA interface is required. Furthermore, CRISPR/Cas9 enables rapid genome-wide interrogation of gene function by generating large gRNA libraries (Koike-Yusa *et al*., 2014) for genomic screening.

1.3.7 The future of CRISPR/Cas9

The rapid progress in developing Cas9 into a set of tools for cell and molecular biology research has been remarkable and is attributable to the simplicity, high efficiency and versatility of the system. Of the designer nuclease systems currently available for precision genome engineering, the CRISPR/Cas system is by far the most user friendly. It is now evidently becoming clear that Cas9's potential reaches beyond DNA cleavage, and its usefulness for genome locus-specific recruitment of proteins will likely only be limited by our imagination.

1.4 Aim of thesis

Projects pursued at the Chair of Livestock Biotechnology involve genetic modification of pigs to model colorectal, pancreatic and other cancers, various cardiovascular diseases and also the modification of pigs as donors for xenotransplantation. Considering the advantages offered by the new CRISPR/Cas 9 system, development of a robust and efficient CRISPR based microinjection technique in pig has been envisaged. This would significantly aid much of this work by circumventing the time consuming steps of designing and generating large gene-targeting vectors, isolating and characterising targeted cell clones, and most importantly the need for somatic cell nuclear transfer. Realisation of this objective requires a steady supply of porcine embryos, which is best achieved by establishing robust and efficient porcine in vitro embryo production. The success of porcine embryo culture is strongly influenced by the quality of oocytes selected for culture and the particular ovaries chosen as the starting material. Work described in this thesis focussed on these fundamental aspects of the system (chapter III), and investigated the potential value of new techniques to predict the quality of oocytes (Chapter IV). These included using state of the art technologies such as selective plane illumination microscopy and multispectral optoacoustic tomography (Chapter V).

The experiments described in this thesis are primarily works of methodology development. The main experimental methodology; techniques and results are documented for each chapter individually. This allows the reader to assess the newly developed methods and the results based on those methods. This is followed by a discussion for all experiments.

Chapter II

Material

2.1 Chemicals

Sodium chloride (S-9888)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
D-glucose (G-7021)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Polyvinyl Chloride	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Polyvinyl Alcohol (P-8136)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Glutamine (G-5763)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Cysteine (C-3752)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Sodium Bicarbonate (S-5761)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Calcium Lactate.5H_2O (C-8356)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Na H_2 PO_4.H_2O (S-9638)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
$MgCl_2$.6H_2O (M-2393)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
KCl (P-5405)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Kanamycin (K-1377)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Sodium lactate (L-7900)	Sigma-Aldrich and Merck KGaA, Darmstadt,

	Germany
BSA (A-9647)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Sodium pyruvate (P-2256)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Caffeine (C-0750)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
KH_2PO_4 (P-5655)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
$CaCl_2.2H_2O$ (C-7902)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
$MgSO_4.7H_2O$ (M-1880)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
D-Sorbitol (D-1876)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Penicillin G (P-3032)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Streptomycin (S-9137)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
β Mercaptoethanol (M-7522)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Heparin (H -3149).	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Hyaluronidase (H-3506)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
ITS (recombinant human insulin, human transferrin, and sodium selenite, I -3146)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Epidermal growth factor (E-	Sigma-Aldrich and Merck KGaA, Darmstadt,

4127),	Germany
Chlortetracycline hydrochloride (C -4881)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Glutaraldehyde G-5882	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Hoechst (B2261)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Ca-ionophore A23187 (C7522)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
6-dimethylaminopurine, D2629	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Mineral oil (M8410)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Propidium Iodide (P4170-10MG)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Glacial acetic acid 695092-100ML	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
EDTA (E9884-500G)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
SDS (L3771-100G)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Ethanol absolute	Riedel-de-Haen, Seelze, Germany
Isopropanol	Roth, Karlsruhe, Germany
GenAgarose LE	Genaxxon BioScience, Ulm, Germany
Mineral oil (M8410)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany
Lissamine B (199583)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany

Brilliant Cresyl Blue (16030-10G)	Sigma-Aldrich and Merck KGaA, Darmstadt, Germany

2.2 Media and components

Advanced Dulbecco's Modified Eagle's Medium (DMEM)	PAA, Pasching, Austria
Amino acids, non-essential (100x)	PAA, Pasching, Austria
Amphotericin B (250 µg/ml)	PAA, Pasching, Austria
Cell culture water	PAA, Pasching, Austria
Dimethyl sulphoxide (DMSO)	Sigma, Steinheim, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	PAA, Pasching, Austria
Dulbecco's Phosphate buffered saline (PBS), w/o Ca, Mg	PAA, Pasching, Austria
Foetal calf serum (FCS)	PAA, Pasching, Austria
Hank's buffered salt solution (HBSS), w/o phenol red, with Ca, Mg	Biochrom, Berlin, Germany
Heparin sodium salt	Sigma, Steinheim, Germany
Penicillin/Streptomycin	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria
TCM 199 (M 2154)	Sigma Aldrich
PZM5	IFP
PFM	IFP
Androcoll P	Sweden
Androhep Plus	Minitube,Germany

2.3 Kits

In situ Apoptosis Detection Kit.	TAKARA BIO
Mitoorange,mitochondrial stain	Abcam
TURBO DNA-free™ Kit	Life Technologies
SuperScript® III First-Strand Synthesis System for RT-PCR	Life Technologies
RNeasy Mini Kit	Qiagen, Europe
KAPA SYBR FAST q PCR Kit	Kapa Biosystems)
REPLI-g whole genome amplification kit	Qiagen, Europe

2.4 Consumables

1.5/2.0 ml microcentrifuge tubes	Zefa Laborservice, Harthausen, Germany
15/50 ml centrifuge tubes	Corning, New York, USA
35/60 mm culture dish	Corning, New York, USA
4 well IVF dish	Nunc, Wiesbaden, Germany
1.8 ml CryoTubes	Nunc, Wiesbaden, Germany
1/2/5/10/25 ml plastic pipettes	Corning, New York, USA
Filter pipette tips	Zefa Laborservice, Harthausen, Germany
Glass pasteur pipettes	Brand, Wertheim, Germany
10/25/50 ml plastic syringes	Becton Dickinson, Heidelberg, Germany
0.22/0.45 µm syringe filter	Sartorius, Göttingen
Borosilicate Tubing with filament capillary	(Sutter Instrument BF100-50-10)
Vacutip	Eppendorf, Germany
Microloader tip	Eppendorf, Germany

2.5 Devices

SpeedMill PLUS ® homogeniser	Analytikjena
Transjector 5246 InjectMan	Eppendorf, Hamburg, Germany
ABI 7300 real time PCR System	Applied Biosystems
Femtojet	Eppendorf, Hamburg, Germany
Celltram Air	Eppendorf
Thermocycler DNA Engine DYAD PCR	Bio-Rad Laboratories, Hercules, CA
Biophotometer 6131 Cuvettes UVette	Eppendorf, Hamburg, Germany
Clean Bench HERASafe	Heraeus Instrument, München, Germany
Gene Genius Bio Imaging System	Syngene, Cambridge, United Kingdom
Incubator BD 115	Binder, Tuttlingen, Germany
Flaming and Brown P 87 model pipette puller	Sutter instruments
Nanodrop Lite	Thermo Scientific, Waltham, Germany
Orbital Shaker 420	Thermo Scientific, Waltham, Germany
pH meter Cyberscan 510	Eutech Instruments, Singapore, Singapore
Steri-Cycle CO2 Incubator	Thermo Electron, Dreieich, Germany
Vortex-Genie	Scientic Industries, Bohemia, NY
Eppendorf MiniSpin centrifuge	Eppendorf, Hamburg, Germany
Sigma 1-15K (Rotor 12024)	Sigma, Steinheim, Germany
Sigma 4K15 (Rotors 11150, 13350)	Sigma, Steinheim, Germany

Sigma 3-16 (Rotor 12024)	Sigma, Steinheim, Germany
Kern 440-33N balance	Kern & Son, Balingen, Germany
APX-1502 balance	Denver Instrument, Göttingen,Germany
AxioCAM Mrc	Zeiss, Oberkochen, Germany
AxioCAM MRm	Zeiss, Oberkochen, Germany
Axiovert 25	Zeiss, Oberkochen, Germany
Axiovert 40 CFL	Zeiss, Oberkochen, Germany
Axiovert 200M	Zeiss, Oberkochen, Germany
Axiovert 10	Zeiss, Oberkochen, Germany
HBO 100	Zeiss, Oberkochen, Germany
spectroscopic measurements Ocean optics	USB4000-FL, Bandwidth: 351-1043nm
Small animal multispectral optoacoustic tomography (MSOT) scanner	MSOT256-TF, iThera Medical GmbH, Munich, Germany
80 mW continuous wave DPSS laser	Frankfurt laser company, Germany
(sCMOS) camera pco.edge	PCO AG, Kelheim, Germany)
EC Epiplan-Neofluar objective	Zeiss, Germany

Chapter III

Standardisation of *in vitro* production of porcine embryos for direct zygote microinjection

In vitro culture of porcine embryos for direct zygote microinjection

3.1 Chapter introduction

The tremendous potential of one-step production of knockout by the latest CRISPR/Cas9 system and other customisable endonuclease based systems in pig is still unmet because of the absence of a reliable supply of developmentally competent embryos. IVF still remains a critical limiting factor in realising the great potential of pig as a model animal in large animal transgenic research as already elaborated earlier. Improvements of *in vitro* maturation and fertilisation systems have overcome some previous limitations and led to high nuclear maturation rates and better male pronucleus formation. Despite the improvements made, the problems of poor cytoplasmic maturation, polyspermy and poor cryopreservability of sperm are still prevalent in most IVP systems. Therefore, the main focus of the study was to improve the *in vitro* production of porcine embryos for direct zygote microinjection of CRISPR /Cas 9. As already mentioned in the aim section (Chapter I), for the purpose of easy and coherent flow of information, all the methods, results and discussion of this experiment are compiled together in the following part.

3.2 Methods

3.2.1 Oocyte collection and classification of oocytes

Slaughterhouse ovaries of prepubertal gilts were brought to the laboratory within one hour of collection in a temperature-controlled box maintained at 39^0C. Ovaries were thoroughly washed with pre-warmed (39^0C) Dulbecco's phosphate-buffered saline (DPBS) solution containing 0.1% polyvinyl alcohol and penicillin, streptomycin solution. For collection of oocytes, oocytes collection medium (Annexure) consisting of TCM-199, 25 mM HEPES, L-glutamine, BSA, gentamicin and 10% FBS was used. Fresh medium

(100 ml) was prepared, filtered through 0.22 µm membrane filter and pH was adjusted to 7.2-7.4. Prior to use it was kept in 5% CO_2 incubator at 39^0C for at least 2 h for equilibration. Slicing method was used for sufficient recovery of oocytes. Ovaries were placed one by one in an oocyte-searching dish containing oocyte collection medium. Then the ovaries were grasped by sterilised forceps and the surface visible all follicles (3-6 mm diameter) were punctured by slicing with sterile scalpel. At a time 10-12 ovaries were processed in this way and the dish was observed under zoom stereomicroscope under 20 X magnification. For oocyte pick up sterilised Pasteur pipettes were used. They were pulled over the flame to reduce the inner diameter to 300 µm. Oocytes along with the cumulus cells (COCs) were picked up gently without damaging the cumulus cells and were kept in a 35 mm petri dish containing washing medium for further washing.

3.2.1.1 Classification of porcine oocytes

The collected oocytes were graded as either A, B or C according to the number of cumulus cell layers surrounding the oocytes and the homogeneity of ooplasm.

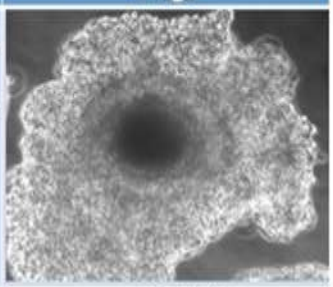
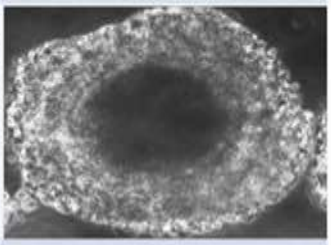
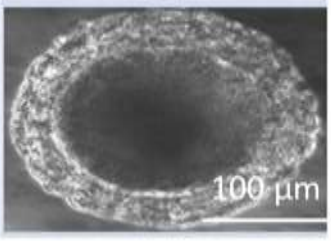
Grade	Criteria	Image
Grade A	The oocytes with cumulus-oocyte-complexes (COCs) having more than five layers of compact cumulus cells and evenly granulated cytoplasm.	
Grade B	The oocytes with cumulus-oocyte-complexes (COCs) having 3-5 layers of cumulus cells and evenly granulated cytoplasm.	
Grade C	The oocytes with fibrous (much expanded) cumulus layers, irregular and dark cytoplasm.	

Fig 9 Grading of porcine COC based visual observation of cumulus cell investments.

3.2.1.2 Oocyte selection by BCB staining

Only developmentally competent oocytes were selected on the basis of glucose 6 phosphate dehydrogenase (G6PDH) activity of the oocytes indicated by brilliant cresyl blue (BCB) staining (Bhojwani *et al*., 2007). The protein G6PDH is active in growing oocyte, but its activity is decreased in oocytes that have finished their growth phase and are then likely to have achieved developmental competence. The enzyme G6PDH can degrade BCB, thus oocyte yielding lower G6PDH (finished growth phase) shows blue cytoplasm (BCB+) after BCB staining, while growing oocytes (unfinished growth phase) have abundant G6PDH and a colourless cytoplasm (BCB-) (fig10). The developmentally competent (BCB+) oocytes were selected for further culturing.

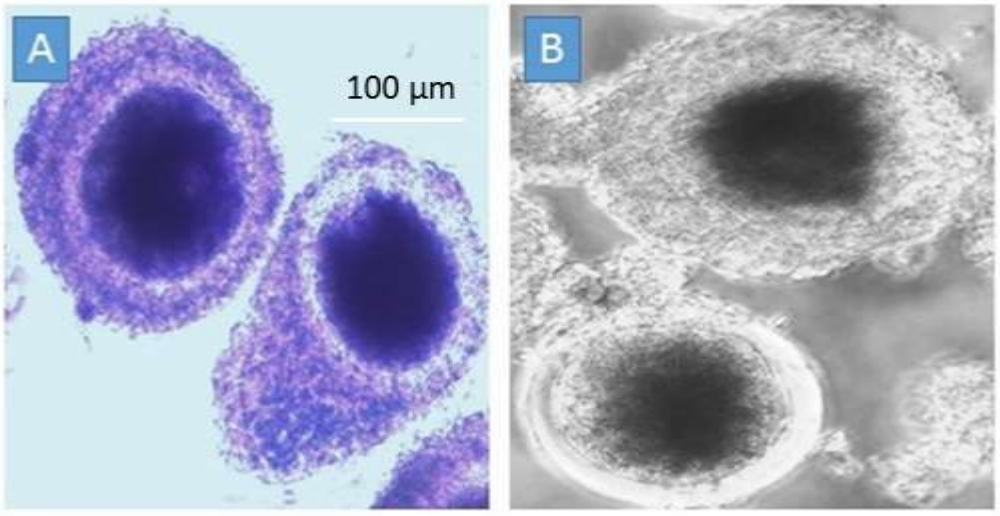


Fig 10 BCB selection of oocyte. A. BCB +: Developmentally competent oocyte stained blue by BCB; B. BCB - **:** The immature or incompetent oocyte remain unstained.

For BCB staining, immediately after oocyte collection, COCs were exposed in 100 µl droplets of Dulbecco's phosphate buffered saline (PBS; Gibco-BRL, Grand Island, NY, USA) containing 13 mM BCB (Sigma), 3.05 mM glucose (sigma), 0.91 mM Na-pyruvate (sigma), 75 mg/L kanamycin (Sigma), and 0.05% PVA covered with mineral oil (Sigma). The COCs were kept in the droplets for 90 min at 39^0C in air. Then, COCs were washed 3 times in fresh DPBS and were classified into two groups with respect to their cytoplasmic coloration: BCB-positive oocytes had a blue-coloured ooplasm (BCB+), and BCB negative oocytes had no blue coloration in their cytoplasm (BCB-).

3.2.2 *In vitro* maturation of oocytes

In the present study existing culture conditions were modified to improve nuclear maturation and cumulus expansion of cumulus-oocyte complexes. A two-step sequential culture method was used for IVM culture of the collected porcine oocytes. A TCM 199 based sequential medium (day 1 and 2 medium) was used for the culture (annexure). Day 2 medium was essentially the same medium like the day 1 medium but without the hormones namely human chorionic gonadotropin (hCG) and pregnant mare

serum gonadotropin (PMSG). Collected COCs were washed 3-4 times in oocyte collection medium (annexure) and 2 times in maturation medium. The maturation medium was supplemented with 50 ng/ml epidermal growth factor. Four drops of 100 µl maturation medium were made in 35 mm dishes and covered with mineral oil. These dishes were placed in the incubator with 5% CO_2 in air at 39^0C prior to use for the equilibration. After washing, the dishes were taken out from the incubator and 15- 16 'A' 'B' and 'C' grade oocytes were placed in each drop of maturation medium. The Culture condition was maintained at temperature-39^0C, time - 48 h, relative humidity-95%, and CO2-5%. For the first 24 h, the COC s were cultured in Day 1 medium. After 24 h post culture the COCs were washed three times in Day 2 medium. The COCs were cultured for further 20 hours in Day 2 medium. After IVM culture, cumulus investments were removed from the oocytes by gentle pipetting. Oocytes were then fixed for 30 minutes in 0.5% glutaraldehyde (500µl PBS+10 µl 25% glutaraldehyde G-5882), washed in 500 µl PBS for 5 minutes then transferred to 500 µl Hoechst (B2261) stain solution (0.1%). The nuclear status was evaluated by epifluorescence microscopy (UV filter and Carl Zeiss) using standard measures like germinal vesicle breakdown, condensation of chromatin and appearance of the first polar body as confirmation of nuclear maturation.

3.2.3 *In vitro* culture of porcine embryo

The most stringent criteria to indicate proper maturation are the ability to undergo fertilisation and cleavage division. The second experiment was design to see if the IVM oocytes can undergo cleavage division.

3.2.3.1 Parthenogenetic activation of porcine embryo

Parthenogenesis is the process of embryo development without involvement of the male counterpart. This technique is a possible way of producing homogeneous

embryos with same ploidy. These embryos can develop up to the blastocyst stage during the cultivation; this makes the process of parthenogenetic activation a commonly used step for standardisation of other assisted reproduction technique like IVF or reproductive cloning. Ovulated or *in vitro* matured bovine oocytes are activated in metaphase II (MII) by spermatozoa or by artificial stimulus (Shirazi *et al*., 2009). When a spermatozoon activates an oocyte, it promotes multiple and periodic oscillations of intracellular free calcium. In mammalian oocytes, activity of maturation promoting factor MPF, which consist of cdc2 kinase and cyclin B, is essential for meiotic arrest at metaphase II (Gordo *et al*., 2000). Porcine oocytes are arrested at the metaphase II stage of meiosis and the activation is the release from this arrested stage and progression to first interphase. The objective of this study was to assess the ability of calcium ionophore with combination of 6-DMAP to parthenogenetic activation of porcine oocyte.

3.2.3.1.1 Chemical activation of oocytes

Chemicals such as calcium ionophore increase intracellular Ca^{2+} in the treated oocytes, which occur during normal fertilisation process. Increased Ca^{2+} destroys the existing Ca-sensitive cytostatic factor (CSF), resulting in the reduction of maturation promoting factor (MPF) activity (Whitaker, 2006). The increased intracellular Ca_2+ and the decreased CSF cause the inactivation of MPF, resulting parthenogenetic activation (Liu *et al*., 2003). The oocytes were activated by incubating in D 2 medium (TCM 199 medium used for day 2 of IVM culture as mentioned above), containing 5 µM calcium ionophore A23187 for 5 min at 39^0C under 5% CO_2 in air. The oocytes were then washed three times in D2 medium and were incubated individually in 5µl droplets containing 2 mM 6-Dimethylaminopurine (6-DMAP) and covered with mineral oil in CO_2 incubator at 39^0C under 5% CO_2 in air for 4 h.

3.2.3.1.2 Assessment of parthenogenetic activation

In order to evaluate the developmental competence of the embryos two mediums were studied namely porcine zygote medium 5 (commercial) and embryo development medium (EDM) (annexure). For the culture system, 500 µl of culture medium was added to each well of a 4 well petri dish overlaid with 500 µl mineral oil. The activated embryos were gently placed (10-15 per well) along the periphery of the well at a distance from each other so as to avoid aggregation. The petri dish was handled carefully so as not to displace the embryos and incubated undisturbed at 39ºC in 5% CO_2 in air for 7 days. The rate of embryo development (cleavage, 8-16 cell and morula) was recorded and percentage of development of each stage calculated.

3.2.4 Standardisation of porcine embryo culture condition

In order to establish a robust culture system for the *in vitro* fertilised porcine zygote two culture mediums were compared namely a TCM-199 based medium with modifications and a commercial medium PZM 5. Three basic modifications were tested. A modified porcine embryo culture medium (annexure) by substituting glucose with 0.2 mM pyruvate and 5.7 mM lactate in various concentrations for the first two days was tried. The second modification was inclusion of non-essential amino acids at a dilution of 1:100 into the medium. The timing for making a medium change was fixed at day two or 16 cell stage, as it was the first time point when embryonic development would be normally assessed. At this time a change was made from a culture medium containing pyruvate and lactate to a culture medium containing glucose 0.6 mM.

3.2.5 *In vitro* fertilisation

Matured oocytes after 48 h of maturation were taken out from maturation media drops and expanded cumulus cells were removed by repeated gentle pipetting in

mTBM (modified Tris buffered medium) medium using sterilised pasteur pipette. The oocytes were washed 5-6 times in mTBM medium. About 10-12 denuded oocytes were placed in 50 µl droplet of mTBM and covered with mineral oil and incubated at 39^0C under 5% CO_2 for the equilibration of medium prior to *in vitro* fertilisation. About 50 µl ($2x10^6$ to $4x10^6$sperm/ml) of capacitated sperm suspension was added to each 50 µl drop of m TBM medium containing denuded oocytes and co-incubated for 8 h at 39^0C in 5% CO_2 incubator.

3.2.5.1 Selection of superior quality spermatozoa

Sperm quality is a crucial parameter that determines the outcome of IVF. Unlike cattle and other domestic species for which high quality frozen semen straws are commercially available, the rich lipid content of porcine sperm makes cryopreservation difficult. This is also an important factor that reduces the efficiency of porcine embryo production. In the current experiment, a new system of selective colloidal centrifugation system was used to select good quality, highly motile spermatozoa. The semen was prepared by single layer centrifugation (SLC) through a colloid (Androcoll-P) to select good quality spermatozoa. Briefly, 4.5 ml extended semen were carefully layered on top of 4 ml Androcoll-P in a 15 ml tube. The tube was centrifuged at 300 × g for 20 min at room temperature and the supernatant removed. The centrifugation forms two distinct layers with non-motile and dead spermatozoa in the middle layer while motile and good quality spermatozoa settle at the bottom, as shown in Figure 11.

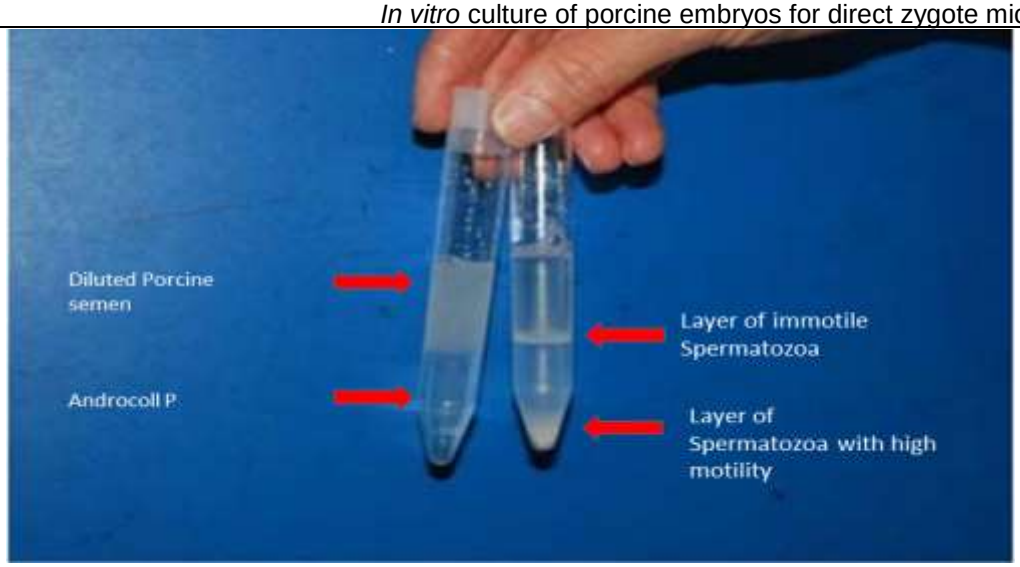


Fig 11. Sperm selection after centrifugation (300 x g, 20 minute). The bottom pallet with the highest motility is used for further processing.

3.2.5.2 Sperm quality analysis

3.2.5.2.1 Sperm chromatin dispersion test

The quality of the porcine sperm was assessed by sperm chromatin dispersion test. When somatic cells or spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinated nuclei show extended halos of DNA dispersion consisting of relaxed DNA loops attached to the residual nuclear structure. Such deproteinated nuclei are termed ‘‘nucleoids.’’ The presence of DNA breaks promotes the expansion of the halo of the nucleoid and is the basis for the halo test to detect DNA damage.

Methodology of SCD test given by Fernandez *et al*. (2003) for buffalo sperm samples with some modification was used. Aliquots of (0.2 ml) of sperm suspension (108 cells/ ml) were placed in a 1.5 ml micro centrifuge tube. The suspensions were mixed with 1% low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration) at 39^0C. Then, 80 µl of the mixture were pipetted onto a glass slide precoated with 0.70% standard agarose dried at 80^0C, covered with a coverslip, and left

to solidify at 4^0C for 5 min. Coverslips were carefully removed, and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 min at 22^0C in the dark, to generate restricted ssDNA motifs from DNA breaks. The denaturation was then stopped, and proteins were removed by transfer of the slides to a tray with neutrali2ing and lysing solution 1 (0.4 M Tris, 0.8 M DTT, 1% SDS, and 50 mM EDTA, pH 7.5) for 10 min at room temperature, followed by incubation in neutralising and lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 min at room temperature. Slides were thoroughly washed in Tris-borate–EDTA buffer (0.09 M Tris–borate and 0.002 M EDTA, pH 7.5) for 2 min, dehydrated in sequential 70, 90, and 100% ethanol baths (2 min each), and air dried. Finally, slides were stained in wright solution: phosphate buffer (1**:**1). In each experiment, observation of 200 sperm cells from 10 different samples was performed in 5– 7 min by single individual. Extent of variation in scoring DNA fragmentation in two different observers was estimated by scoring same slide by the second observer on second day. All experiments were done in triplicate.

3.2.5.2.1.1 Scoring criteria

Three dispersion patterns were clearly observed, similar to those reported by Pawar and Kaul (2011), during scoring SCD test in processed sperm nuclei by bright field microscopy and fluorescence microscopy: (i) nuclei with large DNA dispersion halos – halo width similar to or larger than the diameter of the core of the nucleoid; (ii) nuclei with medium sized halos– their halo size was between those with high and with very small halo;(iii) nuclei with no halo. Sperm cells with fragmented DNA had no halos or very small halos of dispersion of DNA loops, whereas those without DNA fragmentation release peripheral halos from the central.

3.2.5.2.2 Flow cytometry analyses

Porcine sperm samples were analysed using a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, CA, USA; Serial Number: AL300087) with collaboration with Dr. Ludovica Butto. The cytometer was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. The volume and diameter of the spermatozoa were measured directly with the Cell Lab Quanta® SC cytometer using the Coulter principle for volume assessment, based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system has, thus, forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10µm Flow-Check fluorospheres (Beckman Coulter) by positioning this size of bead at channel 200 on the volume scale. In this system, the optical characteristics for these filters were: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width 505nm-545nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560 nm-590 nm); and FL3 (red fluorescence): LP filter: 670 nm/730 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR-14, PNA-FITC, YO-PRO-1, Fluo3-AM and H_2DFCDA), while FL3 was used to detect red fluorescence (M540, HE and PI). Sheath flow rate was set at 4.17 µl min^{-1} in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE *vs.* SS dot plots) for a minimum of 10,000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter >12 µm). Therefore, the sperm-specific events were positively gated on the basis of EV and SS distributions, while the others were gated out.

3.2.5.2.2.1 Sample preparation

Pre diluted sperm tubes (75 ml) were brought from commercial supplier. For Androcoll P selection 1 ml of sperm was carefully overlaid on 3 ml of Androcoll-P in a 15 ml tube and centrifuged for 10 minutes at 3000 RPM. The supernatant was discarded. The sperm pallet was dissolved in 2 ml of porcine fertilisation medium. 20 µl of diluted sperm was taken in 1 ml micro tube and 480 µl of Androhep® Plus (13529/6005 Minitube) was added to it to prevent cell clumping. A normal sperm sample without Androcoll P selection was used as a control.

3.2.5.2.2.2 Live and dead count

The viability of the sperm samples was analysed by propidium iodide (PI) staining. Briefly, sperm samples were incubated at 38^0C for 5 min with PI at a final concentration of 10 µM for 5 min at the same temperature. FL-3 was used for measuring the PI fluorescence.

3.2.5.2.2.3 Assessment of capacitation

Plasma membrane integrity was also evaluated through PNA-FITC/PI. In this case, spermatozoa were stained with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocyanate (FITC) and with PI, according to the modified procedure described by Nagy *et al*., 2003. Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5µg/mL) and PI (final concentration: 10µM) and incubated at 38^0C for 5min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3.

3.2.5.3 Co incubation of selected spermatozoa and IVM oocytes for IVF

After sperm-oocyte coincubation, putative zygotes were washed three times in IVC medium, transferred (30-40 embryos) to a Nunc 4-well multi dish containing 500µl

of the same medium per well, and incubated at 39^0C, 5% CO_2 in air. At 48 and 144 h after IVF, cleavage rate was evaluated under a stereomicroscope.

3.2.6 Embryo microinjection

Microinjection was carried out using an Eppendorf Transferman NK2 system. For standardisation of the conditions of microinjection parthenogenetically activated zygotes were used. As a convenient reporter, enhanced green fluorescent protein (EGFP) mRNA (5ng/µl) was injected into the cytoplasm of early stage embryos to identify the conditions favouring the stability and expression of the mRNA.

3.2.6.1 Injection needle fabrication

Needle for injection were made with standard wall borosilicate tubing with filament capillary (Sutter Instrument BF100-50-10). Filamented glass had a small rod of glass annealed to the inner wall and this rod (filament of glass) created the capillary action required to back-fill the pipette with solution. The filament in the glass not only provided capillary action for quick filling of the micropipette; it also helped to reduce the incidence of air bubbles when introducing solution into the pipette.

3.2.6.1.1 Pipette puller settings

For making the injection pipette Flaming and Brown P 87 model pipette puller by Sutter instruments was used. It was very critical to get the correct setting for pulling a perfect injection needle with proper stalk length. A trough filament was used for pulling and a ramp test was run to standardise the puller settings. When a ramp test was executed, the following events took place:

1. The puller incremented the heat

2. As the heat output began to soften the glass, the puller bars moved apart

3. The heat was then turned off when a certain factory-set ramp test velocity was reached

4. The ramp test value was then shown on the display

A ramp value of 425 volt was obtained using a simple formula provided by Sutter instrument i.e. Heat for pulling =ramp value +15 a voltage of 440 was obtained. A final setting was heat-440 v; pull-70; velocity-70; time-134. A 45^0 angle was given using a blowtorch carefully for proper injection.

3.2.6.2 Micromanipulation of parthenogenetically activated oocytes

As a convenient reporter, enhanced green fluorescent protein (EGFP) mRNA (5ng/µl) (generously supplied by Tatiana and Denise) was injected into the cytoplasm of early stage embryos to identify the conditions favouring the stability and expression of the mRNA. Both the holding pipette (VacuTips Eppendorf) and the injecting pipette were focused under the inverted microscope and brought into same level horizontal to the plan of operation. The upper lid of a 60 mm tissue culture dish was used for manipulation. For manipulation DPBS solution+10% FCS with penicillin/streptomycin was used. A single oocyte was fixed with the holding pipette positioning the polar body at the 6 or 12 o'clock position. The injection pipette was pushed through the zona pellucida and the cell membrane into the cytoplasm. In opaque porcine zygotes, the pronuclei are not discernible. The mixture of eGFP mRNA was deposited 'blindly' in the cytoplasm. An injection pressure between 0.5 and 0.8 hPa was applied depending on the opening size of the injection pipette. The injection pressure can be set at the Eppendorf transjector. An estimated volume of 10 pl of solution was injected by this procedure. Microinjection was verified in each oocyte by observing disturbance in the cytoplasm caused by solution streaming from the capillary. After injection the zygote was moved to the upper part of the elongated drop thus creating a clear demarcation

between injected and not injected zygote as shown in the fig 12. The temperature was maintained at 39^0C throughout the whole procedure using a heated

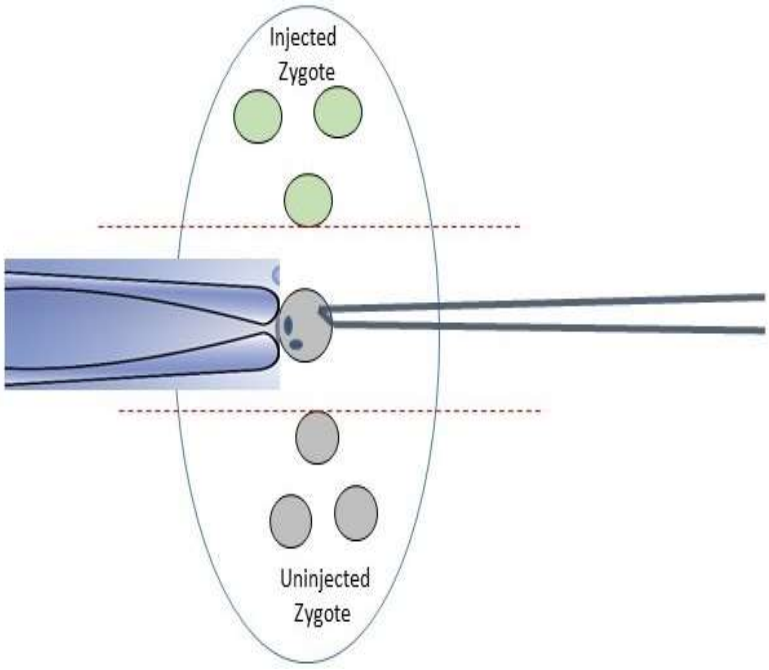


Fig 12 Micromanipulation droplet. The uninjected zygotes are kept at the bottom of the drop, while microinjection is performed at the centre of the droplet and injected zygotes kept at the top of the droplet.

microscope stage. Injected embryos were then cultured and further development monitored. Embryos were observed to develop up to the morula stage.

3.2.7 Microinjection of CRISPR /Cas 9

3.2.7.1 Designing CRISPR against *GGTA*1 and *CMAH*

CRISPR/Cas9 enzymes were designed to target porcine *GGTA1* (α1, 3-galactosyltransferase) and *CMAH* (cytidine monophosphate-N-acetylneuraminic acid hydroxylase) genes (done by Beate Reiblinger) using the CRISPR design web tool at CRISPR.mit.edu. Exposure of pig organs to human blood results in hyperacute rejection (HAR). This is caused by alpha Gal epitopes on the pig vascular endothelium being recognised by natural anti-Gal antibodies in human serum. Antibody binding to the alpha Gal epitopes results in complement activation and cell death. In an effort to avoid

HAR, the *GGTA1* gene (glycoprotein, alpha-galactosyltransferase 1) responsible for alpha Gal synthesis has been inactivated by several researchers, and is now considered to be the critical first step to producing genetically modified donor animals for xenotransplantation (Phelps *et al.,*2003; Milland *et al*., 2005).

Similar to alpha Gal, the N-Glycolylneuraminic acid (Neu5Gc) epitope synthesised by CMP-Neu5Ac hydroxylase (*CMAH*) is widely expressed on endothelial cells of most mammals except humans. A mutation in the *CMAH* gene, unique to humans, causes the absence of Neu5Gc in human, which is present in pigs and elevated in the non-human primates, chimpanzees and baboons (Wang *et al*., 2014). A large proportion of non-Gal xenoreactive antibodies in most healthy human sera is directed toward Neu5Gc epitopes. Neu5Gc is present on many pig cells including vascular endothelial cells, peripheral blood mononuclear cell (PBMC) and erythrocytes, so eliminating porcine *CMAH* gene expression is very important to produce organ donors for xenotransplantation (Kwon *et al*., 2013).

A sgRNA targeting the exon 10 region of *CMAH* and exon 8 of *GGTA1* gene was designed and expressed using the pBS U6 chimaeric expression vector described by Jinek *et al*. (2012). Oligonucleotides containing the recognition site and the T7 promoter sequence as well as suitable overhangs were cloned into pBS U6 chimaeric previously digested with BbsI. The obtained plasmids were sequenced; a correctly assembled vector was then used as a template for two subsequent rounds of PCR with primers T7 FW and Trac RV. The purified PCR product of the second PCR was then transcribed with the MEGA short script T7 kit. All components including Cas9 were transcribed *in vitro* into mRNA; to synthesise Cas9 mRNA, the m7 G (5') G RNA cap structure analog (NEB) was additionally added to stabilise the transcribed mRNA. Prepared RNAs were purified using MicroElute® RNA Clean-Up Kit (Omega) and recovered in DEPC water.

3.2.7.2 Cytoplasmic microinjection of Cas9 mRNAs

The cytoplasmic mRNA microinjection was performed according to parameters already optimised for eGFP m RNA injection. To test the viability of pig embryos after Cas9 injection, mock microinjection was performed with *in vitro* produced parthenogenetic embryos. For this purpose, parthenogenetic embryos were produced by chemical activation using 5 µM calcium ionophore and 2 mM 6 DMAP as already explained in the thesis. For the actual Cas 9 microinjection, putative *in vitro* fertilised zygotes cultured for 18 h, were transferred to manipulation medium and subjected to a single cytoplasmic microinjection of 2-10 pl 125 ng/µl Cas9 mRNA and 12.5 ng/µl sgRNA. After injection, the zygotes were cultured to morula stage in PZM5 medium for 72 hours under 5% CO_2, 39^0C. At morula stage, DNA was isolated from the single zygotes using REPLI-g kit (Qiagen) and mutations were found via PCR and subsequent sequencing.

3.3 Results

3.3.1 Optimisation of *in vitro* maturation culture

3.3.1.1 Nuclear maturation

Of the 191 COCs in the nuclear maturation group, 63 assessed as grade A (32.99%), 67 grade B (35.07%) and 61 grade C (31.93%). Nuclear maturation of each oocyte was scored by the attainment of metaphase II, as judged by the presence of condensed chromosomes in an equatorial position and extrusion of the first polar body (Fig. 13). Nuclear maturation data (Table 3) showed no significant difference between morphological grade A (79.9% ± 3.82), grade B (86.48% ± 3.07), or grade C (82.00% ± 3.00) oocytes. The maturation status was evaluated by epifluorescence microscopy (UV filter and Carl Zeiss) using standard parameters such as germinal vesicle breakdown,

condensation of chromatin and appearance of the first polar body as confirmation of nuclear maturation.

Table 3 Rate of nuclear maturation of porcine oocytes after 48 hours maturation *in vitro*, as measured by Hoechst staining.

	Nuclear maturation (%) of *in vitro* matured oocytes		
	Grade A	Grade B	Grade C
Nuclear maturation (%)	79.9±3.8[a]	86.48±3.07[a]	82.00±3.00[a]

Values are arithmetic mean ± standard deviation. Oocytes were graded by the morphology of their surrounding cumulus investments: grade A, at least three layers; grade B, less than three layers; or grade C, only corona radiata.

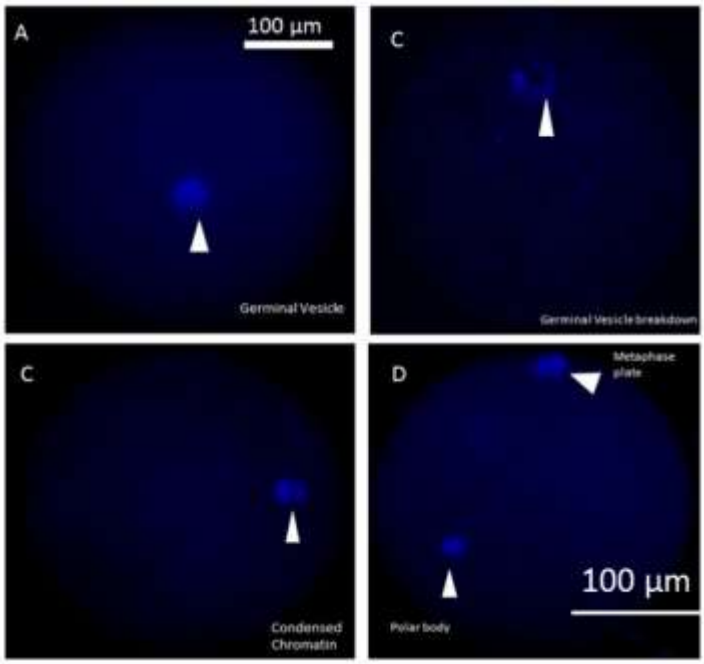


Fig 13 Progression of *in vitro* cultured oocytes through different stages of nuclear maturation. A, Immature oocyte. B, Germinal vesicle breakdown. C, Appearance of first polar body. D, Mature oocyte with visible polar body and metaphase plate.

3.3.1.2 Developmental competence of oocytes

The parthenogenetic activation group comprised 204 oocytes. 204 were graded by morphology as 67 grade A (32.84%), 71 grade B (34.80%) and 66 grade C (32.35%); Development was scored as the number of embryos consisting of two to eight evenly sized blastomeres 48 h after parthenogenetic activation as shown in fig 14. There was no significant difference between morphological grades A (84.55% ± 4.45), grade B (85.06% ± 4.49), or grade C (81.60% ± 4.64) oocytes in terms of rate of activation.

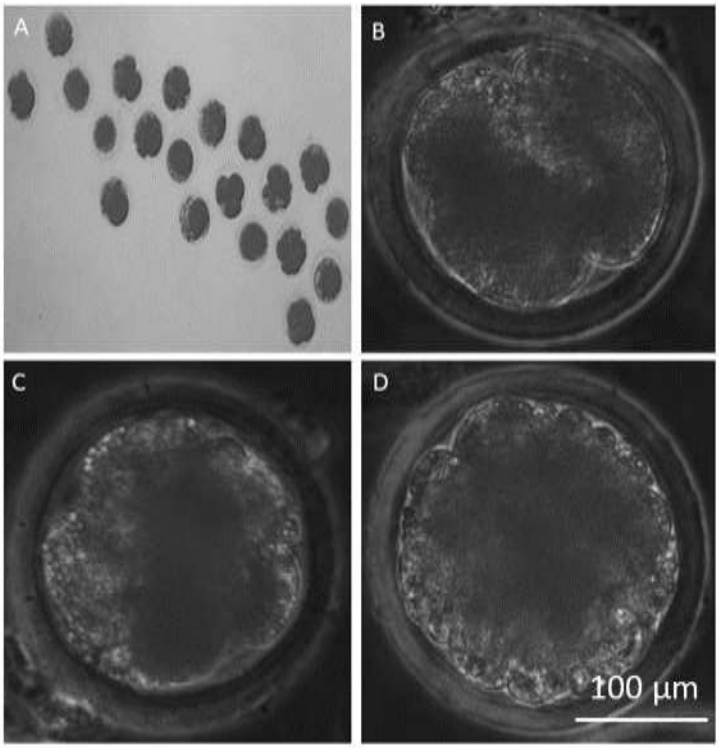


Fig 14 A. a group of embryo at different stages of development, B. 2-cell stage embryo, C. an embryo at 4-8 cell stage D. a compact morula stage embryo.

Table 4 Rate of parthenogenetic activation of porcine oocytes calculated retrospectively after 96 hours of *in vitro* culture.

Proportion of IVM oocytes developing to morula stage embryo after parthenogenetic activation			
Parameter	Grade A	Grade B	Grade C
Activation (%)	84.55±4.4[a]	85.06±4.49[a]	81.60±4.64[a]

Values are expressed as arithmetic mean ± standard deviation

3.3.1.3 Effect of different culture medium on embryo development

The effect of medium on embryo development was calculated retrospectively by counting the number of morula stage embryos developed after activation. There was no significant difference between PZM 5 and TCM-199 based medium in terms of embryo growth.

Table 5 Comparison of embryo development rate in PZM 5 and TCM-199 based EDM.

Medium	Oocyte (N)	Morula (N)	Cleavage (%)
PZM5	400	332	83±3.66[a]
TCM-199 based	310	256	82.58±2.78[a]

*there was no significant difference between the medium at $p<0.05$ (analysed by Paired t test)

3.3.2 *In vitro* fertilisation

3.3.2.1 Selection of superior quality spermatozoa

3.3.2.1.1 Sperm quality analysis using sperm chromatin dispersion test

The quality of the porcine sperm was assessed by a sperm chromatin dispersion test. When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinated nuclei show extended halos of DNA dispersion. The presence of DNA breaks promotes the expansion of the halo of the nucleoid (Fig 15). Sperm selected using the Androcoll P system showed a lower DNA breakage index than untreated sperm. Interestingly, a direct correlation was obtained between the rate of DNA breakage and the rate of fertilisation.

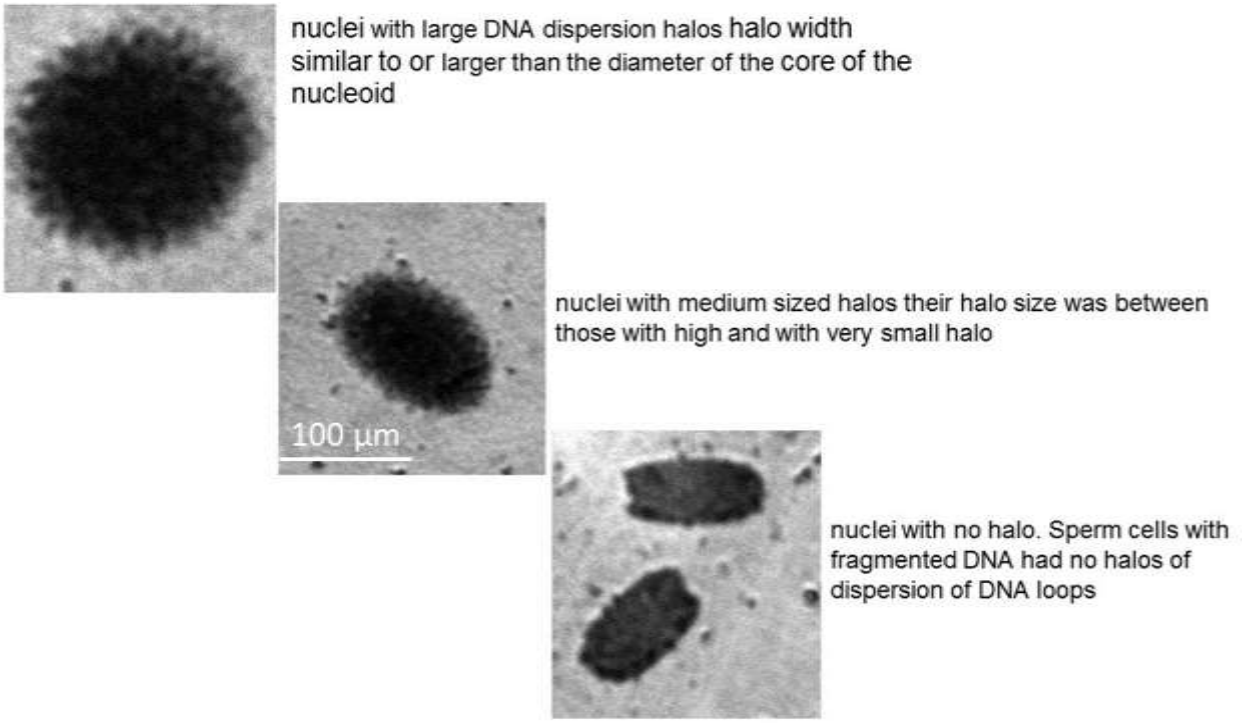


Fig 15 Expansion pattern of the halo of the three different types of nucleoid observed in SCD test.

The dispersion pattern revealed for Androcoll P selected porcine sperm 62 ± 5.7%, 21.8 ± 3.76% and 16.2 ± 2.77% sperm with a large, medium or small, no halo respectively. The same for control sperm was 20.4±2.30%, 13.8±2.17%, 65.8±3.11% sperm with a large, medium or small and no halo respectively. The DNA fragmentation index (DFI) was calculated as percentage of sperm head with no halo i.e. 16.2 for Androcoll P selected sperm and 65.8% for control sample. Result obtained in the SCD was compared and correlated with the data obtained in the fertilisation experiment (80.20%). There was good correlations between low DFI 16.2 ± 2.77 and rate of fertilisation ($r = -0.29$, $p < 0.05$).

3.3.2.1.2 Sperm quality analysis by flow cytometry

Androcoll P selected sperm were further analysed using flow cytometry. The parameter analysed were viability (live and dead), status of capacitation and mitochondrial status. The treated samples when observed under fluorescent microscope revealed the pattern as shown in fig 16

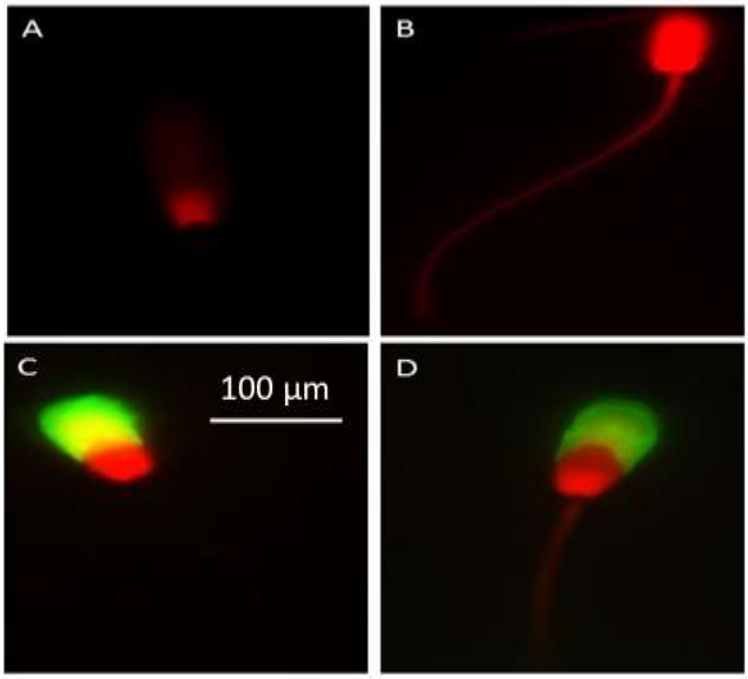


Fig 16 Fluorescent staining pattern of porcine sperm. A-Live spermatozoa with active mitochondrial function, B-Dead spermatozoa stained with propidium iodide, C-PNA +ve; Mito +ve acrosome reacted (capacitated sperm) with mitochondrial activity, D-PNA +ve; PI +ve acrosome reacted (capacitated sperm) dead sperm.

3.3.2.1.2.1 Live and dead percentage

The viability of the Androcoll P selected sperm samples were analysed by propidium iodide (PI) staining after this assessment, two sperm populations were identified: i. viable unstained spermatozoa (PI-); ii. Non-viable red-stained spermatozoa (PI+). Out of the total population, 87.9% were viable live spermatozoa and 10.6% were dead spermatozoa (fig 17).

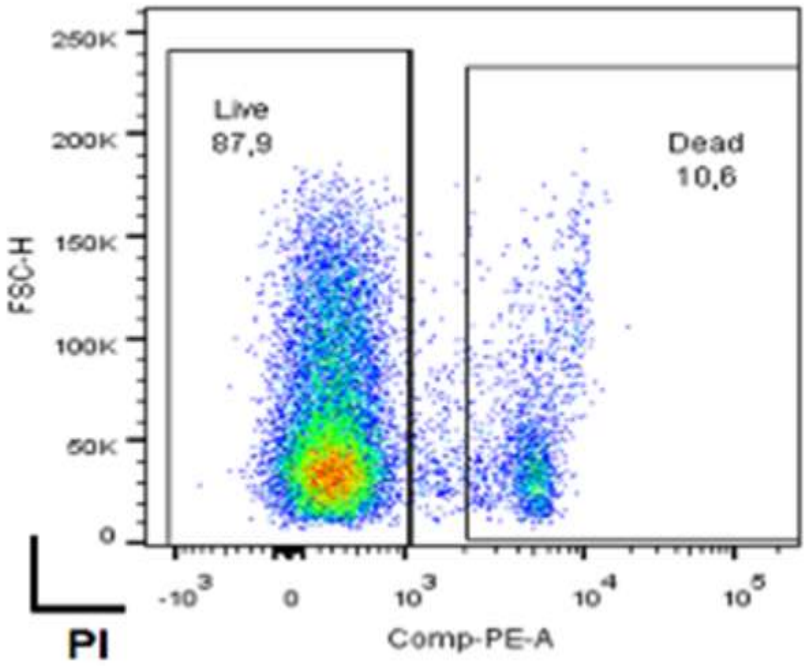


Fig 17 Distribution of live and dead sperm population as stained by propidium iodide as separated by Flow cytometry.

3.3.2.1.2.2 Mitochondrial status

The Androcoll p selected sperms were analysed for their mitochondrial status with a mitochondria specific stain called Mitotracker orange. The stain Mitotracker orange when combined with PNA-FITC revealed 3 subpopulations of spermatozoa namely-(Mito +/PNA-FITC+), (Mito +/ PNA-FITC –) and (Mito -/PNA-FITC –) as seen in fig 18.

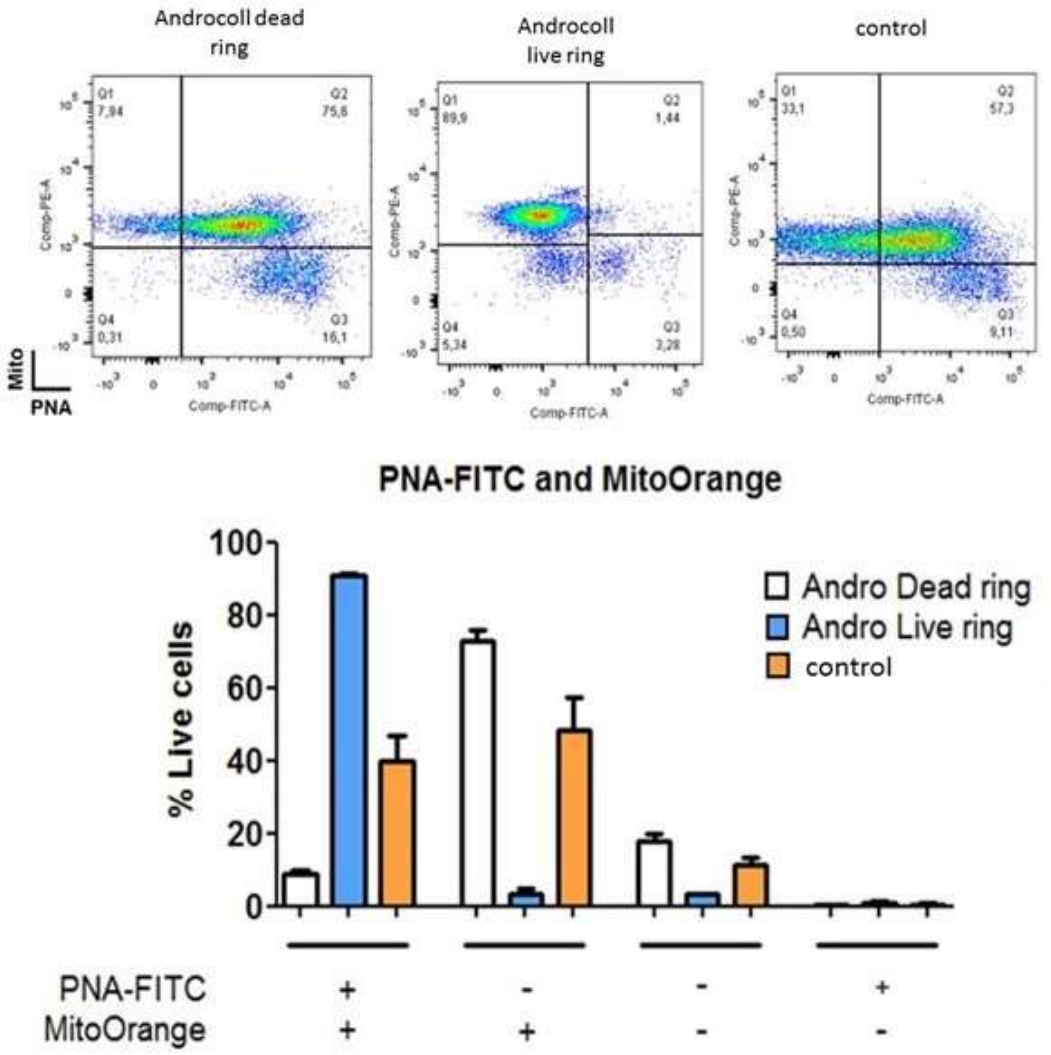


Fig 18 Distribution of 3 subpopulations of spermatozoa namely-(Mito +/PNA-FITC+), (Mito +/ PNA-FITC –) and (Mito -/PNA-FITC –)

3.3.2.1.2.3 Capacitation status

The capacitation status was detected using PNA-FITC staining. When combined with propidium iodide three subpopulations of spermatozoa were identified. i. Spermatozoa with intact plasma membrane (PNA-FITC-/PI-) ii. Spermatozoa with damaged plasma membrane that presented an outer acrosome membrane that could not be fully intact (PNA-FITC+/PI+) iii. Spermatozoa with damaged plasma membrane and intact outer acrosome membrane (PNA-FITC+/PI+) as seen in fig 19.

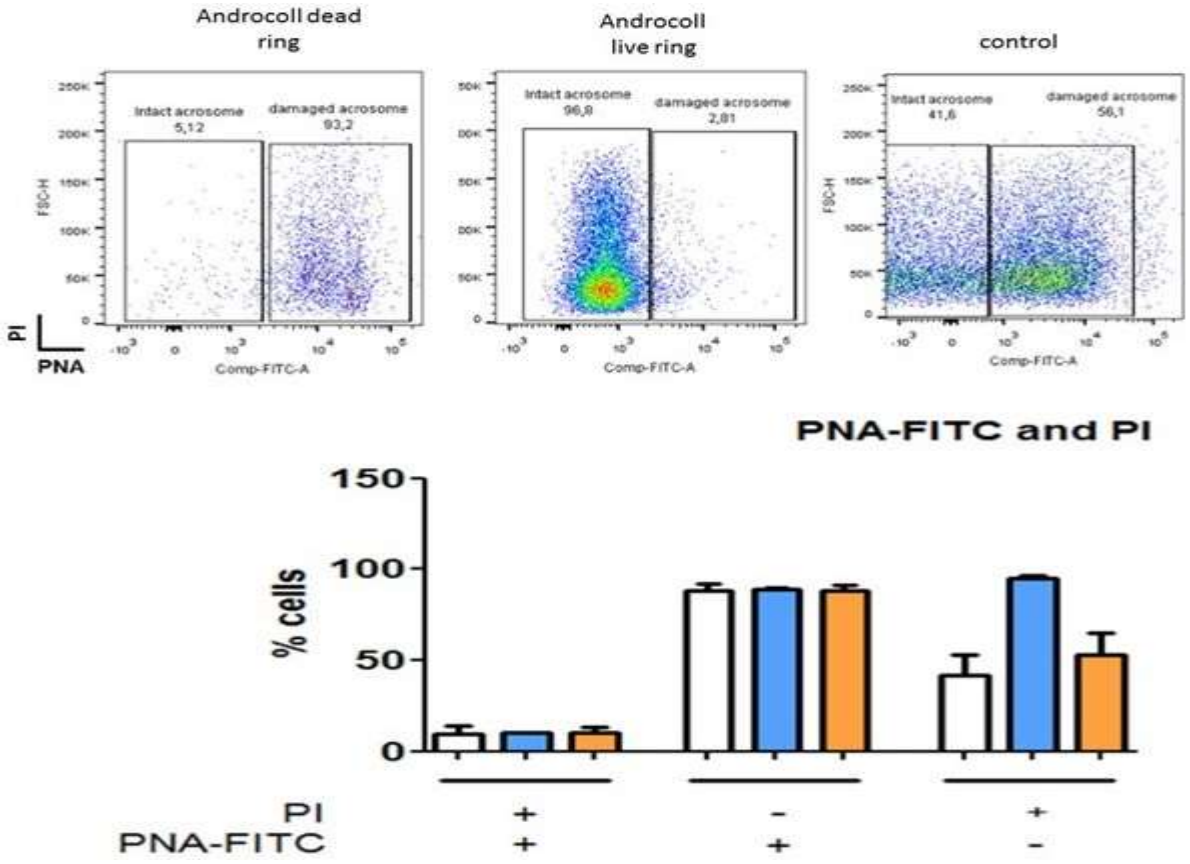


Fig 19 Distribution of 3 subpopulations of spermatozoa based on capacitation namely- (PI +/PNA-FITC+), (PI+/ PNA-FITC –) and (PI -/PNA-FITC +)

3.3.2.3 Optimisation of *in vitro* fertilisation

Samples of Androcoll P selected spermatozoa were diluted in IVF medium 1:50 corresponding to 4 × 103 cells/ml. After washing, 30-40 IVM oocytes were placed in 50µl drops of the same medium and covered with paraffin oil in a 35 mm dish (Corning). After appropriate dilution, 50 µl of sperm suspension was added to the drop containing the oocytes and coincubated at 39^0C for 6 hours. After coincubation, remaining cumulus cells and excess spermatozoa were removed by vortexing for 1 min and presumptive fertilised oocytes transferred to 500 µl *in vitro* culture medium (PZM-5). The numbers of early cleaved embryos at 24 h after fertilisation and total cleavage rates (48 h post insemination, hpi) were assessed by Hoechst 33342 staining. Oocytes were considered

as fertilised when they contained one or more swollen sperm heads and /or male pronuclei. Fertilisation rates were calculated retrospectively and included the percentage of non-cleaved oocytes with ≥ 2 pronuclei-like structures (thus, including polyspermic oocytes) that were arrested and were unable to cleave, plus the embryos found at 48 hpi. An overall fertilisation rate of 78.92% was obtained and 68.91 % of embryos developed to morula stage.

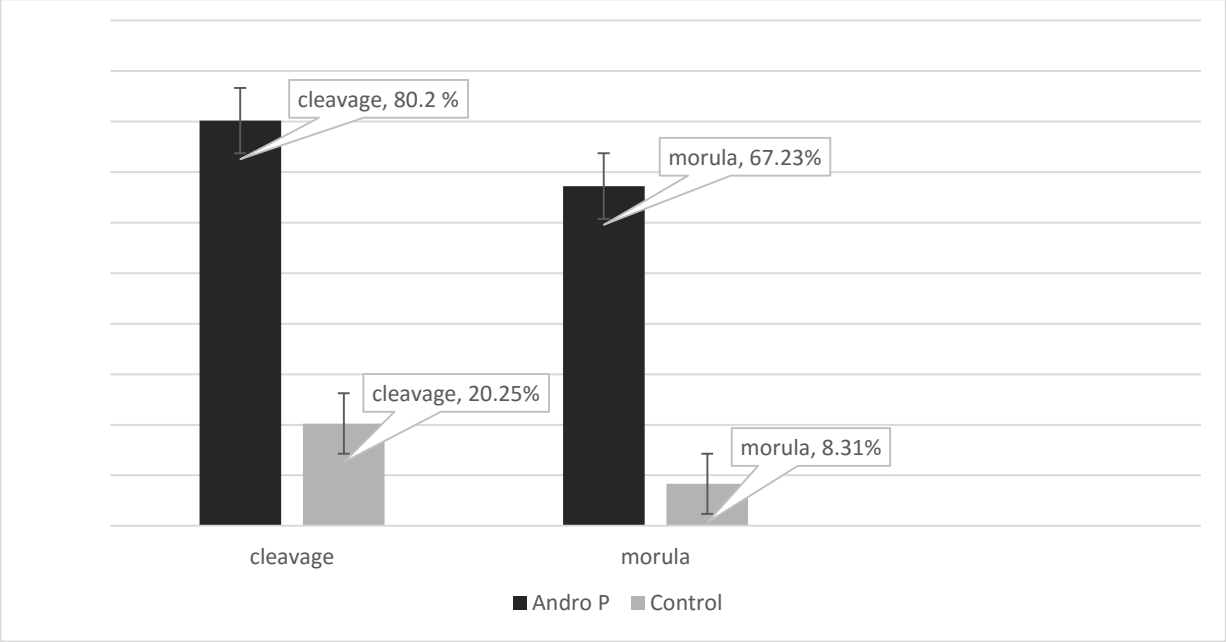


Fig 20 Cleavage and morula development rate among Androcoll P selected and control spermatozoa after IVF

However, >75% of the developed embryos were polyspermic, as shown in Figure 21. Polyspermy is a common occurrence in porcine IVF and is well recognised to be a major obstacle to establishing porcine embryo IVP.

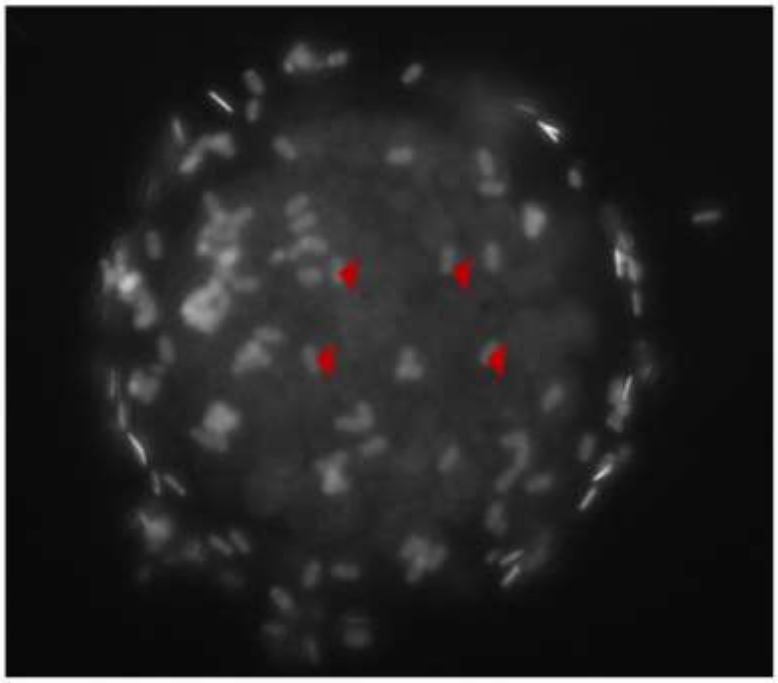


Fig 21 IVF zygote showing polyspermic penetration. The red arrows pointing at the swollen sperm heads.

3.3.3 Embryo microinjection

Microinjection was carried out using an Eppendorf transferman NK2 system Enhanced green fluorescent protein (EGFP) mRNA (5ng/µl) was injected into the cytoplasm of early stage embryos to identify the conditions favouring the stability and expression of the mRNA. Microinjection was verified in each oocyte by observing slight swelling of the pronucleus, or disturbance in the cytoplasm caused by solution streaming from the capillary. Injected embryos were then cultured and further development monitored. Embryos were observed to develop up to the morula stage and green fluorescent signal was detected under microscope (Fig 22).

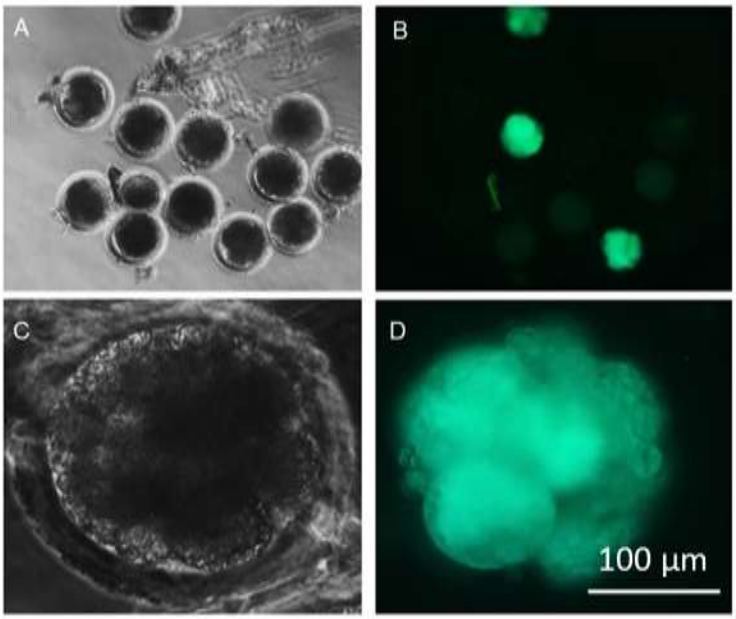


Fig 22 Embryo development following microinjection. A-B. A group of injected embryos under bright field and GFP fluorescence; C-D. A single embryo under bright field and GFP fluorescence. (excitation = 489 nm, emission = 509 nm)

3.3.4 CRISPR/ Cas 9 microinjection

A sgRNA targeting the exon 10 region of *CMAH* (CMP-Neu5Ac hydroxylase) and exon 8 *GGTA*1 (glycoprotein galactosyltransferase α 1, 3) gene was designed and expressed using the expression vector described by Jinek *et al*. 2012 (Pham-Thi, 2014). 125 ng/µl Cas9 mRNA and 12.5 ng/µl sgRNA targeting both the gene of interest were injected in to the putative *in vitro* fertilised zygotes from cultured for 18 h. After, injection at zero cell stage of embryo development post IVF, 82 % of injected embryos developed up to the morula stage. While mutations were found via PCR and subsequent sequencing 20% of the embryos were double knockouts. 64% of the embryos homozygote knockouts for *CMAH* Ex10 and 30% were for *GGTA* 1Ex 8 as seen in table 6.

Table 6 Summary of CRISPR /CAS9 targeting of *CMAH* and *GGTA*1 gene

Genotype	*CMAH* Ex10	*GGTA*1 Ex 8
Homozygous	64%	30%
Heterozygous	14%	10%
Wild-type	21%	60%

3.4 Chapter summary

3.4.1 Optimisation of IVM culture condition against different variables

The TCM 199 based medium used for culture was found to support a very efficient *in vitro* maturation rate of 86.48%. The maturation status was evaluated by epifluorescence microscopy (UV filter and Carl Zeiss) using standard parameters such as germinal vesicle breakdown, condensation of chromatin and appearance of the first polar body as confirmation of nuclear maturation. The rate is comparable to the maturation rate reported in porcine by other researchers using NCSU 37 (Abeydeera and Day, 1997). For maturation culture, the oocytes were chosen based on the traditional grading system of Grade A, B and C on the basis of the thickness of the cumulus cell layers. A nuclear maturation rate among A, B and C grade oocytes were calculated respectively as 79.9%±3.8, 86.48%±3.07, and 82.00%±3.00. It was clearly evident that the rate was not statistically different among the different grades of oocytes graded based on cumulus investment. While higher rates of nuclear maturation, fertilisation and development after IVP have been reported in Grade A and B bovine oocytes, the current data showed that this cannot automatically be extended to porcine oocytes. The findings clearly reaffirmed the importance of a cumulus layer for oocyte maturation and development (Davachi *et al*., 2012, Prates *et al*., 2014), but suggest that measures of cumulus cell integrity are more useful than morphological parameters alone.

3.4.2 Achieving both nuclear and cytoplasmic maturation

Although the protocol used has given a very high rate of nuclear maturation, the same cannot be said about the cytoplasmic maturation with certainty. It is noteworthy that there is currently no method available to measure completion of cytoplasmic maturation other than successful fertilisation and embryonic development (Yuan and Krisher, 2012). An efficient *in vitro* environment must support both nuclear and cytoplasmic maturation. While nuclear maturation, which entails the re-initiation and successful completion of meiosis I, can be easily assessed, cytoplasmic maturation is a much more complex, multifaceted process that is difficult to measure, but equally critical to successful oocyte competence and development. Even oocytes that complete nuclear maturation may be incompetent for further development following fertilisation (Edwards *et al*., 1969). Thus, careful attention to the culture environment during oocyte maturation is critical to optimise oocyte developmental potential.

3.4.3 Developmental competence of IVM cultured oocytes

A stringent measure of successful *in vitro* maturation of the cultured oocytes is their ability to develop into embryos. To assess their developmental competence IVM cultured porcine oocytes were parthenogenetically activated chemically using 5 µM calcium ionophore A23187 in combination with 2 mM 6-dimethylaminopurine (6-DMAP). A total of 204 IVM oocytes were parthenogenetically activated out of which 173 developed to morula stage embryo, which further confirmed the successful *in vitro* maturation protocol. Activation rate among A, B and C grade oocytes were calculated respectively as 84.55%±4.4, 85.06%±4.49, and 81.60%±4.64. It was clearly evident that the activation rate was not statistically different among the different grades of oocytes graded based on cumulus investment.

3.4.4 Optimisation of *in vitro* fertilisation

3.4.4.1 Need for selecting superior quality sperm

Due to the unavailability of commercial frozen porcine sperm attempts were made to use pre-diluted commercial sperm for the IVF experiments. In order to select the sperm with highest quality in terms of motility and fertility single layer centrifugation (SLC) was used. It has been reported as a useful technique to select porcine spermatozoa for further artificial insemination practices. The goal of the experiment was to evaluate the suitability and effectiveness of single layer centrifugation (SLC), using the pig-specific colloid Androcoll-P, as a routine procedure for selecting boar spermatozoa for IVF procedure. The study focused special attention on the effectiveness of SLC for processing a commercially available pre diluted sperm tubes. One sperm fraction (one per boar) was split into two aliquots. One aliquot of 3 mL was SLC-processed (500 × *g* for 20 min) using 6 ml of Androcoll-P-Large and the other aliquot remained un-processed as a control.

3.4.4.2 Sperm quality analysis using sperm chromatin dispersion test

The quality of the porcine sperm was assessed by a sperm chromatin dispersion test. Androcoll P selected porcine sperm revealed a dispersion pattern as 62 ± 5.7%, 21.8 ± 3.76% and 16.2 ± 2.77% sperm with a large, medium or small, no halo respectively. The same for control sperm is 20.4±2.30%, 13.8±2.17%, 65.8±3.11% sperm with a large, medium or small and no halo respectively. Sperm selected using the Androcoll P system showed a lower DFI of 16.2 than untreated sperm with DFI of 65.8. Interestingly a direct correlation was obtained between the rate of DNA breakage and the rate of fertilisation. There was good correlations between low DFI 16.2 ± 2.77 and rate of fertilisation ($r = -0.29$, $p < 0.05$).

3.4.4.3 FACS analysis of Androcoll P selected sperm

The Androcoll P selected sperm were analysed with flow cytometry for parameters of viability (live and dead), capacitation and mitochondrial status. The recovery rates of total, motile, viable (flow cytometric evaluated after staining with PI and FITC-PNA) and morphologically normal spermatozoa ranged between 20 and 100% and those with intact nuclear DNA ranged between 60 and 100%, irrespective of the sample variability. The viability and intact nuclear DNA were higher ($P < 0.05$) in SLC-processed than in control semen samples, irrespective of the Androcoll-P used.

The Androcoll P system selected a distinctly superior population of sperm for IVF trial. The selected spermatozoa had better viability as well mitochondrial activity. However, the Androcoll selection did not have distinguishable impact on the capacitation status. Sperm selected using the Androcoll P system showed a lower DFI, which in turn improved the fertilisation rate.

3.4.5 *In vitro* fertilisation

After co-incubation with Androcoll P selected sperm (the *in vitro* matured oocytes inseminated with a viable sperm: oocyte ratio of 300:1 and coincubated for 6 h), the *in vitro* fertili2ing ability was measured as the percentage of penetrated oocytes and the mean number of swollen sperm heads and/or male pronuclei in penetrated oocytes. Fertilisation rates were calculated retrospectively and included the percentage of non-cleaved oocytes with ≥ 2 pronuclei-like structures (thus, including polyspermic oocytes) that were arrested and were unable to cleave, plus the embryos found at 48 hpi. An overall fertilisation rate of 78.92% was obtained and 68.91 % of embryos developed to morula stage. The rate of fertilisation is at per with other reports (Grupen, 2014; Abeydera *et al*., 2002).

3.4.6 Direct microinjection into porcine zygote

After optimising a robust *in vitro* culture protocol, a microinjection protocol was successfully established for direct cytoplasmic injection of CRISPR /Cas 9 mRNA into *in vitro* fertilised porcine zygotes. The standardisation of the protocol was done using m eGFP injection into parthenogenetically activated porcine zygote as parthenogenesis was used as a convenient and valid functional assay of developmental competence (Rougier and Werb 2011, McElroy *et al*., 2010). After injection green fluorescence was observed in the injected embryos. Once conditions for microinjection were standardised the CRISPR/Cas 9 injections were performed in IVF produced zygote. To ensure a successful transfer of foreign genetic material into porcine zygotes, and to increase the chance of genome editing, a number of parameters were carefully evaluated and applied when performing microinjection. The time for injection was chosen at 18 h post IVF before the first cleavage division as it is the window for genome activation and missing the window meant chimeric integration. It is very important to have an optimum concentration of injected mRNA into the zygote. A concentration that is too high may cause zygotes to lyse, and a concentration that is too low may yield poor or no activity of Cas9. A concentration of 125 ng/µl Cas9 mRNA and 12.5 ng/µl sgRNA was used for the microinjection and it gave the best transgenic efficiency. Moreover, the injection pressure with which the mRNA was injected into the cytoplasm was maintained low enough not to damage the zygote. A flow rate of 0.5 and 0.8 hPa was used to deliver the m RNA using an Eppendorf transjector. For a successful outcome the manner in which the injection is performed is very important. Gentle yet swift injections minimise the trauma to the zygotes. This was done first by carefully breaking the tip of the needle against the holding pipet in order to create ideal fluid flow out of the injection needle. The zona pellucida punctured very gently and injection was performed as quickly as

possible. Upon injection 79.5% of the injected zero stage embryos developed up to the morula stage. The rate of development was at par with earlier reports in pig. Close to 20 % of the embryos either did not undergo cleavage or stopped after cleavage at 2-4-cell stage. Earlier some reports suggested nearly lysis of ~20% zygotes after injection (Ivics *et al*., 2014). This lysis was not seen during the study. This high percentage of successful development indicated that Cas9 mRNA/sgRNA had little toxicity to pig embryonic development as reported earlier (Hai *et al*., 2014).

3.5 Chapter conclusion

In conclusion, this study established a simple, efficient and easily reproducible *in vitro* culture system for porcine embryos that can be used for direct zygote microinjection. An optimised IVM system that supports very high rate of nuclear maturation and developmental competence was standardised. A relatively high rate of *in vitro* fertilisation was also obtained. Injection of CRISPR/Cas 9 enzymes targeted to *CMAH* exon 10 and *GGTA 1* exon 8 resulted in 64% homozygous knockout and 14% heterozygous knockout for *CMAH* and 30% homozygous knockout and 10% heterozygous knockout for *GGTA1* respectively. 20% of the embryos were double knockouts. However, there were some problems of batch-to-batch sample variation, developmental block, difficulty in sperm freezing and most importantly polyspermy. These issues have been elaborated further in the discussion chapter of this thesis.

Chapter IV

Non-invasive assessment of porcine oocyte quality by supravital staining of cumulus-oocyte complexes with lissamine green B

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4 Supravital staining of cumulus-oocyte complexes with lissamine green B

4.1 Chapter Introduction

The cumulus layer is undoubtedly vital for oocyte development, but visual observation of cumulus layers of oocyte grading provides only limited information about its functional competence. Alternative methods of PCR based analysis of cumulus cells are too time consuming and expensive for routine laboratory use. Propidium iodide staining has also been used as a non-invasive method of determining cumulus cell integrity (Uchikura *et al*., 2011), but this requires fluorescent microscopy, which may not be available for some researchers and may be potentially mutagenic. In this experiment the usefulness of lissamine green B (LB) staining of cumulus-oocyte complexes (COC) as a non-invasive method of predicting maturational and developmental competence of slaughterhouse-derived porcine oocytes cultured *in vitro* was evaluated. LB is used in ophthalmology to evaluate ocular membrane integrity (Hamrah *et al*., 2011), but it has never previously been used in reproductive technology. LB is a non-toxic synthetic organic acid dye that can be used as a supravital stain and, unlike propidium iodide, is directly visible, making staining rapid, simple, and very useful. As already mentioned in the aim section (Chapter I), for the purpose of easy and coherent flow of information, all the methods, results and discussion of this experiment are compiled in the following part.

4.2 Methods

4.2.1 Lissamine green B staining

A stock solution of 1% LB (199583) in DPBS (pH=7.0) was prepared and further diluted into TCM-199 medium. All staining was performed at 39^0C. Suitable staining

conditions to detect cumulus cell damage were determined using COCs artificially damaged by treatment with 0.1% hyaluronidase (H3506) for 30 minutes at room temperature. Treated COCs were placed in groups of 25 in 500µl droplets at four different LB concentrations (1%, 0.5%, 0.25%, 0.1%) under mineral oil for 2.5, 5 and 10 minutes. Each treatment was repeated 3 times. Following staining, COCs were washed in PBS to remove residual stain, observed by zoom stereomicroscope, and the number of stained COCs recorded.

4.2.2 Determination of nuclear maturation after IVM culture

After quick assessment of cumulus membrane integrity, oocytes were washed twice in *in vitro* maturation (IVM) medium and placed in groups of 40 to 60 in a final volume of 500 µl IVM medium in 4-well dishes (Nunc GmbH, Co.KG, Germany). Oocytes were cultured at 39^0C under 5% CO_2 in air and maximum humidity for 46 h. The maturation medium consisted of TCM 199 (M 2154) with 1 mM L-glutamine, 1 mM sodium pyruvate (P 3662), 0.1 mM 2-mercaptoethanol (M 7522), 50 ng/ml epidermal growth factor (E 4127), 50 µg/ml gentamicin and 0.1% (w/v) BSA (A 3311). During the first 24 h of IVM, the medium was supplemented with 10 IU/ ml PMSG and 5 IU/ ml hCG (MSD Tiergesundheit, Germany) and 5 µl/ml ITS (recombinant human insulin, human transferrin, and sodium selenite, I 3146) (Sjunnesson *et al*., 2013).

After IVM culture, cumulus investments were removed from the oocytes by gentle pipetting. Oocytes were then fixed for 30 minutes in 0.5% glutaraldehyde (500µl PBS+10 µl 25% glutaraldehyde G-5882), washed in 500 µl PBS for 5 minutes then transferred to 500 µl Hoechst (B2261) stain solution (0.1%). The nuclear status was evaluated by epifluorescence microscopy (UV filter and Carl Zeiss) using standard measures like germinal vesicle breakdown, condensation of chromatin and appearance of the first polar body as confirmation of nuclear maturation.

4.2.3 Parthenogenetic activation of porcine oocytes

Oocytes were activated chemically to assess their developmental competence. After 46 h IVM culture, COCs were manually denuded by mouth pipetting, then activated by incubating in IVM medium containing 5 µM Ca-ionophore A23187 (C7522) for 5 min at 39ºC under 5% CO_2 in air. Oocytes were washed three times in IVM culture medium and incubated in 500 µl droplets containing 2 mM 6-DMAP (6-dimethylaminopurine, D2629) and covered with mineral oil in an incubator at 39ºC under 5% CO_2 in air for 4 h. After activation, oocytes were cultured in 500 µl porcine zygote medium (PZM 5 IFP) in 4 well dishes (Nunc GmbH, Co.KG, Germany) overlaid with 500 µl mineral oil (M8410). The dish was incubated undisturbed at 39ºC in 5% CO_2 in air for 7 days. Embryo development was observed microscopically and cleavage assessed at 48 h post-activation.

4.2.4 RNA isolation and reverse-transcription

Apoptotic gene expression was evaluated in groups of 50 to 60 COCs. Total RNA was isolated using an RNeasy mini kit (Qiagen, Europe) according to the manufacturer's instructions with slight modification. During isolation lysing the zona pellucida proved difficult to achieve using the kit. Therefore, this problem was solved using SpeedMill PLUS ® homogeniser (analytic jena). Briefly, the group of COCs were transferred to a 0.50 ml lysis tube with metal beads (supplied by manufacturer) and homogenised for 30 seconds in a lysis buffer provided in the RNeasy mini kit. During the process temperature was maintained at 0^0C. After the homogenisation step the sample was processed using the mini kit protocol. Sample purity was assessed using the A260/A280 nm ratio with expected values between 1.8 and 2.0, RNA samples were treated with TURBO DNA-free™ Kit (Life Technologies) to remove genomic DNA.

Reverse-transcription reactions were performed with 0.5-1 µg total RNA using the SuperScript® III first-strand synthesis system for RT-PCR (Life Technologies) and random hexamer as a primer, according to the manufacturer's instructions. For each sample, negative controls lacking reverse transcriptase were prepared using the identical procedure.

4.2.5 Quantitative real-time PCR

Gene expression was assessed by quantitative RT-PCR. Primers for *BAK*, *CASP3* and *TP53*, and a housekeeping gene *ACTB* (beta-actin) were designed using 'Primer3' software. The predicted fragment sizes are listed in Table 7. Real-time PCR amplification was conducted using an ABI 7300 real time PCR System (Applied Biosystems). A KAPA SYBR FAST q PCR Kit (Kapa Biosystems) was used to provide real-time quantification of the amplified product. Three replicates of each reaction were measured and the mRNA level of each sample was normalised to that of beta-actin. Relative mRNA levels were determined by the ΔΔCt method (Livak and Schmittgen, 2001).

Table 7 Primer sequences and annealing temperature

Genes	Primer Sequences	Annealing Temperature	Product Size(bp)	Accession no.
β- Actin	F-5'GTGGACATCAGGAAGGACCTCTA 3' R-5'ATGATCTTGATCTTCATGGTGCT 3'	58 ^{0}C	135	U07786
Bak	F-5'CAGCACCATGGGGCAGGTAG3' R-5'AGGCTGGAGGCGATCTTGGT3'	58 ^{0}C	150	AJ001204.1
Caspase3	F-5'GCCGAGGCACAGAATTGGAC 3' R-5'GCGCTGCACAAAGTGACTGG3'	58 ^{0}C	180	AB029345.1
TP53	F-5'CTTTGAGGTGCGTGTTTGTG3' R-5'CGGATCTGGAGGGTGAAATA3'	60 ^{0}C	152	AF098067.1

4.2.6 Measurement of DNA fragmentation

The TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) procedure was used to fluorescein-dUTP label 3'-OH ends of DNA fragments generated by apoptosis, allowing detection of apoptotic cells by fluorescence microscopy. Fixed and permeabilised embryos were subjected to the TUNEL assay procedure using an *in situ* apoptosis detection kit (Takara Bio INC., Japan) according to the manufacturer's instructions with minor modifications. COCs were fixed for 30 minutes at room temperature in PBS (pH 7.4), permeabilised for 5 min on ice in permeabilisation buffer, and then washed once in PBS. COCs were then incubated with terminal transferase and labelled nucleotide solution in a CO_2 incubator at 37^{0}C for 1 h. Slides were overlaid with a cover slip and the edges sealed with quick drying nail polish, then evaluated using an epifluorescence microscopy (green excitation filter and Carl Zeiss). Oocytes showing discrete pinpoint fluorescent green signals were judged to be

positive for DNA fragmentation. The apoptosis score was expressed as a percentage of the total.

4.3 Results

4.3.1 Lissamine B staining

A staining regimen of 0.50% LB for 5 minutes was found to be optimum for staining in terms of specificity and easily detectable colouration. Under light microscopy, damaged cumulus cells were stained greenish blue, while healthy cumulus cells remained unstained. The ooplasm of denuded oocytes that incurred damage to the Zona pellucida stained greenish blue. The LB stained oocytes were scored according to a system previously used for propidium iodide staining (Uchikura *et al*., 2011;Ruppert-Lingham *et al*., 2006), with grade 1 having less than 25% cumulus cells stained; grade 2, 25% to 50% cumulus cells stained; and grade 3, more than 50% cumulus cells stained, as shown in Fig 23.

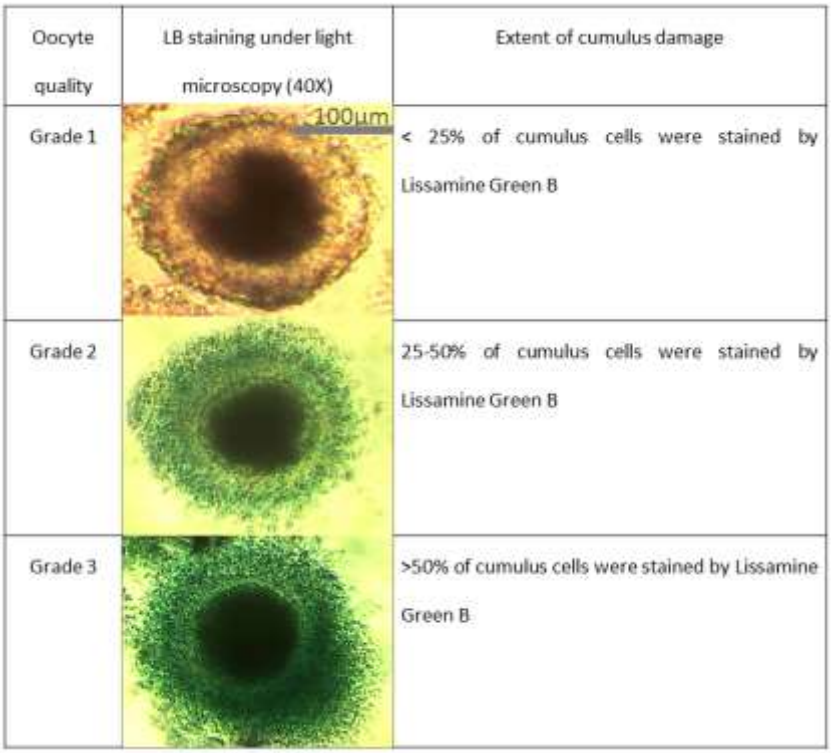

Fig 23 Classification of COCs by lissamine green B staining.

4.3.2 Nuclear maturation

Of the 372 COCs in the nuclear maturation group, 191 were graded by morphology, with 63 assessed as grade A (32.99%), 67 grade B (35.07%) and 61 grade C (31.93%); 181 were graded by LB staining as 59 grade 1 (32.59%), 65 grade 2 (35.91%, and 57 grade 3 (31.49%). Nuclear maturation of each oocyte was scored by the attainment of metaphase II, as judged by the presence of condensed chromosomes in an equatorial position and extrusion of the first polar body. Nuclear maturation data (Table 8) showed no significant difference between morphological grade A (79.9% ± 3.82), grade B (86.48% ± 3.07), or grade C (82.00% ± 3.00) oocytes. The nuclear maturation rates of LB graded oocytes were: grade 1 (84.70% ± 3.36), grade 2 (83.61 ± 3.44) and grade 3 (62.26 ± 3.69). The difference between grade 3 and grades 1 and 2 was significant ($P<0.05$).

4.3.3 Developmental competence of oocytes

The parthenogenetic activation group comprised 395 oocytes. 204 were graded by morphology as 67 grade A (32.84%), 71 grade B (34.80%) and 66 grade C (32.35%); 191 were graded by LB staining as 64 grade 1 (33.50%), 65 grade 2 (34.03%), and 62 grade 3 (32.46%). Development was scored as the number of embryos consisting of two to eight evenly sized blastomeres 48 h after parthenogenetic activation. There was no significant difference between morphological grade A (84.55% ± 4.45), grade B (85.06% ± 4.49), or grade C (81.60% ± 4.64) oocytes. The rates of parthenogenetic activation in LB graded oocytes were: grade 1 (87.71% ± 2.46), grade 2 (82.72% ± 3.39) and grade 3 (62.88% ± 3.85). Again this shows a statistically significant difference between grade 3 and grades 1 and 2 ($P<0.05$) (Table 8).

Table 8 Effects of cumulus morphological categories on maturational and developmental ability of porcine oocytes

Item	Nuclear maturation and Parthenogenetic Activation					
	Grades based on cumulus investment			Grades based on membrane integrity		
	Grade A	Grade B	Grade C	Grade 1	Grade 2	Grade 3
Nuclear maturation (%)	79.9±3.8[a]	86.48±3.07[a]	82.00±3.00[a]	84.70±3.36[a]	83.61±3.44[a]	62.26±3.69[b]
Activation (%)	84.55±4.4[a]	85.06±4.49[a]	81.60±4.64[a]	87.71±2.46[a]	82.72±3.39[a]	62.88±3.85[a]

a, b Value with different superscripts differ significantly ($p < 0.05$). Values are means ± standard deviation (SD).

4.3.4 TUNEL assay for oocytes and cumulus cells

A total of 176 COCs were graded by either morphology or LB staining. For each grade a TUNEL score was determined as the percentage of observed COCs showing signs of DNA fragmentation, as indicated by a fluorescent signal (Table 9, Fig.24). Throughout, fluorescent signals were observed only in the ooplasm and not in the cumulus cells. There was no significant difference between morphological grade A (20.97% ± 3.52) and grade B (22.69% ± 4.05) oocytes, but grade C oocytes showed significantly higher (38.42% ± 4.03) incidence of DNA fragmentation ($P<0.05$). The TUNEL score in LB graded oocytes was similar: grade 1 (21.66% ± 4.08), grade 2 (21.82% ± 5.85), with grade 3 significantly higher (37.08% ± 2.76) ($P<0.05$). There was no significant difference in TUNEL score between morphological grade C and LB grade 3 COCs.

Table 9 Rate of DNA fragmentations as detected by TUNEL assay in different category of porcine oocytes.

Item	DNA fragmentation assay					
	Grades based on cumulus investment			Grades based on membrane integrity		
	Grade A	Grade B	Grade C	Grade 1	Grade 2	Grade 3
Apoptosis (%)	20.97±3.52[a]	22.69±4.05[a]	38.42±4.03[b]	21.66±4.08[a]	21.82±5.85[a]	37.08±2.76[b]

a, b Value with different superscripts differ significantly ($p < 0.05$). Values of DNA fragmentation are means ± standard deviation (SD).

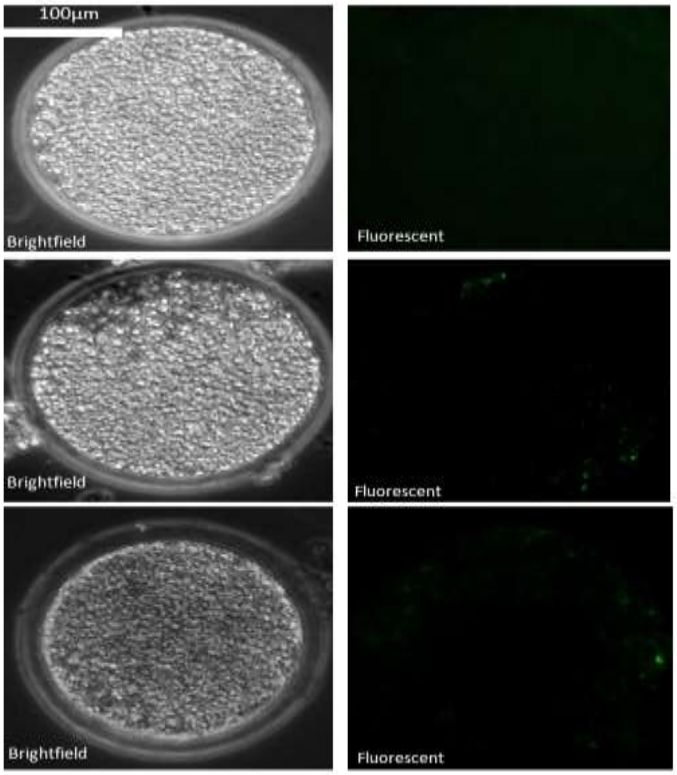

Fig 24 DNA fragmentation detection by TUNEL assay. A, porcine oocyte with no detectable DNA fragmentation. B, Porcine oocyte with moderate DNA fragmentation. C, Porcine oocyte with extensive DNA fragmentation

4.3.5 Expression of stress and apoptosis related genes in lissamine B graded porcine COCs

Differential expression of the stress-associated gene *TP53* and two genes related to apoptosis, *BAK* (BCL2-antagonist/killer) and *CASP3* (Caspase 3), was analysed by quantitative real-time RT-PCR in three pools of 50 to 60 COCs representing LB-stained grades 1, 2 and 3 (Fig 25). A total of three replicates were

conducted for each experiment. Fig. 25 B shows that *BAK* and *CASP3* expression was similar in all grades of LB-stained COCs, but *TP53* expression was significantly greater ($p<0.05$), in grade 3 COCs than grades 1 and 2.

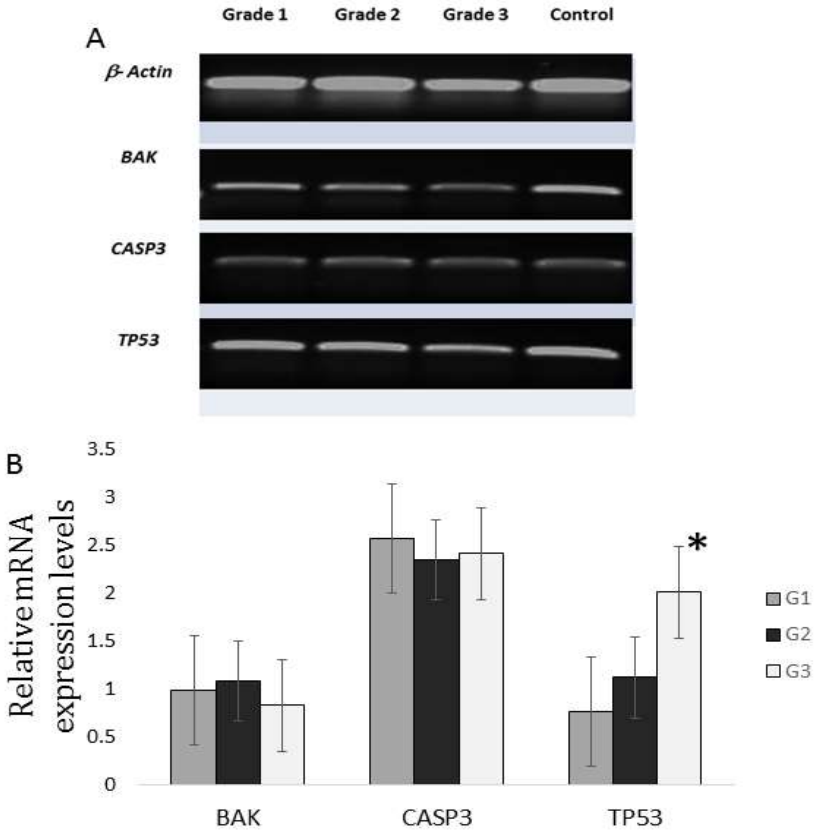

Fig 25 A, Reverse transcription PCR of apoptosis associated genes in lissamine B stained oocytes. Relative gene expression levels of lissamine B stained porcine oocytes. B, The m RNA levels of BAK, Caspase 3 and TP53 were analysed in porcine oocytes. Results are displayed as fold changes in different grades of lissamine B stained oocytes relative to each other. Significant difference is marked with an asterisk (*) ($p<0.05$).

4.4 Chapter summary

The cumulus layer is extremely vital for oocyte development, however its visual observation yields only limited useful information about its functional competence. During the present investigation also, the visual grading scheme of A, B and C proved to ineffective in predicting the developmental competence of the porcine oocytes. Alternative methods of analysing cumulus cells are available but are too time

consuming and expensive for routine laboratory use. The other less commonly used method of propidium iodide staining is fluorescent microscopy dependent, which may be cost inhibitory and may be potentially mutagenic. Therefore, the usefulness of lissamine green B (LB) staining for non-invasive prediction of maturational competence of porcine oocytes was evaluated.

4.4.1 Nuclear maturation and developmental competence of LB stained porcine oocyte

While higher rates of nuclear maturation, fertilisation and development after IVP have been reported in A and B grade bovine oocytes graded on the basis of the thickness of the cumulus cell layers, current findings showed that this cannot automatically be extended to porcine oocytes. Nuclear maturation data showed no significant difference between morphological grade A (79.9% ± 3.82), grade B (86.48% ± 3.07), or grade C (82.00% ± 3.00) oocytes. The nuclear maturation rates of LB graded oocytes were: grade 1 (84.70% ± 3.36), grade 2 (83.61 ± 3.44) and grade 3 (62.26 ± 3.69). The difference between grade 3 and grades 1 and 2 was significant ($P<0.05$). Our data clearly reaffirmed the importance of a cumulus layer for oocyte maturation and development (Davachi *et al*., 2012, Prates *et al*., 2014), but suggest that measures of cumulus cell integrity are more useful than morphological parameters alone. There was no significant difference between morphological grades A (84.55% ± 4.45), grade B (85.06% ± 4.49), or grade C (81.60% ± 4.64) oocytes. The rates of parthenogenetic activation in LB graded oocytes were: grade 1 (87.71% ± 2.46), grade 2 (82.72% ± 3.39) and grade 3 (62.88% ± 3.85). Again this showed a statistically significant difference between grade 3 and grades 1 and 2 ($P<0.05$) (Table 2). The data indicated that LB staining of cumulus oocyte complexes (COCs) immediately after collection is

superior to morphological grading as a predictor of oocyte viability, nuclear maturation after IVM culture, and successful parthenogenetic activation and development.

4.4.2 Assessment of DNA fragmentation measured by TUNEL assay

The investigation of the role of apoptosis in COC quality revealed some interesting findings. As expected, DNA fragmentation measured by TUNEL assay showed that LB-grade 3 COCs were more damaged than higher grade COCs, consistent with their developmental properties and also in line with morphological grade C COCs. DNA fragmentation in immature oocytes (in particular, GV and MI stages) could be a consequence of stress during maturation of the ovarian follicle or hypoxia resulting from compromised microcirculation and correlate with aneuploidy or other chromosomal abnormalities (Van Blerkom, 1996). It is notable that no evidence of DNA fragmentation in the cumulus layer in immature porcine COCs was found. This is consistent with a similar finding in cattle where no TUNEL signal was obtained in cumulus cells of immature oocyte (Yuan *et al*., 2005).

4.4.3 Relative mRNA expression of stress related genes

Gene expression analysis of three genes namely *TP 53*, *BAK* and *CASP3* were chosen for their well-known roles in stress and apoptosis however revealed an interesting and unexpected distinction. The expression of *TP53*, a stress response gene induced by DNA damage, was significantly greater in LB-stained grade 3 COCs, consistent with TUNEL assay findings and the poorer developmental parameters of this grade. Studies in humans and mice have revealed a clear relationship between *in vitro* cultures related stress and *TP53* expression in embryos (Chandrakanthan *et al*., 2006). In mice, genetic inactivation of Mdm2, a negative regulator of p53, results in early embryonic lethality (Jones *et al*., 1995) that can be reversed by deletion of Trp53

(Montes de Oca Luna *et al*., 1995), demonstrating that the p53 response pathway plays a direct role in embryonic loss.

It was expected that *BAK* and *CASP3* expression would also be greater in the lowest quality COCs, but this was not the case. *BAK* and *CASP3* are members of two important regulatory families involved in apoptosis. *BAK* is a pro-apoptotic member of the Bcl-2 family whose cytoplasmic elevation is sufficient to cause induction of oocyte apoptosis (Morita and Tilly, 1999). The lack of any clear correlation between the RNA level of these genes and COC grade, despite evident DNA damage, could be due to a variety of reasons. One is that there is abundant maternal *CASP3* mRNA in the oocytes (Weil *et al*., 1996), and this may mask any differential expression in the cumulus cells of different grades. Another is that regulation of Caspase 3 activity is known to occur at the protein level, by modification of an inactive proenzyme. In which case direct immunocytological detection of the active form of Caspase-3 may be a more reliable measure. It is also known that Caspase cleavage and DNA fragmentation do not take place simultaneously (Yuan *et al*., 2005). However, the findings are also consistent with similar observations in bovine embryos treated with the apoptosis inducer staurosporine, which showed no difference in the expression of several apoptosis-related genes including *CASP3* and other Bcl family genes (Vandaele *et al*., 2008).

4.5 Chapter conclusion

In conclusion, it is pertinent to state that, during the study a very easy quality control assay was developed for selection of good quality porcine oocytes for IVP. LB is used traditionally in ophthalmology to evaluate ocular membrane integrity (Hamrah *et al*., 2011), it has never previously been used in reproductive technology. LB is a non-toxic synthetic organic acid dye that can be used as a supravital stain and, unlike propidium iodide, is directly visible, making staining rapid and simple. LB offers an

efficient, objective, non-invasive and directly visible means of selecting good quality COCs and oocytes. This study for the first time reported the use of lissamine green B dye to visually assess the quality of COCs used for *in vitro* culture. However, LB may not be useful as a predictor of apoptosis status of the selected oocytes. This point has been elaborated in the discussion chapter of this thesis.

Chapter V

Improvement of ovarian follicle imaging and classification

5.1 Chapter introduction

As previously mentioned in this introduction *in vitro* production of embryo is heavily dependent on a supply of slaughterhouse ovaries. For *in vitro* culture porcine oocytes are isolated from a cohort of dominant and subordinate follicles, most commonly by aspiration. Follicles of size roughly 3-6 mm are chosen for aspiration simply based on the visual observation. The developmental competence of the *in vitro* isolated oocytes varies according to the developmental stage of the ovarian follicle. As a result it leads to a batch-to-batch variability during *in vitro* embryo production. At present there is no other way of identifying those follicles with the best quality oocytes. Therefore with the broader objective of optimising porcine IVP in mind, a non-invasive strategy to determine the quality of the follicle for oocyte aspiration using new imaging modalities were investigated in collaboration with the group of Prof Dr. Daniel Razansky, Institute of Biological and Medical Imaging, HelmholtzZentrum München. As already mentioned in the aim section (Chapter I), for the purpose of easy and coherent flow of information, all the methods, results and discussion of this experiment are compiled together in the following part.

5.2 Methods

5.2.1 Porcine ovarian folliculometry with selective plane illumination microscopy (SPIM)

The size of ovarian follicle, the size of the developing oocyte within, and the thickness of the follicular wall are all important folliculometric parameters for selection of superior quality porcine ovaries for oocyte aspiration. With the commonly used ultrasound scans, the growing follicle appears as a black bubble on the ultrasound image without much folliculometric information. In ultrasound, most of the folliculometric

information including follicular wall thickness is determined by manual or semi-automated segmentation. A study was undertaken to explore selective plane illumination microscopy (SPIM) as an imaging technique for more accurate and informative folliculometric data from the scanned ovary. In this imaging modality, a single plane of the sample is optically excited by thin laser light-sheets. This allows 2D fluorescent images to be captured by placing a camera orthogonal to the light-sheet, and by translating either the light-sheet or the sample, 3D volumetric imaging stacks can be obtained (Huisken *et al*., 2004a).

5.2.1.1 Selective-plane illumination microscopy (SPIM) system

A state-of-the-art SPIM system was built in-house to optimally support imaging of ovarian follicles. The light source was an 80 mW continuous wave diode-pumped solid-state (DPSS) laser at 670 nm (Frankfurt laser company, Germany), with a beam quality factor M2=1.10. A 5X telescope system was used to expand the beam diameter to 10 mm to allow excitation of the full width of the ovary samples. A beam splitter divided the main beam to allow double-sided illumination and reduce effects of light attenuation due to absorption and scattering when imaging through large samples.

A side-view of the SPIM system can be seen in Fig 26. Thin sheets of light were generated using cylindrical lenses with focal lengths of 40 mm. The light-sheets were oriented horizontally, with the sample centered at the beam waist. To capture 2D fluorescent images, a 5-MP scientific complementary metal oxide semiconductor (sCMOS) camera (Model: pco.edge, PCO AG, Kelheim, Germany) was placed directly above the sample, facing downwards. The high-end scientific camera allowed acquisition of up to 100 frames per second with 2560 x 2160 pixel resolution and high sensitivity supported by dynamic range of 14 bits and 1.0 e- readout noise. An EC

epiplan-neofluar objective (Zeiss, Germany) with a magnification of 2.5X and a NA of 0.06 was used because it provided a large working distance of 35 mm, and 220 µm depth of field. Fluorescence signals were filtered using a 690/10 fluorescence filter (Chroma, USA), and a tube lens used to relay the fluorescent image into the camera. The imaging system was characterised using line pair targets, and determined to have an effective field-of view (FOV) of 10x9 mm with <6 µm lateral resolution. The light sheets were characterised by rotating the cylindrical lens by 90˚and filling the sample

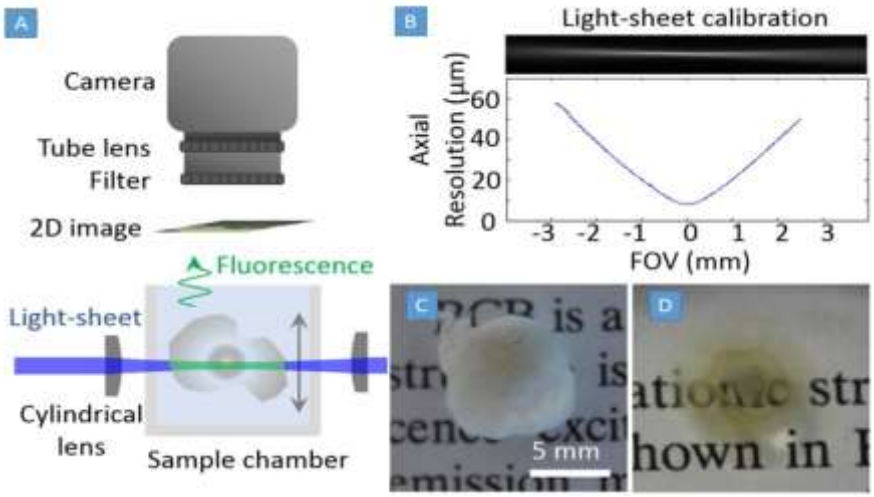

Fig 26 Schematic of the SPIM system used for ovarian follicular imaging

chamber with fluorescent dye Alexa Fluor 488 (Life Technologies, USA) diluted in deionised water, which allowed the light sheets to be imaged by the camera, as shown in Fig. 26 B. The axial resolution was defined by the light-sheet thickness, and it was found to be 11 µm at the beam waist expanding to about 60 µm at a 3 mm distance from the centre of the FOV.

5.2.1.2 Preparation of ovary sample

Ovaries from prepubertal gilts were brought from a local slaughterhouse within one hour of collection in a temperature-controlled box maintained at 39^0C. Due to the FOV limitation of the SPIM system, individual follicles of 5-8mm diameter and small

groups of follicles were dissected out using ultrafine surgical blades. The samples were stained with brilliant cresyl blue (BCB), then chemically cleared using an alcohol-based method (Jährling *et al*., 2009;Pfeifer *et al*., 2009) Follicles were dehydrated using 50%, 80%, and 100% ethanol for 12 hours each, repeating the last step twice, then cleared in 2:1 benzyl alcohol/ benzyl benzoate (BABB) solution for 6 hours. This rendered the samples optically transparent and ready for imaging. Fig 26 D and Fig 26 E shows a sample before and after clearing.

5.2.1.3 SPIM imaging

Samples were fixed on a holder and placed inside the sample chamber. A linear motorised stage (MTS25/Thorlabs, USA) was used to translate the sample vertically, allowing different planes to be excited by the light sheet. Rapid translation enabled stacks of up to 600 cross-sectional SPIM images to be collected within two minutes. The sample was translated by 40 µm between successive images. During imaging, the sample chamber was filled with BABB solution to match the refractive index of the medium to the sample, avoiding any image distortion. A glass sample chamber was used to avoid any reaction with BABB. Image analyses to extract morphological measurements were carried out using ImageJ software. Haematoxylin and eosin (H&E) stained porcine ovary sections were used as standards for comparison. Their irregular shape complicated measurement of follicle size, so two orthogonal measurements intersecting at the midpoint of the first measurement were taken and the average used as the diameter. All analyses were carried out manually. Dimensions of several follicles and their (COC) were measured.

5.2.2 Multi spectral optoacoustic tomography of porcine ovary

Selection of developmentally competent oocytes for *in vitro* culture can significantly improve the outcome of IVF. BCB stain is known to be a simple test that allows the selection of developmentally competent oocytes. The BCB test determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), a pentose phosphate pathway enzyme that gradually decreases its activity as oocytes reach their growth phase. BCB dye can be reduced by G6PDH activity; therefore oocytes that have reached their growth phase cannot reduce BCB to a colourless compound and exhibit a blue coloured cytoplasm (BCB+). However, growing oocytes are expected to have a high level of G6PDH activity and be able to reduce the blue compound, resulting in a colourless oocyte cytoplasm (BCB−). It has been shown that the developmental potential of the oocytes are not affected by this dye. In an exploratory study, BCB was successfully used to enhance contrast of porcine ovarian follicles by MSOT imaging. MSOT is a relatively new technique based on the principle of multi-wavelength optoacoustics that is capable of high resolution three dimensional (3D) visualisation of molecular probes located deep in scattering living tissues, with resolution and speed representative of ultrasound. This method can simultaneously deliver anatomical, functional and molecular information with both high resolution and penetration capabilities. With this method, in future there would be a possibility of visualising developmentally competent oocytes in the follicle which in turn may help in improved selection of the best follicles.

5.2.2.1 BCB dilution and spectrum calculation

The optical and optoacoustic response of BCB was characterised using spectroscopic measurements (Ocean optics USB4000-FL, Bandwidth: 351-1043nm) and direct optoacoustic measurements with BCB solutions (diluted with PBS) at

different molar concentrations. The spectroscopic measurements show the absorption peak to be at 620 nm. However, the said wavelength could not be used for optoacoustic measurement, given the laser was optimised in the range 680-900 nm for maximum penetration in tissues. The optoacoustic spectrum was measured by placing a fine bore polyethylene tubing (0.86mm ID and 1.27mm OD) embedded within a diffusing agar block (for uniform illumination) and perfusing BCB solutions at 6 different concentrations. The stock solutions (1 M) were diluted in PBS to achieve the desired concentrations 13 Mm as reported in earlier reports (Mohammadi-Sangcheshmeh *et al*., 2012). The signals were acquired by an array of 256 detectors with 4 averages using 20 wavelengths, and reconstructed using IMMI method. The signals were normalised for laser power over all 20 WLs and the lumen of the tubing was segmented. Thereafter the spectral signal amplitude was obtained as an average of the integral intensity value across the segmented lumen, and plotted for different concentrations of solution. From the optoacoustic spectral evaluations, it was observed that concentration volumes of 150 µl of the stock solution (13 mM) gives better signal recovery and causes no quenching. Thus, this concentration of PBS based BCB solution was used throughout the experimentation.

5.2.2.2 MSOT imaging sample preparation

Slaughterhouse porcine ovaries were brought to the laboratory within one hour of collection in a temperature-controlled box maintained at a temperature of 39^0C. Ovaries were thoroughly washed with pre-warmed (39^0C) Dulbecco's phosphate-buffered saline (DPBS) solution containing 0.1% polyvinyl alcohol and penicillin, streptomycin solution The BCB dye at a concentration of 13 mM BCB was carefully injected into follicles size of 4-6 mm using a fine needle (29 G). The concentration of BCB dye has been reported in different report across multiple species. In order to inject the dye, the needle was

pushed opposite to the target follicle and slowly injected into the follicle without rupturing it, a volume of 50 µl dye was injected into each follicle for study. The ovary was fixed using a polythene film inside the MSOT scanner.

5.2.2.3 MSOT setup and protocol

To image ovaries (*ex-vivo*) cross-sectional optoacoustic acquisition geometry was employed using a commercial small animal multispectral optoacoustic tomography (MSOT) scanner (Model: MSOT256-TF, iThera Medical GmbH, Munich, Germany). The scanning system consisted of a custom-made 256-element array of cylindrically focused piezocomposite transducers with 5 MHz central frequency for simultaneous acquisition of the signals generated with each laser pulse. The transducer array covered an angle of approximately 270° and has a radius of curvature of 40 mm. Light excitation was provided with the output laser beam from a wavelength-tunable optical parametric oscillator (OPO)-based laser, which was shaped to attain ring-type uniform illumination on the surface of the phantoms by means of a custom-made fibre bundle. The detected optoacoustic signals were simultaneously digitised at 40 Mega samples per second. The scanner was capable of rendering 10 cross-sectional images per second but here the images were averaged 10 times in order to improve SNR performance in acquiring entire ovary cross sections. The acquired signals were initially band-pass filtered with cut-off frequencies between 0.1 and 7 MHz for removing low frequency offsets and high frequency noise, and subsequently input to a reconstruction algorithm rendering a cross-sectional distribution of the optical absorption.

The acquired images were reconstructed with the exact numerical model-based reconstruction algorithm, termed interpolated-matrix-model inversion IMMI. The choice of IMMI was inspired by need to have more quantitative optoacoustic reconstructions by taking into account the various experimental imperfection, retaining frequency

information and mitigating artefacts, which are characteristic of the named class of algorithms. It was based on a least-squares minimisation between the measured pressure at a set of locations and instants (expressed in a vector form as $\mathbf{p}$) and the equivalent theoretical pressure predicted by a linear model obtained from a discretisation of the optoacoustic forward solution. The optical absorption at the pixels of the ROI, expressed as vector form$\mathbf{F}$, was calculated as follows

$$\mathbf{F} = \mathrm{argmin}_{\mathbf{f}} \|\mathbf{Af} - \mathbf{p}\|^2 + \lambda^2 \|\mathbf{Lf}\|^2,$$

Where A is the linear operator (or model matrix) mapping the optical absorption to the acoustic pressure. Standard Tikhonov regularisation was employed to minimise the high-frequency noise in the inversion process, which is particularly beneficial in presence of limited view problems. The matrix L represents a high-pass filter operation. The model-based inversion procedure can be further modified to account for speed of sound variations in the medium or to minimise the artefacts due to internal reflections in case these effects cause undesired distortion in the images. In the experimental implementation, the computational time of model-based reconstruction was approximately 10 s in an Intel Core2 Duo CPU E8400 operating at 3.00 GHz and with 8 GB of RAM, and was accelerated with GPU-based processing. In the experiments performed herein, a grid of 200x200 pixels corresponding to a field of view of 25 mm x 25 mm (125 µm pixel size) was employed, which was adapted to the actual resolution of the system [DR NProt].

5.2.2.4 Spectral unmixing to identify BCB contrast

The MSOT was capable of selectively quantifying the distribution of specific biomarkers using multispectral information. In this experiment, a vertex component analysis (VCA) based fast blind unmixing method to map the distribution of BCB was used. Since the internal molecular composition of fluid inside the follicles was not

spectrally evaluated, mathematically the problem was treated as a two-object problem – the tissue and the BCB contrast. Thus, in this approach unmixing was done specifically for presence of BCB within the ovarian follicle, given the fact the presence of BCB+ structure would help better identify the competent follicles. The VCA method was able to arbitrarily identify two channels and display the corresponding spectrum. The spectra were then matched with the optoacoustic spectrum of BCB experimentally measured and mapped to the corresponding tissue components and the BCB channel. For mathematical details of the method Dean Ben *et al* 2014 can be referred.

5.2.2.5 *In vitro* culture of oocytes aspirated for ovaries after MSOT scanning

The important part of the experiment was to see if MSOT scanning could hamper the viability of the oocytes. In order to assess the effect, after MSOT was performed and the sample was rushed back to the lab within 2 hours maintaining a temperature of 39^0 C for further culture. After MSOT imaging BCB infused follicles with a diameter of 3-8mm were aspirated using a vacuum suction machine 18-gauge needle. A group of unscanned ovaries maintained at 39^0C were also infused with BCB at similar concentration formed the control group. Aspiration medium consisted of HEPES-buffered TCM 199 (M 2520) with 26 mM $NaHCO_3$ (S 5761), 1 mM L-glutamine (G 8540), 0. 5 mg/ml polyvinyl alcohol (P 8136), 50-µg/ml gentamicin (G 1264) and 20 U/ml heparin (H 3149). Oocytes (COCs) were retrieved in the same medium without heparin using a stereomicroscope. A total of 130 BCB+ve and 120 BCB-ve oocytes were isolated from the MSOT scanned ovarian follicle. The control group oocyte 134 BCB +ve and126 BCB –ve oocytes were also aspirated. The aspirated oocytes were then kept for *In vitro* maturation culture using the protocol already describes in the thesis. They were further subjected to parthenogenetic activation and TUNEL assay as previously reported in the thesis.

5.3 Results

5.3.1 Porcine ovarian folliculometry with selective plane illumination microscopy (SPIM)

All the ovary samples were imaged in their entirety, as their sizes matched the camera FOV. Wide ranges of individual follicles were extracted from two different sets of image stacks. Fig 27 shows single image slices taken at two different planes of the same sample. Follicular antrum and *theca interna* layers are visible, allowing individual follicles to be readily identified. Follicles of all sizes can be seen and additional morphological details such as atretic follicles, blood vessels and interstitial connective tissue between adjacent follicles could be identified. SPIM images were analysed for the most clinically or diagnostically relevant morphological features, specifically: size and number of follicles, size of the developing cumulus oophorus complexes (COC), and follicle wall thickness.

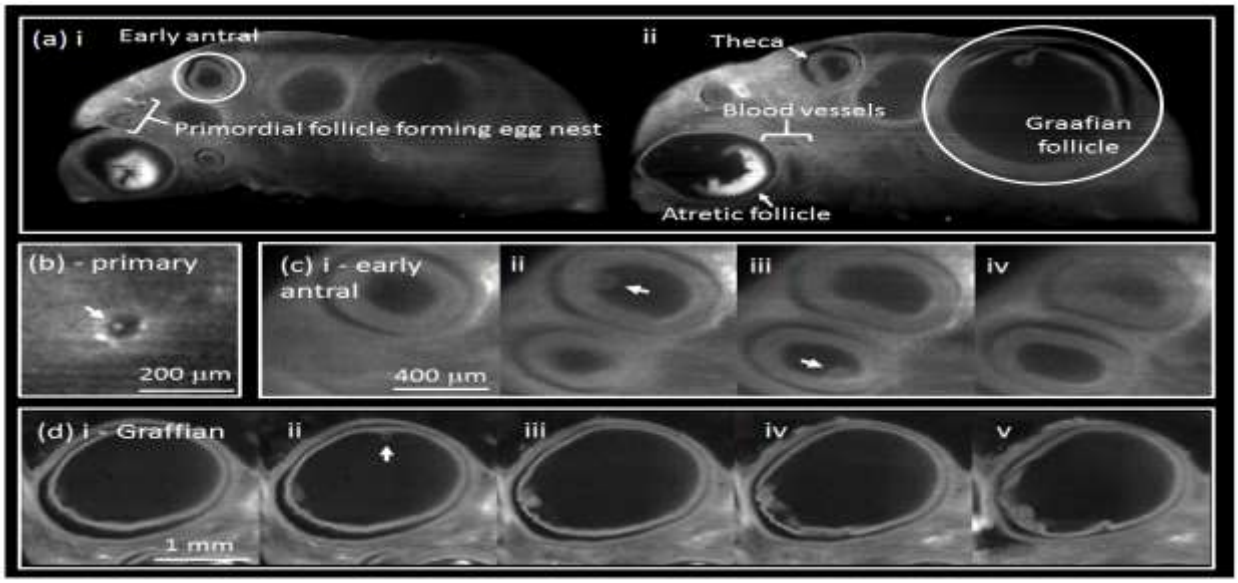

Fig 27 (a) Full FOV cross-sectional SPIM images taken at two different planes of a cleared follicle cluster. A few features are indicated: (i) early antral follicle and primordial follicle forming egg nest; (ii) Graafian and atretic follicles, theca layer, and blood vessels. (b) Primary follicle - diameter: 73 µm, COC: 29 µm. (c) Early antral follicles – diameter 586 µm (top) and 476 µm (bottom). Arrows indicate COCs, which were measured to be 101 µm (top) and 98 µm (bottom). (d) Graafian follicle - diameter: 1788 µm, COC: 104 µm.

5.3.1.1 Relationship between oocyte size and follicle size

The relationship between oocyte and follicle size was analysed. 30 data points were taken from the SPIM data of two different samples, and their results are compared to published histological results of porcine ovarian follicles (Griffin *et al*., 2006). The adjusted coefficient of determination (R-squared) between SPIM data and the literature-fitted curve was 0.76, indicating good agreement. SPIM is thus suitable for quantitative follicle characterisation.

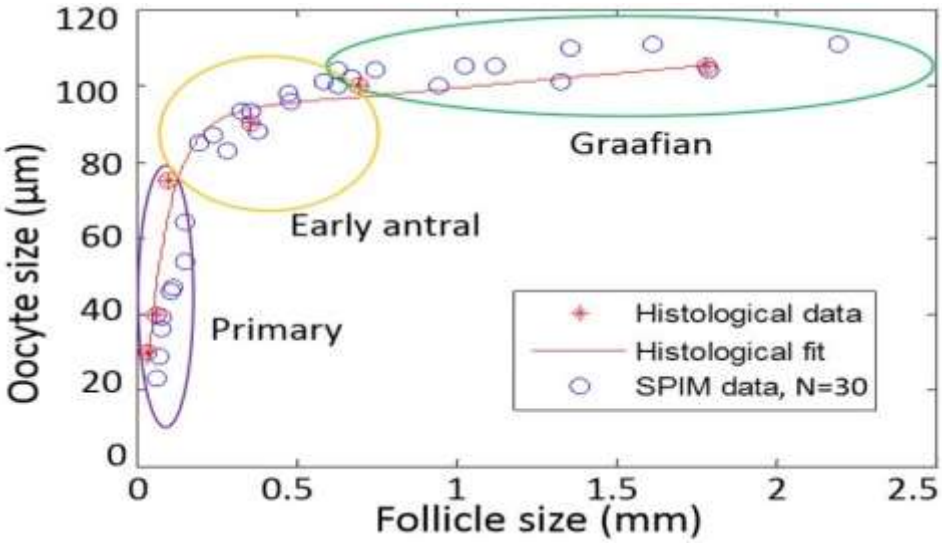

Fig 28 Quantitative analysis of the oocyte size versus the follicle size. Blue circles are data points taken from the SPIM images. 30 data points were taken from SPIM results of two different samples. Histological data of porcine ovarian follicles previously reported in literature are shown in red. R^2 (adjusted coefficient of determination) = 0.76, indicating good agreement.

5.3.1.2 Relationship between thecal wall thickness and follicle size

Thecal wall thicknesses of 10 early antral and graafian follicles from two samples were measured, Wall thickness is however not uniform, so a distribution was measured for each follicle. A maximum and minimum value was taken from the SPIM images, and a linear fit performed on the averaged values. A clear correlation between follicle size and thecal wall thickness was evident. For the largest Graafian follicle, wall thicknesses were around 90 µm, while antral follicle walls were around 120 µm. Identification of the

follicle wall thickness is useful in characterising different classes of developing follicles because it varies with developmental stage (Krivanek and Sonka, 1998a).

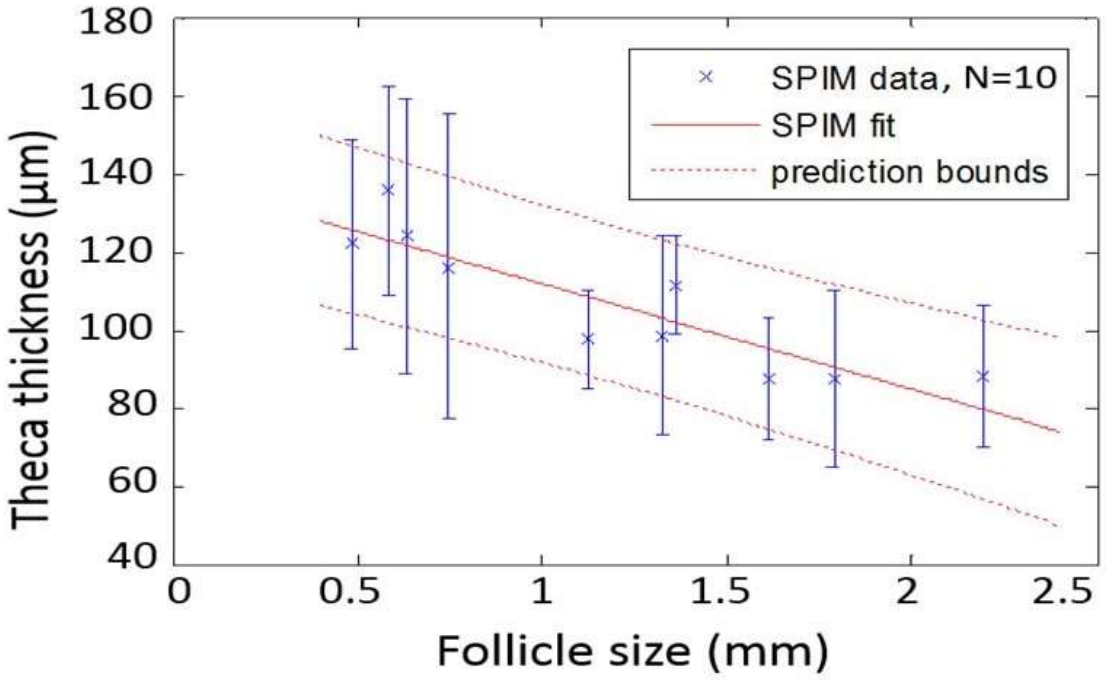

Fig 29 Theca wall thickness versus follicle size measured from the SPIM data. 10 follicles from two different samples were analysed. A maximum and minimum were taken for each follicle, and the average calculated. A linear fit was performed on the averaged values, along with the prediction bounds.

5.3.2 Multi spectral optoacoustic tomography (MSOT) of porcine ovary using brilliant cresyl blue (BCB) as a contrast medium

In this experiment, a supravital dye brilliant cresyl blue (BCB) was injected in to porcine ovarian follicle as a contrast medium. The BCB contrast was spectrally unmixed and enhancement of contrast was quantified with MSOT. Subsequently in order to validate the non-invasive nature of the treatment oocyte were isolated from that follicle for further culture. The results presented a proof of principle of the fact that BCB is capable of being used as a contrasting agent and when coupled with MSOT a really simple, gentle and very efficient imaging method can be developed. BCB finding were

consistent with other findings that it is nontoxic and does not kill the oocytes. This improves the quality of visualisation manifold.

5.3.2.1 BCB dilution and spectrum calculation

The available system setting was able to probe a short window of BCB contrast approximately between 680-750nm. In the optoacoustic spectral evaluations, as illustrated in figure 30, it can be observed that concentration volumes of 13mM stock solution gives better signal recovery and causes no quenching. Thus, this determined concentration of PBS based BCB solution was used throughout the experimentation. It is also to be noted that since the follicle were fluid filled structure and BCB injections were further diluted, the injection concentrations were conservatively overestimated by 10% by volume.

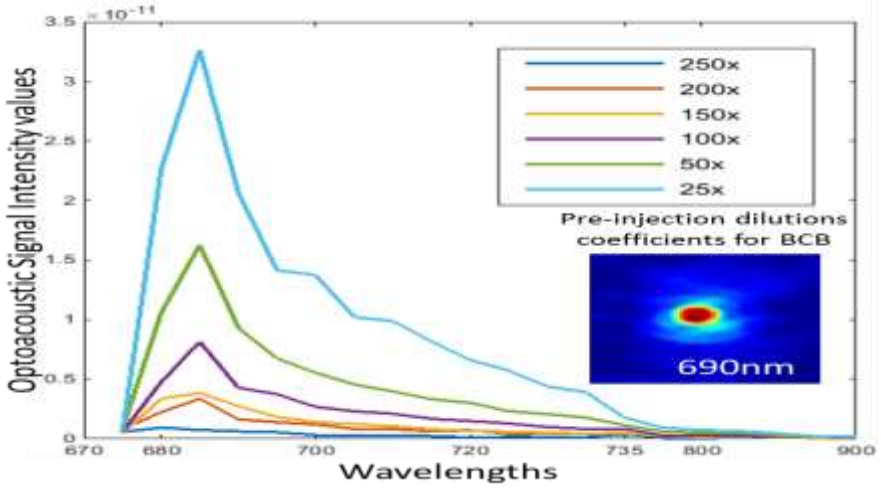

Fig 30 Wavelength dependent optoacoustic signal extinction curve for BCB: The optoacoustic signal responses were measured at several pre-injection dilution coefficients using the MSOT (cross-sectional) imaging at varying wavelengths. The BCB solution was perfused through a transparent fine bore polyurethane tubing (0.86mm ID and 1.27mm OD) embedded inside a scattering agar block (7% intra lipid by volume). A representative reconstructed image is shown in an insert.

5.3.2.2 MSOT imaging of ovarian follicle

The BCB dye at a concentration of 13 mM BCB was carefully injected into follicles size of 4-6 mm using a fine needle (29 G). The concentration of BCB dye has been reported in different report across multiple species (Su *et al*., 2012; Shabankareh *et al*., 2014). The ovary was fixed using a polythene film inside the MSOT scanner. The single wavelength image at 690 nm (fig 31) showed the internal anatomy of the ovary (*ex-vivo*) and also the BCB contrast owing (fig 31 shows high signal intensity at the used wavelength). A vertex component analysis (VCA) based blind unmixing algorithm was employed to isolate the chromophores. Further a single follicle (BCB+) was isolated and the three dimensional rendering is seen in (fig 31 B). Two channels (BCB and tissue) were assumed so as to clearly identify the locations where BCB is deposited vis-à-vis the tissue morphology. The BCB channel as impressed in fig 31 shows the deposition along the walls of the follicles. The derived spectra for tissue (showing heamoglobin contrast) and BCB are shown in fig 31 D, the blindly annotated BCB curve was seen to closely reproduce the experimentally derived spectral curve. A three dimensional rendering for the whole ovary imaging is shown in fig 31 C, which was obtained by stacking multiple scan slices along Z-direction scanned at a distance of 0.1mm. The 3D scan was able to provide suitable anatomical land marking and allow for the computation of follicular volume, which is an important marker of follicle maturity. At the determined wavelength of 690 nm, both the anatomy and contrast agents were visualised distinctly, and the images could provide a better understanding of follicular content, volume, anatomical position and sizes.

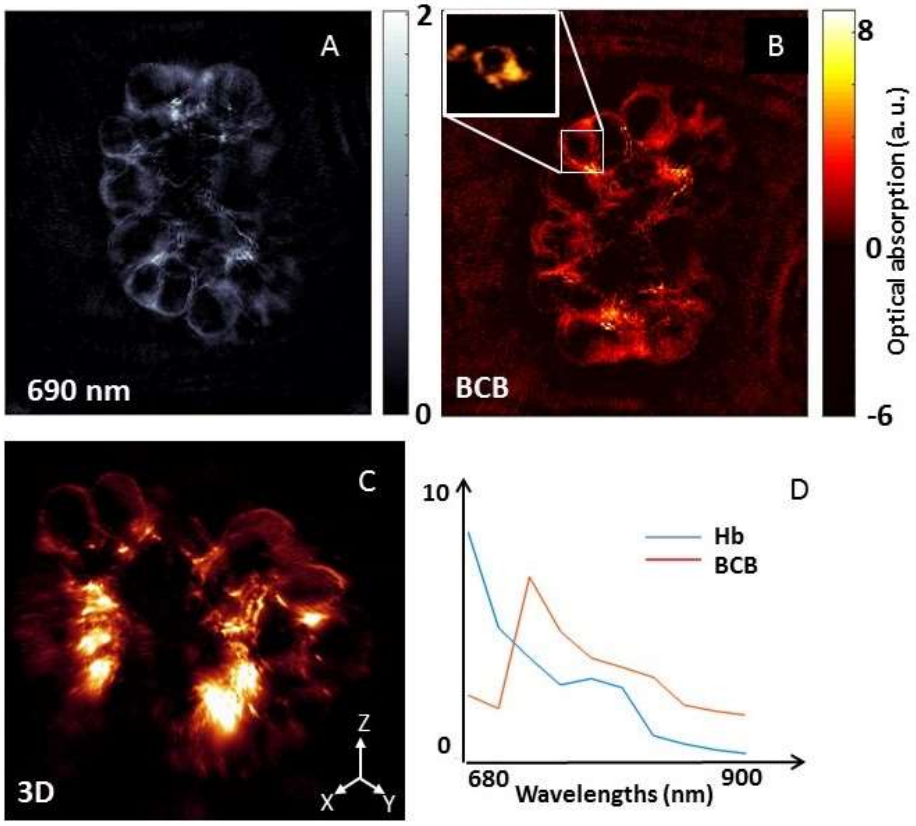

Fig 31 Single wavelength (690 nm) image of BCB contrast enhanced ovarian follicles are shown in (A), the BCB molecules is spectrally resolved in (B) through a blind unmixing process using 10 wavelengths, zoom in reconstruction of a follicle is shown in the insert. In (C) a 3D volume rendering is shown and (D) shows the reference spectrum recovered from the blind unmixing process and is mapped to suitable chromophores.

5.3.2.3 Nuclear maturation

Of the 510 COCs in the nuclear maturation group, 250 were aspirated from control group ovaries, with 130 assessed as BCB+ve (52%) and 120 BCB -ve (48 %); 260 were aspirated from control group ovaries, with 134 assessed as BCB+ve (51.53%) and 126 BCB -ve (48.46%). Nuclear maturation of each oocyte was scored by the attainment of metaphase II, as judged by the presence of condensed chromosomes in an equatorial position and extrusion of the first polar body. Nuclear maturation data (Table 10) showed BCB+ MSOT scanned oocyte showed significantly higher rate of nuclear maturation (85.52%±2.92) than the BCB –ve MSOT scanned group

(78.41%±3.91). Similarly BCB+ control group oocytes showed significantly higher rate of nuclear maturation (86.48% ± 3.07) than BCB-ve control group (79.9±3.82). Moreover, there was no significant difference between the respective groups among the MSOT scanned and control group.

5.3.2.4 Developmental competence of oocytes

The parthenogenetic activation group comprised of 244 oocytes. 124 were aspirated from MSOT scanned ovaries and further classified by BCB selection as 63 BCB+ oocytes (51.21%), 61 BCB –ve oocytes (48.79%) and 120 were aspirated from control group ovaries and further classified by BCB selection as 59 BCB+ oocytes (49.17%), 61 BCB –ve oocytes (50.83%). Development was scored as the number of embryos consisting of two to eight evenly sized blastomeres 48 h after parthenogenetic activation. There was rate of parthenogenetic activation was significantly higher in MSOT scanned BCB +ve oocytes (88.91%±3.65), than in MSOT scanned BCB -ve oocytes (70.57%±3.28) oocytes at ($P<0.05$). The rates of parthenogenetic activation in control oocytes also showed a statistically significant difference: BCB+ve (87.98%±4.91), BCB-ve (69.03% ± 5.26) at ($P<0.05$) (Table 2). However, there was no statistically significant difference between the MSOT scanned BCB +ve oocytes and controlled BCB +ve oocytes. (Table.10)

Table 10 Developmental competence of oocytes isolated from MSOT scanned ovaries

Item	Nuclear maturation and Parthenogenetic Activation			
	MSOT scanned		Control group	
	BCB+ve	BCB-ve	BCB+ve	BCB-ve
Maturation(%)	85.52%±2.92[a]	78.41%±3.91[b]	86.48% ± 3.07[a]	79.9%±3.82[b]
Activation (%)	88.91%±3.65[a]	70.57%±3.28[b]	87.98%±4.91[a]	69.03% ±5.26[b]

a, b Value with different superscripts differ significantly ($p < 0.05$). Values are means ± standard deviation (SD).

5.3.2.5 TUNEL assay

A total of 134 COCs were randomly selected from MSOT scanned and control ovaries for TUNEL assay without considering their developmental competence. A TUNEL score was determined as the percentage of observed COCs showing signs of DNA fragmentation, as indicated by a fluorescent signal. An oocyte was scored as TUNEL positive when a distinct, green fluorescence signal was observed. Throughout, fluorescent signals were observed only in the ooplasm and not in the cumulus cells. There was no significant difference in TUNEL score between MSOT scanned oocytes (31.26% ± 4.23) and control oocytes (30.11%± 2.97) oocytes.

5.4 Chapter summary

For *in vitro* culture porcine oocytes are isolated from a cohort of dominant and subordinate follicles, most commonly by aspiration of slaughterhouse obtained ovaries. Follicles of size roughly 3-6 mm are chosen for aspiration simply based on the visual observation. Since the ovarian oocytes available for IVM are primarily those present in mid-size antral follicles of prepubertal gilts, more research is needed to gain an improved understanding of the classification and characterisation of preantral and antral follicles in addition to modifications in IVF systems to overcome the problem of polyspermic penetration. The developmental competence of the *in vitro* isolated oocytes varies according to the developmental stage of the ovarian follicle. As mentioned earlier in the thesis a lesser developmental competence of *in vitro* matured oocytes following *in vitro* culture (IVC) appears to be due the fact that the COCs at the time of aspiration have not undergone the stage of dominant follicular growth before ovulation. As a result, it leads to a batch-to-batch variability during *in vitro* embryo production. Presently, technologies to identify follicles with the best quality oocytes are not available. In order to improve the overall success rate of porcine IVF it is very important to develop newer

methods, to characterise the porcine ovarian follicle used for oocyte collection, must be developed. Therefore, to further enhance optimisation porcine IVP, a new strategy was investigated to characterise porcine ovary on basis of most important criterion like size of the ovarian follicle and the developing oocyte within, and the thickness of the follicular wall.

5.4.1 Porcine ovarian folliculometry with selective plane illumination microscopy (SPIM)

In the study use of selective plane illumination microscopy (SPIM) was explored to gain accurate and informative folliculometric data from scanned ovaries. SPIM technology is increasingly being used in developmental biology as it enables rapid visualisation of both chemically-cleared and living specimens (Huisken *et al*., 2004b, Santi, 2011, Buytaert *et al*., 2012). In this technique, a single plane of the sample is optically excited by a thin light sheet and 2D fluorescent images captured by a camera placed orthogonally to the light sheet, allowing assembly of 3D volumetric image stacks. The biological sample remains intact, allowing repeated imaged from multiple angles, to increase the quality of the combined 3D rendering. Any reduction of image quality due to physical destruction of the tissue was also avoided. SPIM is time- and labor-efficient and well suited for producing high resolution imaging stacks of 3D biological samples.

5.4.2 Improvement of image quality without destroying the follicle

In the experiment, wide ranges of individual follicles were extracted from two different sets of image stacks and the images demonstrated the high contrast (signal-to-noise ratio) and image resolution achievable. SPIM images were analysed for the most clinically or diagnostically relevant morphological features, specifically: size and number of follicles, size of the developing cumulus oophorus complexes (COC), and follicle wall

thickness. Follicles of all sizes can be seen and additional morphological details such as atretic follicles, blood vessels and interstitial connective tissue between adjacent follicles could be identified.

5.4.3 Multi spectral optoacoustic tomography (MSOT) of porcine ovary using brilliant cresyl blue (BCB) as a contrast medium

Ultrasonographic follicular imaging which has long been the primary tool for oocyte retrieval *in vitro* fertilisation laboratories reveals the growing follicle only as a black bubble with the most folliculometric information, including follicular wall thickness, is determined by manual or semi-automated segmentation. Thus there is immense scope of improvement in the existing techniques as the methods provide only crude information with questionable predictive value. Despite the fact that quality of oocyte is probably the one of the most important factors that determines the outcome of an ART oocyte quality, a method for assessment without disrupting the follicle is non-existent. Contrast enhancement in ovarian follicle imaging has never been tried primarily due to possible toxicity of the contrast agent to the oocytes. A non-invasive method that could reliably predict quality of the developing oocyte inside a follicle will be of great benefit for the IVF. Therefore, the study was undertaken using BCB as a contrast medium in combination with MSOT to improve the quality of visualisation manifold. This method can simultaneously deliver anatomical, functional and molecular information with both high resolution and penetration capabilities.

5.4.4 Optimal dilution factor for BCB for spectrum calculation

The optical and optoacoustic response of BCB was characterised using spectroscopic measurements (Ocean optics USB4000-FL, Bandwidth: 351-1043nm) and the spectroscopic measurements showed the absorption peak to be at 620 nm.

However, the said wavelength cannot be used for optoacoustic a measurement given the laser is optimised in the range 680-900 nm for maximum penetration in tissues. In this study a vertex component analysis (VCA) based fast blind unmixing method to map the distribution of BCB. Thus, in this approach unmixing was done specifically for presence of BCB within the ovarian follicle, given the fact the presence of BCB+ ve COC would help to better identify the developmentally competent follicles. The VCA methods were able to arbitrarily identify two channels and display the corresponding spectrum. The spectra were then matched with the optoacoustic spectrum of BCB experimentally measured and mapped to the corresponding tissue components and the BCB channel.

5.4.5 MSOT scanning of ovarian follicle

The single wavelength image at 690 nm showed the internal anatomy of the ovary (*ex-vivo*) clearly and showed good BCB contrast with high signal intensity at the used wavelength. Two channels (BCB and tissue) were assumed so as to clearly identify the locations where BCB is deposited vis-à-vis the tissue morphology. The BCB channel in the obtained images showed their clear deposition along the walls of the follicles. The spectrum for tissue (showing hemoglobin contrast) and BCB were clearly distinguishable from each other. The blindly annotated BCB curve was seen to closely reproduce the experimentally derived spectral curve. At the calculated wavelength, both the anatomy and contrast agents were detectable, and could provide a better visualisation of folliculate, volume, anatomical position and sizes.

5.5 Chapter conclusion

During the experiment, a novel combination of clearing and imaging of porcine ovarian follicles with SPIM was developed. The follicular antrum and theca interna

layers were distinctly visible, allowing individual follicles to be easily identified. Follicles of all developmental stages were identified and their diameters characterised, ranging from small primordial follicles up to 2.5 mm graafian follicles. Clearly distinguishable COCs protruding into the follicular antrum of several antral and graafian follicles were measured at 40-110 µm in size, and their correlation with the developmental stage of the follicles agreed with the literature. A remarkable correlation was obtained between the thecal wall thickness and the developmental stage of the follicles. Moreover, during the study, BCB was explored as a nontoxic contrast medium for imaging porcine ovarian follicle and it has substantially improved the quality of follicular visualisation. The findings have provided the proof of principle that BCB can be used as a contrast medium and when coupled with MSOT a really simple, gentle and very efficient imaging method can be developed. The other salient findings of these experiments are elaborated in greater length in the discussion chapter. But with this new approach, in future, there would be a possibility of better characterisation of ovarian follicles and thus improving the outcome of any ART approach including IVF.

Chapter VI

Discussion

Genome editing directly in mammalian embryos by CRISPR/Cas9 system is a significant advance that is now being widely used as an alternative to traditional embryonic stem cell targeting-based KO production. However, the tremendous potential of one-step production of knockout pig is yet to be realised because of the lack of a reliable supply of developmentally competent embryos. In case of large animals developing *in vitro* produced (IVP) embryos have many bottlenecks including *in vitro* maturation (IVM) and the culture systems available are still unacceptably inefficient, especially in species like pig (Grupen, 2014). The production of an *in vitro*–derived embryo involves multiple critical steps. These include *in vitro* oocyte maturation, sperm preparation, IVF, and further culture of the putative zygotes. IVF still remains a critical limiting factor in realising the tremendous potential of pig as a model animal in large animal transgenic research. Improvements of *in vitro* maturation and fertilisation systems have overcome some previous limitations and led to high nuclear maturation rates and better male pronucleus formation. Despite the improvements made in the past decade, polyspermy is still a prevalent obstacle in most IVP systems. Most of the current IVF systems hardly reach 50% to 60% efficiency and any progression in porcine ARTs requires an unavoidable improvement in the monospermy rate (Romar *et al*.2015).

Taking into account all the above-mentioned considerations, the main focus of the study was to improve the *in vitro* production of porcine embryos for direct zygote microinjection of CRISPR /Cas 9. During the study, a few novel techniques for improving embryo culture by selecting superior quality oocytes and improving ovarian follicle characterisation were also developed. The section below discusses the major findings and problems associated with *in vitro* porcine embryo production as observed during the course of the experimental study.

6.1 Optimisation of IVM culture condition against different variables

In vitro oocyte maturation is the foundation for multiple assisted reproductive technologies in swine, including nuclear transfer and transgenesis (Yuan and Krisher, 2012). Generation of porcine zygotes by IVF requires a large number of *in vitro* matured oocytes. In practice, this necessitates the use of slaughterhouse-derived material because of the cost and time required to obtain *in vivo* matured oocytes and the difficulty of ovum pick up in pig. However, slaughterhouse ovaries can be subjected to stresses that result in poor maturation and subsequent embryo development *in vitro*, leading to considerable batch-to-batch variation of the samples. Nevertheless, if such materials are to be used, then the success rate needs to be improved as far as possible by optimising each stage of the procedure. With this in mind the first experiment was designed to develop an efficient IVM protocol. A TCM-199 based medium supplemented with 20 % porcine follicular fluid was used. In this study TCM-199 was chosen over the other commonly used medium for porcine IVM culture i.e. NCSU-37 for being a chemically defined medium. A chemically defined medium is useful for analysing the physical action of substances, such as inorganic compounds, energy substrates, hormones, cytokines, and vitamins, on the development of preimplantation embryos, because it eliminates undefined factors present in serum or serum albumin (Yoshioka, 2011). For IVM of porcine oocytes, media supplemented with porcine follicular fluid (FF) is generally been used. However, FF contains many unknown factors and often have considerable variability among sources or even among batches from the same source. Furthermore, there is a possibility that these fluids may contain occult viruses (Stringfellow and Givens, 2000). Although defined IVP systems (using media not containing serum or FF) has been developed in many laboratories (Gomez *et al*., 2002; Mizushima and Fukui, 2001), the successful use of defined media for porcine IVP has

not yet been reported. At present, porcine FF is one of the variables, which is beyond control of the researchers leading to lack of reproducibility.

6.2 Developmental blocks and overcoming the block

Two different mediums namely a commercial porcine zygote medium (PZM5) and a TCM-199 based embryo development medium (EDM) were compared in order to standardise a better medium for IVF embryo production in future. The PZM 5 produced a cleavage rate of 83 %±3.66 while that of EDM was 82.58% ± 2.78. As it was clearly seen there was no statistically significant difference between the two media tried. During this study none of the morula stage embryos developed up to the blastocyst stage by 6 d of culture, it may be possible that the media compared in this experiment are not suitable to successfully develop from single cell embryos to blastocysts. But some reports of blastocyst production are available using PZM 5 medium (Grupen, 2014). So it could be a form of developmental arrest as reported earlier in other species. The phenomenon of developmental arrest has been studied most extensively in the mouse, in which 1-cell embryos from most outbred and inbred strains do not develop to blastocysts when cultured in a chemically denned medium, but arrest at the 2-cell stage, a phenomenon referred to as the '2-cell block' (Goddard and Pratt, 1983). Other species also experience a similar block in development; for rat it occurs at 8-cells; hamster, 2-cells and 8-cells; and cow, 8- to 16-cells (Reed *et al*., 1992). In pigs 4-cell block was mostly reported. In fact development of *in vivo* obtained porcine embryo is likewise arrested at the four-cell stage (Polge, 1982). This may be due to substrate and metabolic changes as the embryo enters the uterine horn from the oviduct at the four-cell stage (Lindner and Wright, 1978; Bavister, 1988) and begins synthesis of new proteins (Polge, 1982). The block occurs during the S phase of the cell cycle (Eyestone *et al*., 1986).

However, in the current study, most of the embryos did grow beyond the 4-cell stage to reach the compact morula stage. So it may be concluded that this was not a case of much widely reported 4-cell block. The probable reasons could be poor oocyte quality. For *in vitro* production of porcine embryos, the cumulus-oocyte complexes (COC) are collected from ovarian follicles that are 3–6 mm in diameter, meaning that the COC have not undergone the stage of dominant follicular growth before ovulation. A lesser developmental competence of *in vitro* matured oocytes following *in vitro* fertilisation (IVF) appears to be due to a heterogeneous population of oocytes at the start of *in vitro* maturation (IVM) and/or the absence of the final phase of oocyte growth (Funahashi and Day, 1997; Coy and Romar, 2002). Unfortunately, this problem is unavoidable while using pre pubertal gilt ovaries obtained from slaughterhouse. Therefore, much attention has to be paid in characterisation of ovarian follicles and ovarian reserve.

Another reason for failure to produce blastocyst could be low cytoplasmic maturity of IVM oocytes. Cytoplasmic maturation along with nuclear maturation is not well described in porcine, compared to other animals. Therefore, there are still many difficulties in securing high quality embryos due to the developmental retardation and stoppage in the 4-cell stage during *in vitro* culture because of unstable *in vitro* maturation (Wang *et al*., 1997). So further development of the IVM protocol may be desirable for optimal growth.

During the embryo culture, low oxygen tension as used by many researchers was not maintained which might be another reason for the developmental failure. High oxygen tensions *in vitro* can influence the balance between the synthesis of glycogen (from exogenous glucose) and its degradation, probably via accumulation of ATP (Bavister, 1987). These disturbances in glucose metabolism could lead to the formation

of free radicals by generating NADPH via the pentose phosphate shunt, indeed glucose has been reported as deleterious for development through the 2-cell stage, its omission from the media improving development. However, in the current study the oxygen concentration was not controlled to a 5% level. This could also be a cause for the developmental block noticed during culture. Therefore, a recommended optimal culture condition for porcine embryo culture is temperature 39^0 C, CO_2 5%, O_2 5% and relative humidity-95%, for 5-6 days.

6.3 Optimisation of *in vitro* fertilisation

6.3.1 Lack of cryopreservability of porcine sperm

One of the major problems associated with porcine IVF is the difficulty in cryopreservation of porcine sperm. Freezing of boar spermatozoa started in the 1960's and their fertility was reassured using cervical artificial insemination (AI) by 1971 (Co, 1990). Still, despite documented efforts to reach acceptable fertility and prolificacy after AI, overall boar sperm cryosurvival is consistently low in comparison to other species, owing to damage during a processing that is time consuming, costly and yields few doses per ejaculate. The relatively low fertility of frozen thawed (FT) boar semen is associated with many factors including a highly sensitive plasma membrane of boar spermatozoa against the changing in temperature during cooling, freezing and thawing process (Rodriguez-Martinez *et al*., 2010; Holt 2010). This problem is related to the lipid composition of the sperm plasma membrane. The plasma membrane of the boar spermatozoa contains a high level of polyunsaturated fatty acids (PUFAs) i.e., docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and had a low cholesterol to phospholipids ratio. DPA and DHA are dominant fatty acids in the plasma membrane of boar spermatozoa (Johnson *et al*., 2000).

6.3.2 The problem of polyspermy

Although rate of fertilisation of Androcoll P selected sperm were significantly (80.2%) improved over the control group (20.25%) but the rate of polyspermy was also high. The techniques for achieving *in vitro* maturation and *in vitro* fertilisation (IVF) of pig oocytes have been progressively improved in recent years, despite this, a high incidence of polyspermy is still one of the major problems in the pig IVF program (Abeydeera and Day, 1997; Yoshida *et al*., 1997, Niwa 1993; Kikuchi *et al*., 1999). The number of polyspermic embryos often exceeds 60% of total embryos produced by IVF (Romar *et al*., 2015). In comparison to other species, polyspermic fertilisation occurs more frequently in the pig, even for *in vivo* fertilisation under diverse experimental conditions. This rate is significantly higher in *in vitro* matures oocytes than that of *in vivo* matured and fertilised oocytes (<5%) (Hunter, 1990).

6.3.3 The probable causes and solution

Causes for this problem likely include the quality of semen at fertilisation (Kim *et al*., 1996a; Pavlok, 2000; Sirard *et al*., 1993). Large variations among individual males have been noted in fertilisation rates with fresh (Sirard *et al*., 1993) and frozen sperm (Nagai *et al*., 1988). In pigs, the high incidence of polyspermy is still an unresolved problem (Abeydeera, 2001) which has been associated with “failures” in the cortical reaction (CR). The cortical reaction is a process initiated during fertilisation by the release of cortical granules (CG) from the oocyte, which prevents polyspermy. The probable cause of polyspermy is i) delayed and incomplete CR, ii) lack of distribution of released CG contents into the perivitelline space iii) delayed zona reaction iv) undefined differences between *in vitro* (IVM) and *in vivo* (IVV) matured oocytes such as incomplete zona pellucida maturation in the former ones (Abeydeera, 2001). Under *in*

vivo conditions, it is believed that the oviduct microenvironment participates in the completion of oocytes to block polyspermic fertilisation (Hunter, 1967). However, during IVF, high concentrations of spermatozoa are usually introduced to micro drops in which oocytes have been placed. Hence, the oocytes are exposed to an excess number of spermatozoa, which causes simultaneous sperm penetration into an oocyte and results in polyspermic fertilisation, even in a condition that the oocytes have the ability to block the polyspermic penetration.

Some approaches by mimicking *in vivo* conditions, such as preincubation of spermatozoa (Nagai and Moor, 1990) or oocytes (Kano *et al*., 1994) in cultured oviductal epithelial cells or follicle somatic cells (Wang *et al*., 1997) or preincubation of oocytes in collected oviductal fluid (Kim *et al*., 1996), have been reported to be able to reduce polyspermic penetration of pig oocytes matured and inseminated *in vitro* but a satisfactory IVF system in the pigs has not been established.

Therefore, it may be noted that a further improvement of IVM culture may be desirable as incomplete cytoplasmic maturation may contribute to higher incidence of polyspermy (Grupen, 2014). Using frozen sperm in place of prediluted sperm selected with Androcoll P may be helpful. However, attempts to freeze porcine sperm were not successful. Other culture condition improvement like reducing the concentration of sperm during co incubation and reducing the time of co incubation time from 6h to shorter period may also be helpful.

6.4 Gene targeting by microinjection of CRISPR /Cas 9

A sgRNA targeting the exon 10 region of *CMAH* (CMP-Neu5Ac hydroxylase) and exon 8 *GGTA1* (glycoprotein galactosyltransferase α1, 3) gene was designed and expressed using the expression vector described by Jinek *et al*.2012. 125 ng/µl Cas9

mRNA and 12.5 ng/µl sgRNA targeting both the gene of interest were injected in to the putative *in vitro* fertilised zygotes from cultured for 18 h. The results indicated 64% homozygous knockout and 14% heterozygous knockout for *CMAH* Ex10. While for *GGTA1* Ex 8 30% knockouts were homozygous and 10% were heterozygous. While mutations were found via PCR and subsequent sequencing. 20% of the embryos were double knockouts. The efficiency of targeting was at per with earlier reports (Hai *et al*., 2014; Whitworth *et al*., 2014). Although no blastocyst were produced it has been demonstrated that one- to four-cell porcine embryos cultured for 72 h resulted in pregnancy after transfer (Davis, 1985; Yoshida *et al*., 1990). The gene targeted embryos were not subsequently used for embryo transfer but the results of this exploratory study showed that if transferred into surrogate mother there is possibility of producing transgenic animal using the current protocol.

6.5 Cumulus layer viability as indicator of oocyte quality

The usefulness of lissamine green B (LB) staining for non-invasive prediction of maturational competence of porcine oocytes was evaluated. LB preferentially stains membrane-damaged or devitalised cells (Kim and Foulks, 1999). Studies using LB to stain rabbit and human corneal epithelial cells in vitro show that it does not stain healthy, proliferating cells and has minimal effect on cell viability (Kim, 2000). Evidence to date indicates no carcinogenic or cytotoxic properties (Clode *et al*., 1987, Moorhouse *et al*., 1987, Bramton *et al*., 1987). The results indicated that LB staining of cumulus oocyte complexes (COCs) immediately after collection is superior to morphological grading as a predictor of oocyte viability, nuclear maturation after IVM culture, and successful parthenogenetic activation and development. Parthenogenesis was used as a convenient and valid functional assay of developmental competence (Rougier and Werb, 2001, McElroy *et al*., 2010).

Investigations of the role of apoptosis in COC quality revealed some interesting findings. As expected, DNA fragmentation measured by TUNEL assay showed that LB-grade 3 COCs were more damaged than higher grade COCs, consistent with their developmental properties and also in line with morphological grade C COCs. DNA fragmentation in immature oocytes (in particular, GV and MI stages) could be a consequence of stress during maturation of the ovarian follicle or hypoxia resulting from compromised microcirculation and correlate with aneuploidy or other chromosomal abnormalities (Van Blerkom, 1996). It is notable that no evidence of DNA fragmentation in the cumulus layer was found in immature porcine COCs. This is consistent with a similar finding in cattle where no TUNEL signal was obtained in cumulus cells of immature oocytes (Yuan *et al*., 2005).

Cumulus cells play a critical role in oocyte maturation and fertilisation by releasing and mediating signals to oocytes (Vandaele *et al*., 2008), but the relationship between cumulus cell apoptosis and the development potential of the oocyte is unclear. There are in fact contradictory reports in various species as to whether definitive apoptosis actually takes place in COCs (Tanghe *et al*., 2002). For example, some studies have shown that human and bovine cumulus cells undergo apoptosis (Yuan *et al*., 2005, Lee *et al*., 2001, Mikkelsen *et al*., 2001), While others report the absence of apoptosis in cumulus cells of rats (Zeuner *et al*., 2003), pigs (Szołtys *et al*., 2000), and cattle (Manabe *et al*., 1996).

The simplicity and non-invasive nature of LB staining makes it straightforward to adopt as a quality control test for embryological labs. LB staining can give valuable information about the quality of oocytes in terms of maturational ability, developmental potential and extent of DNA damage. Further studies are clearly required to assess any long-term effects of LB on embryo survival and foetal health.

6.6 Ovarian follicle classification for isolation of superior quality oocytes

For *in vitro* culture porcine oocytes are isolated from follicles of size roughly 3-6 mm are simply based on the visual observation. Since, the ovarian oocytes available for IVM are primarily those present in mid-size antral follicles of prepubertal gilts, more research is needed to gain an improved understanding of the classification and characterisation of preantral and antral follicles, in addition to modifications in IVF systems to overcome the problem of polyspermic penetration. The developmental competence of the *in vitro* isolated oocytes varies according to the developmental stage of the ovarian follicle. This is a major cause of variability during *in vitro* embryo production. Therefore, in order to improve the overall success rate of porcine IVF it is pertinent to develop newer methods to characterise the porcine ovarian follicle and hence, a novel and innovative approach was adopted to improve the characterisation of ovarian follicle.

6.6.1 Correlation between follicle size thecal wall thickness and COC size

An important feature of the SPIM images was the distinct visualisation of the follicular thecal walls. During the clearing phase there was delamination of the granulosa layer from the thecal layer, delineated by a black area in the SPIM images. This proved to be particularly advantageous in clearly demarcating the thecal layer from the granulosa layer. Wall thickness was however not uniform, so a distribution was measured for each follicle. A clear correlation between follicle size and thecal wall thickness was evident. For the largest Graafian follicle, wall thicknesses were around 90 µm, while antral follicle walls were around 120 µm. Identification of the follicle wall thickness is useful in characterising different classes of developing follicles because it

varies with developmental stage (Krivanek and Sonka, 1998b). A plot of the relationship between oocyte and follicle size. 30 data points were taken from the SPIM data of two different samples, and there results are compared to published histological results of porcine ovarian follicles (Griffin *et al*., 2006). The adjusted coefficient of determination (R-squared) between SPIM data and the literature-fitted curve was 0.76, indicating good agreement. SPIM is thus suitable for quantitative follicle characterisation.

While SPIM cannot fully replace the current imaging methods used for ovarian studies, it has unique advantages and compatibility with this specific size and type of sample to offer supplementary information for biological studies. Ultrasonic biomicroscopy is conventionally used for non-invasive monitoring of ovarian follicles, with the main advantage that it can be used *in vivo*, but the images are lower contrast and poorer resolution than the SPIM images obtained. Ultrasound fails to detect primordial or primary follicles in the ovary and can seldom detect the growing COC in graafian follicles (Pfeifer *et al*., 2012). Although SPIM cannot yet be used for *in vivo* biological studies, it offers a level of morphological detail that will greatly aid developmental studies. Here, the smallest anatomical feature that could be imaged was ~10 µm, which was well supported by the 6 µm in-plane resolution offered by SPIM.

6.6.2 Multi Spectral Optoacoustic Tomography (MSOT) of porcine ovary using brilliant cresyl blue (BCB) as a contrast medium

Ultrasonographic follicular imaging which has long been the primary tool for oocyte retrieval and there is immense scope of improvement in the existing techniques as the methods provide only crude information with questionable predictive value. Since quality of oocyte is one of the most important factors that determine the outcome of an ART experiment, a non-invasive method that could reliably predict quality of the

developing oocyte inside a follicle would be of great benefit for the IVF. Therefore, the study was undertaken using BCB as a contrast medium in combination with multispectral optoacoustic tomography (MSOT) to improve the quality of ovarian visualisation manifold.

6.6.2.1 Viability of porcine oocytes after MSOT scanning

Viability of oocyte was one of the most important criteria for choosing BCB as the contrast medium. The viability of the oocytes were assessed by their competency to develop further in *in vitro* culture The experimental data indicated that MSOT scanning after BCB injection into ovarian follicle is not detrimental to oocyte viability. As expected the nuclear maturation and parthenogenetic activation rate was significantly higher in BCB+ oocytes than the BCB oocytes similar to previous reports in goats (Rodríguez-González *et al*., 2002), heifers (Castaneda *et al*., 2013) and pigs (Santos *et al*., 2015). The low nuclear maturation rate of BCB –ve oocytes could be due to the incomplete or abnormal cytoplasmic maturation. It is not clear whether the BCB test can serve as an indirect marker of oocyte apoptosis. Investigation of the probable effect of MSOT scanning on oocyte quality revealed no cellular stress of the scanning on the oocyte. The DNA fragmentation measured by TUNEL assay showed that there is no significant difference between the oocytes isolated from MSOT scanned ovaries and control ovaries. The observed DNA fragmentation in immature oocytes (in particular, GV and MI stages) could be a consequence of stress during maturation of the ovarian follicle or hypoxia resulting from compromised microcirculation and correlate with aneuploidy or other chromosomal abnormalities (Van Blerkom, 1996). It is notable that no evidence of DNA fragmentation was found in the cumulus layer in immature porcine COCs. This is consistent with a similar finding in cattle where no TUNEL signal was obtained in cumulus cells of immature oocytes.

This approach in future can open newer dimension of follicular imaging where useful and additional information relevant for IVF can be obtained without hampering integrity of the follicle. The relatively low success rate of porcine IVF may be attributed to poor quality of retrieved oocytes, which in turn is due to the inaccurate timing of oocyte retrieval. With this approach, in future, there would be a possibility of visualising developmentally competent oocyte in the follicle; this information can help researchers to make informed diagnosis about quality of oocytes at time of retrieval. Besides a regular imaging, the possible utility of this approach can be extended to diagnosis of follicular cyst, even empty follicle syndrome and other developmental studies.

Chapter VII

Conclusion and future direction

During the research, an efficient and easily reproducible *in vitro* culture system for porcine embryos meant for direct zygote microinjection was established. An optimised IVM system that support very high rate of nuclear maturation (86.48%) was standardised. The developmental competence of those IVM cultured oocytes was corroborated by high rate (83%) of parthenogenetic activation. A relatively high *in vitro* fertilisation rate (80.2%) was also achieved. By injecting CRISPR/Cas 9 targeting the exon 10 region of *CMAH* and exon 8 of *GGTA* 1gene respectively into IVF produced embryos successful gene targeting was achieved. The results indicated 64% homozygous knockout and 14% heterozygous knockout for *CMAH* Ex10. While for *GGTA*1 Ex 8 30% knockouts were homozygous and 10% were heterozygous. 20% of the embryos were double knockouts. 79.5% of the injected zero stage embryos developed up to the morula stage. The gene targeted embryos were not subsequently used for embryo transfer but the results of this exploratory study showed that if transferred into surrogate mother there is possibility of producing transgenic animal using the current protocol.

With the objective of further improving the selection good porcine oocytes for *in vitro* embryo production, the usefulness of a novel dye lissamine green B (LB) staining for non-invasive prediction of maturational competence of porcine oocytes was evaluated. The simplicity and non-invasive nature of LB staining makes it straightforward to adopt as a quality control test for embryological labs. LB staining can give valuable information about the quality of oocytes in terms of maturational ability, developmental potential and extent of DNA damage.

A novel combination of clearing and imaging of porcine ovarian follicles with SPIM to better characterise the porcine ovarian follicle was developed. Using the method, follicles of all developmental stages were identified and their diameters characterised, ranging from small primordial follicles up to 2.5 mm Graafian follicles.

Clearly distinguishable COCs protruding into the follicular antrum of several antral and Graafian follicles were measured at 40-110 μm in size, and their correlation with the developmental stage of the follicles agreed with the literature. The first measurements of theca thickness for follicles of different developmental stages were conducted, yielding results ranging from 90 to 120 μm. The good spatial resolution and high contrast offered by SPIM provided more quantitative anatomical information than ultrasound biomicroscopy. The unique capability to image 3D follicle anatomy while retaining morphological integrity, coupled with high throughput labor-saving nature of the SPIM system, also with ease and speed makes it superior to conventional H&E histology. SPIM was further shown to be suitable for characterising developmental stages of ovarian follicles, without the need to aspirate oocytes. Abnormalities in the physical dimensions could indicate possible ovarian diseases and precise folliculometric measurements will be of immense importance in improving the understanding of ovarian physiology and developing new applications to better manage ovarian diseases.

During experiment, BCB was explored as a nontoxic contrast medium for imaging porcine ovarian follicle. This has improved the quality of follicular visualisation manifold. Subsequently in order to validate the non-invasive nature of the treatment oocyte were isolated from that follicle for further culture. The results have provided the proof of principle that BCB can be used as a contrast medium and when coupled with MSOT a really simple, gentle and very efficient imaging method can be developed. MSOT images can clearly interpret the follicular structure. This approach in future can open newer dimension of follicular imaging where useful and additional information can be obtained without hampering integrity of the follicle. As previously reported, present relatively low success rate of porcine IVF may be attributed to poor quality of retrieved oocytes which in turn is due to lack of proper tools for oocyte imaging. But with this new

approach, in future, there would be a possibility of visualizing developmentally competent oocyte in the follicle, this information may help improve outcome of porcine IVF by making informed diagnosis at the time of oocyte retrieval.

In recent times, there have been some reports about direct zygote electroporation protocol that brings high efficiency, high throughput genome engineering in animal models within closer reach (Kaneko *et al*., 2014; Hashimoto & Takemoto 2015; Quin *et al*., 2015). However, these protocol fail to address the most significant bottleneck of transgenic animal production, i.e. the *in vitro* embryo production. By combining the simplicity and robustness of the CRISPR/Cas9 system with the throughput of the microinjection technology, we may now be in a position to generate animal models of human disease alleles with unprecedented efficiency. This would significantly increase the range and number of genes that can be investigated, reduce the time necessary, and reduce the number of animals needed in accordance with the principle of 'replacement, reduction and refinement' for work with experimental animals.

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Annexure

Embryo development medium (10 ml) pH: 7.2-7.4

Components	Quantity(per 10 ml)
TCM 199 (HEPES modification)	9 ml
Sodium pyruvate	0.3 mg
L-glutamine	1 mg
Gentamycin	0.5 mg
Essential amino acids	100 µl
Non-Essential amino acids	50 µl
BSA (Fraction-V)	100 mg
Cysteamine	50 µM
FBS	1 ml

mTBM (modified tris buffered medium) for 50 ml

Component	Quantity (per 50 ml)
NaCl	113.1m
KCl	3.0 M
$CaCl_2$. $2H_2O$	7.5mM
Tris	20mM
Glucose	11mM
Sodium pyruvate	5mM
Caffeine	1mM

Oocyte collection medium (100ml) pH: 7.2-7.4

Component	Quantity (per 100 ml)
TCM 199 (HEPES modification)	90 ml
L-glutamine	10 mg
FBS	10 ml
Gentamicine	5 mg
BSA (Fraction-V)	300 mg

Oocyte Maturation Medium (10 ml) pH: 7.2-7.4

Components	Quantity (per 10ml)
TCM 199 (HEPES modification)	9 ml
hCG	5IU/ml
PMSG	10IU/ml
Cysteamine	100μM
Glutamax	50μL
Porcine Follicular Fluid	100μL
Epidermal Growth factor	10μg/ml

ABBREVIATIONS

%	=	Percent
μg	=	Micro gram
μl	=	Microlitre
μm	=	Micrometer
μm	=	Micromolar
6-DMAP	=	6-dimethyl amino purine
ANOVA	=	Analysis of variance
ATP	=	Adenosine triphosphate
B	=	Blastocysts
BCB	=	Brilliant Cresyl Blue
bp	=	base pair
BSA (FAF)	=	Bovine serum albumin (Fatty acid free)
BSA	=	Bovine serum albumin
cm	=	Centimetre
COC	=	Cumulus-oocyte-complex
CMAH	=	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase
DIC	=	Differential Interference Contrast
DFI	=	DNA fragmentation Index
DMEM	=	Dulbecco's modified eagle's medium
DMSO	=	Dimethyl sulphoxide
DNA	=	Deoxyribonucleic acid
DPBS	=	Dulbecco's phosphate buffered saline
DPSS	=	Diode-pumped solid-state
EDM	=	Embryo development medium
EDTA	=	Ethylene diamine tetra-acetic acid
eGFP	=	Enhanced Green Fluorescent Protein
FBS	=	Foetal bovine serum
FoV	=	Field of view
Fig	=	Figure
FSH	=	Follicle stimulating hormone
ft	=	Feet
FITC	=	Fluorescein Isothiocyanate

g	=	Gram
G0	=	Gap zero
G1	=	Gap 1
*GGTA*1	=	α1, 3-galactosyltransferase
h	=	Hour
HCG	=	Human Chorionic Gonadotropin
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hPa	=	Hecto pascal
I.U.	=	International units
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilisation
IVM	=	*In vitro* maturation
LB	=	Lissamine green B
LH	=	Luteinising hormone
LSD	=	Least significant difference
M I	=	Meiosis I
M II	=	Meiosis II
M	=	Morulae, Molar
MAPK	=	Mitogen activated protein kinase
MBCT	=	Micromanipulator based cloning technique
mCR2	=	Modified Charles Rosenkrans medium
MD	=	Micro drop
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
mm	=	millimetre
mM	=	Mill molar
MPF	=	Maturation promoting factor
MPF	=	Maturation promoting factor
mSOF	=	Modified synthetic oviductal fluid
MSOT	=	Multi Spectral Optoacoustic Tomography
n	=	Number
ng	=	Nano gram
NEBD	=	Nuclear envelope breakdown
NT	=	Nucleus transfer

^{o}C	=	Degree Celsius
OPO	=	optical parametric oscillator
PA	=	Parthenogenetically activated
PFF	=	Porcine follicular fluid
PFM	=	Porcine fertilisation Medium
PI	=	Propidium Iodide
Pl	=	Pico liter
PMSG	=	Pregnant Mare Serum Gonadotropin
PNA	=	Peanut Agglutinin
PVA	=	Poly Vinyl Alcohol
PZM	=	Porcine Zygote Medium
rpm	=	revolutions per minute
SCNT	=	Somatic cell nuclear transfer
sCMOS	=	Scientific complementary metal oxide semiconductor
SE	=	Standard error
SEm	=	Standard error of mean
SLC	=	Single layer centrifugation
SNR	=	Signal to noise ratio
SPIM	=	Selective Plane Illumination Microscopy
TCM-199	=	Tissue culture medium-199
TUNEL	=	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
UV	=	Ultraviolet
V	=	Volts
VCA	=	Vertex component analyses
v/v	=	volume/ volume
vs.	=	Versus

Bibliography

Abeydeera, L.R. (2001). *In vitro* fertilisation and embryo development in pigs. Reprod. Camb. Engl. Suppl. 58, 159–173.

Abeydeera, L.R. (2002). *In vitro* production of embryos in swine. Theriogenology 57, 256–273.

Abeydeera, L.R., and Day, B.N. (1997). *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 48, 537–544.

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A., and Day, B.N. (1998). Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after *in vitro* fertilisation: relevance to intracellular glutathione. Biol. Reprod. 58, 213–218.

Assou, S., Haouzi, D., De Vos, J., and Hamamah, S. (2010). Human cumulus cells as biomarkers for embryo and pregnancy outcomes. Mol. Hum. Reprod. 16, 531–538.

Bavister, B.D. (1987). Studies on the Developmental Blocks in Cultured Hamster Embryos. In The Mammalian Preimplantation Embryo, B.D. Bavister, ed. (Springer US), pp. 219–249.

Bavister, B.D. (1995). Culture of preimplantation embryos: facts and artifacts. Hum. Reprod. Update 1, 91–148.

Beckmann, L.S., and Day, B.N. (1993). Effects of media NaCl concentration and osmolality on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium. Theriogenology 39, 611–622.

Betthauser, J., Forsberg, E., Augenstein, M., Childs, L., Eilertsen, K., Enos, J., Forsythe, T., Golueke, P., Jurgella, G., Koppang, R., et al. (2000). Production of cloned pigs from in vitro systems. Nat. Biotechnol. 18, 1055–1059.

Bhojwani, S., Alm, H., Torner, H., Kanitz, W., and Poehland, R. (2007). Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. Theriogenology 67, 341–345.

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

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326, 1509–1512.

Boissel, S., Jarjour, J., Astrakhan, A., Adey, A., Gouble, A., Duchateau, P., Shendure, J., Stoddard, B.L., Certo, M.T., Baker, D., *et al*. (2014). megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. Nucleic Acids Res. 42, 2591–2601.

Brantom, P.G., Creasy, D.M., and Gaunt, I.F. (1987). Long-term toxicity study of Green S in mice. Food Chem. Toxicol. 25, 977–983.

Brüssow, K.P., Torner, H., Kanitz, W., and Rátky, J. (2000). *In vitro* technologies related to pig embryo transfer. Reprod. Nutr. Dev. 40, 469–480.

Buytaert, J.A.N., Descamps, E., Adriaens, D., and Dirckx, J.J.J. (2012). The OPFOS Microscopy Family: High-Resolution Optical Sectioning of Biomedical Specimens. Anat. Res. Int. 2012, 206238.

Carrol, D.J. (2009). Microinjection methods and applications. Humana press.

Castaneda, C.A., Kaye, P., Pantaleon, M., Phillips, N., Norman, S., Fry, R., and D'Occhio, M.J. (2013). Lipid content, active mitochondria and brilliant cresyl blue staining in bovine oocytes. Theriogenology 79, 417–422.

Chandrakanthan, V., Li, A., Chami, O., and O'Neill, C. (2006). Effects of *in vitro* fertilisation and embryo culture on TRP53 and Bax expression in B6 mouse embryos. Reprod. Biol. Endocrinol. RBE 4, 61.

Chen, B., Gilbert, L.A., Cimini, B.A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E.H., Weissman, J.S., Qi, L.S., *et al*. (2013). Dynamic imaging of genomic loci in living human cells by an optimised CRISPR/Cas system. Cell 155, 1479–1491.

Cheng, E.-H., Chen, S.-U., Lee, T.-H., Pai, Y.-P., Huang, L.-S., Huang, C.-C., and Lee, M.-S. (2013). Evaluation of telomere length in cumulus cells as a potential biomarker of oocyte and embryo quality. Hum. Reprod. 28, 929–936.

Clode, S.A. (1987). Teratogenicity and embryotoxicity study of Green S in rats. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 25, 995–997.

Co, B. (1990). Cryopreservation of boar semen. I: A literature review. Acta Vet. Scand. 32, 431–453.

Cognié, Y., Poulin, N., Locatelli, Y., and Mermillod, P. (2004). State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. Reprod. Fertil. Dev. 16, 437–445.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., *et al*. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823.

Corn, C.M., Hauser-Kronberger, C., Moser, M., Tews, G., and Ebner, T. (2005). Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. Fertil. Steril. 84, 627–633.

Coticchio, G., Sereni, E., Serrao, L., Mazzone, S., Iadarola, I., and Borini, A. (2004). What criteria for the definition of oocyte quality? Ann. N. Y. Acad. Sci. 1034, 132–144.

Cox, D.B.T., Platt, R.J., and Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. Nat. Med. 21, 121–131.

Cruz, M.H.C., Saraiva, N.Z., da Cruz, J.F., Oliveira, C.S., Del Collado, M., Fernandes, H., Castro, F.C. de, and Garcia, J.M. (2014). Effect of follicular fluid supplementation during *in vitro* maturation on total cell number in bovine blastocysts produced *in vitro*. Rev. Bras. Zootec. 43, 120–126.

Dickinson, D.J., Ward, J.D., Reiner, D.J., and Goldstein, B. (2013). Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nat. Methods 10, 1028–1034.

Dobrinsky, J.R., Johnson, L.A., and Rath, D. (1996). Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. Biol. Reprod. 55, 1069–1074.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. *72*, 8463–8471.

Dunn, D.A., Pinkert, C.A., and Kooyman, D.L. (2005). Foundation Review: Transgenic animals and their impact on the drug discovery industry. Drug Discov. Today 10, 757–767.

Edwards, R.G., Bavister, B.D., and Steptoe, P.C. (1969). Early Stages of Fertilisation *in vitro* of Human Oocytes Matured *in vitro*. Nature 221, 632–635.

Fabre, L., Le Hello, S., Roux, C., Issenhuth-Jeanjean, S., and Weill, F.-X. (2014). CRISPR Is an Optimal Target for the Design of Specific PCR Assays for Salmonella enterica Serotypes Typhi and Paratyphi A. PLoS Negl. Trop. Dis. 8.

Flisikowska, T., Kind, A., and Schnieke, A. (2013). The new pig on the block: modelling cancer in pigs. Transgenic Res. 22, 673–680.

FS Beebe, L. (2013). Development of an Improved Porcine Embryo Culture Medium for Cloning, Transgenesis and Embryonic Stem Cell Isolation. Cloning Transgenesis 02.

Galeati, G., Modina, S., Lauria, A., and Mattioli, M. (1991). Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. Mol. Reprod. Dev. 29, 40–46.

Gajda, B. (2009). Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology. Reprod. Biol. 9, 97–112.

Gandhi, A.P., Lane, M., Gardner, D.K., and Krisher, R.L. (2001). Substrate utilization in porcine embryos cultured in NCSU-23 and G1.2/G2.2 sequential culture media. Mol. Reprod. Dev. 58, 269–275.

Galli, C., Duchi, R., Crotti, G., Turini, P., Ponderato, N., Colleoni, S., Lagutina, I., and Lazzari, G. (2003). Bovine embryo technologies. Theriogenology 59, 599–616.

Garg, S., Dutta, R., Malakar, D., Jena, M.K., Kumar, D., Sahu, S., and Prakash, B. (2012). Cardiomyocytes rhythmically beating generated from goat embryonic stem cell. Theriogenology 77, 829–839.

Gil, M.A., Almiñana, C., Roca, J., Vázquez, J.M., and Martínez, E.A. (2008). Boar semen variability and its effects on IVF efficiency. Theriogenology 70, 1260–1268.

Goddard, M.J., and Pratt, H.P. (1983). Control of events during early cleavage of the mouse embryo: an analysis of the “2-cell block.” J. Embryol. Exp. Morphol. 73, 111–133.

Gordo, A.C., Wu, H., He, C.L., and Fissore, R.A. (2000). Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of [Ca (2+)] (i) oscillations and oocyte age. Biol. Reprod. 62, 1370–1379.

Gosden, R. and Bownes, M (1995). Molecular and cellular aspects of oocyte development In: Grudzinskas J, Yovich J. (Eds) Gametes - The Oocyte. Cambridge: Cambridge University Press, 23-53-

Gratz, S.J., Ukken, F.P., Rubinstein, C.D., Thiede, G., Donohue, L.K., Cummings, A.M., and O’Connor-Giles, K.M. (2014). Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in Drosophila. Genetics genetics.113.160713.

Griffin, J., Emery, B.R., Huang, I., Peterson, C.M., and Carrell, D.T. (2006). Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). J. Exp. Clin. Assist. Reprod. 3, 2.

Grupen, C.G. (2014). The evolution of porcine embryo *in vitro* production. Theriogenology 81, 24–37.

Gün, G., and Kues, W.A. (2014). Current Progress of Genetically Engineered Pig Models for Biomedical Research. BioResearch Open Access 3, 255–264.

Hamrah, P., Alipour, F., Jiang, S., Sohn, J.-H., and Foulks, G.N. (2011). Optimizing evaluation of Lissamine Green parameters for ocular surface staining. Eye Lond. Engl. 25, 1429–1434.

Hardy, K., Wright, C.S., Franks, S., and Winston, R.M.L. (2000). *In vitro* maturation of oocytes. Br. Med. Bull. 56, 588–602.

Harrison, M.M., Jenkins, B.V., O'Connor-Giles, K.M., and Wildonger, J. (2014). A CRISPR view of development. Genes Dev. 28, 1859–1872.

Houdebine, L.-M. (2002). The methods to generate transgenic animals and to control transgene expression. J. Biotechnol. 98, 145–160.

Houdebine, L.-M. (2007). Transgenic animal models in biomedical research. Methods Mol. Biol. Clifton NJ 360, 163–202.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278.

Hu, J., Lei, Y., Wong, W.-K., Liu, S., Lee, K.-C., He, X., You, W., Zhou, R., Guo, J.-T., Chen, X., *et al*. (2014). Direct activation of human and mouse Oct4 genes using engineered TALE and Cas9 transcription factors. Nucleic Acids Res. 42, 4375–4390.

Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., and Stelzer, E.H.K. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. Science 305, 1007–1009.

Hunter, R.H. (1967). The effects of delayed insemination on fertilisation and early cleavage in the pig. J. Reprod. Fertil. 13, 133–147.

Hunter, R.H. (1990). Fertilisation of pig eggs *in vivo* and *in vitro*. J. Reprod. Fertil. Suppl. 40, 211–226.

Hunter, R.H.F. (1991). Oviduct function in pigs, with particular reference to the pathological condition of polyspermy. Mol. Reprod. Dev. 29, 385–391.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Kaini, P., Sander, J.D., Joung, J.K., Peterson, R.T., and Yeh, J.-R.J. (2013). Heritable and Precise Zebrafish Genome Editing Using a CRISPR-Cas System. PLoS ONE 8, e68708.

Ivics, Z., Garrels, W., Mátés, L., Yau, T.Y., Bashir, S., Zidek, V., Landa, V., Geurts, A., Pravenec, M., Rülicke, T., *et al*. (2014). Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nat. Protoc. 9, 810–827.

Johnson, L.A., Weitze, K.F., Fiser, P., and Maxwell, W.M. (2000). Storage of boar semen. Anim. Reprod. Sci. 62, 143–172.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 378, 206–208.

Joo, J.Y., Lee, J., Ko, H.Y., Lee, Y.S., Lim, D.-H., Kim, E.-Y., Cho, S., Hong, K.-S., Ko, J.J., Lee, S., *et al*. (2014). Microinjection free delivery of miRNA inhibitor into zygotes. Sci. Rep. 4, 5417.

Jura, J., Smorag, Z., Slomski, R., Gajda, B., and others (2009). Comparison of transfection methods for porcine zygotes. Ann. Anim. Sci. 9, 279–285.

Kano, K., Miyano, T., and Kato, S. (1994). Effect of oviductal epithelial cells on fertilisation of pig oocytes *in vitro*. Theriogenology 42, 1061–1068.

Karami Shabankareh, H., Azimi, G., and Torki, M. (2014). Developmental competence of bovine oocytes selected based on follicle size and using the brilliant cresyl blue (BCB) test. Iran. J. Reprod. Med. 12, 771–778.

Kim, N.H., Funahashi, H., Abeydeera, L.R., Moon, S.J., Prather, R.S., and Day, B.N. (1996). Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilisation of pig oocytes *in vitro*. J. Reprod. Fertil. 107, 79–86.

Kim, J., and Foulks, G.N. (1999). Evaluation of the effect of lissamine green and rose bengal on human corneal epithelial cells. Cornea 18, 328–332.

Kim, J. (2000). The use of vital dyes in corneal disease. Curr. Opin. Ophthalmol. 11, 241–247.

Koike-Yusa, H., Li, Y., Tan, E.-P., Velasco-Herrera, M.D.C., and Yusa, K. (2014). Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat. Biotechnol. 32, 267–273.

Krivanek, A., and Sonka, M. (1998). Ovarian ultrasound image analysis: follicle segmentation. IEEE Trans. Med. Imaging 17, 935–944.

Kues, W.A., and Niemann, H. (2004). The contribution of farm animals to human health. Trends Biotechnol. 22, 286–294.

Kwon, D.-N., Lee, K., Kang, M.-J., Choi, Y.-J., Park, C., Whyte, J.J., Brown, A.N., Kim, J.-H., Samuel, M., Mao, J., *et al*. (2013). Production of biallelic CMP-Neu5Ac hydroxylase knock-out pigs. Sci. Rep. 3, 1981.

Lee, K.S., Joo, B.S., Na, Y.J., Yoon, M.S., Choi, O.H., and Kim, W.W. (2001). Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. J. Assist. Reprod. Genet. 18, 490–498.

Lillico, S.G., Proudfoot, C., Carlson, D.F., Stverakova, D., Neil, C., Blain, C., King, T.J., Ritchie, W.A., Tan, W., Mileham, A.J., *et al*. (2013). Live pigs produced from genome edited zygotes. Sci. Rep. 3, 2847.

Lim, K.T., Jang, G., Ko, K.H., Lee, W.W., Park, H.J., Kim, J.J., Lee, S.H., Hwang, W.S., Lee, B.C., and Kang, S.K. (2007). Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. Theriogenology 67, 293–302.

Liu, L., Deng, M., Tian, X.C., and Yang, X. (2003). Activation of Mammalian Oocytes: Principles and Practice. In Introduction to Mammalian Reproduction, D.R.P. Tulsiani, ed. (Springer US), pp. 319–345.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods San Diego Calif 25, 402–408.

Ma, Y., Zhang, X., Shen, B., Lu, Y., Chen, W., Ma, J., Bai, L., Huang, X., and Zhang, L. (2014). Generating rats with conditional alleles using CRISPR/Cas9. Cell Res. 24, 122–125.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science 339, 823–826.

Manabe, N., Imai, Y., Ohno, H., Takahagi, Y., Sugimoto, M., and Miyamoto, H. (1996). Apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in pig ovaries. Experientia 52, 647–651.

Marchal, R., Peláez, J., Terqui, M., and Mermillod, P. (2002b). Effect of sperm survival and CTC staining pattern on *in vitro* fertilisation of porcine oocytes. Theriogenology 57, 1917–1927.

Marchal, R., Vigneron, C., Perreau, C., Bali-Papp, A., and Mermillod, P. (2002a). Effect of follicular size on meiotic and developmental competence of porcine oocytes. Theriogenology 57, 1523–1532.

Mashiko, D., Young, S.A.M., Muto, M., Kato, H., Nozawa, K., Ogawa, M., Noda, T., Kim, Y.-J., Satouh, Y., Fujihara, Y., *et al*. (2014). Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. Dev. Growth Differ. 56, 122–129.

Mattioli, M., Galeati, G., Bacci, M.L., and Seren, E. (1988). Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. Gamete Res. 21, 223–232.

McElroy, S.L., Byrne, J.A., Chavez, S.L., Behr, B., Hsueh, A.J., Westphal, L.M., and Pera, R.A.R. (2010). Parthenogenic blastocysts derived from cumulus-free in vitro matured human oocytes. PloS One 5, e10979.

Milland, J., Christiansen, D., and Sandrin, M.S. (2005). Alpha1,3-galactosyltransferase knockout pigs are available for xenotransplantation: are glycosyltransferases still relevant? Immunol. Cell Biol. 83, 687–693.

Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.-L., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S., Kim, K.A., *et al*. (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. Nat. Biotechnol. 25, 778–785.

Miyano, T., and Manabe, N. (2007). Oocyte growth and acquisition of meiotic competence. Soc. Reprod. Fertil. Suppl. 63, 531–538.

Mohammadi-Sangcheshmeh, A., Soleimani, M., Deldar, H., Salehi, M., Soudi, S., Hashemi, S.M., Schellander, K., and Hoelker, M. (2012). Prediction of oocyte

developmental competence in ovine using glucose-6-phosphate dehydrogenase (G6PDH) activity determined at retrieval time. J. Assist. Reprod. Genet. 29, 153–158.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378, 203–206.

Moor, R.M., Mattioli, M., Ding, J., and Nagai, T. (1990). Maturation of pig oocytes *in vivo* and *in vitro*. J. Reprod. Fertil. Suppl. 40, 197–210.

Moorhouse, S.R., Creasy, D.M., and Gaunt, I.F. (1987). Three-generation toxicity study of rats ingesting Green S in the diet. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 25, 985–993.

Morita, Y., and Tilly, J.L. (1999). Oocyte apoptosis: like sand through an hourglass. Dev. Biol. 213, 1–17.

Mussolino, C., and Cathomen, T. (2012). TALE nucleases: tailored genome engineering made easy. Curr. Opin. Biotechnol. 23, 644–650.

Nagai, T., and Moor, R.M. (1990). Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilised *in vitro*. Mol. Reprod. Dev. 26, 377–382.

Nagai, T., Takahashi, T., Masuda, H., Shioya, Y., Kuwayama, M., Fukushima, M., Iwasaki, S., and Hanada, A. (1988). In-vitro fertilisation of pig oocytes by frozen boar spermatozoa. J. Reprod. Fertil. 84, 585–591.

Nakai, M., Kashiwazaski, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D.-I., Noguchi, J., Kaneko, H., Shino, M., and Kikuchi, K. (2003). Viable piglets generated from porcine oocytes matured *in vitro* and fertilised by intracytoplasmic sperm head injection. Biol. Reprod. 68, 1003–1008.

Nishimasu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S.I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156, 935–949.

Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., *et al*. (2014). Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156, 836–843.

Niwa, K. (1993). Effectiveness of *in vitro* maturation and *in vitro* fertilisation techniques in pigs. J. Reprod. Fertil. Suppl. 48, 49–59.

Perez-Pinera, P., Ousterout, D.G., Brunger, J.M., Farin, A.M., Glass, K.A., Guilak, F., Crawford, G.E., Hartemink, A.J., and Gersbach, C.A. (2013). Synergistic and tunable human gene activation by combinations of synthetic transcription factors. Nat. Methods 10, 239–242.

Perreault, S.D., Barbee, R.R., and Slott, V.L. (1988). Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev. Biol. 125, 181–186.

Petters, R.M., and Wells, K.D. (1993). Culture of pig embryos. J. Reprod. Fertil. Suppl. 48, 61–73.

Pfeifer, L.F.M., Siqueira, L.G.B., Adams, G.P., Pierson, R.A., and Singh, J. (2012). *In vivo* imaging of cumulus-oocyte-complexes and small ovarian follicles in cattle using ultrasonic biomicroscopy. Anim. Reprod. Sci. 131, 88–94.

Phelps, C.J., Koike, C., Vaught, T.D., Boone, J., Wells, K.D., Chen, S.-H., Ball, S., Specht, S.M., Polejaeva, I.A., Monahan, J.A., et al. (2003). Production of alpha 1, 3-galactosyltransferase-deficient pigs. Science 299, 411–414.

Picton, H.M., Harris, S.E., Muruvi, W., and Chambers, E.L. (2008). The *in vitro* growth and maturation of follicles. Reproduction 136, 703–715.

Platt, R.J., Chen, S., Zhou, Y., Yim, M.J., Swiech, L., Kempton, H.R., Dahlman, J.E., Parnas, O., Eisenhaure, T.M., Jovanovic, M., *et al*. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159, 440–455.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308.

Reed, M.L., Jin, D.I., and Petters, R.M. (1992). Glucose and inorganic phosphate inhibits rat 8-cell embryo development *in vitro.* Theriogenology 37, 282.

Robl, J.M., Wang, Z., Kasinathan, P., and Kuroiwa, Y. (2007). Transgenic animal production and animal biotechnology. Theriogenology 67, 127–133.

Rodríguez-González, E., López-Béjar, M., Velilla, E., and Paramio, M.T. (2002). Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 57, 1397–1409.

Romar, R., Funahashi, H., and Coy, P. (2015). *In vitro* fertilisation in pigs: New molecules and protocols to consider in the forthcoming years. Theriogenology.

Rose, D.W. (2007). Genetic manipulation of Mammalian cells by microinjection. CSH Protoc. 2007, pdb.prot4754.

Rougier, N., and Werb, Z. (2001). Minireview: Parthenogenesis in mammals. Mol. Reprod. Dev. 59, 468–474.

Rouet, P., Smih, F., and Jasin, M. (1994). Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. 14, 8096–8106.

Ruan, J., Li, H., Xu, K., Wu, T., Wei, J., Zhou, R., Liu, Z., Mu, Y., Yang, S., Ouyang, H., *et al*. (2015). Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Sci. Rep. 5, 14253.

Ruppert-Lingham, C.J., Paynter, S.J., Godfrey, J., Fuller, B.J., and Shaw, R.W. (2006). Membrane integrity and development of immature murine cumulus–oocyte complexes following slow cooling to −60°C: The effect of immediate rewarming, plunging into LN2 and two-controlled-rate-stage cooling. Cryobiology 52, 219–227.

Santi, P.A. (2011). Light sheet fluorescence microscopy: a review. J. Histochem. Cytochem. Off. J. Histochem. Soc. 59, 129–138.

Santos, E.C.S., Sato, D., Lucia, T., and Iwata, H. (2015). Brilliant cresyl blue staining negatively affects mitochondrial functions in porcine oocytes. Zygote Camb. Engl. 23, 352–359.

Shirazi, A., Bahiraee, A., Ahmadi, E., Nazari, H., Heidari, B., and Borjian, S. (2009). The Effect of the Duration of *In vitro* Maturation (IVM) on Parthenogenetic Development of Ovine Oocytes. Avicenna J. Med. Biotechnol. 1, 181–191.

Sjunnesson, Y.C.B., Morrell, J.M., and González, R. (2013). Single layer centrifugation-selected boar spermatozoa are capable of fertilisation *in vitro*. Acta Vet. Scand. 55, 20.

Stokes, P.J., Abeydeera, L.R., and Leese, H.J. (2005). Development of porcine embryos *in vivo* and *in vitro*; evidence for embryo “cross talk” *in vitro*. Dev. Biol. 284, 62–71.

Sirard, M.A., Richard, F., Blondin, P. & Robert, C. (2006). Contribution of the oocyte to embryo quality. Theriogenology 65,126–136.

Stringfellow, D.A., and Givens, M.D. (2000). Epidemiologic concerns relative to *in vivo* and *in vitro* production of livestock embryos. Anim. Reprod. Sci. 60-61, 629–642.

Sun, Q.Y., Wu, G.M., Lai, L., Park, K.W., Cabot, R., Cheong, H.T., Day, B.N., Prather, R.S., and Schatten, H. (2001). Translocation of active mitochondria during pig oocyte maturation, fertilisation and early embryo development *in vitro*. Reproduction 122, 155–163.

Szołtys, M., Tabarowski, Z., and Pawlik, A. (2000). Apoptosis of postovulatory cumulus granulosa cells of the rat. Anat. Embryol. (Berl.) 202, 523–529.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and de Kruif, A. (2002). Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol. Reprod. Dev. 61, 414–424.

Tong, G.Q., Heng, B.C., Chen, N.Q., Yip, W.Y., and Ng, S.C. (2004). Effects of elevated temperature *in vivo* on the maturational and developmental competence of porcine germinal vesicle stage oocytes. J. Anim. Sci. 82, 3175–3180.

Uchikura, K., Nagano, M., and Hishinuma, M. (2012). Prediction of maturational competence of feline oocytes using supravital staining of cumulus cells by propidium iodide. Zygote Camb. Engl. 20, 333–337.

Van Blerkom, J. (1996). The influence of intrinsic and extrinsic factors on the developmental potential and chromosomal normality of the human oocyte. J. Soc. Gynecol. Investig. 3, 3–11.

Vandaele, L., Goossens, K., Peelman, L., and Van Soom, A. (2008). mRNA expression of Bcl-2, Bax, Caspase-3 and -7 cannot be used as a marker for apoptosis in bovine blastocysts. Anim. Reprod. Sci. 106, 168–173.

Wallace, W.H.B., and Kelsey, T.W. (2010). Human ovarian reserve from conception to the menopause. PloS One 5, e8772.

Wang, W.H., Sun, Q.Y., Hosoe, M., Shioya, Y., and Day, B.N. (1997). Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. Biol. Reprod. 56, 1376–1382.

Wang, W., Hosoe, M., Li, R., and Shioya, Y. (1997). Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. Dev. Growth Differ. 39, 607–615.

Wang, W.H., Macháty, Z., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1999). Time course of cortical and zona reactions of pig oocytes upon intracellular calcium increase induced by thimerosal. Zygote Camb. Engl. 7, 79–86.

Wang, Z.-Y., Burlak, C., Estrada, J.L., Li, P., Tector, M.F., and Tector, a. J. (2014). Erythrocytes from *GGTA1*/*CMAH* knockout pigs: implications for xenotransfusion and testing in non-human primates. Xenotransplantation 21, 376–384.

Wefers, B., Meyer, M., Ortiz, O., Hrabé de Angelis, M., Hansen, J., Wurst, W., and Kühn, R. (2013). Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. Proc. Natl. Acad. Sci. U. S. A. 110, 3782–3787.

Wei, C., Li, X., Zhang, P., Zhang, Y., Liu, T., Jiang, S., Han, F., and Zhang, Y. (2015). Characterization of porcine partially reprogrammed iPSCs from adipose-derived stem cells. Reprod. Camb. Engl. 149, 485–496.

Weil, M., Jacobson, M.D., Coles, H.S., Davies, T.J., Gardner, R.L., Raff, K.D., and Raff, M.C. (1996). Constitutive expression of the machinery for programmed cell death. J. Cell Biol. 133, 1053–1059.

Whitaker, M. (2006). Calcium at fertilisation and in early development. Physiol. Rev. 86, 25–88.

Wiedenheft, B., van Duijn, E., Bultema, J.B., Waghmare, S.P., Zhou, K., Barendregt, A., Westphal, W., Heck, A.J.R., Boekema, E.J., Dickman, M.J., *et al*. (2011). RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. Proc. Natl. Acad. Sci. 108, 10092–10097.

Yang, D., Xu, J., Zhu, T., Fan, J., Lai, L., Zhang, J., and Chen, Y.E. (2014). Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. J. Mol. Cell Biol. 6, 97–99.

Yoshida, M. (1993). Role of glutathione in the maturation and fertilisation of pig oocytes *in vitro*. Mol. Reprod. Dev. 35, 76–81.

Yoshioka, K. (2011). Development and application of a chemically defined medium for the *in vitro* production of porcine embryos. J. Reprod. Dev. 57, 9–16.

Yuan, Y.Q., Van Soom, A., Leroy, J.L.M.R., Dewulf, J., Van Zeveren, A., de Kruif, A., and Peelman, L.J. (2005). Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. Theriogenology 63, 2147–2163.

Yuan, Y., and Krisher, R.L. (2012). *In vitro* maturation (IVM) of porcine oocytes. Methods Mol. Biol. Clifton NJ 825, 183–198.

Yuge, M., Otoi, T., Nii, M., Murakami, M., Karja, N.W.K., Rajaei, F., Agung, B., Wongsrikeao, P., Murakami, M., and Suzuki, T. (2003). Effects of cooling ovaries before oocyte aspiration on meiotic competence of porcine oocytes and of exposing *in vitro* matured oocytes to ambient temperature on *in vitro* fertilisation and development of the oocytes. Cryobiology 47, 102–108.

Zeuner, A., Müller, K., Reguszynski, K., and Jewgenow, K. (2003). Apoptosis within bovine follicular cells and its effect on oocyte development during in vitro maturation. Theriogenology 59, 1421–1433.

Zhang, S., Guo, Y., Cui, Y., Liu, Y., Yu, T., and Wang, H. (2015). Generation of intermediate porcine iPS cells under culture condition favorable for mesenchymal-to-epithelial transition. Stem Cell Rev. 11, 24–38.

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I hereby declare that I have produced this work without the prohibited assistance of third parties and without making use of aids other than those specifically indicated. This work has not been presented to any other examination board and has not been part of any other thesis.

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Publications

1 **Rahul Dutta**, Shun Li, Konrad Fischer, Alexander Kind, Tatiana Flisikowska, Krzysztof Flisikowski, Oswald Rottmann, Angelika Schnieke (2015). Non-invasive assessment of porcine oocyte quality by supravital staining of cumulus-oocyte complexes with lissamine green B. Zygote (online first)

2. Hsiao-Chun Amy Lin**, Rahul Dutta*,** Subhamoy Mandal, Alexander Kind, Angelika Schnieke, Daniel Razansky (2015) *equal contribution. Advancing ovarian folliculometry with selective plane illumination microscopy. Plos one (Under Review)

3. **Rahul Dutta**, Subhomoy Mandal (2015). Exploring BCB as a contrast medium for ovarian imaging using Multispectral Optoacoustic Tomography. (Manuscript in preparation)

4. Vishwanath PS†,Tobias Weisser†, Phalgun Chintala†,Subhamoy Mandal*, and **Rahul Dutta*** Grading of Mammalian Cumulus Oocyte Complexes by Machine Learning for in vitro Embryo Culture. Manuscript submitted to IEEE International Conference on Biomedical and Health Informatics Feb. 25-28, 2016, Las Vegas, NV, USA.

5. **Rahul Dutta**, Dhruba Malakar, Keviletsu Khate, Shailendra Sahu, Yogesh Akshay and Manishi Mukesh (2011). A comparative study on efficiency of adult fibroblast, putative embryonic stem cell and lymphocyte as donor cells for production of handmade cloned embryos in goat and characterization of putative ntES cells obtained from these embryos. Theriogenology 76 (2011) 851-863.

6. **Rahul Dutta***, Shweta Garg, Dhruba Malakar, Manoj Jena, Dinesh Kumar, Shailendra Sahu and Brahma Prakash (2012).Cardiomyocytes rhythmically beating generated from goat embryonic stem cell. Theriogenology 2012 March15; 77(5):829-39

7. **Rahul Dutta**, Dhruba Malakar, Keviletsu Khate, Shailendra Sahu, Manoj Kumar Jena, Yogesh Akshay, Shweta Garg and Manishi Mukesh (2011). Production and characterization of putative ntES cells from handmade cloned goat embryos derived from adult fibroblast donor cell. Journal of Reproductive and Stem Cell Biotechnology. 2(1): 64-76, 2011.

8. Yogesh S.Akshey, Dhruba Malakar. Arun Kumar De, Manoj Kumar Jena, Shweta Garg, **Rahul Dutta**, Sachin Pawar and Manishi Mukesh. (2010).Handmade cloned goat embryos (Capra hircus)-A

comparision of different donor cells and culture system. Vol 12, No.5.Cellular Reprogramming. October 2010, 12(5): 581-588.

9. Yogesh S. Akshey, Dhruba Malakar, Arun Kumar De, Manoj Kumar Jena, Shailendra Sahu and **Rahul Dutta** (2011). Study of the efficiency of the chemically assisted enucleation method for handmade cloning in goat (Capra hircus). Reproduction in Domestic Animals Volume 46, Issue 3, June 2011

10. Arun Kumar De, Dhruba Malakar, Yogesh S. Akshey, Manoj Kumar Jena, Shweta Garg, **Rahul Dutta** and Shailendra Sahu (2011). In vitro development of goat (Capra hircus) embryos following cysteamine supplementation of the in vitro maturation and in vitro culture media. Small Ruminant Research 96 (2011) 185-190

11. Yogesh S. Akshey, Dhruba Malakar, Arun Kumar De, Manoj Kumar Jena, Sachin Kumar Pawar, **Rahul Dutta** and Shailendra Sahu (2011). Effect of roscovitine treated donor cells and different activation methods on development of handmade cloned goat (Capra hircus) embryos. Theriogenology 75 (2011) 1516-1524

12. Arun Kumar De, Dhuruba Malakar, Yogesh S Akshey, Manoj Kumar Jena and **Rahul Dutta** (2011). Isolation and characterization of embryonic stem cell like cell from in-vitro produced goat (Capra hircus) embryos. Animal Biotechnology 22:4, 181-196.

13. Arun Kumar De, Dhruba Malakar, Manoj Kumar Jena, R**ahul Dutta**, Shweta Garg and Yogesh S Akshey (2012). Zona free and with-zona parthenogenetic embryo production in goat (Capra hircus) — effect of activation methods, culture systems and culture media. Livestock Science Volume 143, Issue 1 January

14. Arun Kumar De, Dhruba Malakar, **Rahul Dutta**, Manoj Kumar Jena, and Shailendra Sahu (2011). Effect of Leukaemia Inhibitory Factor and different types of feeder layers on growth and pluripotent nature of embryonic stem cells from in vitro produced goat (Capra hircus) blastocyst. Journal of applied animal research.

15.Manoj Kumar Jena, Dhruba Malakar, Yogesh S. Akshey, Shweta Garg, **Rahul Dutta** and Arun Kumar De (2012). Handmade cloned and parthenogenetic goat embryos - A comparison of different culture media and donor cells. Small Ruminant Research. Volume 105, Issues 1–3, June 2012

16.Yogesh S Akshey, Dhruba Malakar, Arun Kumar De, **Rahul Dutta**, Manoj Kumar Jena and Shweta Garg (2011). Micromanipulator free approach (Hand-made cloning) for the production of cloned goat (Capra hircus) embryos. Indian Veterinary Journal August 2011, 88(8): 12-15

17. Yogesh S. Akshey, Dhruba Malakar, Arun Kumar De, Shailendra Sahu, Shweta Garg Manoj Kumar Jena, Sachin Pawar and **Rahul Dutta (2010)**. Selection of developmentally competent oocyte using brilliant cresyl blue stain to promote blastocyst development rate after somatic cell nuclear transfer in goat. Indian Journal of Dairy Science, 63(3),

18. Yogesh S Akshey, Dhruba Malakar, Arun Kumar De, **Rahul Dutta** Manoj Kumar Jena and Shailendra Sahu (2011). Effect of pronase concentration and exposure time on zona digestion in goat (Capra hircus) oocytes for hand-made cloning. Indian Journal of Dairy Science.